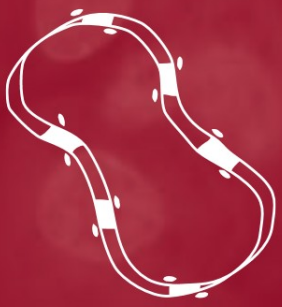
Advanced Techniques in Diagnostic Microbiology



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Charles W. Stratton
Editors

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Advanced Techniques in Diagnostic Microbiology

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Preface

Clinical microbiologists are engaged in the field of diagnostic microbiology to determine whether pathogenic microorganisms are present in clinical specimens collected from patients with suspected infections. If microorganisms are found, these are identified and susceptibility profiles, when indicated, are determined. During the past two decades, technical advances in the field of diagnostic microbiology have made constant and enormous progress in various areas, including bacteriology, mycology, mycobacteriology, parasitology, and virology. The diagnostic capabilities of modern clinical microbiology laboratories have improved rapidly and have expanded greatly due to a technological revolution in molecular aspects of microbiology and immunology. In particular, rapid techniques for nucleic acid amplification and characterization combined with automation and user-friendly software have significantly broadened the diagnostic arsenal for the clinical microbiologist. The conventional diagnostic model for clinical microbiology has been labor-intensive and frequently required days to weeks before test results were available. Moreover, due to the complexity and length of such testing, this service was usually directed at the hospitalized patient population. The physical structure of laboratories, staffing patterns, workflow, and turn-around time all have been influenced profoundly by these technical advances. Such changes will undoubtedly continue and lead the field of diagnostic microbiology inevitably to a truly modern discipline.

Advanced Techniques in Diagnostic Microbiology provides a comprehensive and up-to-date description of advanced methods that have evolved for the diagnosis of infectious diseases in the routine clinical microbiology laboratory. The book is divided into two parts. The first part, "Techniques," covers the principles and characteristics of techniques ranging from rapid antigen testing, to advanced antibody detection, to *in vitro* nucleic acid amplification techniques, to nucleic acid microarray and mass spectrometry. Sufficient space is assigned to cover different nucleic acid amplification formats that are currently being used widely in the diagnostic microbiology field. Within each technique, examples are given regarding its application in the diagnostic field. Commercial product information, if available, is introduced with commentary in each chapter. If several test formats are available for a technique, objective comparisons are given to illustrate the contrasts of their

advantages and disadvantages. The second part, "Applications," provides practical examples of application of these advanced techniques in several "hot spots" in the diagnostic field. A diverse team of authors presents authoritative and comprehensive information on sequence-based bacterial identification, blood and blood product screening, molecular diagnosis of sexually transmitted diseases, advances in mycobacterial diagnosis, novel and rapid emerging microorganism detection and genotyping, and future directions in the diagnostic microbiology field.

We hope our readers like this technique-based approach, and your feedback is greatly appreciated. We want to thank the authors who devoted their time and efforts to produce their chapters. We also thank the staff at Springer, especially Melissa Ramondetta, who initiated the whole project. Finally, we greatly appreciate the constant encouragement of our family members through this long effort. Without their unwavering faith and full support, we would never have had the courage to commence this project.

Yi-Wei Tang
Charles W. Stratton

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Part I
Techniques

1
Automated Blood Cultures

XIANG Y. HAN

Introduction

A clinically suspected infection is ultimately confirmed by isolation or detection of the infectious agent. Subsequent identification of the microorganism and antibiotic susceptibility tests further guide effective antimicrobial therapy. Bloodstream infection is the most severe form of infection and is frequently life-threatening, and blood culture to detect circulating microorganisms has been the diagnostic standard. Much of the scientific and technologic advances in blood culture were made from the 1970s to the 1990s; this chapter briefly reviews various aspects of blood culture with emphasis on automated culturing systems.

Principles

The principles and scientific basis to optimize the diagnostic yield of blood cultures have been reviewed and summarized (Weinstein, 1996; Reimer, 1997). Most parameters were initially established for manual blood culture systems that used basal culture media. A recent study addressed some of these parameters for newer culture systems and media and found them to be mostly valid nowadays (Cockerill et al., 2004). Major features are summarized below.

Host and Microbial Factors

Invasion of the bloodstream by microorganisms reflects the failure of initial host defense, such as the loss of integrity of skin and mucosa and weakening of the innate and acquired immunity. Among those patients bearing an intravascular device or using recreational drugs intravenously, direct blood seeding of a microorganism is also possible. Once in the bloodstream, microbes are constantly attacked by host defenses, such as complements, phagocytic leukocytes, and antibodies. The ability of invading microorganisms to evade or shield off host defense or antimicrobials favors their survival and dissemination in the bloodstream. Therefore, both the host

and microbial factors determine the occurrence, severity, and duration of septic episodes and the yield of culture recovery. The presence of antimicrobial agents in the circulation may also reduce culture recovery.

Timing, Volume, and Frequency of Cultures

Blood culture should be drawn, if at all possible, before initiation of empirical antibiotic therapy. Conversely, persistence of fever during therapy is also a common reason to repeat culture. Timing the blood-draw has bearings on culture recovery. Most bacteremia or fungemia are not constant except in the case of endocarditis; thus, the host responses, such as rising fever, likely herald the best time to draw blood culture. The preferred volume is 20–30 mL; lower volume reduces culture sensitivity, whereas higher volume does not necessarily increase sensitivity, because of more host factors and/or antimicrobials introduced, while adding to the cost and iatrogenic anemia. Generally, for an adult patient, 10 mL of blood should be drawn to each culture bottle (a set of aerobic and anaerobic bottles) to reach a blood/broth ratio of 1:5 to 1:10. For each septic episode, two to three sets of cultures over a 24-h period provide maximum recovery for the offending microorganisms. How frequent to draw blood for follow-up culture is more of a clinical decision depending on the patient's response to initial treatment and host and microbial factors; it may take 2–3 days or even longer for a patient to show improvement.

Atmosphere and Length of Incubation

Traditionally, two aerobic bottles and two anaerobic bottles have been recommended. However, the declining proportion of bacteremias due to obligate anaerobes has led to the suggestion that routine anaerobic cultures are not needed and can be tailored to the needs of an individual institution and patient population. How long to incubate? Several studies on different culturing systems have shown that 5-day culturing and testing is sufficient to recover nearly all significant microorganisms (∼99%) (Doern et al., 1997; Wilson, 1997; Han and Truant, 1999; Cockerill et al., 2004). Most fastidious organisms can also be recovered in 5 days, such as the HACEK organisms (*Haemophilus aphrophilus*, *Actinobacillus actinomycetemcomitans*, *Cardiobacterium hominis*, *Eikenella corrodens*, and *Kingella kingae*), *Brucella* spp., and nutritionally variant streptococci (Doern et al., 1996). A new species, *Cardiobacterium valvarum*, proposed by us as well as a cause of endocarditis, was cultured within 3 days (Han et al., 2004b). The length of culture for *Brucella* spp. had been controversial until the study by Bannatyne et al. (1997), which showed that 90 of 97 such bacteremic patients became culture-positive within 5 days. Blood cultures for *Francisella tularensis*—fewer than a dozen such culture-positive cases in the United States currently—mostly become positive after incubation for 3 to 8 days (reviewed by Doern et al., 1996; Han et al., 2004a).

Interpretation of Significance

Several common blood isolates are almost always significant: *Staphylococcus aureus*, *Escherichia coli*, and other members of the family Enterobacteriaceae, *Pseudomonas aeruginosa*, and *Candida albicans*. In contrast, common skin organisms, such as coagulase-negative staphylococci (CoNS), Coryneform bacilli, alpha-hemolytic streptococci, and *Propionibacterium acne*, are frequent contaminants. However, with many patients carrying an intravascular device that is prone to colonization and infection, each positive culture entails clinical correlation with other findings and sound judgment to make final assessment (Mirrett et al., 2001; Weinstein, 2003).

Recent Trends

Some noticeable trends in the past decades are increasing number and life span of immunocompromised or immunosuppressed patients, and thus emergence of more opportunistic pathogens; more frequent use of antibiotics and associated resistance, in fact, up to 29% of blood cultures came from patients with active antimicrobial therapy; more use of indwelling devices, such as intravascular catheters and others; and emergence of more *Candida* and other fungal infections (Weinstein et al., 1997).

Automated Culturing Systems

Blood culture has evolved over the years from manual methods, now infrequently used, to automated culturing systems. The major advantage of an automated system, such as BACTEC NR660, is the obviation of manual inspection or examination to detect microbial growth because each system automatically does so by monitoring microbial CO_2 production. Agitation of culture bottles also improves mixing and aeration to promote the growth of aerobes and facultative anaerobes. These features make blind subcultures of negative bottles unnecessary, as shown in a few studies reviewed by Reimer et al. (1997). Automation has improved the practice of blood culture enormously.

Continuously monitoring blood culturing systems (CMBCSs) are most commonly used nowadays. Introduced in the early 1990s, CMBCSs have added nearly continuous (every 10 to 12 min) monitoring of bacterial growth to the automated systems. Currently, three CMBCSs are available in the United States, and they are briefly shown in Table 1.1. More detailed description can be found elsewhere (Weinstein and Reller, 2002).

Numerous studies have been published to compare the performance of the systems and associated media with or without various lytic agents or additives to remove blood antimicrobials, and several recent ones are summarized as follows (Table 1.2).

TABLE 1.1. Commercial continuously monitoring blood culturing systems (CMBCSs).

Manufacturer	Current system since early 1990s	Microbial detection mechanism	Test interval (min)	Newer system, year
BioMerieux	BacT/Alert series for varying holding capacity	Colorimetric for CO_2 production	10	BacT/Alert 3D, 2001
Becton-Dickinson	BACTEC series for varying holding capacity	Fluorescent for CO_2 production	10	BACTEC LX, 2004
Trek	ESP series for varying holding capacity	Manometric for CO_2 production	12	VersaTrek, 2004

McDonald et al. (1996) compared the BacT/Alert standard bottle with BacT/Alert FAN bottle that contains Ecosorb, an antimicrobial-absorbing substance, and they found that FAN bottle recovers significantly more microbes from all septic episodes, especially *S. aureus*, CoNS, and members of Enterobacteriaceae. Along with this, however, recovery of all contaminants, including CoNS, is also higher. The performance of the BacT/Alert FAN bottle and the BACTEC Plus aerobic/F bottle (with resins to absorb antimicrobials) were also compared, and the two systems were found comparable (Jorgensen et al., 1997). A recent study compared BacT/Alert standard bottle and BACTEC standard bottle and found the former significantly improved the recovery of *S. aureus*, CoNS, and yeasts (Mirrett et al., 2003). In a study comparing BacT/Alert FAN versus Trek ESP 80A, Doern et al. (1998) found that BacT/Alert FAN recovered more *S. aureus*, enterics, and non–*Pseudomonas aeruginosa* Gram-negative rods, along with more

TABLE 1.2. Performance of culture media with or without lytic agents or additives.

Compared media (bottle)	Findings	Reference
BacT/Alert FAN vs. BACTEC Plus/F	Comparable	Jorgensen et al., 1997
BacT/Alert FAN vs. BacT/Alert standard	BacT/Alert FAN improved recovery of *S. aureus*, CoNS, and enterics	McDonald et al., 1996
BacT/Alert standard vs. BACTEC 9240 standard	BacT/Alert standard improved recovery of *S. aureus*, CoNS, and yeasts	Mirrett et al., 2003
BacT/Alert FAN vs. Trek ESP 80A	BacT/Alert FAN improved recovery of *S. aureus*, enterics, and non–*Pseudomonas aeruginosa* Gram-negative rods	Doern et al., 1998
BacT/Alert FAN vs. Trek ESP 80A, in pediatric patients	Overall comparable. BacT/Alert FAN better for *S. aureus* and antibiotic-treated samples; ESP 80A better for streptococci and enterococci.	Welby-Sellenriek et al., 1997
BacT/Alert FAN vs. BACTEC fungal medium	Comparable with detect fungemia.	McDonald et al., 2001
BACTEC Plus Anaerobic/F bottles vs. Standard Anaerobic/F bottles	BACTEC Plus Anaerobic/F bottles detected more microorganisms	Wilson et al., 2001

contaminants, too. In a similar study in pediatric patients (Welby-Sellenriek et al., 1997), the two systems were found to be overall comparable, with BacT/Alert FAN recovering more *S. aureus* and better for antibiotic-containing samples and ESP 80A recovering more streptococci and enterococci. Because yeasts are an increasing cause of nosocomial bloodstream infections, McDonald et al. (2001) compared BacT/Alert FAN with BACTEC fungal medium for their recovery, and the two systems were found comparable. The anaerobic culture media have also been compared; a recent study by Wilson et al. (2001) found that the BACTEC Plus Anaerobic/F bottles detect more microorganisms and episodes of bacteremia and fungemia than the BACTEC Standard Anaerobic/F bottles.

In summary, CMBCSs, each with cost, strength, and weakness, perform well overall in delivering timely and accurate diagnosis of bloodstream infections. Addition of lytic or antimicrobial-absorbing substances has consistently improved the recovery of *S. aureus* and members of Enterobacteriaceae, particularly from treated patients.

New versions of CMBCSs have been released or are about to be (Table 1.1), which have kept the key elements from earlier versions while refining the hardware, computer system, and data management. The trend is to increase user-friendly features for space, operation, and flexibility. The BACTEC LX system now also uses laser technology to detect microbial CO_2 production. Clinical evaluations are being conducted and results are expected soon. It is reasonable to assume that newer systems should perform just as well as or better than their previous versions.

Blood Culture for Mycobacteria

Bacteremia due to rapidly growing mycobacteria (RGM) can be detected by blood cultures, similar to other fastidious organisms. In our experience with the BACTEC 9240 and the Isolator 10 system (Wampole Laboratories, Princeton, NJ, USA), RGM typically grow in 2–5 days (De et al., 2005). RGM bacteremias are usually associated with use of intravascular catheter (Raad et al., 1991; De et al., 2005). From an analysis of 80 consecutive clinical RGM strains, 24 were isolated from blood and/or catheters, and *Mycobacterium mucogenicum* accounted for most of them (15 of 24) (De et al., 2005).

Blood culture has been useful to detect and monitor *Mycobacterium avium* bacteremia in patients with AIDS. *M. avium* bacteremia usually occurs when the $CD4^+$ cell count falls below 50/mm^3 (Inderlied et al., 1993). Circulating *M. avium*, exclusively within monocytes, usually range 10 to 10^3 colony-forming units (CFU) per milliliter of blood but can be as high as 10^6 CFU/mL (Inderlied et al., 1993). A number of blood culture systems have been used: the earlier BACTEC 13A radiometric system and Isolator 10 system and the more recent CMBCSs, such as BACTEC 9240 with MYCO/F LYTIC medium and BacT/Alert MB. Several studies have evaluated these systems. In a controlled comparison of the performance of these systems, Crump et al. (2003a) found that these systems perform comparably well in detecting *M. avium* bacteremia and other mycobacterial and fungal sepsis.

However, the two CMBCSs detect *M. avium* bacteremia 2–3 days sooner than the earlier systems. On average, an incubation of 14 days is required.

Blood culture also detects *Mycobacterium tuberculosis* bacteremia and usually takes 24 days for incubation (Crump et al., 2003a). Culture of blood is as sensitive as culture of bone marrow to detect *M. tuberculosis*, and its role seems to be expanding (Crump, 2003b). *M. tuberculosis* bacteremia seems particularly common in AIDS patients in developing countries. For example, in Tanzania, it is the most common organism of all sepsis-causing microorganisms, accounting for 48% (57 of 118 patients) (Archibald et al., 1998). In Thailand, it ranks second (27 of 114 patients), following *Cryptococcus neoformans* (30 of 114) and surpassing *M. avium* (24 of 114 patients) (Archibald et al., 1999). In Brazil, it is also the most common cause of mycobacterial sepsis (Grinszejn, 1997). Clinically, knowing these facts will direct empirical antibiotic coverage against these organisms to reduce immediate mortality once the patient is seen at the hospital. These data will also impact public health policies and health care priorities in their respective countries.

Summary

In conclusion, automatic blood cultures have become the diagnostic mainstay for bloodstream infections. The systems are refined and able to cultivate various bacteria, fungi, and mycobacteria. The laboratories have seen improved efficiency through automation and a 5-day culturing cycle. With the vast majority of significant isolates being detected within the first 72 h of culture, the timely care of patients is facilitated. The remaining challenge is that the sooner the identification of cultured organism is rendered, the better the patient care will be.

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2
Urea Breath Tests for Detection of *Helicobacter pylori*

SIHE WANG AND XIAOTIAN ZHENG

Introduction

Helicobacter pylori

The association of *Helicobacter pylori* with peptic ulcer disease and gastric cancer was first proposed by Warren and Marshall in 1983 (Warren and Marshall, 1983). In February 1994, the National Institutes of Health Consensus Development Conference concluded that *H. pylori* infection is the major cause of peptic ulcer disease, and all patients with confirmed peptic ulcer disease associated with *H. pylori* infection should receive treatment with antimicrobial agents (Yamada et al., 1994). The International Agency for Research on Cancer Working Group of the World Health Organization categorized *H. pylori* as a group I, or definite, human carcinogen (Versalovic, 2003). Based on the data retrieved during the National Health Interview Survey of 1989, 10% of adult U.S. residents reported physician-diagnosed ulcer disease, among whom one third had an ulcer in the past year (Sonnenberg and Everhart, 1996). In developing countries, the prevalence of *H. pylori* carriers can be as high as 70–90%. Most patients acquire the infection at childhood. The prevalence of the infection in developed countries is lower, ranging from 25% to 50% (Dunn et al., 1997). Seroprevalence studies demonstrate an increasing rate in adults of 3–4% per decade (Cullen et al., 1993; Sipponen et al., 1996; Kosunen et al., 1997; Versalovic, 2003).

H. pylori–infected patients may develop chronic gastric inflammation that can be asymptomatic. Infection of *H. pylori* is associated with peptic ulcer disease (Dunn et al., 1997). *H. pylori* infection is also associated with gastric adenocarcinoma (Oconnor et al., 1996) and mucosa-associated lymphoid tissue (MALT) lymphoma (Isaacson, 1994). The American Medical Association published guidelines for testing and treatment of *H. pylori*–related disease (Peterson et al., 2000). The panel of experts recommends testing for *H. pylori* in patients with active ulcers, a history of ulcers, or gastric mucosa–associated lymphoid tissue lymphomas, and young patients with ulcer-like dyspepsia and those with family history should also be tested for *H. pylori*. Eradication of the infection leads to cure of the ulcers (Dunn et al., 1997). Treatment of the infection with antibiotics includes twice-daily triple

therapy with a proton pump inhibitor or ranitidine bismuth citrate, clarithromycin, and amoxicillin for 10–14 days (Peterson et al., 2000). A similar recommendation of triple therapy is also recommended by European *Helicobacter pylori* Study Group (Moayyedi, 1999). Multiple therapeutic regimens have been shown to be effective (Harris and Misiewicz, 1996; Dunn et al., 1997; Howden and Hunt, 1998; Gene et al., 2003a, 2003b; Versalovic, 2003). Metronidazole or clarithromycin should be included to achieve higher than 90% eradication rate (Dunn et al., 1997; Versalovic, 2003). The MOC therapy, which includes metronidazole, omeprazole, and clarithromycin for 7–14 days, has also been shown to offer greater than 90% eradication (Versalovic, 2003). The traditional FDA-approved triple therapy includes bismuth subsalicylate (two tablets, 262 mg), metronidazole (250 mg), and tetracycline (500 mg) taken four times daily for 14 days (Dunn et al., 1997). Because of the resistance problems, quadruple therapy (proton pump inhibitor, tetracycline, metronidazole, and a bismuth salt) has been used to improve the efficacy and is associated with fewer side effects (Dunn et al., 1997). However, a later meta-analysis shows only a slightly improved (statistically insignificant) eradication rate of the quadruple therapy compared with the traditional triple therapy, and there are no significant differences in compliance or adverse effects (Gene et al., 2003a).

Laboratory Diagnosis of *H. pylori* Infection

Detection of the Organism in Biopsy Tissue Specimens

Patients infected with *H. pylori* can be diagnosed by examination of biopsy tissue specimens obtained by endoscopy. The organism can be directly demonstrated in silver-stained histology tissue samples or in imprint cytology specimens stained with Giemsa or Gram stain.

H. pylori can be isolated from clinical tissue specimens (Versalovic and Fox, 2003). Special transport medium, microaerophilic culture environment, and extended incubation time (5–7 days) are required. The organism can be presumptively identified based on its microscopic morphology and positive reactions for catalase, oxidase, and urease tests. *H. pylori* can also be indirectly detected in the gastric biopsy tissue by testing its urease activity. This enzyme (organism) present in the specimen converts urea in the testing medium into ammonia. The elevated pH as a result of the reaction can be observed with a color pH indicator in the testing medium. These methods are reasonably sensitive, specific, and easy to perform. However, invasive procedures are required.

Antibody Detection by Serology Assays

H. pylori specific IgG can be detected in infected patient serum samples by using ELISA assays. IgG-negative patient samples can be followed by detecting specific IgA antibodies. These assays are commercially available in both laboratory-based

and point of care–based formats. They are easy to perform, relatively sensitive, and low cost. The disadvantage is that these antibodies may persist for months or years after eradication of the organism, and test results may need careful interpretation.

Urea Breath Tests

Urea breath tests detect current *H. pylori* infection. This test is based on production by *H. pylori* of powerful urease, an enzyme that converts urea to ammonium and carbon dioxide (CO_2) (Bazzoli et al., 1997; Vakil and Vaira, 2004). When infected with *H. pylori*, high urease activity is present in the stomach. A dose of urea labeled with either ^{13}C or ^{14}C is taken by the subject. The urease-catalyzed reaction then takes place in the mucus layer. The labeled CO_2 diffuses to the epithelial cells and then is carried in the bloodstream and ultimately is released in the exhale. The labeled CO_2 in the subject's breath can be measured. The amount of the labeled CO_2 is related to the urease activity, which indicates the presence or absence of *H. pylori* infection (Bazzoli et al., 1997; Logan, 1993; Vakil and Vaira, 2004). The amounts of the isotopic CO_2 can be measured by various techniques, and the results are expressed relative to the endogenous CO_2 production. The sensitivity and specificity of breath tests range from 95% to 97%, although this method has been reported to be less reliable for patients with gastric surgery or in patients who take proton pump inhibitors or ranitidine (Vakil and Vaira, 2004). In a study involving 20 volunteers, Cutler et al. found that ranitidine at standard dose (150 mg b.i.d.) or high dose (300 b.i.d.) does not decline breath test results reproducibly, and ranitidine does not need to be discontinued before a urea breath test (Cutler et al., 1998).

^{14}C-Urea Breath Test

Conventionally, patient preparation for the test requires fasting for at least 4 h and oral ingesting of 5 μCi ^{14}C-urea in 20 mL water. Breath is collected 20 min postdosing in a CO_2-absorbing solution (examples are hyamine-methanol solution with a pH indicator or benzethonium hydroxide–methanol with a pH indicator) (Marshall et al., 1991; Desroches et al., 1997; Rollan et al., 1997). Radioactivity in the sample is measured by a scintillation counter, and the result is expressed as counts per minute (cpm) or as specific activity at a specific postdosing time (AS_{time}) (Marshall et al., 1991; Desroches et al., 1997; Rollan et al., 1997).

$$AS_{\text{time}} = (\%^{14}CO_2 \text{ dose excreted/mmol of } CO_2) \times \text{weight (kg)}$$

where the ^{14}C-urea dose is calculated from measurements of standard solutions with known concentrations of ^{14}C-urea, and $^{14}CO_2$ dose excreted = counts at the specific time − counts at baseline. This parameter is also corrected for the patient's weight (Desroches et al., 1997). The initial ^{14}C-urea test using β-scintillator is suitable for diagnosis of *H. pylori* as well as confirmation of eradication

of *H. pylori* after antibiotic treatment (Marshall et al., 1991; Desroches et al., 1997; Rollan et al., 1997).

The two parameters that have been subjected to modification are the ^{14}C-urea dose and breath-collection times (Kao et al., 1993; Abukhadir et al., 1998). A reduced dose of ^{14}C-urea to 1 μCi has been shown to be highly sensitive and specific (same as the initial test) for both diagnosis and post-treatment confirmation of eradication. (Hegedus et al., 2002; Raju et al., 1994). Further reduction of the collection time to 10 min post–^{14}C-urea dosing has been shown to be appropriate for the clinical diagnosis of *H. pylori* (Ozturk et al., 2003; Peura et al., 1996). Though the dose of radioactive ^{14}C-urea is minimal, strict regulations have to be followed to ensure the patient's safety. The test has not been approved for use in pregnant women and children.

^{13}C-Urea Breath Test

^{13}C-urea breath test is considered a standard noninvasive test for both initial diagnosis and eradication confirmation. Compared with the ^{14}C-urea breath test, ^{13}C-urea is a nonradioactive substance, and no special handling is necessary (Logan, 1993). The general procedure is to take a simple test meal to delay gastric emptying and maximize the distribution of ^{13}C-urea after fasting followed by ingesting the ^{13}C-urea dose in water or tablets. If the ^{13}C-urea dose is taken in water solution, immediate mouth-rinsing with water is recommended to prevent false-positive results caused by oral bacteria with urease activity (Epple et al., 1997; Liao et al., 2002; Ohara et al., 2004; Oksanen et al., 1997; Peng et al., 2001). This mouth-rinsing step can be eliminated by taking a film-coated tablet-formulated ^{13}C-urea dose that is not soluble in the oral cavity but readily soluble in the stomach (Ohara et al., 2004). A breath sample is then taken at both baseline and the specified postdose time points, usually at 20 or 30 min. The conventional detection of the breath is by isotope ratio mass spectrometer (IRMS) that differentiates $^{13}CO_2$ and $^{12}CO_2$. Less expensive gas chromatography–mass spectrometry (GC-MS) has also been used to measure the specimens (Lee et al., 1998). The ^{13}C element is a nonradioactive isotope of ^{12}C with a natural relative abundance of 1.11% (Silverstein and Webster, 1998). The delta over the baseline of $^{13}CO_2$ excess is used as the diagnostic parameter. The formula is expressed as the following (Oksanen et al., 1997):

$$\delta = \frac{(R_{\text{sample}} - R_{\text{ref}})}{R_{\text{ref}}} \times 1000‰$$

where R is the ratio of $^{13}CO_2$ to $^{12}CO_2$ in the sample and in a reference gas. The reference gas is an international primary standard, PD belemnite calcium carbonate (Logan, 1993). The test results are expressed as the difference in relative enrichment between predose and postdose breath samples (delta over baseline, or DOB) (Oksanen et al., 1997). Cutoff values vary with various ^{13}C-urea doses, different test administration methods including formulation of ^{13}C-urea and test meals, sample collection time, and detection techniques.

Other detection techniques have been developed to reduce the initial cost of mass spectrometry. Based on the slightly different absorption spectra between $^{13}CO_2$ and $^{12}CO_2$, the ratio of $^{13}CO_2/^{12}CO_2$ can be accurately determined by nondispersive isotope-selective infrared spectrometer (NISIR). The sensitivity and specificity of the ^{13}C-urea breath test using NISIR are comparable with those measured by mass spectrometer (Braden et al., 1999; Savarino et al., 1999; Isomoto et al., 2003; Kato et al., 2004). This detection technique is less expensive compared with mass spectrometry. It can also be placed in a regular laboratory, clinics, and even in a doctor's office (see "FDA Approved Tests," below).

Another technique to detect ratio of $^{13}CO_2/^{12}CO_2$ is laser-assisted ratio analyzer (LARA). The detection principle is based on the optogalvanic effect, which is an electrical signal in response to optical stimulation of a resonance transition in an electrical discharge species. The optogalvanic effect is due to changes in the effective electrical impedance of the gas discharge, which results from an optically induced change in the electron energy distribution function in the molecules. The laser-induced stimulation modifies ionization rate in the discharge cell, which enables measurement of electron energy to determine the gas concentration in the specimen (Braden et al., 2001; Murnick and Peer, 1994). The LARA is based on two unique light sources: $^{13}CO_2$ and $^{12}CO_2$ charging lamps. The use of the two charging lamps ensures that light absorption is due to the existence of $^{13}CO_2$ and $^{12}CO_2$ only in the gas mixture. It also reduces the background radiation leading to a highly sensitive and specific technique (Shirin et al., 2001). The application of this technique has been proved to be an effective alternative to the traditional IRMS (Minoli et al., 1998; Cave et al., 1999; Savarino et al., 2000; Braden et al., 2001; Shirin et al., 2001).

Since its description using 350 mg of ^{13}C-urea (Graham et al., 1987) the test has been modified extensively on two major areas to reduce the cost and increase the comfort level: ^{13}C-urea dose and duration of the test. Reduction of ^{13}C-urea dose to 100 mg for a test duration of 30 min without a test meal has been shown to be highly sensitive and specific (Oksanen et al., 1997). Tests employing a dose of 100 mg or 75 mg ^{13}C-urea for duration of 30 min have been proved to be as accurate and less expensive compared with larger doses (Epple et al., 1997; Labenz et al., 1996; Liao et al., 2002; Oksanen et al., 1997). The test meal can be milk, orange juice, or a citric acid solution (Epple et al., 1997; Hamlet et al., 1999; Labenz et al., 1996; Liao et al., 2002). Reduction of dose to 50 mg ^{13}C-urea and test duration to 15 min have also proved to be sufficient (Liao et al., 2002). Further modification using a tablet containing 50 mg ^{13}C-urea and 456 mg citric acid without a test meal for duration of as short as 10 min provides sufficient sensitivity and specificity when endoscope was used as a "gold standard" diagnosis of *H. pylori* infection (Gatta et al., 2003; Wong et al., 2003). Ingestion of 100 mg ^{13}C-urea in 50 mL water with no test meal after 6 h fasting, the earliest optimal time for discriminating *H. pylori*–positive and –negative patients is 2 min with endoscopic administration and 6 min with conventional method of administration (Peng et al., 2001). Another study involving 202 patients shows no significant difference between the conventional tests (75 mg ^{13}C-urea in 50 mL water) with and without a test meal (200 mL 0.1 N citric acid) (Wong et al., 2000).

A further modification incorporating the endoscope technique shows highly accurate diagnosis of *H. pylori* and confirmation of eradication (Suto et al., 1999). The most important feature of the technique (endoscopic ^{13}C-urea breath test; EUBT) is the direct spray of ^{13}C-urea over the entire gastric mucosa under observation endoscopically. However, this technique requires a lot of patient preparation, including oral intake of 80 mg dimethylpolysiloxane to remove adherent gastric mucus 10 min before the endoscope, oral intake of 200 mg lidocaine to anesthetize the pharyngeal areas, and intramuscular injection of 20 mg scopolamine butylbromide 5 min before the endoscopy (Suto et al., 1999).

The ^{13}C-urea breath test is not affected by bleeding peptic ulcers, whereas the sensitivity of the rapid urease test is decreased significantly (Wildner-Christensen et al., 2002). One drawback with the ^{13}C-urea breath test is that equivocal or false-negative results often occur in patients on antisecretory medications. This problem could be resolved by taking the ^{13}C-urea in a tablet formulation supplemented with citric acid (Hamlet et al., 1999).

The diagnosis of *H. pylori* using a ^{13}C-urea breath test has been explored in infants and adolescents. The commonly accepted method using 75 mg ^{13}C-urea with breath samples taken at baseline, 20 min, and 30 min was shown to be highly sensitive (100%). The specificity is lower in children less than 6 years of age (88.1% vs. 97.8%) compared with the older group. Because of some overlap, definition of a gray zone seems to be appropriate (Kindermann et al., 2000). This method has also been shown to have excellent sensitivity and specificity for confirmation of eradication of *H. pylori* (100%) in 72 children aged 3–18 years. The diagnostic specificity (95%) and sensitivity (100%) have also been shown to be comparable with histology, rapid urease test, and serology (Yoshimura et al., 2001). Reduction of ^{13}C-urea dose to 50 mg in children is sufficient for diagnosis of *H. pylori* (Bazzoli et al., 2000; Kawakami et al., 2002; Canete et al., 2003). A fatty test meal and 50 mg ^{13}C-urea with breath sampled at 30 min have been shown to give the best sensitivity (98%) and specificity (98%) in a muticenter study (Bazzoli et al., 2000).

FDA-Approved Tests

As shown in Table 2.1, urea breath tests from two companies have been approved by the FDA for *H. pylori* diagnosis (U.S. Food and Drug Administration, 2004).

BreathTek (Meretek Diagnostics, Inc., Lafayette, CO, USA) is an FDA cleared and CLIA nonregulated test (Meretek Diagnostics, 2004). It is claimed to be simple, with no special in-office licenses or personnel needed to perform the test. The test can be administered in a doctor's office, clinic, or patient service center. The patient should abstain from antibiotics, proton pump inhibitors, and bismuth 14 days before the initial testing or 4 weeks prior to testing for confirmation of eradication. Though H_2 antagonists are not in the list, discontinuation of H_2 antagonists 24 h prior to the testing is recommended. The patient is also required not to have anything in his or her mouth 1 h prior to the testing. Immediately after a baseline breath sample is collected by blowing into a collection bag (or duplicate collection

TABLE 2.1. Comparison of ^{13}C-urea breath tests for *H. pylori* based on information at manufacturers' Web sites.

Test name	FDA status	Fasting	Detection	Sample collection time	Instrument time	Sensitivity[a]	Specificity[a]	Manufacturer
BreathTek	IVD[b]	1 h	GIRMS, or UBiT-IR300	0 and 15 min	Sent to specialty lab (GIRMS) 5.5 min (UBiT-IR300)	95%	95%	Mereteck
Helikit	IVD[b]	4 h	IRMS, or ISOMAX 2002	30 min	Sent to specialty lab (IRMS) not available (ISOMAX)	98%	95%	Isodiagnostika

[a] Based on statements in manufacturers' product inserts.
[b] For in vitro diagnostic use.

tubes for GIRMA) to determine the initial ratio of $^{13}CO_2$ and $^{12}CO_2$, the patient is given a lemon-flavored Pranactin-Citric solution by mouth. Each 3-g dose of the Pranactin-Citric powder is supplied in a polyethylene-lined foil pouch containing 75 mg ^{13}C-urea, citric acid, aspartame, and mannitol. The second breath sample is then collected 15 min after the dose ingestion by blowing into the second collection bag (or duplicate collection tubes for GIRMA). Urease produced by *H. pylori* hydrolyzes ^{13}C-Panactin-Citric to form $^{13}CO_2$, which is expelled and detectable in the second breath sample. The system uses a Gas Isotope Ratio Mass Spectrometer (GIRMS) or an UBiT-IR300 Infrared Spectrometer for the measurement of $^{13}CO_2$ and $^{12}CO_2$ in breath samples. GIRMS assay has to be performed by Meretek Clinical Laboratory or other qualified laboratories licensed by Meretek. Quality checks have to be performed on all final results: each specimen must contain at least 1.5% volume CO_2 to assure adequate breath for analysis; the relative abundance of the baseline has to be in the range of −27.0 to −17.0 delta per milliliter; the DOB result must be greater than −1.0. Analysis by UBiT-IR300 spectrometer can be set up and operated by each individual laboratory or test facility. The result is provided as delta over baseline, which is defined as the difference between the ratio $^{13}CO_2$ and $^{12}CO_2$ in the postdose specimen and the corresponding ratio in the baseline specimen. A cutoff of 2.4 is for both initial diagnosis and post-treatment monitoring of *H. pylori*. However, the test performance of persons under 18 years of age has not been established. There is also no established correlation between the number of *H. pylori* organisms in the stomach and the breath test results (Meretek Diagnostics, 2004).

Helikit (Isodiagnostika, Edmonton, Alberta, Canada) also incorporates ^{13}C-urea formulation with possibilities of both IRMS and infrared point-of-care (ISOMAX2002) detections. The postdose breath collection is set at 30 min, and the sensitivity and specificity are claimed to be 98% and 95%, respectively (Isodiagnostika, 2004).

BreathID (Oridion BreathID Ltd., Jerusalem, Israel) has been considered as a test for investigational purposes. The detection of $^{13}C/^{12}C$ is achieved by LARA via continuous breath sampling at a point-of-care environment. The BreathID technology enables health care providers to perform the breath test by pushing a single button, and results are printed within 10 min in most cases. It is also claimed that this technology is suitable for pediatric testing (Oridion BreathID Ltd., 2004).

In summary, urea breath tests for diagnosis of *H. pylori* detect active infection. They are noninvasive and highly accurate. Newer assay formats and instruments are much simpler, more cost effective, and more user friendly and thus are the alternative choices for clinical diagnosis.

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3
Rapid Antigen Tests

SHELDON CAMPBELL AND MARIE L. LANDRY

Introduction

Immunoassays for the detection of the antigens of microorganisms remain important tools for the diagnosis and management of infectious diseases. Great strides have been made since the introduction of the early precipitation and agglutination assays in increasing the sensitivity, specificity, standardization, and automation of antigen tests (Hage, 1999; Carpenter, 2002; Constantine and Lana, 2003; Peruski and Peruski, 2003). Antigen tests have long been used to detect infectious agents that are difficult, slow, or hazardous to culture. However, antigen detection methods are especially useful for rapid diagnosis, whether in the clinic, emergency department, doctor's office, or the central laboratory. Recently, simple one-step assays have been introduced that can provide results in 15 min with dramatic benefits to physician decision-making.

The basis for antigen detection assays is the specific binding of an antigen (protein or glycoprotein) to an antibody. Antigen assays are generally more economical than either culture or molecular techniques; however, they do not amplify their target as culture amplifies infectious organisms or as polymerase chain reaction amplifies nucleic acid. Thus, they are often less sensitive than these other methods. Because antigen immunoassays traditionally detect only the antigen originally present in the sample, optimal sample collection and handling are key to good results.

Antigen detection methods are also very valuable for the rapid and specific identification of infectious agents after amplification in culture. However, because these culture techniques require at least an overnight incubation, they will not be discussed here. In this chapter, we will consider only those tests that detect antigens directly in clinical samples with results available within minutes to several hours after sample receipt. First, we will briefly review the principles and characteristics of major techniques, and then we will discuss their application to detection of microorganisms and viruses in clinical specimens.

Principles of the Techniques

Agglutination

Agglutination methods use the antibody–antigen bond to create clumping (agglutination) of particles. Agglutination tests to detect antigens employ fixed red cells (hemagglutination), latex beads, gelatin, or synthetic microbeads coated with specific antibody as carrier or indicator particles. In a typical agglutination assay for detection of microbial antigen, a drop of liquid suspension of antibody-coated particles is placed on a card, and the specimen is added and mixed. The card is then incubated, often on an oscillating mixer, and read by visually observing the clumping reaction (Fig. 3.1). No washing is required. Agglutination assays can be made semiquantitative by performing serial dilutions of the specimen and reporting the greatest dilution that results in a positive reaction.

A major source of error in agglutination tests is the prozone reaction, which occurs when antigen is in excess. "Prozoning" is observed at high antigen concentrations where excess antigen occupies most antibody binding sites with unique antigen molecules, thus preventing the multiple antibody-binding of each antigen that causes the particles to clump (Fig. 3.1). These false-negative reactions can be detected by repeating the test at a higher dilution of sample, which reduces the antigen concentration into the range that produces agglutination.

Compared with other methods, agglutination tests tend to be very rapid and require minimal training and equipment. However, test sensitivity is usually less than for enzyme immunoassay (EIA) or fluorescent techniques, as a greater quantity of

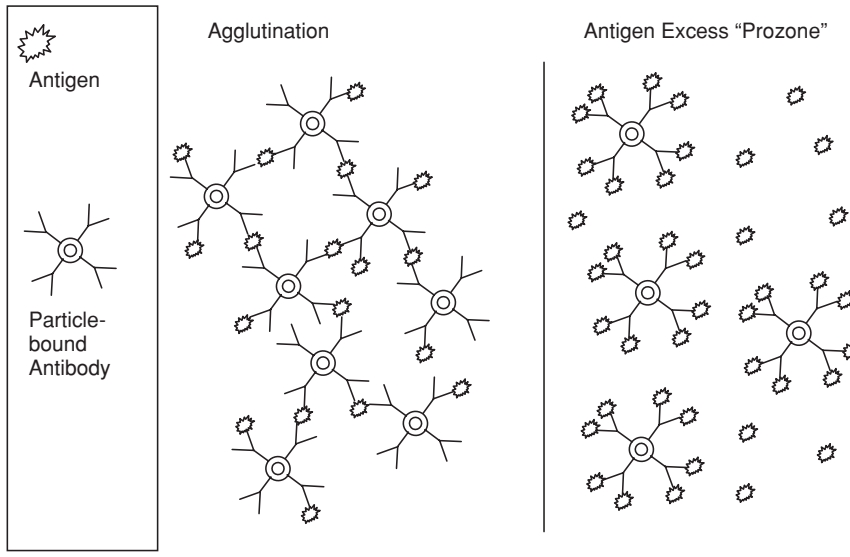

FIGURE 3.1. Particle agglutination and prozoning.

antigen is required to produce visible agglutination. Factors that limit the specificity of agglutination methods include heterophile and rheumatoid factor antibodies, which may cause agglutination in the absence of specific antigen; mucus and other substances, which may agglutinate particles nonspecifically; and lipemia and other opaque materials, which interfere with interpretation.

Immunofluorescence

Immunofluorescence (IF) is a microscopic technique that uses specific antibodies labeled with fluorochromes to detect, localize, or quanitify microorganisms (or proteins expressed in virus-infected cells) in samples applied to slides. A variety of fluorochromes are available, but the most commonly used are fluorescein and rhodamine. Several fluorochromes can be used simultaneously to detect more than one organism. Fluorochromes are excited by UV light, and in returning to their resting state, they emit photons at a specific wavelength. Visualization requires a microscope with a dark-field condenser and filters for each fluorochrome that allow only the emitted fluorescent light to be seen. In the direct method, the primary antibody is labeled with the fluorochrome (Fig. 3.2). In the indirect method, the specific antibody is unlabeled, but a second anti-species antibody that reacts with the antigen–antibody complex is labeled and allows detection. The direct technique is shorter and simpler, whereas the indirect method is cheaper and more sensitive.

Prior to IF, clinical specimens may be washed to remove material that can itself fluoresce or trap stain. After application to a glass slide, the sample is fixed by heat, cold acetone, or occasionally formalin. The sample affixed to the slide is allowed to react with specific antibodies then washed to remove nonreacting materials. Mounting oil and a coverslip are applied. Time to result after fixation is less than 1 h for direct and about 2 h for the indirect method.

IF requires an expensive fluorescence microscope, which must be well-maintained, kept in a dark room, and the bulb life monitored as intensity declines with use. IF allows microscopic visualization of sample quality and thus the

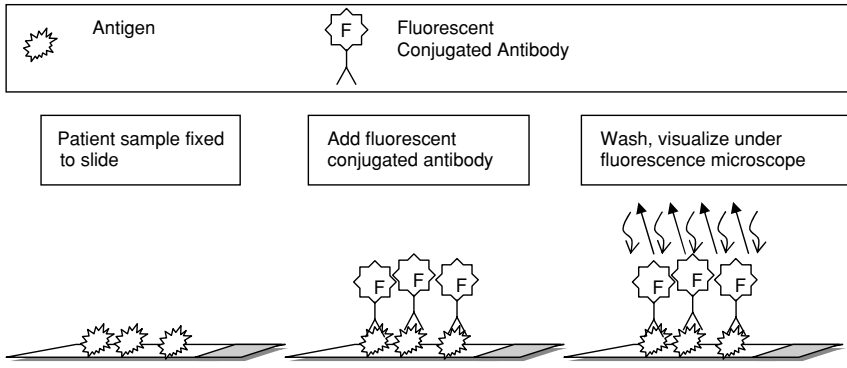

FIGURE 3.2. Direct immunofluorescence.

opportunity to recollect inadequate samples. IF also allows the detection of only 1 or 2 infected cells or few microorganisms, making it potentially more sensitive than other immunoassays. However, significant training and judgment are required to ensure good-quality preparations and accurate interpretation. Performance characteristics for IF must be established by each laboratory, for each reader, and for each analyte. Slides can be saved at 4°C for weeks for quality-control purposes and correlation with culture results.

Enzyme Immunoassay

Enzyme immunoassay (EIA) is the generic term for a large number of methods that link an antigen–antibody reaction to an enzymatic reaction to produce a colorimetric, fluorimetric, or chemiluminescent readout. A variety of enzymes may be used, but the most common are alkaline phosphatase and horseradish peroxidase. EIA methods are used in formats that range from self-contained kits sold for home use to methods that run on high-throughput, random-access laboratory instruments. Typical assay times are 2–3 h, though self-contained membrane EIAs and competitive EIAs can be significantly faster. EIAs thus allow manufacturers to offer tests in a wide variety of formats to suit different clinical applications.

More automated EIA methods such as fluorescent particle immunoassay (FPIA) and chemiluminescent immunoassays tend to be used for higher volume testing such as drug and hormone assays. Testing for some microbial antigens, such as hepatitis B surface Ag (HBsAg), is sufficiently high volume to merit these automated formats.

Enzyme-linked immunosorbent assay (ELISA) is a specific category of EIA in which one of the antibodies is "adsorbed" or bound to a solid phase (immunosorbent). ELISAs typically are implemented in a microwell, tube, or bead format (Fig. 3.3). The label can be carried on a single labeled antibody or a sandwich of an antigen-specific antibody and a label. In the latter case, the label is borne

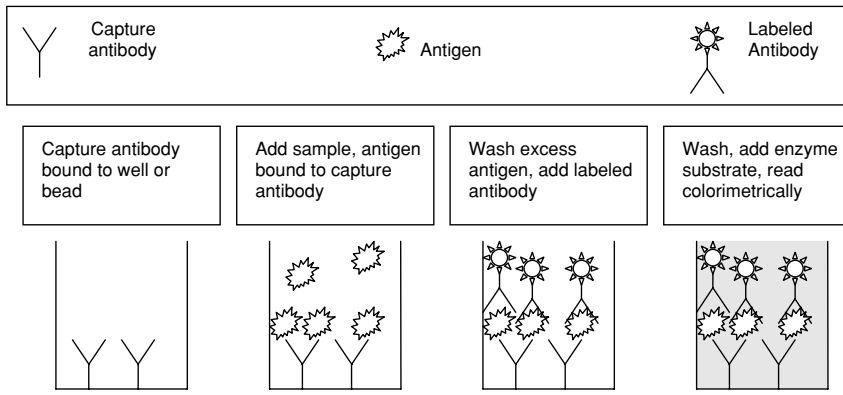

FIGURE 3.3. Antigen capture ELISA.

either on a second anti-species antibody that reacts with the antigen–antibody complex or on an antibody-binding protein such as staphylococcal protein A. Another strategy uses biotin-labeled antibody and streptavidin–horseradish peroxidase conjugate. The sandwich-type methods increase sensitivity but may increase time and cost. Various steps of the process can be automated by plate washers and readers and by more comprehensive automated ELISA systems.

Competitive ELISAs may be set up with either antibody or antigen on the solid phase. Labeled antigen is added either simultaneously with, or after the patient specimen is reacted with the first antibody. The signal generated is inversely proportional to the amount of antigen in the specimen. In comparison with direct or noncompetitive formats, competitive ELISAs tend to be more rapid and specific, but less sensitive.

In qualitative antigen detection, a quantitative cutoff divides positive from negative results. The precise value of the cutoff, which is usually expressed as a signal relative to that generated by a negative control sample, depends on the method and the desired mix of sensitivity and specificity needed for clinical purposes; lower cutoffs provide more sensitivity but less specificity. Receiver–operator curve (ROC) analysis may be used to optimize the cutoff; ROC curves demonstrate the relationship between sensitivity and specificity as cutoff values vary and allow assessment of the effect of changing cutoff values on test performance.

Significant interferences in EIA testing arise from "hook effects," heterophile antibodies in blood, and nonspecific binding of specimen constituents producing high backgrounds. Hook effects arise when extremely high quantities of antigen are present; however, the mechanism of interference with EIAs is not as clearly defined as with agglutination. Heterophile antibodies can produce either negative or positive interference, depending on the details of the assay construction. Nonspecific binding of specimen constituents is particularly troublesome in respiratory specimens, where mucoid specimens may be associated with false-positives.

Advantages of EIAs include ability to run large numbers of samples with minimal hands-on time, modest personnel training requirements, ability to automate, and objective end-points. Disadvantages include inability to assess specimen quality, the need to set sometimes arbitrary cutoffs, hook effects, interfering substances, including rheumatoid factors and heterophile antibodies, and the need for careful and thorough washing to avoid false-positive results.

Chemiluminescent Methods

Chemiluminescence is the emission of light that occurs when a substrate decays to a ground state from an excited state produced by a chemical reaction, most often an oxidation. The emission is read with a luminometer or may be captured on photographic film. Chemiluminescence is the most sensitive reporter system for immunoassays, because light emission can be detected at very low levels, and there are few naturally occurring molecules that emit light under the conditions used for chemiluminescence, leading to very low backgrounds. Chemiluminescent readouts can employ either a chemiluminescent readout from an enzyme assay or a

directly chemiluminescent labeled antibody. The most common chemiluminescent compounds are acridinium esters and derivatives of isoluminol, both of which are excited by sodium hydroxide and hydrogen peroxide. In addition, 1,2-dioxetane molecules are used as substrates for alkaline phosphatase in many commercial immunoassays. Finally, electrochemiluminescent detection of ruthenium-labeled antibodies has been employed in systems for the detection of biological weapons agents in environmental samples.

Other Rapid Formats (Immunogold, Lateral Flow Immunoassay, Immunochromatography, Optical Immunoassay, Endogenous Viral-Encoded Enzyme Assay)

Membrane EIAs usually involve a series of steps: addition of sample, wash step, addition of conjugate, wash step, addition of substrate, and then stop reagent. The result is read as a colored spot or triangle on a solid surface. By substituting an IgG binding dye (e.g., staphylococcal protein A–gold reagent) for the anti-immunoglobulin conjugate, the procedure can be shortened by one step. Like membrane EIAs, most of these tests include a built-in control; if the test differentiates two different agents (e.g., influenza A and B), two controls are included.

Immunochromatographic or lateral flow assays require the addition of only one or no reagent and thus are extremely simple to perform. These tests use antibodies spotted onto nitrocellulose membranes with lateral or vertical flow of sample or reagents to interact with immobilized antibody (Fig. 3.4). Use of an antibody sandwich increases sensitivity. Specific antibody is adsorbed onto a nitrocellulose membrane in the sample line, and a control antibody is adsorbed onto the same membrane as second line. Both antibodies are conjugated to visualizing particles that are dried onto an inert fibrous support. Conjugate pad and striped membrane are combined to construct the test strip. An extracted sample is added at one end

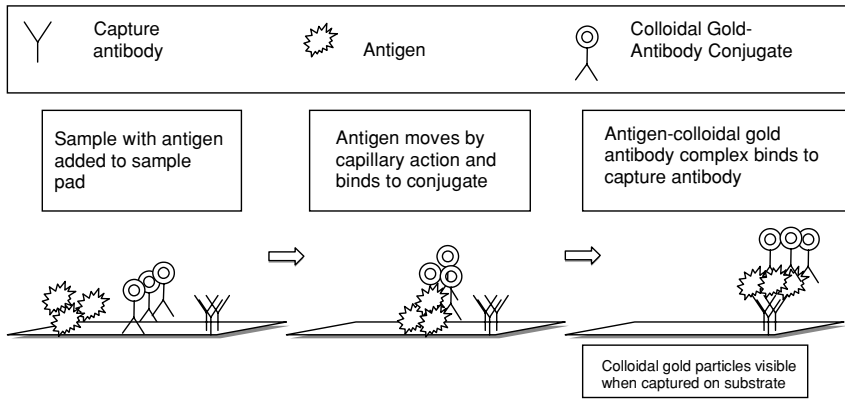

FIGURE 3.4. Lateral flow immunochromatography.

and moves along the membrane by capillary action to reach the immobilized antibody stripes. Alternatively, a test strip can be inserted vertically into a tube containing the extracted sample.

Optical immunoassays allow direct visualization of a physical change in the thickness of molecular thin films (Boivin et al., 2001). The observed physical change is due to antigen–antibody binding on an optical surface of a silicon wafer, on which specific antibodies have been immobilized. When an extracted specimen is placed directly on the optical surface, antigen is captured. After a wash step, substrate is added, and the thickness of the thin film increases. This change in thickness alters the reflected light path and is perceived as a color change.

One rapid test uses a chromogenic substrate that is uniquely recognized by the influenza virus–encoded neuraminidase (NA) enzyme (Hamilton et al., 2002). The unique substrate is coupled to a color-generating molecule, and in the presence of influenza NA, the coupled substrate is cleaved, and a colored product is produced and precipitates. This strategy bypasses the need for the antibody-capture step and wash procedures of other antigen tests.

Disadvantages of rapid membrane assays in general include subjective interpretation, lack of automation, and possible errors if the reader is color-blind. Although simple to perform, lack of attention to technique can lead to errors. Samples must disperse within specified time limits, and pipettes must be held vertically for correct delivery of reagent volumes. Accurate timing of steps can be adversely affected when multiple samples are tested. These formats are useful primarily for small-volume testing. Conventional EIA and similar methods scale up to larger sample volumes more efficiently.

Characteristics of the Techniques

The characteristics of the techniques are presented in Table 3.1, stratified by training requirements. Immunofluorescence requires the most intensive training and quality control, from preparation of slides to staining and reading. When done well, the benefits of IF, especially to viral diagnosis, are significant. However, some staff members may lack the expertise, judgment, and attention to detail that is required to produce consistent, sensitive, and specific results. EIA methods are widely used for many analytes, high-quality commercial kits are available, and automation is common. Implementation requires attention to detail and accurate pipetting, but these skills should be standard in clinical laboratories. Rapid membrane and agglutination assays, though generally simple, vary in number of steps. The newer methods may require no wash steps or reagent additions; however, sensitivity and specificity may suffer somewhat.

Each laboratory needs to evaluate these methods and establish performance characteristics in their own settings and patient populations. Decisions on which tests to employ should take into account clinical needs, test volumes, time to result, cost of materials and labor, equipment required, and staff expertise.

TABLE 3.1. Characteristics of the techniques.

Method	Time to result	Equipment	Training	Advantages	Limitations
Immunofluorescence	1–4 h	Cytospin, centrifuge, fume hood, fluorescence microscope	Extensive	Can assess sample quality; can detect 1–2 infected cells; can multiplex detection of multiple viruses; can quantitate infected cells.	Need adequate target cells, expert slide preparation and interpretation; subjective; performance must be established in each laboratory.
Microwell, tube, or bead ELISA	1 h 15 min to 2 h	Spectrophotometer, pipettors	Moderate	Most suited to high-volume testing; can be automated.	Interference due to hook effects, heterophile antibodies, and nonspecific binding.
Agglutination	15 min	Vortex (optional), oscillating mixer (optional)	Minimal	Very rapid, simple, no wash steps.	Prozone reaction, subjective; may be less sensitive.
Membrane and other rapid EIA	15–30 min	Pipettors or none	Minimal	Rapid, simple, can be used at POC.	Subjective interpretation, lack of automation, possible errors if the reader is color-blind; samples must disperse within specified time limits; inaccurate timing of steps when testing multiple samples.
Other rapid formats[a]	15–20 min	None	Minimal	Rapid, very simple, some have no reagent additions or wash steps; can be used at POC.	Similar to rapid EIA; may be less sensitive and specific.
Endogenous viral enzyme assay (EVEA)	30 min	Heating block	Minimal	Rapid, simple, can be used at POC.	Similar to rapid EIA; may be less sensitive and specific.

ELISA, enzyme linked immunosorbent assay; EIA, enzyme immunoassay; POC, point of care.
[a] immunogold, lateral flow immunoassay, immunochromatography, optical immunoassay.

Applications of the Techniques

A summary of the applications of antigen techniques to specific pathogens is given in Table 3.2, and common uses are discussed below.

Bacteria

Rapid antigen testing is routine for diagnosis of group A streptococcal pharyngitis. Although rapid antigen tests offer less than 100% sensitivity, their wide availability at the point of care (POC) allows practitioners to diagnose and treat this common childhood illness in a single office visit in most cases, reserving culture for antigen-negative patients (Bisno et al., 2002).

The value of detection of *Streptococcus pneumoniae* antigen in urine for the diagnosis of pneumonia is limited by the positive results obtained in patients with mere oropharyngeal colonization, occurring especially in children, and by sensitivities of only 50–85%. The role of this test in management of patients with community-acquired pneumonia is still evolving (Smith et al., 2003; Roson et al., 2004).

Antigen detection in urine is a major diagnostic procedure for *Legionella* infections. Although available tests detect only 80–90% of the serotypes associated with human disease, the method is sensitive and specific for those serotypes and is much more rapid than culture. Urinary antigen can remain positive for days to weeks after therapy is begun and thus can be performed on treated patients. Direct fluorescent antibody (DFA) testing of respiratory specimens for *Legionella* is insensitive, even relative to culture, and requires a skilled reader to limit false-positives. Monoclonal reagents are more specific than polyclonal reagents, but both have been described to cross-react with non-*Legionella* species. The true sensitivity and specificity of antigen detection in *Legionella* infections is difficult to determine, because culture itself is insensitive, and molecular methods are still in development (Waterer et al., 2001).

For diagnosis of enterocolitis due to *Clostridium difficile* toxins, there is no gold standard. Rather, a variety of diagnostic techniques are employed, including toxigenic culture, tissue culture cytotoxicity with antibody neutralization, and both rapid and conventional toxin EIAs. The various EIA methods are the most widely employed because of their modest technical requirements and rapid time-to-result; newer tests that detect both toxin A and toxin B are more sensitive than methods that detect only toxin A. Older latex agglutination and membrane EIA tests that detect *C. difficile* glutamate dehydrogenase (a.k.a. "common antigen") do not distinguish between toxigenic and nontoxigenic strains and lack specificity but may be used as screening tests to select specimens for further, definitive testing (Wilkins and Lyerly, 2003).

Antigen testing of stool for *Helicobacter pylori* has recently become an option to the urea breath test and serology. It may be particularly useful in children, where the urea breath test may be difficult to perform, and in patients in whom serologic testing is likely to be problematic, such as steroid-treated or HIV-infected patients (Versalovic, 2003).

TABLE 3.2. Application of techniques to detection of specific pathogens.

Pathogen	Methods	Specimen	Sensitivity	Specificity	Comments
Bacteria					
Streptococcus group A	Agglutination, rapid EIA, OIA	Throat swab	70–90+%	>95%	Often performed at POC. Negatives must be evaluated by culture.
Streptococcus pneumoniae	Rapid EIA	Urine	50–85%	94%	Clinical role still evolving. Provides adjunct, but not definitive, diagnostic information in patients at risk for *S. pneumoniae* disease.
Legionella spp.	IF	Respiratory	25–75%	90%+	Requires FA microscope. Cross-reactions with some other bacteria, especially with polyclonal reagents. No gold standard for comparison.
	EIA or IC	Urine	80–99%	99%	Test characteristics well-established only for *L. pneumophilia* group 1.
Clostridium difficile	Agglutination, rapid EIA, ELISA, OIA	Stool	65–100%	88–100%	Measured sensitivity and specificity are relative to tissue culture cytotoxicity. Tests detecting Toxin A + B are more sensitive than those detecting Toxin A only.
Helicobacter pylori	ELISA	Stool	89%	90–94%	Used as an alternative to serology and urea breath testing.
Chlamydia trachomatis	ELISA	Genital	60–70%	97%	Being phased out, but POC versions might be valuable if sensitivity improves. No single-test format available. Not useful for screening low-prevalence populations due to poor specificity.
Meningitis panel (*H. influenzae*, *N. meningitidis*, *S. pneumoniae*, group B *Streptococcus*)	Agglutination	CSF, urine			Inadequate sensitivity/specificity for routine clinical use. Empirical therapy given for CSF neutrophilia covers these pathogens, until culture results available. Positive predictive value of antigen tests is very low in patients without CSF leukocytosis.

Fungi					
Cryptococcus	Agglutination, ELISA	CSF, serum	99%+	Very high if heat or pronase pretreatment used	Sensitivity may exceed culture. Cross-reactivity with (very rare) systemic *Trichosporon* infections. Prozone is a problem in high-level infections.
Pneumocystis jiroveci (formerly *P. carinii*)	IF	Respiratory	Variable	High	Requires fluorescence microscope. Sensitivity is highest for antibodies that detect antigens present in trophozoites and cysts. No significant sensitivity or specificity advantages over conventional and Calcifluor white stains.
Parasites					
Giardia	IF, ELISA, rapid EIA	Stool	Higher than microscopy	100%	No gold standard available for comparison. Specimen treatment (e.g., fixed, unfixed, or frozen) varies with different tests.
Cryptosporidium	IF, ELISA, rapid EIA	Stool	Higher than microscopy	93–100%	No gold standard available for comparison. Some kits detect both *Giardia* and *Cryptosporidium*.
Entamoeba histolytica/dispar group	ELISA	Stool	Higher than microscopy	>95%	Not widely used. Reagents are available to distinguish between *E. histolytica* and *E. dispar*.
Trichomonas vaginalis	IF, LA	Genital	85%	High	Alternatives include wet prep (60% sensitivity relative to culture), culture, molecular detection. Wet prep is limited by specimen stability.
Plasmodium falciparum	Rapid EIA and other rapid formats	Blood	Similar to microscopy	High	Three dipstick-format rapid tests available. Cost limits use in endemic areas.
Lymphatic filariases	ELISA, rapid EIA	Blood	Equivalent to microscopy; similar to or higher than concentration methods	>95%	No gold standard. Cost limits use in endemic areas.

(*continued*)

TABLE 3.2 (*Continued*)

Pathogen	Methods	Specimen	Sensitivity	Specificity	Comments
Viruses					
Respiratory syncytial	ELISA, IC	NP swab or aspirate, BAL, sputum	80–95%	97–99%	Very sensitive in young infants who shed high titers of virus. Mucoid samples may not disperse properly and may give rise to erroneous results.
	IF	NP swab or aspirate, BAL, sputum	90–100%	>99%	More sensitive than culture or other antigen tests. Can be multiplexed with other antibodies. IF allows assessment of sample quality.
Influenza A and B	ELISA, rapid EIA, lateral flow IA, IC, OIA, EVEA	NP swab or aspirate, BAL, sputum, throat swab	50–90%	95–99%	Sensitivity higher in children and with NP aspirates and washes. Some kits require use of special swab. Many tests do not differentiate between influenza A and B. Some new rapid tests less specific than older methods. Simpler rapid tests suitable for POC. Mucoid samples may not disperse properly and may give rise to erroneous results.
	IF	NP swab, NP aspirate, nasal wash, BAL	85–98%	95–99%	Performance must be established in each laboratory. Can be more sensitive than other rapid tests. Cytospin preparation of slides improves results. Use of pooled antibodies can be used to screen a single cell spot for multiple respiratory viruses. IF allows assessment of sample quality.
Parainfluenza	IF	NP swab or aspirate, BAL, sputum	80–95%	95–99%	Only rapid method available. Cytospin preparation of slides improves results. Antibodies to types 1, 2, 3, but not type 4, are included in commercial antibody pool.
Adenovirus	IF	NP swab or aspirate, BAL, sputum	50–70%	99%	IF for adenovirus not as sensitive as for other respiratory viruses. Cytospin preparation of slides improves results.

	EIA	Stool	90%	99%	Test available for detection of all adenovirus types in culture fluids or stools; does not differentiate among types.
Adenovirus, enteric types 40,41	EIA, agglutination	Stool	98%	99%	Test available to detect only enteric types 40 and 41. Ad40 and Ad41 do not grow in routine cell cultures.
Rotavirus	EIA, agglutination, immunogold	Stool	90–98%	90–98%	Rotavirus does not grow in routine cell cultures, so rapid tests are compared with EM. Rotavirus shed in high titers in stools of infants and young children. Titers decline after day 8.
Astrovius	EIA	Stool	97%	99%	Rapid tests compared to EM.
Norovirus	EIA	Stool	85–96%	94–99%	Limited by antigenic variation; rapid onset and resolution of illness.
Herpes simplex	IF	Skin lesions, genital lesions, oral lesions, BAL, brain tissue	80–95%	>99%	Sensitivity enhanced by cytospin preparation of slides. Sensitivity is higher for skin lesions than for mucosal lesions. HSV and VZV antibodies labeled with different fluorochromes can be used to test for both viruses in a single cell spot.
	EIA	Skin or genital lesions	35–95%	99%	EIA available in reference laboratories. Most sensitive for fresh vesicular skin lesions. Amenable to automation.
Varicella zoster	IF	Skin lesions, BAL	>99%	>99%	IF for VZV in skin lesions is more sensitive than culture. VZV and HSV antibodies can be pooled for dual detection using two fluorochromes.
Cytomegalovirus	IF, IP	Blood leukocytes	90–97%	>99%	Quantitative detection of CMV pp65 antigenemia is very useful in rapid diagnosis and in monitoring therapy. More sensitive than culture and equivalent to PCR in plasma.

(*continued*)

TABLE 3.2 (*Continued*)

Pathogen	Methods	Specimen	Sensitivity	Specificity	Comments
Human immunodeficiency virus	EIA	Blood	50–99%	95–99%	Once antibody appears in blood, sensitivity of antigen detection decreases. Immune complex dissociation and signal amplification boost sensitivity. Rheumatoid factor can cause false-positive results. Neutralization test needed to confirm specificity of result.
Human immunodeficiency virus	Real-time immuno-PCR	Blood	<50 to 6000 viral copies/mL plasma	N/A	Detects ultralow level of protein. Combines traditional ELISA with PCR. Much less expensive than current molecular tests.
Hepatitis B surface antigen (HBsAg) and e antigen (HBeAg)	EIA	Blood	99%	>99%	Free HBsAg is produced in 100- to 1000-fold excess over complete virus particles. Thus HBsAg is generally more sensitive than DNA techniques. HBeAg has been the standard marker for high levels of viral replication.

POC, point of care; ELISA, enzyme linked immunosorbent assay; EIA, enzyme immunoassay; IF, immunofluorescence; PCR; polymerase chain reaction.
Other rapid formats: IA, immunoassay; IC, immunochromatography; OIA, optical immunoassay; EVEA, endogenous viral encoded enzyme assay. LA, latex agglutination; NP, nasopharyngeal; BAL, bronchoalveolar lavage; EM, electron microscopy.

Antigen testing for genital *Chlamydia* infections has been almost entirely replaced by nucleic acid testing, which is substantially more sensitive and specific. Rapid tests have the potential for POC use, but none is yet FDA approved (Mahony et al., 2003).

Bacterial antigen testing for meningitis is rapid but has fallen out of use in recent years due to inadequate sensitivity and specificity and the use of empirical antibiotic therapy. The presence of neutrophils in cerebrospinal fluid (CSF) generally leads to therapy in patients with compatible syndromes, whereas the positive predictive value of antigen testing performed on patients with acellular CSF is dismal. Empirical antibiotic choices cover the organisms detected by the antigen tests (Kiska et al., 1995; Thompson et al., 2003).

Fungi

For *Cryptococcus*, antigen testing is the mainstay of diagnosis. The sensitivity in cryptococcal meningitis approaches that of culture while providing more rapid diagnosis (Perfect and Casadevall, 2002).

By contrast, IF staining of respiratory specimens for *Pneumocystis jiroveci* is one of several techniques of similar sensitivity for detection of *Pneumocystis* pneumonia. The choice of IF, conventional stain, or Calcifluor white depends on the laboratory. IF and Calcifluor require fluorescent microscopes, and IF reagents are expensive. Calcifluor and conventional staining methods require the reader to discriminate between *Pneumocystis*, yeasts, other pathogens, and cellular structures morphologically, which requires more interpretive skill than IF staining (Cruciani et al., 2002; Thomas et al., 2004).

Parasites

For infections by *Giardia* and *Cryptosporidium*, antigen testing has become the method of choice, with sensitivities that exceed that of routine microscopic exam (Garcia, 2001). Cost-saving strategies using pooled specimens screened with antigen detection have been described. Many different formats are available, and laboratories select a method based on technical (e.g., availability of fluorescence microscope, test format) and operational (e.g., specimen requirements, test volume) differences (CDC, 2004).

EIA methods are also available for *Entamoeba histolytica*. *E. histolytica* is morphologically indistinguishable from a nonpathogenic relative, *Entamoeba dispar*. Several tests are available, but the EIA from Techlab (*E. histolytica* II) is comparatively specific for pathogenic *E. histolytica* and is useful for distinguishing it from *E. dispar*. Because *E. histolytica* is comparatively rare in the United States, antigen tests are not as widely used as for *Giardia* and *Cryptosporidium*.

Because *Trichomonas* rapidly loses motility below body temperature, the wet prep has always been an insensitive approach to diagnosis, particularly if specimens need to be transported prior to viewing. Commercially available DFA and latex agglutination methods provide better sensitivity.

Rapid diagnosis of malaria by antigen detection is a promising approach to field diagnosis. The proliferation of chloroquine-resistant strains and the expense of newer antimalarial drugs may make these tests economical in endemic regions. Tests are available for *Plasmodium falciparum* and *Plasmodium vivax*.

Rapid antigen tests have also been evaluated for *Wuchereria bancrofti* infections. They appear to be more sensitive than direct microscopy and approach or exceed the sensitivity of concentration techniques in some studies (Chandrasena et al., 2002).

Viruses

Hospitals that serve infants and children have long provided rapid antigen testing for respiratory syncytial virus (RSV) and rotavirus (Wilhelmi et al., 2001; Slinger et al., 2004). Recently, testing for influenza using membrane EIA or other rapid formats has increased in clinics, emergency departments, and hospitals in high-risk adults and in pediatric patients. Having a test result available within 15 to 30 min has been shown to reduce use of antibiotics and other tests and to allow administration of antiviral agents when needed (Barenfanger et al., 2000; Bonner et al., 2003). Antigen tests are the only methods that can currently provide such rapid results, and the number of commercial rapid influenza tests has increased dramatically (Storch, 2003). Some detect only influenza A, whereas others detect both A and B but may not differentiate between them. Although less sensitive than cell culture or IF, these tests perform very well in young children because children shed high titers of virus. There is concern however that the simpler and more rapid tests are less specific, and false positives have been reported.

IF is slower than other rapid antigen tests but has advantages of a broader array of analytes available, ability to multiplex and ability to quantitate infected cells. Because IF is commonly done in virology laboratories for identification of culture isolates, the equipment and expertise are usually available. Obtaining excellent results using IF directly on clinical samples, however, requires a significant commitment to training, monitoring, and quality control. Performance characteristics must be established in each laboratory, usually by comparing IF results with culture results. Without careful attention to detail and extensive training, nonspecific staining can be misinterpreted as positive, or small numbers of positive cells can be overlooked. Use of cytocentrifugation to prepare slides enhances slide quality and test sensitivity.

Respiratory virus screening by use of pooled antibodies can test for 7 viruses in a single cell spot (Landry and Ferguson, 2000b). Because the same symptoms can be caused by many viruses, this provides an advantage similar to culture. Likewise, antibodies to herpes simplex virus (HSV) and varicella-zoster virus (VZV) can be pooled to screen skin lesions (Scicchitano et al., 1999).

The use of IF to rapidly detect and quantitate cytomegalovirus (CMV) in peripheral blood revolutionized the diagnosis and monitoring of CMV infections, especially in transplant patients (Gerna et al., 1992). Even with the increasing use of polymerase chain reaction (PCR) to monitor viral load, CMV antigenemia

retains advantages for economical on-site testing. IF takes 1–2 h to complete, and it can be done repeatedly during laboratory hours (Landry and Ferguson, 2000a).

The lack of sensitivity of direct antigen detection compared with methods that amplify the target, such as culture and PCR, may be addressed by the recently reported method of real-time immuno-PCR, which amplifies signal, can detect ultralow levels of proteins, and is cheap and simple to perform (Barletta et al., 2004).

Finally, virology laboratories using antigen testing to provide results within 15 min to 2 h can also save resources by canceling cultures on most samples that have a positive rapid test result.

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4
Advanced Antibody Detection

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Introduction

In addition to basic microbiological methods, such as microscopy and culture, to detect pathologic organisms, antigen or antibody detection methods by immunoassay and nucleic acid detection by amplification technology have been developed, are commonly used, and will be expanded further for rapid and accurate diagnosis of the common or newly emerging infection-causing agents, such as viruses, in clinical as well as public health laboratories. Since the first competitive radioimmunoassay was developed more than 40 years ago for human insulin detection (Yalow and Berson, 1960), immunoassays have been developed with emphasis on fast and sensitive detection technologies and automated systems. Due to the demand of large-scale screening for epidemiology, blood bank, prenatal care, and diagnosis of HIV and hepatitis, more immunodiagnostic procedures are performed using instruments and reagents similar to traditional immunochemistry platforms, including tests for oncology, toxicology, cardiology, and endocrinology. Immunoassays for detection of host-produced antibodies directed against microorganisms, particularly viruses, is now one of the most widely used analytical techniques in laboratory medicine (Andreotti et al., 2003; Peruski and Peruski, 2003).

This chapter will review the antibody detection assays, limitations for detection and identification of infectious agents, and look into the application of those technologies with emphasis on development of detection methods such as chemiluminescent and multianalyte profile (xMAP), automation and their clinical application in the areas of diagnosing HIV, hepatitis, and other viral infections.

Principles and Characteristics of Techniques

Immunoassays for antibody (Ab) detection rely upon three important factors: (1) the specific antigen used to capture target antibody; (2) the target antibody if present, and the detector or secondary antibody used for indirect detection of antibody; and (3) the detection method. The first two factors are important for the

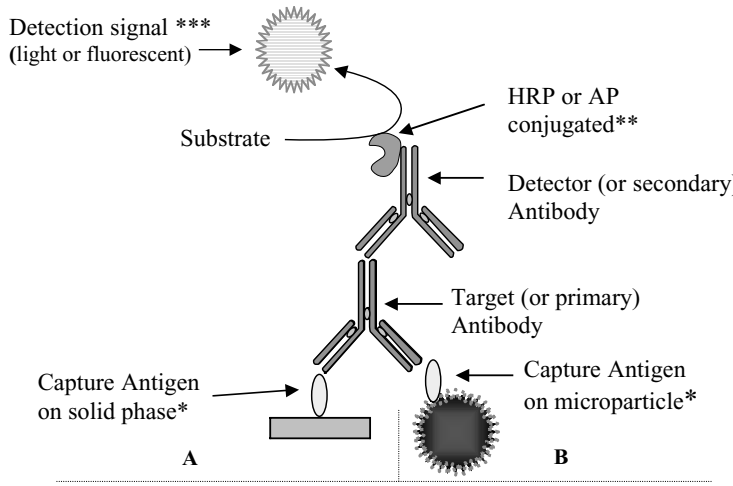

FIGURE 4.1. Model for EIA detection method. Model for target antibody detection in two typical indirect immunoassays. *Capture antigen is bound to solid phase such as microwell plate (A), or antigen can be labeled or bound to microparticle or microsphere in liquid phase (B). **Horseradish peroxidase (HRP) or alkaline phosphatase (AP) conjugated second antibody (usually anti-human IgG). ***Detection signal can be generated by colorimetric, chemiluminescent, or fluorescent methods.

efficiency of antigen–antibody complex formation and the third one concerns the ability to detect these complexes.

An ideal immunoassay to detect antibodies against infectious agents will have high sensitivity so to detect low concentrations of antibodies, as well as high specificity to avoid cross-recognition of antigenically related antigens and reduce the possibility of no false-positive results. In reality, a highly sensitive assay has a low chance of producing false-negative findings and is suitable for screening large numbers of samples. The specific antigens such as the killed or neutralized virus lysate, synthetic peptides, or recombinant proteins are usually developed in the research and development phase for specificity. For clinical testing, it is the detection methods that are most important for automation and throughput.

Immunoassays can be grouped according to the method of analysis, such as direct or indirect assays, or competitive inhibition assays. Because most direct immunoassays are used for antigen detection, and most indirect immunoassays can be used as competitive inhibition assays, in this chapter we will only cover the indirect immunoassays. The indirect immunoassay, the most commonly used type of immunoassay, is illustrated in Fig. 4.1. In brief, the capture antigen used can be either bound on solid phase (Fig. 4.1A) or microparticle in liquid phase (Fig. 4.1B). The target antibody that needs to be detected shall bind to specific antigen. The detector or so-called secondary antibody is conjugated for signal detection. The signal detection system, such as conjugate, substrate, and detection methods such as color or fluorescent, is critical in the immunoassay. The immunoassays can be

grouped into several categories according to the type of detection systems used (Table 4.1): (1) colorimetric, (2) radiometric, (3) chemiluminescent, or (4) fluorescent (Engvall and Perlmann, 1971, 1972; Kricka, 1991; Peruski and Peruski, 2003).

Enzymes are effective labels because they catalyze chemical reactions, which can produce a signal. Because a single enzyme molecule can catalyze many chemical reactions without being consumed in the reaction, these labels are effective at amplifying assay signals. Most enzyme–substrate reactions used for immunoassays use chromogenic, chemiluminescent, or fluorescent substrates that produce a signal detectable with the naked eye, a spectrophotometer, luminometer, or fluorometer (Table 4.1).

Colorimetric or Chromogenic Susbstrate

The colorimetric method involves a substrate color change that can be detected by the naked eye or by optical density using a specific wavelength of light detected by a spectrophotometer.

Latex agglutination is a photometric immunoassay that is used more in antigen detection than in antibody detection and thus is not covered in this chapter.

Enzyme-Linked Immunosorbent Assay (ELISA)

ELISA is an indirect or colorimetric enzyme immunoassay (EIA). Solid-phase enzyme-coupled reagent assays were developed 30 years ago (Engvall and Perlmann, 1971). In principle of indirect ELISA (Fig. 4.1), antibody, if present in test sample, forms immune complex first with the capture antigen affixed to the solid phase (plastic microwell plate or tube). The primary or target antibodies in serum sample can bind to the target or capture antigens immobilized on plate wells by using enzyme-linked detector (or secondary, conjugate) antibodies, such as goat, mouse, or rabbit anti-human IgG antibodies. Secondary antibody labeled by chemical conjugation of an enzyme binds the immune complex. The enzyme "fixed" on the solid phase through immune complex interacts with the substrate, then catalyzes a chemical reaction, and yields a colored product. The colored product can then be visualized and measured by optical density measured by a spectrophotometer. The intensity of substrate color change is proportional to the amount of enzyme-linked secondary antibodies present in the sample wells, which is proportional to the amount of primary antibodies in the sample that are bound to the immobilized antigen.

Use of indirect ELISA can reduce or eliminate the nonspecific Ab binding and interfering serum factors (e.g., rheumatoid factors), thus providing low background and high sensitivity and specificity. Some indirect ELISA use avidin–biotin complexes between antibodies and antigens to increase assay sensitivities. The most commonly used enzymes for EIA are alkaline phosphatase (AP) and horseradish peroxidase (HRP). These are effective detection enzymes because of their stability, turnover number, and lack of interferences. HRP is a relatively small enzyme with a high turnover and is derived from nonmammalian sources. When used with a

TABLE 4.1. Types of antibody detection methods.

Method	Capture antigen (Ag)	Sample target antibody	Lable conjugated on detector (secondary) antibody	Detection method
Colorimetric or chromogenic			Usually goat, mouse, or rabbit anti-human IgG	
Latex agglutination	Ag on bead suspension	Serum		Visible agglutination
ELISA	Ag on solid phase or microparticle	Serum	Enzyme (HRP or AP[a])	Visible color, optic density (OD) by spetrophotometer
Immunoblotting	Ag on nitrocellulose membrane	Serum	Enzyme (HRP or AP[a])	Visible band
Lateral flow diffusion (handheld)	Ag colloidal gold-labeled on nitrocellulose or nylon membrane	Serum, blood, oral fluid	Colloidal gold (chromatographic lateral flow)	Visible line
Radioimmunoassay (RIA)	Ag on bead (or radiolabeled)	Serum	radiolabeled (^{125}I, ^{14}C, ^{3}H)	Radioactivity by gamma counter
Chemiluminescence (CLIA)	Ag on solid phase or microparticle	Serum	Luminol (dioxetane through HRP or AP[a]) or acridinium ester	Photon output or light by luminometer
Enhanced CLIA				
Electrochemiluminescence (ECL)	Ag on magnetic beads	Serum	Chelate ruthenium (Ru) as electron carrier (TPA as substrate)	Photon output by flow cell with photon detector
Fluorescence Indirect fluorescence (IFA)	Ag bound on slide or microparticle	Serum	Fluorescein isothiocyanate (FITC) conjugated	Fluorescence by microscope under UV light or fluoromter
Time-resolved fluoroescence (TRF)	Ag bound on microparticle	Serum	Fluorescein biotinylated with lanthanide chelate (europium) in low pH	Fluorescence by fluorometer
Flow cytometry (FC) Multianalyte profile (xMAP)	Ag-coated dyed microsphere	Serum	Fluorescein (through biotin-Ab to streptavidin)	Fluorescence cell scanner (flow cytometer), or with FlowMetrix (Luminex)

[a] Horseradish peroxidase (HRP) or alkaline phosphatase (AP).

variety of substrates such as 2,2′ azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) or ABTS, HRP generates large signals from the production of the colored products (a deep-green color) in the presence of hydrogen peroxide, which can be seen without a spectrophotometer. The amount of color generated is then measured after a fixed incubation time at a specific wavelength such as 405 nm. The optical density obtained is then related back to the concentration of the antigen in the sample.

Immunoblotting

Immunoblotting which includes Western blot, is another technique for antibody detection. Capture antigens such as proteins are electrotransferred to a nitrocellulose membrane. If target antibodies are present in the specimen, they will bind to the antigens present on the nitrocellulose strips. Visualization of the antibodies bound to antigen is accomplished using a series of reactions with goat anti-human IgG conjugated with biotin, avidin conjugated with HRP, and the HRP substrate. The bands corresponding to the antigens will be seen on the nitrocellulose strip.

Lateral Flow Diffusion (Handheld, Portable Device) Method

Lateral flow diffusion (handheld, portable device) method has been designed more for the antigen-specific immunoassay than for antibody detection. It uses colloidal gold, carbon, paramagnetic, or color latex beads to create a visible line in the capture zone where there is a nitrocellulose or nylon membrane. Labeled capture antigen–antibody complex migrates by capillary action.

Immunochromatographic lateral flow assay can be used for antibody detection. Typical handheld assay devices contain a colloidal gold (or other)-labeled antigen dried onto a filter pad affixed to a nitrocellulose strip. A capture antibody is applied in a line.

Lateral flow assays have been available on the commercial market since the assey was developed for drug and pregnancy testing 20 years ago (Zuk et al., 1985). Also known as “handheld” assays, they are simple to use, require minimal training, and require no special storage conditions. In most cases, the manufacturer provides simple instructions that include pictures of positive and negative results. The assays are typically designed on nitrocellulose or nylon membranes contained within a plastic or cardboard housing. In the antibody detection format, a capture antigen is bound to the membrane, and a second labeled antibody is placed on a sample application pad. As the sample migrates down the membrane by capillary action, antibody present in the sample binds to the labeled antigen and is captured as the complex passes. Colloidal gold, carbon, paramagnetic, or colored latex beads are commonly used particles that create a visible line in the capture zone of the assay membrane for a positive result.

Radioimmunoassay (*RIA*)

RIA uses radiolabels for measurement of antigen-binding antibody in a fluid phase. Antibody in a test serum binds radiolabeled antigen to form antigen–antibody

complex in liquid phase. Subsequent protein A–Sepharose or protein G–Sepharose beads bind Ag–Ab complexes. RIAs use ^{125}I, ^{14}C, or ^{3}H labeled antigens as so-called tracers. Radioactivity can be measured by collecting beads after centrifugation and by gamma counter. In a direct immunoassay, detector (secondary) antibody is radiolabeled.

Chemiluminescence Immunoassay (CLIA)

CLIA and enhanced chemiluminescence represent the chemical generation of visible light by a reaction and as such do not use any light source and can be measured by a luminometer (Kricka, 1991, 1996). Thus the need for optical wavelength filtering systems is eliminated. Chemiluminescent systems fall into three classes: (1) indirect CLIA, (2) direct CLIA, and (3) enhanced CLIA.

Indirect CLIA uses an enzyme as the label. The enzymes are used to produce the chemiluminescent signal. Typically either HRP or AP is used and the amount present is determined by the addition of substrates that under the influence of the enzyme system give rise to visible emission. One example is 1·2-dioxetane compound which converts to a metastable intermediate by alkaline phosphatase and emits "glow" light. The chemiluminescent substrate, a phosphate ester of adamantyl dioxetane, undergoes hydrolysis in the presence of AP to yield an unstable intermediate. The continuous production of the intermediate results in the sustained emission of light for photon output signal measured by the luminometer. Use of this type of signal enhancement has allowed the development of immunoassays that are faster and more sensitive than any traditional colorimetric assay. Light intensity is a linear function of the amount of labeled enzyme, and the luminescence intensity at any time point is a direct measure of the concentration of the enzyme. The low background signal of the system allows a high degree of discrimination between negative and (true) positive serum samples (Schaap et al., 1987). Luminol is the substrate to the horseradish peroxidase.

Direct CLIA is a nonenzymatic system. Substrate linked to antibody/antigen is the label. One oxidation event liberates one molecule of label with release of a set number of photons. A nonenzymatic system uses direct chemiluminescent labels, which have lower background signals than the enzyme systems, and will typically give rise to very fast times to elicit signals. Luminol reaction is widely used as a chemiluminescent fast or "flash" reaction, but unlike the peroxyoxalate system, it does not require an organic/mixed solvent system. The chemiluminescent emitter is a "direct descendant" of the oxidation of luminol by an oxidant in basic aqueous solution. Probably the most useful oxidant is hydrogen peroxide (H_2O_2). With the acridinium ester system, after the immunological binding and subsequent wash step, the signal takes only 2 min to develop, compared with 30 min or longer for an enzyme generated system. This molecule has been used to label a number of different antibodies to develop super-sensitive assays (Weeks and Woodhead, 1991). The labeled molecule can be easily detected. In general, turning to chemiluminescence will speed up most assays by making an assay 10-fold or more sensitive.

One reason accounting for the growing popularity of chemiluminescent assays is their exquisite detection sensitivity. Unlike absorbance (colorimetric) or fluorescent measurements, assay samples typically contribute little or no native background chemiluminescence. The lack of inherent background and the ability to easily measure very low and very high light intensities with simple instrumentation provide a large potential dynamic range of measurement. Linear measurement over a dynamic range of 10^6 or 10^7 using purified compounds and standards is routine.

In Enhanced CLIA, like indirect or enzymatic CLIA, HRP enzyme is the label, luminol is the substrate; it has so-called enhancers act as catalysts. Enhancers speed the oxidation of the luminol by HRP by as much as 1000 times. Thus, HRP oxidation of luminol as enhancement leads to eventual light by luminol.

Electrochemiluminescence (ECL)

ECL is a promising new technology for antibody detection, which is similar to ELISA except that the secondary antibody is labeled with a chemiluminescent label ruthenium (Ru). Magnetic beads provide greater surface for target capture (Peruski and Peruski, 2003). Electron transfer between the Ru atom and the substrate tripropylamine (TPA) results in photon production, and excitation results in light emission that is detected by a photon detector which detects a electrochemiluminescent signal in electrochemical flow cell for magnetic bead–Ru-tagged immune complex. The advantage of magnetc beads that contain paramagnetic magnetite (FE_3O_4) is the capability for rapid separation of captured antigen–antibody complex when placed in a magnetic field. The beads are usually small spherical and range from a few nanometers to micrometers in sizes.

An example is an ECL assay using immunomagnetic separation (IMS by ORIGEN system, IGEN) with a magnetic ECL detection system (Blackburn, 1991; Haukanes and Kyam, 1993; Yu, 1998). Detection of ECL is accompanied by heavy metal chelate ruthenium (Ru) conjugated to a detector antibody. Initially, Ru and tripropylamine (TPA) in the buffer are oxidized at the surface of an anode when an electric field is applied to the electrode. TPA loses a proton and becomes a reducer, which causes Ru to enter a high-energy state by a high-energy electron transfer from the electron carrier TPA. A rapid electron transfer reaction between the substrate TPA and the Ru atom occurs, resulting in the production of photons in light transmission, which in turn is sensed by the photon detector at 620 nm. A linear dynamic ranges spanning six orders of magnitude (Yang et al., 1994).

Fluorescent Immunoassays

Fluorescent immunoassays can be categorized into five groups: (1) direct fluorescent assay (DFA), (2) indirect immunofluorescence (IFA), (3) time-resolved fluorescence (TRF), (4) flow cytometry (FC), and (5) multianalyte profile (xMAP) technology.

Like latex agglutination, DFA is commonly used for antigen testing and will not be covered here. IFA such as the slide method for microscopic examination under

UV light is used much less in antibody detection than in antigen detection and will not be covered as well. However, IFA techniques such as those used in TRF, FC, and xMAP (Table 4.1) are discussed below.

TRF assays use a lanthanide chelate such as europium (Eu^{3+}) or samarium labels. These labels have unique properties such as a long fluorescence decay time so to lower background interference. TRF is similar to ELISA, except that the capture antigen affixed to the solid phase is mixed with the test sample, and the complex if any is mixed with diluted detector antibody that is labeled with lanthanide chelate. A low-pH enhancement solution added can cause lanthanide to dissociate from the labeled compound and is highy fluorescent (Aggerbeck et al., 1996; Peruski et al., 2002).

TRF exploits the differential fluorescence life span of lanthanide chelate labels compared with background fluorescence. The labels have an intense long-lived fluorescence signal and a large Stokes shift, resulting in assays with a very high signal-to-noise ratio and excellent sensitivity (Hemmila et al., 1984). TRF produces its signal through the excitation of the lanthanide chelate by a specific wavelength of light. Fluorescence is initiated in TRF with a pulse of excitation energy, repeatedly and reproducibly.

FC is a commonly used IFA. The first use of flow cytometry for analysis of microsphere-based immunoassays was published in 1977 (Horan and Wheeless, 1977; McHugh, 1994). Initially, different-sized microspheres were used for simultaneous analysis of different analytes (Horan and Wheeless, 1977). A fluorescent probe is added to a liquid suspension with sample, which is then streamed past a laser beam where the probe is excited. A detector analyzes the fluorescent properties of the sample as it passes through the laser beam. Using the same laser excitation source, the fluorescence may be split into different color components so that several different fluorophores can be measured simultaneously and analyzed by specialized software. A flow cytometer has the ability to discriminate different particles on the basis of size or color, thus making the multiplexed analysis possible with different microsphere populations in a single tube and in the same sample at the same time.

xMAP is definitely an emerging antibody detection method and has been referred to as, multiplexed particle-based flow cytometric assays technology, fluorescent microsphere immunoassay (MIA), fluorescence covalent microbead immunosorbent assay (FCMIA), multiplexed indirect immunofluorescence assay, or multiplex flow cytometry. This two-step suspension method is based on fluorescent detection using the FlowMetrix analysis system (Fulton et al., 1997). Systems using xMAP technology perform assays on the surface of color-coded beads (microspheres) that are covered with capture antigens that react with the target antibodies. The microbeads have surface-binding characteristics and a dyeing process to create up to 100 unique dye ratios, which are used to identify an individual microsphere in a single well.

Specific dyes permeate the polystyrene microspheres that are 5.5 μm in diameter and are composed of polystyrene and methacrylate to provide surface carboxylate functional groups on the surface. Each antigen is covalently linked

by a carbodiimide conjugation method (Staros et al., 1986) such as 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide EDC coupling method to beads of uniform size, which are colored with different amounts of red and orange fluorescent dyes (in a unique ratio) to allow for discrimination based on the relative emission intensities at the wavelengths of the two fluorescent dyes. Currently, there are 64 different ratios of red and orange fluorescence, which identify 64 distinctly colored sets of microspheres. Differently colored microsphere sets can be individually coupled via the surface carboxylate moiety to a specific probe for a unique target.

The flow cytometer analyzes individual microspheres by size and fluorescence, simultaneously distinguishing three fluorescent colors: green (530 nm), orange (585 nm), and red (>650 nm). Microsphere size, determined by 90-degree light scatter, is used to eliminate microsphere aggregates from the analysis. All fluorescent molecules are labeled with a green-emitting fluorophore. Any green-emitting fluorochrome can be use as a reporter; however, each fluorochrome has a characteristic emission spectrum, requiring a unique compensation setting for spillover into the orange fluorescence channel.

Antigen-conjugated microspheres are added to the well, in the sample, as well as the fluorescein-conjugate [red-phycoerythrin (R-PE) through biotin and streptavidin] antispecies detector or secondary antibody (Jones et al., 2002). The red laser excites specific dyes to identify the analyte [red and orange fluorescent dyes (detected by FL2/FL3]; the green laser excites a different dye to quantify the result (a green fluorescent reporter dye FL1) (Vignali, 2000; Mandy, 2001). The fluorescence emission of each bead of the specific antigen was determined with a fluorescence-activated cell scanner (FACScan, Becton-Dickinson, San José, CA, USA), a benchtop flow cytometer (multiparameter flow cytometer that is based on a single 488-nm excitation laser), with FlowMetrix hardware for data acquisition and analysis (Luminex Corp., Austin, TX, USA). The software allows rapid classification of microsphere sets on the basis of the simultaneous gating on orange and red fluorescence.

The Luminex instrument is a dual-laser flow analyzer. The first laser excites the fluorochrome mixture intrinsic to the microspheres, enabling the bead identity to be determined as the beads pass single file through the laser path in the flow cell. The second laser excites the extrinsic fluorochrome (R-PE) that is covalently attached to the secondary antibodies. The dual lasers allow the operator to mix beads with different antigens together in a well of a filter plate, thus enabling multiplex analysis of different antibody specificities at one time. Orange and red fluorescence are used for microsphere classification, and green fluorescence is used for analyte measurement (Fulton et al., 1997).

Contrast of These Techniques

The contrast with immunoassay techniques is shown in Table 4.1.

- ELISA assays are relatively inexpensive, can be adapted for high-throughput use, and thus are commonly used in research and clinical laboratories. The enzymes

and substrates used for ELISA might be unstable and require specialized storage to maintain activity. Most commercial products have been validated and have overcome this issue. Manual format of ELISA have more hands-on time and can only measure one analyte at a time. Automated ELISA system using batched samples are useful for large-scale sceening purposes.

- Handheld system: Lower sensitivity as compared with regular ELISA is always a concern. As with other highly sensitive assays, signal-to-noise ratio and limit of detection should be studied. However, sensitivity in handheld system has been improved (e.g., handheld assay such as OraSure assay for HIV). The better the avidity and affinity of the antibody, the more sensitive and specific the assay. A key limitation of HHAs is that assessment of a result is qualitative and subjected to interpretation. In addition, only one or two agents can be detected per assay strip with certain sensitivity levels.
- Radioimmunoassay: RIA has a few advantages including minor changes to the structure of antigen by radio labeling process. However, RIA is relatively slow and difficult to automate (some require centrifugation or microfiltration). It is susceptible to interfering IgM rheumatoid factor or high backgrounds with some sera. The labile nature of some radioactive molecules (some might decay quicker), and the regulatory constraints in their use (particularly exposure potential and disposal regulation) in the clinical laboratory makes radioactivity no longer the test of choice. RIA has been largely replaced by ELISA methods in the clinical setting.
- Chemiluminescence detection: In general, the use of a more sensitive detection system such as chemiluminescence allows for a faster assay system, as well as a lower detection system. Assays are often more sensitive than enzyme-based immunoassays. CLIA techniques have been widely accepted and implemented for automation because assay samples typically contribute little or no native background chemiluminescence and the detection procedure is simple. It requires no excitation source (as does fluorescence and phosphorescence) and only a single light (photon) detector such as a photomultiplier tube. Most samples have no 'background' signal (i.e., they do not themselves emit light). No interfering signal limits sensitivity (Campbell, 1988; Berthold, 1990; Nieman, 1995). Most chemiluminescent reactions are labeled either with a chemiluminescent compound or with an enzyme and using a chemiluminescent substrate as are most commercially developed immunoassays that are of the CLIA type, as shown in Table 4.2 (Kricka, 1991, 1996).
- Electrochemiluminescence: As compared with the colorimetric background signal that accumulates with time, ECL background signal is constant with time, and steady-state ECL signal is proportional to rate of substrate turnover. In CLIA, light is the consequence of chemical reaction, luminol undergoes oxidative bond cleavage to yield an excited state species that decays by a radiative pathway, and HRP (in the conjugate reagent) catalyzes the one-electron oxidation of luminol and expends hydrogen peroxide. The magnetic beads provide a greater surface area than that of conventional ELISA, so the reaction does not suffer from the same surface steric and diffusion limitations.

TABLE 4.2. Type of commercially available automated antibody detection systems.

Method	Detection method	Automated system (Company)	High throughput	Full automation
Colorimetric	Enzyme colorimetric	Evolis (Bio-Rad) ETI-Max (DiaSorin) Triturus (Grifols)	Yes	Yes
Radioimmunoassay (RIA)	Radioactivity			
Chemiluminescence (CL) immunoassay	CLIA	ACCESS (Beckman) ADVIA Centaur (Bayer) Architect (Abbott) Immunlite (DPC) Liaison (DiaSorin)	Yes	Yes
	Enhanced CLIA	VITROS ECi (Ortho)	Yes	Yes
	Electro-CLIA (ECL)	Elecsys (Roche) ORIGEN (IGEN)	Yes	Yes
Fluorescence	Fluorescence	AxSYM (Abbott) VIDAS (bioMerieux) Nexgen Four (Adaltis)	Yes	Yes
	Flow cytometry (FC) Multianalyte profile (xMAP)	FACScan (Becton-Dickinson) HTS (Luminex) Bio-Plex (Bio-Rad)		
Dual technology	EIA & IFA	PARSEC (Diamedix)		

Note: Many can handle antibody detection assays such as anti-HIV, anti-HAV, anti-HCV, anti-HBs, anti-HBc, CMV, and rubella. Rubella is a disease caused by Rubivirus genus that is within the Togaviridae family. Throughput is generally high from 80 to 400 tests per hour.

- Fluorescence immunoassay: In general, fluorescence detection will allow more sensitive or faster detection than colorimetric methods. However, it could suffer from possible high background contamination due to the intrinsic fluorescence of some proteins and light-scattering effects. Thus, indirect assay is commonly used.
- Indirect fluorescence assay: Although simple to perform and requiring minimal equipment and reagents, significant expertise is necessary to interpret the results of IFA by slide microscopic method (Nuwayhid, 1995).
- Time-resolved fluorescence: The limitation for TRF is similar to ELISA. In addition, dedicated measuring instrument and rigorous washing techniques are important to avoid lanthanide contamination, because lanthanide label is highly fluorescent (Aggerbeck, 1996; Peruski, 2002).
- Flow cytometry: A major strength of FC technology is its ability to be multiplexed with little or no loss of sensitivity (Carson and Vignali, 1999; Vignali, 2000). FC by BD Biosciences (San Jose, CA, USA) has many applications in biomedical research and is commonplace in most large clinical laboratories. However, FC has several disadvantages. Assays typically lack the sensitivity of those based on ECL or TRF. The system itself is relatively complicated, requiring training

and expertise to operate. Optimization of the assays can be tedious, and many user-defined parameters must be adjusted individually.

- Multianalyte Profile: Traditional ELISA and other immunoassays allow one test for each specific antibody at one time. However, many antibodies can be measured at the same time, in a single well or tube by using xMAP multiplexed technology (Luminex, Austin, TX, USA). The xMAP technology was originally developed using the principles of flow cytometry (FACScan) that has multiparametric resolving power. Unlike general flow cytometry on different sizes of beads, the xMAP technology detects identically sized microspheres with two different dyes, emitting in two different wavelengths, allows aggregates to be distinguished, and permits discrimination of at least 64 different sets of microspheres. Due to multiplexing, xMAP technology potentially delivers more data with results comparable to ELISA, simultaneously, within the same sample.

Application of the Techniques in Diagnostic Microbiology

Clinical Applications

It is difficult to cover all areas of clinical applications by using antibody detection. However, clinical application of immunodiagnostics can be best demonstrated in available immunoassays for HIV (Nielsen and Bryson, 2000) and hepatitis. Immunoassays have been developed to detect anti-HIV antibodies or viral antigens present in serum, plasma, dried blood spots, urine, and saliva. Assay formats range from EIAs, ELISA-based Western blot assays, IFA assays, and even rapid handheld immunoassays. In general, however, the EIA remains the most widely used serologic test for detecting antibodies to HIV-1. Thus, HIV-1 immunoassays represent the advances in antibody detection technologies to detect and identify infectious agents. Another study comparing ELISA methods with Western blotting, microagglutination, IFA, and FC for detection of antibodies to *Francisella tularensis* and diagnosis of tularemia is another source to demonstrate the use of antibody detection techniques (Porsch-Ozcurumez et al., 2004). In this study, the combined use of ELISA and confirmatory blotting seems to be the most suitable approach for serodiagnosis of tularemia (Porsch-Ozcurumez et al., 2004).

Immunoanalyzers for broad application range (automation, random access, multiplexing, and high throughput) will help meet the challenges of immunodiagnosis of infectious diseases. The main focus of this section of clinical application will be general use of recent application and automation in terms of detection method.

EIA Detection

ELISA assays are still the methods of choice for large-scale investigations during outbreak or epidemiological surveillance studies. Because of its relative simplicity and good sensitivity, ELISA has been used for screening large numbers of

small-volume samples and has had great impact in epidemiology and in the diagnosis of infection, particularly in the diagnosis of the difficult bacteria and viruses such as West Nile (WN) Virus, not to mention that these assays have been used extensively in AIDS and hepatitis testing.

In a typical ELISA for HIV antibody test, HIV antigens (often a purified viral lysate) attached to a microtiter plate or bead serves as the test platform. The anti-HIV antibody in the sample can be tested by incubating with antigens followed by incubation with labeled conjugate secondary antibody and substrate and detection by using colorimetric method (Nielsen and Bryson, 2000).

Eight EIAs including two single-use EIAs and six plate-type EIAs were evaluated for the detection of IgM and IgG antibodies to *Mycoplasma pneumoniae*, an important etiologic agent of primary atypical pneumonia in children and adults (Talkington et al., 2004). Interestingly, the two single-use EIA methods were more reliable than the plate-type EIAs.

Serologic testing is the primary method of diagnosing WN virus infection. The recommended immunoassays are the immunoglobulin M (IgM) antibody ELISA and the indirect IgG ELISA (Davis et al., 2001). Positive ELISA results are confirmed by flavivirus plaque reduction neutralization tests (Lindsey et al., 1976). This combination of assays is highly sensitive and specific, but performing a complete panel of ELISAs requires 2 to 3 working days to complete, as overnight incubations are deemed necessary to enhance sensitivity. IFAs may also be used for diagnosis, but they are not suitable for a high throughput of specimens and they are less sensitive than ELISA.

Immunoblotting Method

The cross-reactivity of an antibody is prevented by using high-affinity antibody, thus to improve the quality of an immunoassay. Cross-reactivity could result from an antibody that binds to structurally distinct but similar epitopes present on different antigens or result from an antibody that binds to structurally identical epitopes on different antigens. This is why confirmatory tests are needed in certain tests such as HIV using more specific assays such as the Western blot (Jackson et al., 1997). The separated HIV-1 proteins are electrotransferred to a nitrocellulose membrane. If antibodies to any of the major HIV-1 antigens are present in the specimen, bands corresponding to the HIV-1 proteins (p) or glycoproteins (gp) such as gp24, gp41, or gp120 will be seen on the nitrocellulose strip. Antibodies can thus be detected by using enzyme-conjugated secondary antibody (to human IgG) and demonstrated by darkly colored lines on the membrane under the substrate.

Other than HIV, the RIBA Strip Immunoblot Assay (SIA) for detecting NS5 and c33c recombinant proteins and c100p, 5-1-1p, and c22p synthetic peptides of hepatitis C virus (HCV) is intended as a supplemental test for human serum or plasma specimens found to be repeatedly reactive in HCV antibody screening test (Martin et al., 1998). Semiautomated or automated processing instrumentation is available for immunoblotting.

Handheld Assay

Handheld immunoassays are on the horizon. Development of self-contained miniaturized devices will allow an immunoassay to be performed in a field or point-of-care setting. The OraQuick HIV-1 immunochromatographic card assay has nearly equivalent sensitivity and specificity for HIV-1 as EIA. Two more rapid assays, one a lateral flow immunoassay device and the other a membrane immunoreactive test device, have been approved for non-blood donor diagnostic screening (Ketema et al., 2001, 2005). Lateral flow assays were developed for rapid serodiagnosis of human brucellosis by using the lateral flow assay to detect antibodies against lipopolysaccharide (LPS) of *Brucella*-specific capture antigen (Smits et al., 2003).

RIA Application

Although unpopular in the clinical setting, RIA is still available for research settings. One example is the Human Papilloma Virus (HPV) type-specific competitive RIA (cRIA) used to evaluate HPV type-specific antibody titers. Briefly, HPV L1 virus-like particle (VLP) antigens (HPV-6 and HPV-11) are coated onto solid-phase polystyrene beads and incubated with equal volumes of sera and diluted Mab, as well as the ^{125}I-labeled secondary antibody (Opalka et al., 2003).

Chemiluminescence

Chemiluminescence will be discussed in the "Automation" section, below.

Fluorescence Immunoassay

In addition to TRF (McKie et al., 2002; Peruski et al., 2002), another type of fluorescent technology is fluorescence polarization (FP). FP is a phenomenon seen when polarized light excites a fluorescent dye causing photons to be emitted in the same plane as the exciting light. FP assays can be used for detecting antibodies (Nielson et al., 1996). Because of the limited need for sample processing, FP antibody detection assays are particularly useful for high-throughput screening such as AxSYM (Abbott Laboratories, Abbot Park, IL, USA).

Flow Cytometry

Two distinct sizes of microspheres were used for simultaneous detection of two different antibodies and subsequently expanded to the use of four different sizes of microspheres to detect four different antibodies to cytomegalovirus and herpes simplex virus (McHugh et al., 1988) or antibodies against HIV proteins (Scillian et al., 1989). Size discrimination of microspheres allows simultaneous detection of small numbers of analytes, but the inability to distinguish aggregates of smaller microspheres from larger microspheres limits the extent of multiplexing that can be achieved.

Multiplexed Bead Assay

Diagnosis of infection often requires testing for multiple antibodies. The xMAP technique applications include detection of antibodies to a panel of seven respiratory viruses, including influenza A and B viruses; adenovirus; parainfluenza viruses 1, 2, and 3; and respiratory syncytial virus (Martins, 2002), and for *Bacillus anthracis* anti-protective antigen (PA)-specific immunoglobulin G (anti-PA IgG) (Biagini et al., 2004). When compared with the ELISA method (Quinn et al., 2002), xMAP method for anti-PA IgG had a good positive correlation, better sensitivity and speed, and enhanced dynamic range. It uses smaller sample volume and can be multiplexed, that is, measure more than one analyte simultaneously (Biagini et al., 2003). In addition, the Luminex technology was used to simultaneously measure antibodies to HIV-1 p24, gp160, and gp120 eluted from dried blood-spot specimens from newborns (Bellisario et al., 2001; Faucher et al., 2004), and even the HCV antibody and HBs antigen with HIV antibodies (Lukacs et al., 2005).

Simultaneous measurement of antibodies to 23 pneumococcal capsular polysaccharides (PnPs) was developed recently (Biagini et al., 2003), which showed results similar to another xMAP assay developed for antibodies to PnPs (Pickering et al., 2002a). The assay simultaneously determines serum IgG concentrations to 14 PnPs serotypes. The multiplexed assay showed good overall agreement with a well-established ELISA that is currently recommended for evaluation of pneumococcal vaccine immunogenicity.

A Luminex xMAP based technology was compared with ELISA for quantitation of antibodies to the toxoids of *Clostridium tetani* (Tet) for tetanus, *Corynebacterium diphtheriae* (Dip) for diphtheria, and *Haemophilus influenzae* type b (Hib) polysaccharide. The correlations (R^2) between ELISA and Luminex of the 81 samples were 0.96, 0.96, and 0.91 for Tet, Dip, and Hib, respectively. Both methods detected strong postvaccination responses (Pickering et al., 2002b).

Using xMAP technology (Mandy et al., 2001), a new test was developed to measure antibodies induced by flavivirus infection. This assay is based on a recombinant WN virus envelope (E) glycoprotein antigen (rWNV-E). This first-generation test for serodiagnosis of flavivirus infection provides the basis for multiplex system for simultaneously measuring antibodies to several recombinant flavivirus antigens.

A multiplex assay was developed for detection of strain-specific antibodies against the two variable regions of the G protein of respiratory syncytial virus (RSV), which is the single most important lower respiratory tract pathogen of infants and young children worldwide (Jones et al., 2002).

A West Nile virus recombinant antigen microsphere (suspended-microsphere) diagnostic immunoassay was developed for detection of human anti-flavivirus antibodies (Wong et al., 2004). The microsphere immunofluorescence assay can be performed in less than 3 h on specimens of $\leq 30\,\mu L$. Retrospective testing of 833 sera from New York patients with suspected viral encephalitis demonstrated concordance with results obtained with the traditional ELISA for immunoglobulin G antibodies to WN virus. The assay also detects antibodies to E proteins from

related flaviviruses, including St. Louis encephalitis, Japanese encephalitis, and dengue viruses. The new microsphere immunoassay provides a sensitive and rapid alternative to traditional ELISAs.

Cytokines were measured as mediators for or effectors against rotavirus disease, the most common cause of severe gastroenteritis in young children. In a pilot study by using bead-based Luminex assay, an overall increased cytokine response was demonstrated in children with acute rotavirus diarrhea compared with those in control children (Jiang et al., 2003).

Simultaneous quantitation of antibodies to neutralizing epitopes on virus-like particles (VLPs) for human papillomavirus (HPV) types 6, 11, 16, and 18 in 50 μL of serum was achieved by a multiplexed Luminex assay (Opalka et al., 2003). The HPV competitive immunoassay measures titers of polyclonal antibodies and was found to be as sensitive and precise as the currently used cRIAs.

An advantage of the 96-well plate Luminex assay format is that it avails itself to automation, such as the Tecan Genesis liquid handler to automate the assay. The automation such as Bio-Plex system (Bio-Rad Laboratories, Hercules, CA, USA) employing the Luminex multianalyte profiling technology (xMAP) allows individual and multiplex analysis of up to 100 different analytes in a single microtiter well (Vignali, 2000) and is used for detecting 15 human cytokines (de Jager et al., 2003). A multiplexed bead assay was eveluated for assessment of Epstein-Barr virus immunologic status using BioPlex 2200 system (Bio-Rad). Concordance between results generated by the BioPlex system and conventional assays showed 97% agreement with conventional heterophile and anti-nuclear antigen assays (Klutts et al., 2004).

Automation

Automated immunoanalyzers have been widely used to facilitate the analysis of large numbers of samples (Table 4.2). The first generation of immunoassay systems was developed 10 years ago to automate what had been labor-intensive manual laboratory tests. Advances in clinical immunology, and the demand for faster turnaround times and reduced costs, has helped technology developments in immunoassay, as well as the integrated immunochemistry analyzers. The high-volume immunoassay analyzers will have a significant impact on laboratory performance by reducing errors, reducing turnaround times, and reducing the labor requirements for those tests.

The ideal immunoassay system will have the following capabilities to provide optimal productivity and a comprehensive disease-focused menu: no-pause loading of all reagents, samples, and supplies; continuous sample loading for fast turnaround time; high-throughput process efficiency; random access; reduced operator intervention; minimal hands-on time with large on-board capacity for reagents; ability to interface with the laboratory information system for increased efficiency with easy-to-use software; and assays available for HIV and complete hepatitis panels.

Any technology and system, as sophisticated as it may appear, if untested, needs to be validated. Despite the literature purporting excellent clinical utility,

the reliability of these assays, when used under real-time clinical conditions, has not been well studied. The decision to switch will be made on the basis of adequate quality through validation of assays and cost. As methods change, the new automated assays must be validated against the existing ones for better sensitivity, specificity, predictive values, and clinical utility.

Most chemiluminescent reactions can be adapted to this assay format by labeling either with a chemiluminescent compound or with an enzyme and using a chemiluminescent substrate. Most commercially developed immunoassays are of this type (Table 4.2). For example, Lumi-Phos 530 of Luminol CLIA is used as the detection reagent in the Access immunoassay analyzer (Beckman Coulter Inc., Fullerton, CA, USA). Lumigen PPD and enhancer are incorporated in the chemiluminescent detection reagent used in the Immulite Immunoassay Analyzer from Diagnostic Products Corporation (DPC). The AxSYM immunoassay system (Abbott) is based on the microparticle enzyme immunoassay technology (Fiore et al., 1988; Hennig et al., 2000, Lazzarotto et al., 2001). The DPC Immulite (Diagnostic Products Corporation) is a benchtop immunoassay analyzer with continuous random-access capabilities that uses enzyme-amplified chemiluminescence chemistry for antibody or antigen detection (Schaap et al., 1987).

As shown in Table 4.2, several high-throughput systems that can provide streamlined operations to reduce total processing time are available in the market. Many types of immunoassays can be developed on the automated system for hepatitis virus A, B, and C, cytomegalovirus, and HIV assays.

Summary

Over the past 20 years, immunodiagnostic technologies have been developed to identify infectious agents with better sensitivity and specificity to ensure that every true-positive case is diagnosed. Antibody-based methods used to be the tool for the detection and epidemiological analysis of slow-growing, difficult-to-culture, uncultivatable, or emerging infectious agents.

Conventional ELISA has been the predominant technology used for such assays, with CLIA, ECL, and TRF detection formats becoming more promising technologies for automated antibody detection. Handheld assay and multiplexed flow cytometry methods are also emerging as the next generation of rapid laboratory-based technologies.

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5
Phenotypic Testing of Bacterial Antimicrobial Susceptibility

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Introduction

Phenotypic testing of bacterial antimicrobial resistance has been widely used in clinical and diagnostic microbiology laboratories. These methods have been well studied and standardized. They have the advantages of being low cost, easy to perform (automated systems), and interpretation criteria readily available for commonly encountered organisms. These assays also are essential for new resistance discovery.

Direct testing of clinical isolates against antimicrobial agents *in vitro* is the most practical way to assess the *in vivo* activity of drugs routinely in the clinical setting (Greenwood, 1981). In the United States, dilution and disk diffusion tests are two basic methodologies that have been standardized by the Clinical and Laboratory Standards Institute (CLSI), formerly known as the National Committee for Clinical Laboratory Standards (NCCLS). Dilution tests are performed by detecting bacterial growth in broth or agar containing antimicrobial agents in a series of twofold dilutions. The lowest concentration that inhibits the visible growth of an organism is the MIC (minimum inhibitory concentration) value. MICs provide a quantitative evaluation of bacterial growth inhibition by antimicrobial agents. In the disk diffusion method, the drug concentrations are created by diffusion of the testing drug through the agar from filter paper disk containing a single concentration (Barry, 1991). The size of the growth inhibition zone is used to determine the susceptibility of the organism to the drug qualitatively. Based on the pharmacokinetic and pharmacodynamic properties of the drug, the clinical and bacteriological response rates of organisms to the drug, and the population distributions of MICs, the CLSI provides guidelines for interpretative criteria that give the values of MICs or growth inhibition zone sizes to determine the categories of susceptible, intermediate, and resistant (NCCLS, 2001). The susceptible category is defined as that when infection due to the strain tested may be appropriately treated with the dose of antimicrobial agents recommended for that type of infection. The intermediate category indicates that the strain tested can be effectively inhibited if the drugs are physiologically concentrated at the infected body sites or when a high dosage of a drug can be safely administered. Resistant strains are not inhibited by the

usually achievable systemic concentrations of the agent with the normal dosage schedules and/or treatment failures are likely caused by specific microbial resistant mechanisms (CLSI, 2005). The interpretative criteria are specific for each organism/antimicrobial combination along with the specimen type. To achieve the best possible correlation between the *in vitro* test results and clinical outcome, the test procedures and quality controls suggested by CLSI must be closely followed.

The CLSI guidelines offer standardized methods and interpretative standards for antimicrobial susceptibility testing for organisms commonly encountered in clinical microbiology laboratories, including members of the *Enterobacteriaceae*, Gram-negative bacilli that are not members of the *Enterobacteriaceae* such as *Acinetobacter* spp., *Stenotrophomonas maltophilia*, *Pseudomonas* spp., and other nonfastidious, glucose-nonfermenters, *Staphylococcus* spp., *Enterococcus* spp., *Streptococcus* spp., *Haemophilus* spp., *Neisseria gonorrhoeae, Vibrio cholerae, Helicobacter pylori, Listeria monocytogenes*, and four potential agents of bioterrorism: *Bacillus anthracis, Yersinia pestis, Burkholderia mallei*, and *Burkholderia pseudomallei* (CLSI, 2005). For the other clinical isolated organisms that are not described, susceptibility testing is not routinely performed in most diagnostic laboratories due to lack of standardized testing methods, or lack of resistance to the drugs of choice for the treatment and interpretation criteria, or lack of the correlation between *in vitro* susceptibility tests and clinical response.

Agar Dilution

Agar dilution is one of the standardized antimicrobial testing methods. Mueller–Hinton agar (MHA) is used for testing nonfastidious aerobic and facultatively anaerobic bacteria that require no special supplement for growth. To prevent the interference to drug activity, any calcium and magnesium containing supplement should not be added (NCCLS, 1996). Culture medium mentioned above in dehydrated form is commercially available. Preparation of the agar plates should follow the manufacturer's recommendations. Drugs are tested at serials of twofold dilutions with each plate containing one concentration. The range of concentration tested for each drug should cover the CLSI break points and the expected MICs for quality control reference strains. Studies show that the oxacillin MIC for *Staphylococcus* spp. carrying the *mecA* gene are detected with increased sensitivity by the agar containing NaCl (Huang et al., 1993). Therefore, MHA with 2% NaCl is recommended for the testing of staphylococci against methicillin, oxacillin, and nafcillin. Plates containing certain agents such as imipenem, cefaclor, and clavulanic acid combination have short shelf-lives and should be prepared freshly each time used (Murray, 2003).

Inoculation size is critical in obtaining an accurate MIC value. For standardized agar dilution method, a final inoculum of 10^4 CFU (colony-forming units) per spot is recommended. A simple way to quantify bacteria numbers in the inoculum is to measure the turbidity of the bacterial suspension used in preparing the inoculum. By either growing several colonies from an overnight culture in a liquid broth

or directly suspending colonies from an overnight culture on nonselective agar medium, the bacterial suspension with a turbidity equivalent to an 0.5 McFarland turbidity standard is made to reach a concentration of 10^8 CFU/mL. The former method is required for testing staphylococci (CLSI, 2005). Approximately 1 to 2 μL of 1:10 dilution of the suspension with either sterile broth or saline is used to inoculate the agar in order to achieve 10^4 CFU per spot. To maintain the consistent inoculation size, bacterial suspensions have to be plated onto the plates within 30 min of preparation. By using an inoculum device, multiple samples can be plated on the same plate simultaneously. Whenever susceptibility tests are performed, tested organisms from the prepared suspension have to be grown on the plates without antimicrobial agents to ensure the viability and purity. After the inoculation, testing plates are incubated in ambient air at 35°C for 16 to 18 h. When testing staphylococci, incubation temperature between 33°C and ~35°C should be maintained to ensure reliable results. Extended incubation time (24 h) is required to detect the vancomycin-resistant enterococci and oxacillin-resistant staphylococci. MIC values are determined by examining growth of the bacteria on plates containing various concentrations of antimicrobial agents. The drug dilution in the first plate showing no bacteria growth is recorded as the MIC. For bacteriostatic agents, the drug dilution that inhibits 80% of growth is regarded as the MIC.

Broth Dilution

Similar to agar dilution method, broth dilution methods test the organisms in medium containing antimicrobial agents in serials of twofold dilutions. Instead of growing bacteria on solidified medium, bacteria are grown in liquid medium during susceptibility test process, and at the end of the test, bacterial growth is evaluated by the turbidity of broth. Macrodilution testing is performed in serials of 13 × 100 mm tubes with each one containing 2 mL of broth. Microdilution testing uses multiwell microdilution trays with each well containing 0.1 mL of broth. Because the microdilution trays with prepared panels of antimicrobial dilutions either frozen or freeze-dried are commercially available, allowing testing of multiple organisms simultaneously, the method has replaced macrodilution and has been widely used in clinical microbiology laboratories. Cation-adjusted Mueller–Hinton broth (CAMHB) is recommended for standardized broth dilution methods (NCCLS, 2003). The cations Ca^{2+}(20 to 25 mg/L) and Mg^{2+}(10 to 12.5 mg/L) in the broth are critical for the activity of aminoglycosides tested against *P. aeruginosa* as well as for tetracycline tested against other bacteria (D'Amato et al., 1975). For convenience, CAMHB is adopted as the standardized testing medium. The final inoculum for microdilution broth methods is 5×10^5 CFU/mL. As the first step, the turbidity equal to 0.5 McFarland standard (approximately 10^8 CFU/mL) of bacteria suspension containing tested isolate is made either by growing in broth or direct suspension. Bacterial suspension prepared by direct inoculation is required for testing staphylococci (CLSI, 2005). To make bacteria suspension directly, only the colonies from overnight growth on a nonselective agar plate should be used.

The standard suspension is diluted 1:10 with sterile saline or broth to 10^7 CFU/mL, and 5 μL of the diluted suspension is added to each well containing 100 μL of broth with tested drug. As the inoculum volume is less than 10% of the total volume, the change in drug concentration after inoculation is minimal, and there is no need to increase the final drug concentration. The bacteria suspension has to be inoculated within 30 min of preparation to maintain the desired inoculation size. The control well without antibiotics should also be inoculated to determine the viability of the tested organism. To confirm the inoculum density and purity, 5 μL of bacteria suspension should be plated on a nonselective agar plate. All tubes and plates are incubated at 35°C for 16 to 20 h before the MICs are determined. The recommended incubation temperature for testing staphylococci in agar dilution should be used in broth dilution. The incubation time should be extended to 24 h in order to detect vancomycin-resistant enterococci and oxacillin-resistant staphylococci (NCCLS, 2003). As with agar dilution, the drug concentration in the first well showing no bacterial growth indicated by broth turbidity is the MIC value. For bacteriostatic agents, the drug concentration that inhibits 80% of growth is regarded as the MIC.

Both agar dilution and broth dilution are well standardized methods. They are reliable and have served as gold standards for antimicrobial susceptibility testing methods. They allow testing multiple isolates simultaneously and allow flexibility in selecting the drug combinations for testing to best fit the institution formulary. When fastidious organisms are tested, necessary supplement can be added into the agar or broth to provide better support for bacteria growth. However, both methods are labor intensive and require certain levels of experience to read the MIC results consistently. These methods are no longer used routinely in most clinical laboratories.

Disk Diffusion Testing

Like agar dilution and broth dilution methods of susceptibility testing, the disk diffusion method tests the inhibitory effect of antimicrobial agents against microorganisms. The test is carried out by placing filter paper disks with a known concentration of an antimicrobial agent on the surface of agar plates inoculated with a test organism. The drug on disks diffuses through the agar, creating a concentration gradient decreasing along the distance from the center of the disk (Barry, 1991). The areas with drug concentration inhibiting bacteria growth will show no growth, whereas the areas with the drug concentration insufficient for bacterial growth inhibition show confluent growth. As the result, there is a growth inhibition zone formed around the disk and the zone size is generally inversely proportional to the MIC. Based on the correlation between the zone-of-inhibition diameters produced by disk diffusion and the corresponding MIC break points of the same organism–drug combination obtained by broth dilution, CLSI provides interpretation category of the organisms as sensitive, intermediate, or resistant to test antimicrobial agents (NCCLS, 2001). In order to use the interpretation criteria,

testing conditions including the medium, the amount of the antimicrobial agents on disks, inoculum size, the culture conditions, and the test organisms have to closely follow the CLSI guideline. The same medium used for agar dilution testing has been recommended for disk diffusion testing. To inoculate the plate, bacteria suspension with the turbidity equal to 0.5 McFarland is prepared in saline by either growing in broth or direct suspension of the colonies from an overnight growth on nonselective plate, and the suspension should be used within 15 min after preparation. The plate is streaked three times evenly in three different directions throughout the entire surface with a cotton swab dipped in bacteria suspension. The disks are placed on the plate after inoculation with at least 24 mm between them to avoid overlapping of the inhibition zones. The recommended incubation time and conditions are the same as that for agar dilution testing.

The diameters of growth inhibition zone should be measured from the edge of the ring with no bacteria growth. Discrete colonies within a clear inhibition zone are the results of either heterogeneous resistance among the bacteria population or contaminated culture. The heterogeneous resistance has been observed in staphylococci tested with oxacillin (Brown, 2001) and vancomycin (Liu and Chambers, 2003; Rybak et al., 2005), enterococci with vancomycin, and *Enterobacter* spp. with penicillins and cephalosporins (Hsieh, 2000). When such organism–drug combinations are tested, any amount of colony growth in the inhibition zone is an indication of resistance. Swarming *Proteus* spp. sometimes produce a thin film of swarming growth inside the inhibition zone. The margin around the heavy growth should be used for measuring the diameters of zones of inhibition. For bacteriostatic agents, the zone diameters of 80% growth inhibition are measured.

The disk diffusion test is easy to perform. It allows any combinations of antimicrobial agents tested simultaneously against the same organism. However, the test only gives category results, which is not useful when quantitative susceptibility (MIC) is required to follow the change in the antimicrobial susceptibilities. This limitation can be partially overcome by using the BIOMIC VIDEO system, which reads and interprets diameter of inhibition zone automatically. The system also calculates discrete MICs using linear regression analysis to compare the zone of inhibition diameters with MICs from broth dilution. However, linear regression analysis is not valid when isolates are either susceptible or resistant to a test antimicrobial agent (Korgenski and Daly, 1998). Other limitations of the disk diffusion method include its reported questionable reliability in detecting vancomycin intermediate and resistant staphylococci (Tenover et al., 1998) and vancomycin-resistant enterococci (Hageman et al., 2003). For bacteria showing inconsistent growth rate, such as some members of nonfermentative Gram-negative bacteria, the disk diffusion method also has limited application.

E-test

E-test is another form of agar diffusion test. Different from the disk diffusion method in which disks containing a single concentration of antimicrobial agent are used to create a drug concentration gradient, the E-test uses a nonporous plastic

strip covered with preformed exponential gradient of an antimicrobial along the 60 mm of length (Andrews et al., 1993). The gradient of agent covers a concentration range of 0.002 to 32 mg/L, 0.016 to 256 mg/L, or 0.064 to 1024 mg/L, depending on the agent. This range corresponds to 15 twofold dilutions in a conventional MIC method. On the other side of the strip, calibrated MIC values covering 15 twofold dilutions are marked according to the antimicrobial gradient coated. When the strips are applied on the surface of agar plate inoculated with the test organism, the continuous drug gradient is formed on the agar by diffusion. The areas with inhibitory concentration of the antimicrobial to the test organism show no bacteria growth while the confluent lawn growth covers the rest of the area on the plate. As the result of the response of the bacteria to the test drug in different concentrations, an elliptic growth inhibitory zone is formed around the strip. The point on the E-strip at which the inhibition zone intersects is determined as the MIC.

E-test requires the same medium used for agar dilution method. The agar plate is swab inoculated with an adjusted bacteria suspension in the same way as that for a disk diffusion test. The inoculum prepared from colonies grown after 24- or 48-h is standardized to the density of a McFarland standard recommended by the manufacturer for the particular organism–antimicrobial combination tested. Different inocula are required for different organism–antimicrobial agent combinations according to bacteria growth rates. Like agar dilution, the undiluted inoculum must be used within 15 min after preparation. The E-strips are stored at $-20^{\circ}C$ or $-70^{\circ}C$ to prevent loss of drug activity. They should be removed from the freezer and equilibrated to the room temperature for 30 min before use. After overnight incubation, the MIC is read at the point of intersection of the elliptical zone with the strip. Read at the point of inhibition of all growth, including hazes and isolated colonies, except when bacteriostatic agents are tested, 80% end points are used.

E-test gives similar results to the agar dilution method when a standardized technique recommended by the manufacturer is used and care is taken in reading results (Jones, 2001). The method is useful in clinical laboratories for confirmation of unusual resistances, for checking equivocal results, for testing slower growing organisms, and for organisms where a quantitative result is required.

Automated Methods

There are three automated antimicrobial susceptibility test systems available and widely used at this time. All of them are MIC-based systems that follow the principle of determining the MICs by the broth microdilution method. The results provided by the systems are considered to be equivalent to that derived from broth dilution method. The procedure of microdilution test method involves making bacteria suspension to the standard concentration, preparing inoculum, rehydrating and incubating culture media containing tested antimicrobials in various concentrations with the inoculum, reading bacteria growth pattern in the presence

and absence of antibiotics, and finally reporting the MIC values. Most of those steps are performed by the devices within a self-contained instrument in the automated systems. The major differences among the systems are the methods to detect the bacteria growth and to obtain the MIC values.

MicroScan WalkAway 40/96S Systems

Microscan WalkAway (Dade MicroScan Inc., West Sacramento, CA, USA) is a conventional overnight incubation system that uses the reference broth microdilution method. There are two configurations with different test capacities. The small one tests 40 panels and the large one tests 96 panels simultaneously. The system uses standard-size microdilution trays containing dehydrated media and antimicrobials. Bacteria suspension is made manually with saline, and the microdilution trays are inoculated manually with a multiprong device using undiluted suspension. The instrument incubates the trays, detects bacteria growth with a photometer, and determines the growth end-points when the turbidity reaches a predetermined value. The company offers multiple antimicrobic configurations with different antimicrobial combinations in various concentration ranges for Gram-negative and Gram-positive organisms. As the classic microdilution method, the end points of growth inhibition are determined as the MICs by the system.

VITEK 1 and VITEK 2 Systems

In VITEK systems (bioMerieux Vitek, Hazelwood, μ0, USA), antimicrobials are placed on plastic reagent cards that can hold microliter quantities of test media. Each VITEK test card contains up to 64 microwells with 1 well containing only culture media and the rest of the wells containing premeasured amounts of a specific antibiotic combined with culture medium. The bacteria suspension standardized to 0.5 to 0.63 McFarland is made with colonies from overnight culture plates in 0.45% saline. This inoculum is diluted 100 times automatically by the instrument before being used to rehydrate the antimicrobial medium within the card. The card is filled, sealed, and placed into VITEK incubator/reader automatically. The instrument monitors the kinetic growth of each well with photometric detection of turbidity over a defined time period (up to 18 h). Linear regression analysis of the growth rate corresponding to the antimicrobial concentrations is used to determine algorithm-derived MICs. There are four antimicrobial panels that can be chosen for routine susceptibility test for Gram-negative organisms and two panels for Gram-positive organisms. VITEK 2 is a more advanced system than VITEK 1. It automates the initial sample processing, including initial inoculum dilution, density verification, and card-filling and card-sealing steps. In addition, the VITEK 2 cards contain 64 wells and the VITEK 1 cards have 30 to 45 wells.

VITEK systems have limitations in testing the following common drug–organism combinations, ampicillin–*Citrobacter* spp., ampicillin–*Enterobacter* spp., ampicillin–*Serratia* spp., ampicillin/sulbactam–*Citrobacter* spp., ampicillin/

sulbactam–*Enterobacter* spp., ampicillin/sulbactam–*Serratia* spp., aztreonam–*Pseudomonas* spp., imipenem–*Proteus* spp., meropenem–*Acinetobacter* spp., piperacillin–*Acinetobacter* spp., piperacillin/tazobactam–*Acinetobacter* spp., linezolid–*Enterococcus* spp., for resistance and linezolid–*Staphylococcus* spp for resistance. Alternative methods have to be used for testing these drug–organism combinations.

BD Phoenix System

The Phoenix (BD Diagnostics, Sparks, MD, USA) is the newest FDA-approved system. Each susceptibility test panel contains 85 wells with 16 to 25 different antimicrobials in double dilution. Unlike VITEK, all reported antimicrobial concentrations are included on the panel. The test panels are manually inoculated with bacteria suspension equivalent to 0.5 McFarland standard, and the instrument carries out the rest of the steps from incubating the plates, detecting the growth, to reporting MIC and category results. The method used by the Phoenix to detect bacteria growth is different from the other two systems. An oxidation reduction indicated is added to the inoculation broth. Bacteria growth is determined by monitoring reduction of a modified resazurin indicator. In combination with the turbidometric growth detection, the system reports the MICs after 6 to 16 h incubation. The system determines the MICs in the manner similar to the conventional microbroth dilution method. Only one drug panel is available for either Gram-positive or Gram-negative organisms. Limited studies find the system effective in routine susceptibility testing of commonly encountered Gram- positive and Gram-negative bacteria (Donay et al., 2004).

All three systems suffer from the same disadvantage of having limited choices of the antimicrobial combinations for routine use. Due to insufficient growth achieved in the machines, the automated systems have trouble in testing slowly growing organisms, such as nonfermentive Gram-negative bacilli, fastidious organisms, and anaerobic bacteria (Jorgensen and Ferraro, 2000). The problem of insufficient growth can be overcome partially by supplying additional nutritional supplements and extending the incubation time in the Microscan system. Because this system uses standard microdilution trays, results can be read manually. For the more advanced automated systems, such as VITEK and Phoenix, it is impossible to do any manual manipulation. The automated systems only test few dilutions of a drug, which is only enough to cover the break points. Insufficiently tested antimicrobial dilutions make it difficult to monitor the gradual increase in resistance in a specific species over time.

The automated susceptibility testing (AST) systems have gradually replaced the CLSI standard methods in most clinical laboratories. One of the important reasons is that all systems contain computer-based data-management systems. These systems allow the susceptibility test systems to directly interface with institutional LST system to report the antimicrobial test results. Direct data transfer not only saves time used for manual data input but also eliminates errors generated during result recording and data transferring.

Susceptibility Tests for Fastidious Organisms

Fastidious organisms such as *Streptococcus pneumoniae*, viridians streptococci, *Haemophilus* spp., *Neisseria gonorrhoeae, Helicobacter pylori*, and anaerobic bacteria require enriched media and special incubation conditions. For these organisms, sufficient growth cannot be achieved in unsupplemented Mueller–Hinton media normally used for susceptibility testing of rapid growing (grow in less than 24 h) aerobic and facultative anaerobic organisms (Jorgensen and Ferraro, 2000). In the reference test methods provided by CLSI, the media additives and incubation atmospheres are specified for testing some of these species while other test conditions and procedures for testing nonfastidious organisms are maintained.

Table 5.1 lists all the information about the media and methods recommended by CLSI for susceptibility testing of fastidious organisms.

Because the prevalence of resistance to recommended empirical treatment regimens for *N. gonorrhoeae* and *H. pylori* varies in different geographic locations and patient populations, selective susceptibility testing may be performed for special needs. Reference disk diffusion and broth dilution methods for testing *Haemophilus* spp. were developed by CLSI against appropriate antimicrobials. *Haemophilus* test medium (HTM), which is Mueller–Hinton broth or agar supplied with hemin, yeast extract, and nicotinamide adenine dinucleotide (NAD), is required for both methods. Surveillance studies of antimicrobial resistance in *H. influenzae* in North America showed that the majority of isolates of *H. influenzae* were resistant to ampicillin and amoxicillin by producing a TEM-type β-lactamase, and the prevalence of resistance to other antimicrobial agents such as the cephalosporins, β-lactamase inhibitor combinations, macrolides, tetracycline, chloramphenicol, trimethoprim-sulfamethoxazole, and the fluoroquinolones

TABLE 5.1. Media and methods recommended for testing commonly encountered fastidious organisms.

Organism/test method	Recommended medium	Incubation atmosphere
Haemophilus spp.		
Broth dilution	*Haemophilus* test medium	Ambient air
E-test	*Haemophilus* test medium	CO_2
Disk diffusion	*Haemophilus* test medium	CO_2
Neisseria gonorrhoeae		
Agar dilution	Gonococcus agar with XV-like supplement	5% CO_2
E-test	Gonococcus agar with XV-like supplement	5% CO_2
Streptococcus pneumonia and other *Streptococcus* spp.		
Broth dilution	MHB + 3% lysed horse blood	Ambient air
E-test	Mueller-Hinton agar + 3% lysed horse blood	5% CO_2
Disk diffusion	Mueller-Hinton agar + 3% lysed horse blood	5% CO_2
Anaerobic bacteria		
Agar dilution	*Brucella* agar + 5% sheep blood	Anaerobic
E-test	*Brucella* agar + 5% sheep blood	Anaerobic
Broth dilution	Wilkins–Chalgren	Anaerobic

remained low (Doern et al., 1999; Richter et al., 1999). Based on these studies, antimicrobial susceptibility testing of *Haemophilus* from nonsterile sources is not performed routinely in many clinical laboratories. Instead, a direct β-lactamase test is used to provide a rapid means of detecting ampicillin and amoxicillin resistance. Susceptibility testing of obligate anaerobic bacteria by agar dilution and broth dilution requires large inoculum (10^5 CFU/spot and 1×10^6 CFU/mL, respectively) and 48 h incubation. In most clinical laboratories, the E-test is selected as the primary testing method for anaerobic bacteria because of its convenience.

Detection of Specific Antimicrobial Resistance Mechanisms

Not all resistance phenotypes are readily detected by directly measuring MIC values. Some resistance mechanisms are poorly expressed *in vitro*. For some drugs, the resistance phenotype is only induced under certain conditions. The antibiotics tested may be poor substrates for detection of certain resistance mechanisms. In addition, because routine *in vitro* assays only test for the bacteriostatic activity of antimicrobials, the mechanisms of resistance that affect bacteriocidal activity are not detected. For these reasons, conventional broth or agar dilution MIC procedures are not able to detect the complicated resistance mechanisms. Special testing methods using alternative drugs and certain drug combinations are developed to test resistance among several common pathogens encountered in the clinical laboratory.

Extended-Spectrum β-Lactamases (ESBLs) in Some Enterobacteriaceae

Production of β-lactamases, the enzymes that destroy the β-lactam ring, is one of the major mechanisms adopted by Gram-negative bacteria to cause resistance to β-lactam drugs. There are many types of β-lactamases, which differ in their ability to inactivate a given β-lactam as well as in their susceptibility to β-lactamase inhibitors (Bradford, 2001a,b).ESBLs are plasmid-mediated β-lactamases that are capable of hydrolyzing all cephalosporins, penicillins, and aztreonam, but they are generally susceptible to β-lactamase inhibitors (clavulanate, sulbactam, tazobactam). These enzymes are evolved from point mutations around the active site of parental TEM and SHV β-lactamases that normally only inactivate ampicillin, penicillin, and carbenicillin. Production of ESBLs most commonly occur in *Escherrchia coli* and *Klebsiella* spp. but is also found in other members of Gram-negative bacteria, especially various species of *Enterobacteriaceae*. Development of ESBLs in *Enterobacteriaceae* reflects the process that the selective pressure on the β-lactamase producing bacteria posed by the β-lactams results in the continuous mutation of β-lactamases and expending their activities (Bradford, 2001). Because the genes mediating other resistance mechanisms often reside on the

same plasmid, the strains expressing ESBLs typically show multidrug resistance to aminoglycosides, tetracyclines, chloroamphenicol, and trimethoprim (Bradford, 2001).

In vitro, strains carrying ESBLs don't always show resistance to oxyimino-β-lactams under the standard testing conditions using CLSI break points (Katsanis et al., 1994; Paterson et al., 2001a,b). The activities of ESBLs depend on the substrate and inoculum size of the organism. β-lactamases produced by Gram-negative bacteria are accumulated in the periplasmic space to attack the β-lactam drugs before they interact with their targets. Ceftazidime, for instance, has difficulty reaching periplasmic space due to its large size and charge. When it is tested against ESBLs carrying bacteria *in vitro*, it is readily hydrolyzed by the enzyme, which results in a high MIC at the standard inoculum size. However, the resistance to cefotaxime can only be illustrated among ESBLs producing bacteria when the inoculum is increased 100-fold (Rice et al., 1991; Medeiros and Crellin, 1997; Thauvin-Eliopoulos et al., 1997). In patients with serious infections due to ESBL-producing bacteria, poor outcome with cephalosporin treatment has been well documented (Paterson et al., 2001).

CLSI/NCCLS developed the standard methods to screen for the presence of ESBLs initially among *Klebsiella pneumoniae, Klebsiella oxytoca*, and *Escherichia coli* (NCCLS, 2000), and later on expanded to *Proteus mirabilis* (CLSI 2005). The methods are based on MIC obtained through broth dilution or inhibition zone size in disk diffusion using the selective antimicrobial concentrations under the standard test conditions. The screening and confirmatory testing of drugs and interpretations have been well established (CLSI, 2005).

Screening for ESBLs-producing *Proteus mirabilis*, different MIC break points (ceftazidime MIC $\geq$2 μg/mL, cefotaxime MIC $\geq$2 μg/mL, or cefpodoxime MIC $\geq$2 μg/mL) are indicated. The use of more than one of the five antimicrobials increases the sensitivity of the screening (CLSI, 2005). Confirmation test consists of testing for the presence of inhibitory effect of clavulanic acid on cefotazidime and cefotaxime. In broth dilution, MICs of both drugs with or without the presence of 4 μg/mL clavulanic acid are determined. A threefold or more concentration decrease in an MIC for either antimicrobial in the presence of clavulanic acid is a positive result. Disk diffusion test uses disks containing 10 μg of ceftazidime or cefotaxime with or without the addition of 1 μg of clavulanic acid. An increase of equal to or more than 5 mm in the zone of inhibition by addition of clavulanic acid is considered to be a positive result. For all ESBL-producing strains, results of all penicillins, cephalosporins, and aztreonam are reported as resistant regardless of the MICs or inhibition zoom sizes produced in the tests. The ESBL confirmation tests recently became available for several commercial systems including E-test and all three automated systems. ESBL screening E-test strip is based on recognition of a reduction in ceftazidime MICs in the presence of 2 μg/mL clavulanic acid. A greater than 3 dilution reduction in the MIC is a positive result. The automated systems use either ceftazidime and cefotaxime alone and in combination with 4 μg/mL clavulanic acid. A predetermined growth reduction in wells containing the inhibitor compared with those containing drug alone indicates the presence of

ESBL. The ESBL confirmation test doesn't detect all ESBLs. Sometimes, other β-lactamases present in ESBL-containing organisms mask ESBL production in the phenotypic test (Bush, 2001). False-negative results of ESBL confirmation test are also reported for strains with ESBL presence (Queenan et al., 2004). Accurate detection of ESBLs in other species requires further investigation.

Fluoroquinolones and Salmonella

Fluoroquinolones are the first-line drug for treatment of serious *Salmonella* infections. A decrease in susceptibility of fluoroquinolones among clinical *Salmonella* isolates has been observed (Parry, 2003; Rabatsky-Ehr et al., 2004). This change in susceptibility is suspected to associate with an increased usage of fluoroquinolones for treatment of *Salmonella* infections in both humans and animals. Cases of human ciprofloxacin treatment failure have been reported (Chandel and Chaudhry, 2001). Fluoroquinolone resistance is mediated by the mutations occurring in DNA gyrase and topoisomerase 4, which are the targets of quinolones (Casin et al., 2003). The *Salmonella* strains that are resistant to nalidixic acid are found to have higher fluoroquinolone MICs compared with nalidixic acid–susceptible strains. Genetic studies of *Salmonella* strains with or without nalidixic acid resistance in the correlation with the fluoroquinolone treatment outcomes suggest that fluoroquinolones may have an impaired effect for treating *Salmonella*-caused infections that have been determined to be fluoroquinolone susceptible by using CLSI break points. These strains often have only one mutation in the *gyrA* gene. It is speculated that additional mutations that arise during the treatment may result in resistance (Albayrak et al., 2004). It is recommended that clinical microbiology labs should test for the presence of nalidixic acid resistance, using either broth dilution or disk diffusion, for extraintestinal isolates of *Salmonella* that are susceptible to fluoroquinolones. For isolates that test susceptible to fluoroquinolones and resistant to nalidixic acid, the physician should be informed that the isolate may not be eradicated by fluoroquinolones (CLSI, 2005).

Methicillin Resistance in Staphylococci

Methicillin resistance in clinical isolated staphylococci is mostly mediated through acquisition of *mecA* gene encoding a mutant penicillin binding protein (PBP)2a by bacterial genome. PBPs are the enzymes that catalyze the reaction that cross-links the peptidoglycan of the bacterial cell wall. Binding of PBP to β-lactam antimicrobials inhibits the enzyme activity and prevents bacteria growth by interfering with cell wall formation. In contrast to the PBPs in methicillin-susceptible strains, which have high affinity for most β-lactam antimicrobials, PBP2a has low affinity for binding β-lactams. In methicillin-resistant strains, the essential function of PBP is undertaken by PBP2a to maintain survival of the bacterium in the presence of antimicrobials (Chambers, 2003). Heterogeneity is an important feature of methicillin-resistant staphylococci. The level of resistance varies according

to the culture conditions and β-lactams being used. Under routine susceptibility test conditions, most clinical isolates exhibit this heterogeneous pattern of resistance in which the majority of the cells are susceptible to methicillin and only a small proportion of cells show resistance (Chambers, 1997). Change in culture conditions such as prolonged incubation, growth in culture medium supplied with 2∼4% NaCl, and replacing methicillin with oxacillin in test have been shown to greatly increase the sensitivity of detection for resistant isolates (Chambers, 1993, 1997).

Due to the heterogeneous nature of the methicillin resistance, testing of the presence of *mecA* gene by PCR remains the most sensitive method for identification of resistant isolates, although this molecular method is unable to detect *mecA*-negative methicillin-resistant strains (Tomasz et al., 1989). Based on the detection of *mecA* gene product PBP2a, a latex agglutination test with latex particles coated with monoclone antibodies was also developed and reported to have similar sensitivity and specificity to PCR (van Griethuysen et al., 1999; Sakoulas et al., 2001; Louie et al., 2002).

Modification of several conventional testing conditions is necessary for MIC methods to reliably detect methicillin-resistant staphylococci. The modification includes replacing methicillin with oxacillin because of its better stability at storage and high sensitivity in detection of heteroresistance, addition of 2% NaCl to the standard testing medium, preparing bacterial suspension by direct colony suspending, maintaining the incubation temperature of tests at no more than 35°C, and extending the incubation time to a full 24 h. Even with the modified conditions, the oxacillin disk diffusion method was reported to have low specificity compared with broth-based methods (Unal et al., 1994; York et al., 1996; Ghoshal et al., 2004). Separated interpretation criteria have been established for methicillin-resistant *Staphylococcus aureus*, *Staphylococcus lugdunensis,* and other coagulase-negative staphylococci based on their differences in the sensitivity to oxacillin/methicillin. When using the break point for coagulase-negative staphylococci, CLSI MIC methods have low specificity detecting *mecA*-positive low-level methicillin-resistant strains (Gradelski et al., 2001). For serious infections with coagulase-negative staphylococci other than *S. epidermidis*, testing for *mecA* gene or PBP2a protein is necessary for isolates with oxacillin MICs of 0.5 to 2 μg/mL. *mecA* or PBP2a negative strains with oxacillin MIC of $\leq 2\mu g/mL$ are reported as oxacillin susceptible. For methicillin/oxacillin resistant staphylococci, some β-lactams may appear active *in vitro* but are not effective clinically. Results for all those drugs should be regarded as resistant (Chambers, 1997).

Expression of *mecA* gene is regulated by two transcriptional regulators *mecI* and *MecR1* located immediately upstream from the *mecA* promoter in staphylococci (Ryffel et al., 1992; Suzuki et al., 1993). *MecI* is a DNA binding protein that represses the transcription from *mecA* promoter. *MecR1* is a signal transducer that is responsible for activating *mecA* gene transcription in the presence of β-lactams. The mechanism of *mecA* gene activation by β-lactams is postulated to be the result of cleavage of *MecI* protein by activated *MecR1* gene product (Ryffel

et al., 1992; Suzuki et al., 1993). In addition to *MecI* and *MecR1*, two β-lactamase regulatory elements *blaI* and *blaR1* that have similar molecular organization and function as *MecI* and *MecR* are also involved in the transcriptional regulation of *mecA* gene expression (Gregory et al., 1997; Zhang et al., 2001). By comparing the relative induction of *mecA* gene expression through BlaR1 and MecR1 by different β-lactams, cefoxitin is found to be a better inducer of the regulatory system than penicillins (McKinney et al., 2001). Based on these results, a disk diffusion screening test for prediction of *mecA*-mediated resistance using a 30 μg cefoxitin disk for staphylococci has been developed. Using standard disk diffusion testing conditions and 24 h incubation, *S. aureus* with cefoxitin disk diffusion zones of ≤19 mm should be reported as oxacillin resistance and those for which cefoxitin zones are ≥20 mm should be reported as oxacillin susceptible. Coagulase-negative staphylococci for which cefoxitin disk diffusion zones are ≤24 mm should be reported as oxacillin resistant and those for which cefoxitin zones are ≥25 mm should be reported as oxacillin susceptible compared to PBPII detection assay. The cefoxitin disk test is equivalent in sensitivity and specificity for *S. aureus* but shows higher specificity and equal sensitivity to oxacillin disk diffusion for coagulase-negative staphylococci (Boutiba-Ben Boubaker et al., 2004).

To screen for methicillin-resistant *S. aureus*, oxacillin-salt agar is very useful (Sakoulas et al., 2001). The agar is the Mueller–Hinton agar containing 4% NaCl and 6 μg/mL oxacillin. The bacteria suspension equal to 0.5 McFarland turbidity prepared from colonies grown on plate is inoculated on the agar as a spot 10 to 15 mm in diameter. After culturing in ambient air at 35°C 24 for h, any amount of growth is considered to be resistant.

Recent studies of E-test, Vitek, Microscan, and Phoenix showed comparable sensitivity and specificity compared with the reference MIC methods in testing staphylococcal species (Sakoulas et al., 2001; Caierao et al., 2004; Horstkotte et al., 2004; Tveten et al., 2004; Nonhoff et al., 2005). However, low specificity has been reported for less commonly encountered coagulase-negative species (Gradelski et al., 2001; Caierao et al., 2004).

Inducible Clindamycin Resistance in Staphylococci and Streptococci

Some staphylococcal and streptococcal strains that are resistant to erythromycin and susceptible to clindamycin may have inducible clindamycin resistance, referred to as macrolide–lincosamide–streptogramin B (MLS_B) resistance, due to the presence of erythromycin ribosomal methylase *erm*(A) or *erm*(B) (Hamilton-Miller and Shah, 2000). This family of enzymes methylates the N-amino group of adenine residue 2058 in 23S rRNA, which prevents access of the antimicrobial to its binding site on the ribosome. The resistance is referred to as macrolide–lincosamide–streptogramin resistance, as it affects the activities of all three drug groups (Eady et al., 1993; Siberry et al., 2003). The second mechanism that

produces macrolide resistance in staphylococci is mediated by *msr*(A) gene. The gene encodes an ATP-dependent efflux pump that only confers resistance to macrolides and streptogramin B but not to lincosamide, such as clindamycin (Eady et al., 1993; Siberry et al., 2003). Although some strains that have either *erm* gene or *msr*(A) show similar susceptibility pattern for macrolides and clindamycin (i.e., resistant to macrolides but susceptible to clindamycin in standard susceptibility testing), the concern of clindamycin treatment failure for erm-positive organisms warrants the need of distinguishing the *erm* gene containing strains from the *msr*(A) gene containing strains (Eady et al., 1993; Drinkovic et al., 2001; Siberry et al., 2003).

The phenotypic testing for detection of MLS_Bi in staphylococci and streptococci is referred to as "D" test. In this double disk diffusion test, a 15 μg erythromycin disk and 2 μg clindamycin disk are placed on the plate in the area streaked for confluent growth, with a distance from disk edge to disk edge of 15∼20 mm for staphylococci or 12 mm for streptococci. After incubation at 35°C for 16∼20 h, the presence of MLS_Bi results in a flattened shape of the clindamycin zone (D-zone). The standard "D" test requires the test to be done on Mueller–Hinton agar for staphylococci or on Mueller–Hinton with 5% sheep blood agar for streptococci with a 0.5 McFarland standard bacteria suspension. Studies also show that the "D" test performed on sheep blood agar inoculum purity plates used with the VITEK 2 also detects the MLS_Bi reliably in staphylococci (Jorgensen et al., 2004). The strain that shows a positive "D" test is reported as presumptive resistant to clindamycin, and clindamycin may still be effective in some patients.

Staphylococcus aureus with Decreased Susceptibility to Vancomycin

Clinical emergence of vancomycin intermediate or resistant *S. aureus* has been reported, even though the prevalence of the strains with reduced vancomycin susceptibility is still low (Srinivasan et al., 2002; Liu and Chambers, 2003). Although the molecular mechanism of the intermediate resistance in staphylococci is not yet established, studies have suggested that a novel mechanism that differs from the one used by enterococci are employed by staphylococci (Walsh and Howe, 2002) Currently, CLSI defines staphylococci with vancomycin MIC of ≤ 4 μg/mL as susceptible, isolates with MIC of vancomycin 8∼16 μg/mL as intermediate, and isolates with vancomycin MIC of ≥ 32 μg/mL as resistant. Accordingly, VISA and VRSA refer to *S. aureus* with a vancomycin MIC of 8–16 μg/mL and a MIC of ≥ 32 μg/mL, respectively. Detection of VISA requires MIC methods with 24 h incubation. Disk diffusion tests with the standard 30 μg vancomycin disk have been shown to have low sensitivity (Tenover et al., 1998). At present, identification of VISA strains is based on the three criteria proposed by the Centers for Disease Control and Prevention (CDC): broth microdilution MIC of 8–16 μg/mL, E-test MIC of ≥ 6 μg/mL, and growth on commercial brain heart infusion agar

screen plates containing 6 μg/mL vancomycin within 24 h (Tenover et al., 1998). Based on the performance of the different methods in testing three vancomycin-resistant *S. aureus* clinical isolates collected by CDC, none of the three automated systems can detect resistance in all three isolates consistently. However, all three isolates are successfully identified as vancomycin resistant by the standard broth or agar dilution method with overnight incubation, the Mueller–Hinton agar plate containing 6 μg vancomycin, and E-test (Centers for Disease Control and Prevention, www.CDC.gov/ncidod/dhgp/ar_visavrsa_labFAQ_html#5).Like MRSA, heteroresistance to vancomycin was observed in some *S. aureus* strains sensitive to vancomycin measured by MIC methods. In these strains, a subpopulation of cells are capable of growing on the brain–heart infusion agar plates containing more than 4 μg/mL of vancomycin (Hiramatsu et al., 1997). Although the heteroresistant strains may be associated with treatment failure, and may be precursors of VISA, proper methods for detection of these strains have not been developed.

Vancomycin-Resistant Enterococci

Vancomycin resistance in enterococci is classified by six types, VanA, VanB, VanC, VanD, VanE, and VanG, according to their inducibility, antimicrobial specificity, and levels of resistance. Production of pentapeptide other than *N*-acetylmuramyl-L-Ala-D-Glu-Lys-D-Ala-D-Ala, which is found in vancomycin-sensitive enterococci for synthesis of peptidoglycan, is the major mechanism in all six types (Pootoolal et al., 2002). D-Ala-D-Ala is the target of glycopeptide antibiotics. The interaction between dipeptide and glycopeptides inhibits the action of transpeptidases that cross-linked peptidoglycan necessary for the appropriate tensile strength required by the organism. Synthesis of peptidoglycan terminating in either D-Ala-D-lactate or D-Ala-D-Ser in resistant organisms abolishes glycopeptide binding (Pootoolal et al., 2002). Enterococcus strains carrying *VanA* produce the peptidoglycan terminated with D-Ala-D-lactate (Roper et al., 2000). They show a high level of vancomycin resistance (MIC $\geq$64 μg/mL) with teicoplanin resistance (MIC $\geq$16 μg/mL). VanB phenotype is expressed as moderate to high level vancomycin resistance (MIC 6–512 μg/mL) without teicoplanin resistance. They also produce the peptidoglycan terminating with D-Ala-D-lactate (Garnier et al., 2000). Both VanA and VanB phenotypes are inducible and responsible for the vancomycin resistance in most *E. faecalis* and *E. faecium*. VanC phenotype shows low-level vancomycin MICs ranging from 2 to 32 by generating the peptidoglycan terminating with D-Ala-D-Ser (Navarro and Courvalin, 1994). It is identified in *E. gallinarum* and *E. casseliflavus. VanD* gene confers constitutive intermediate to high level resistance to *E. faecium* (Perichon et al., 1997). Phenotypes VanD and VanG are found in *E. faecalis* (Fines et al., 1999; McKessar et al., 2000). Vancomycin screening agar, which is brain–heart infusion (BHI) agar containing 6 μg vancomycin recommended by NCCLS, shows high sensitivity in detection of *vanA, vanB*, or *vanC* types of resistance. MIC methods including conventional broth or agar dilution with overnight incubation, E-test, and automated systems are generally reliable for detection of vancomycin resistance.

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6
Biochemical Profile-Based Microbial Identification Systems

JABER ASLANZADEH

Introduction

The first step in microbial identification is the phenotypic assessment of the growing colony. In many cases, the colonial morphology such as color, shape, size, hemolytic reaction, and growth characteristics on various selective and differential media can place an organism in a single family, genus, or even species level. In fact, assessing the ability of an organism to grow in various laboratory media and their oxygen requirement coupled with Gram-stain morphology and a few rapid tests such as catalase, oxidase, coagulase, and indole can provide preliminary identification for most of the clinically significant isolates. For example, it is very likely that an organism that grows on MacConkey agar plate and ferments lactose is a member of the family Enterobacteriaceae or that an oxidase-positive non–lactose fermenting Gram-negative rod that has distinct grape odor is *Pseudomonas aeruginosa.*

Overall, the biochemical identification tests may be classified into two major groups: the conventional microbial identification systems and commercial microbial identification systems. The identification schemes among the various laboratories are not uniform in part due to the availably of numerous choices, varied complexity of the testing laboratories, volume, experience of technical staff, and cost. In general, most laboratories rely on a combination of both conventional and commercial identification systems. Figures 6.1–6.6 presents flow charts for presumptive identification of clinically significant organisms.

Conventional Microbial Identification Systems

Single-Enzyme Rapid Tests

The single-enzyme rapid tests are a group of tests that detect the presence or absence of a single enzyme or a biochemical reaction within seconds to minutes. These tests are fairly inexpensive, easy to perform, and often provide important initial information that is used to determine the subsequent steps in the microbial identification scheme. Rapid enzyme tests are an important part of both conventional as well as

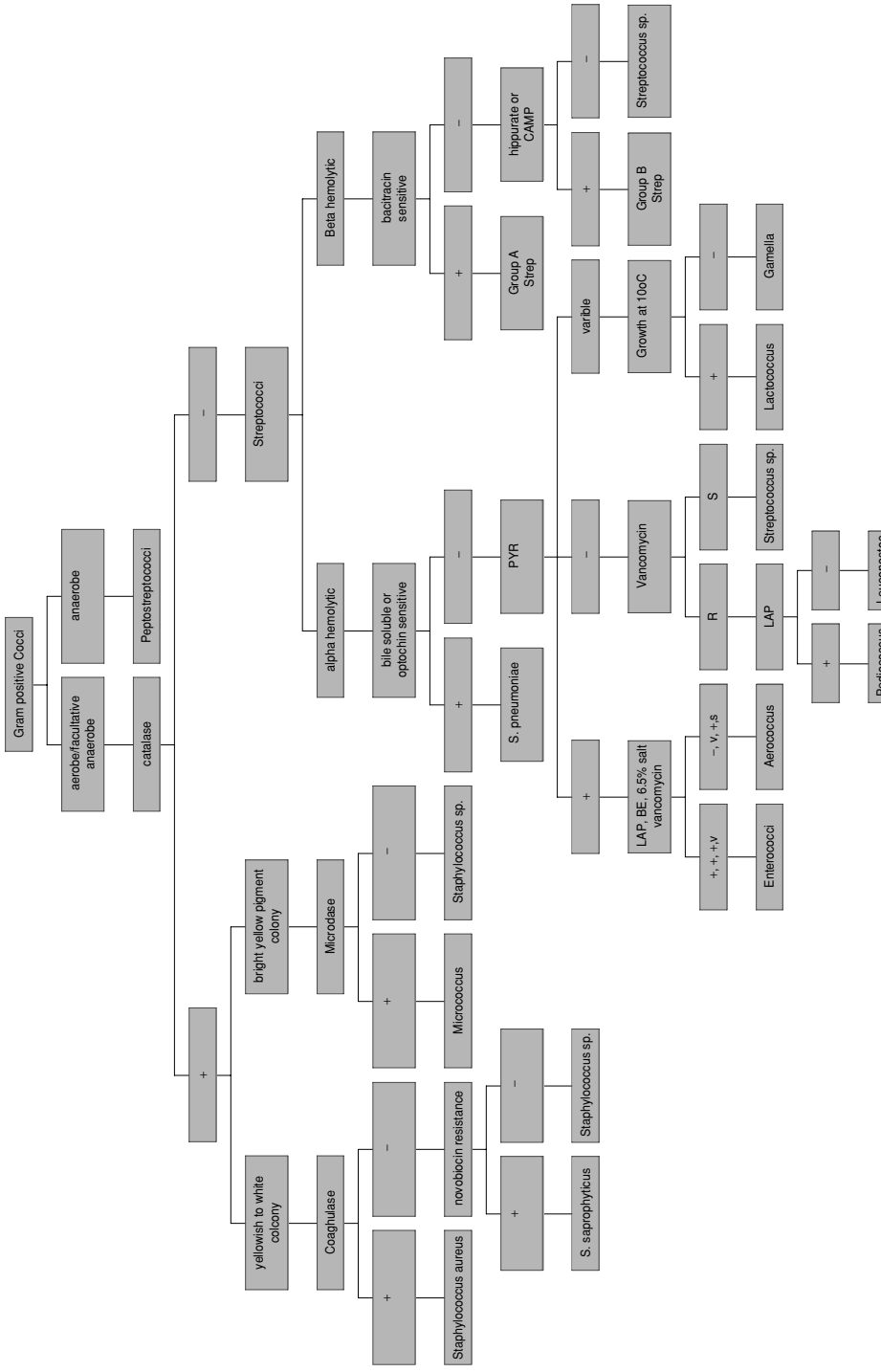

FIGURE 6.1. Flow chart for presumptive identification of Gram positive cocci.

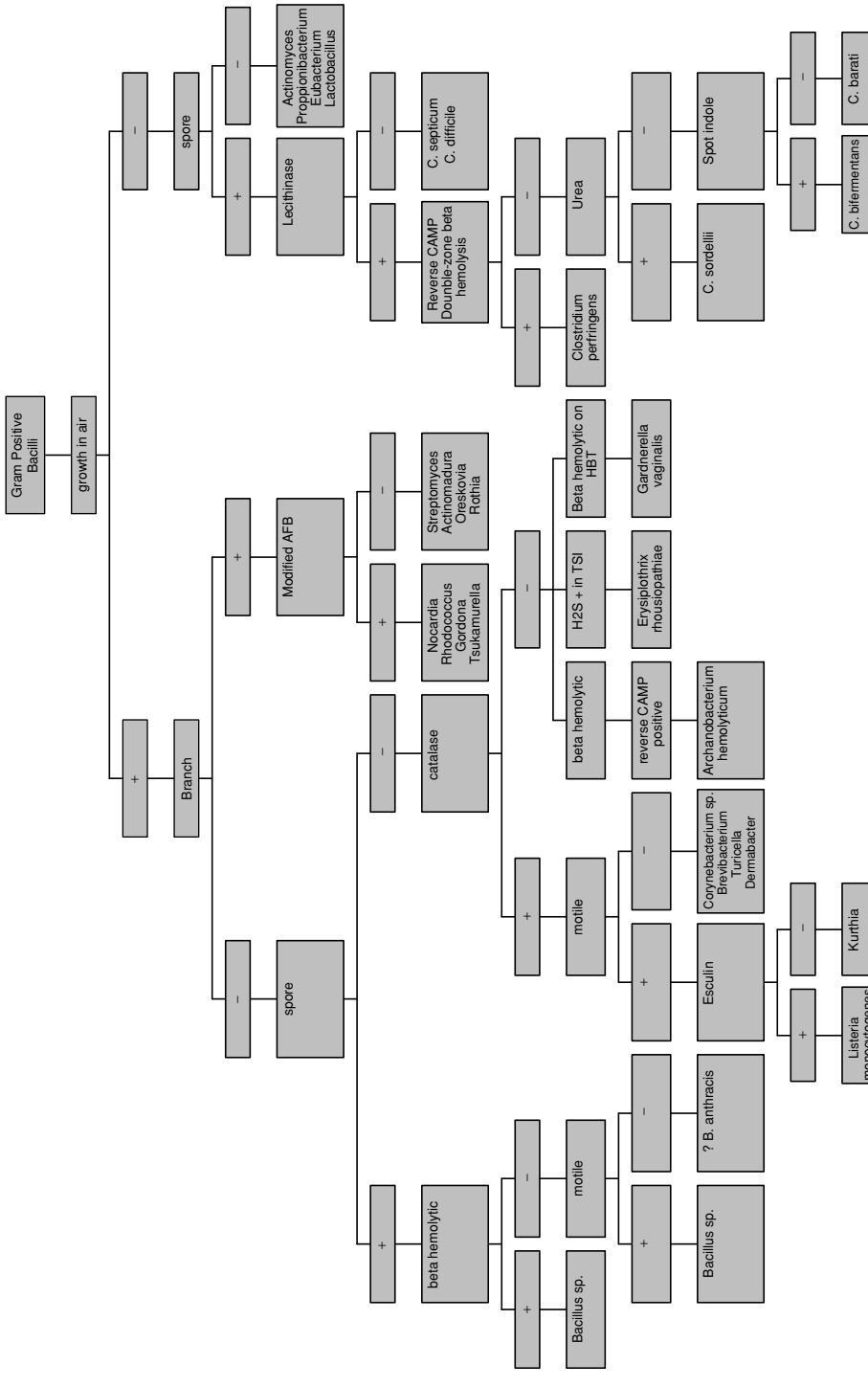

FIGURE 6.2. Flow chart for presumptive identification of Gram positive bacilli.

FIGURE 6.3. Flow chart for presumptive identification of aerobic and facultative anaerobic oxidase negative Gram negative rod.

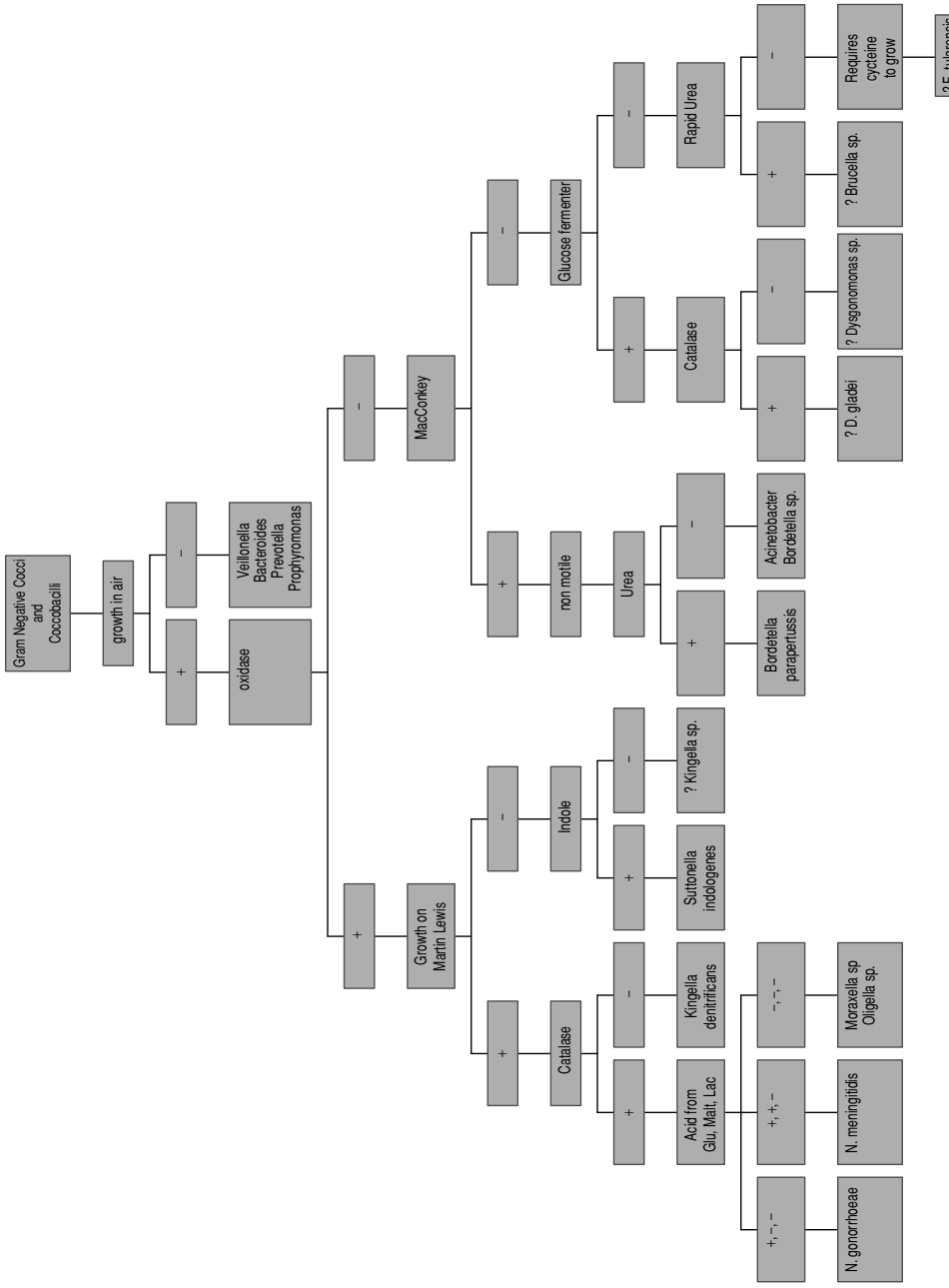

FIGURE 6.4. Flow chart for presumptive identification of Gram negative cocci and cocobacilli.

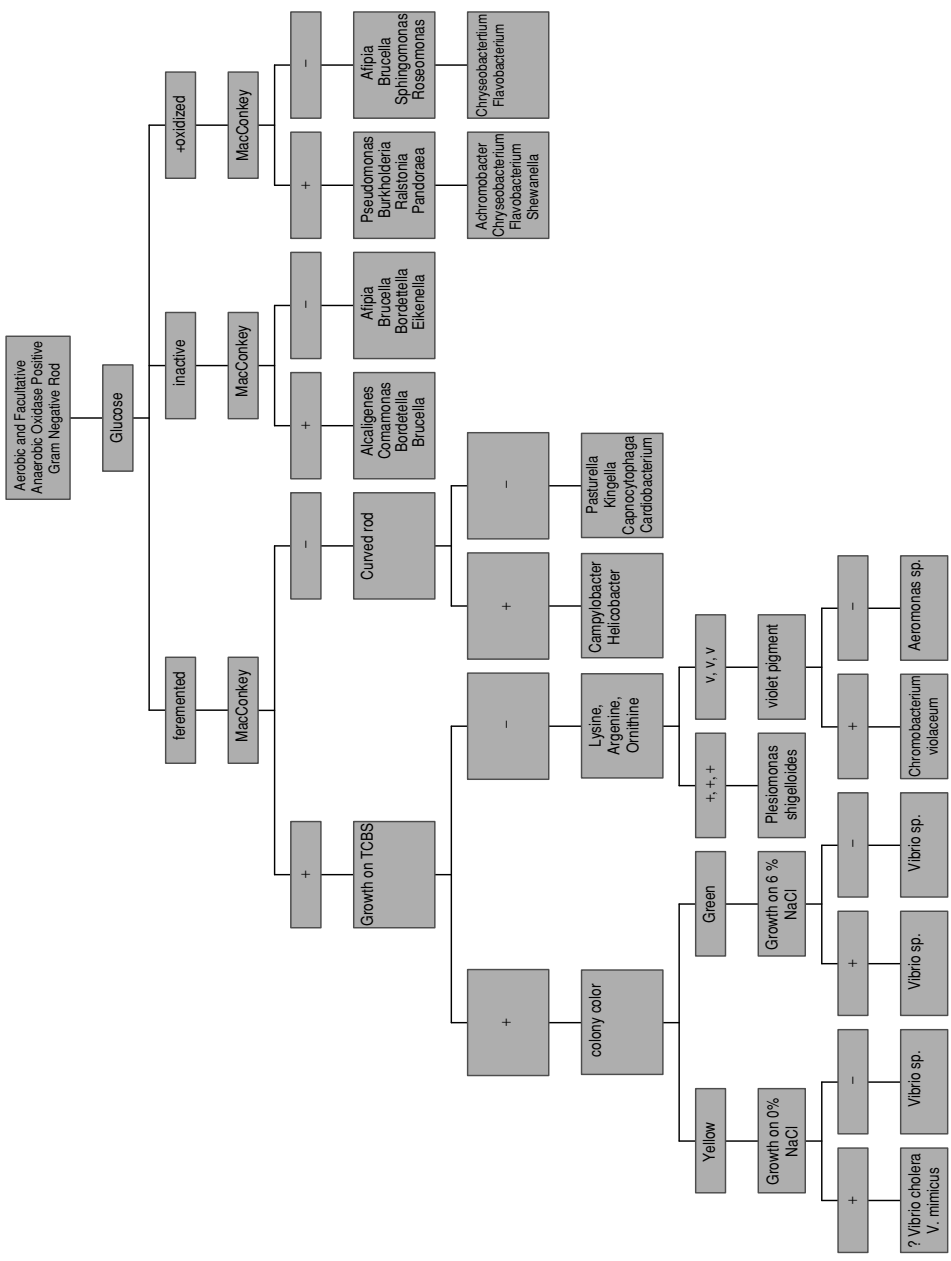

FIGURE 6.5. Flow chart for presumptive identification of aerobic and facultative anaerobic oxidase positive Gram negative rod.

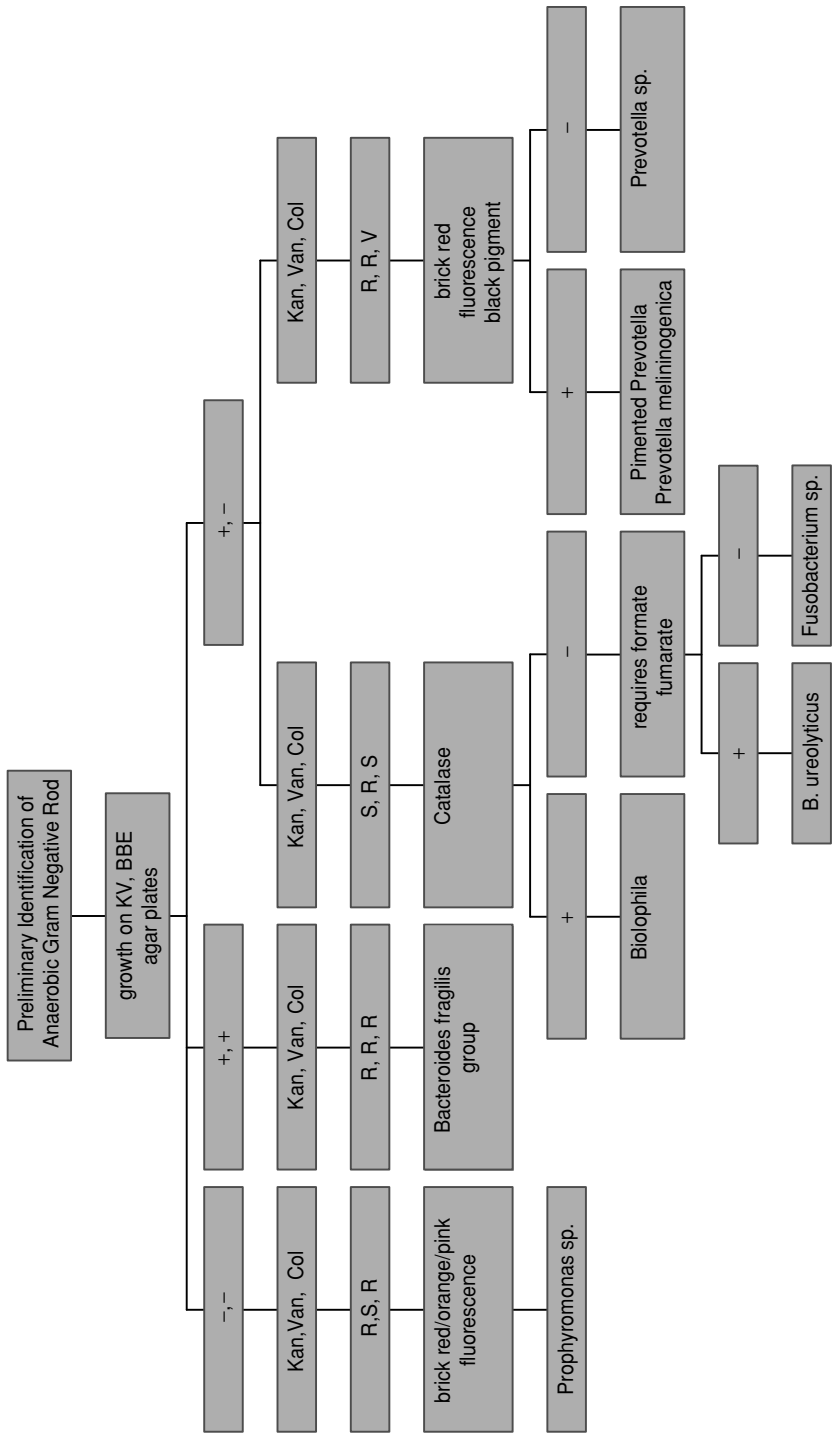

FIGURE 6.6. Flow chart for presumptive identification of anaerobic Gram negative rod.

commercial microbial identification systems. In addition, these tests may be used for presumptive identification of certain organisms to the genus or even species level. For example, a positive catalase test can establish if a Gram-positive cocci is staphylococci or a positive coagulase test can determine if a catalase positive cocci is *S. aureus.*

Catalase Test

Catalase, an enzyme within the cytochrome enzyme system, is responsible for the decomposition of hydrogen peroxide (H_2O_2) formed during aerobic respiration. All organisms using the cytochrome system of respiration will give a positive catalase reaction when tested. Those organisms using a different system will not produce catalase and will yield a negative reaction.

The mechanism of action is as follows:

$$H_2O_2 + \text{catalase} = H_2O + \tfrac{1}{2}O_2$$

The possession of the catalase enzyme helps to distinguish staphylococci from streptococci and is useful in the identification of many other bacteria. A positive test is a bubbling reaction caused by the release of O_2 from the H_2O_2 in the presence of catalase. A negative test is the absence of any bubbling reaction.

Despite the simplicity of the test, false-positive reaction is seen if the test is performed on colonies selected from blood agar plate (BAP), colonies selected from the first quadrant of a blood culture plate, or use of nickel loops (Koneman et al., 1997; MIDI, 2004).

Oxidase Test

The oxidase test is based on the production of the enzyme indophenol oxidase by organisms containing cytochrome C. Indophenol oxidase, in the presence of atmospheric oxygen, oxidizes a redox dye (*N,N,N′,N′*-tetramethyl-*p*-phenylene diamine dihydrochloride) to form a dark-purple indophenol compound.

Filter paper impregnated with the reagent is allowed to dry completely; smear of a loopful of bacteria from a nonselective plate is placed onto the paper with an inoculating loop and examined for development of a violet or purple color (positive reaction). No color change indicates a negative result.

Wire loops containing iron may give a false-positive reaction and reactions from weak oxidase-positive organisms may be inaccurate. Colonies growing on selective media or differential media containing glucose cannot be used for oxidase determination because fermentation inhibits indophenol oxidase activity resulting in false negative results (Koneman et al., 1997; Becton Dickinson, 2003).

Spot Indole Test

The indole test is based on the ability of an organism to hydrolyze tryptophane to glycine and indole. Certain organisms are able to remove the glycine radical from

tryptophane resulting in the production of indole. This test can be performed on organisms grown on a BAP after 24 h incubation.

Filter paper is placed in a Petri plate and saturated with 3–4 drops of 1% solution of *p*-dimethylaminocinn-amaldehyde. Isolated colony(ies) from a 24 h culture grown on a BAP is rubbed into the filter paper with a wooden applicator stick or inoculating loop. Appearance of a blue color immediately or within 30 s of inoculation indicates a positive reaction; no blue color within 30 s indicates a negative reaction.

The test must be performed from BAP. False-negative results will occur from MacConkey agar and TSI slants because there is not a sufficient source of tryptophane in these media, and false positives will occur if indole-positive organisms are present in mixed cultures (Forbes et al., 2002).

Slide Coagulase Test

Coagulase is a thermostable enzyme found primarily in *S. aureus* and is used to differentiate *S. aureus* from other commonly isolated staphylococci. Two forms of coagulase exist: one is bound to the cell wall, and one is liberated by the cell as "free coagulase." Slide coagulase test detects the bound coagulase (clumping factor), which acts directly on the fibrinogen in plasma and causes clumping of bacteria. Slide coagulase test results agree approximately 96% with tube coagulase test results. Coagulase-positive organism forms clumps within 10 s, but coagulase-negative organism remains uniformly suspended.

Using a sterile pipette, a drop of sterile saline is placed on a glass slide. One to two colonies of the organism is emulsified in the saline and tested for autoagglutination. A drop of rabbit plasma is placed on the slide and mixed for few seconds and observed for clumping within 10 s.

A positive slide coagulase test result is valid only for strains of *Staphylococcus* spp. that are negative for autoagglutination or stickiness. Coagulase is also present in *S. intermedius* and *S. hyicus*, but these species are infrequent clinical isolates. Similarly, clomping factor is produced by *S. scheiferi* and *S. lugdunensis* and may give false-positive reactions (Isenberg, 1992; Koneman et al., 1997).

Microdase

Microdase disk is a reagent-impregnated disk used in the differentiation of *Staphylococcus* from *Micrococcus* by the detection of the oxidase enzyme. In the presence of atmospheric oxygen, the oxidase enzyme reacts with tetramethyl-*p*-phenylenediamine (TMPD) in the disk and cytochrome C in the organism to form a colored compound. All micrococci contain cytochrome C, whereas most staphylococci lack cytochrome C. The oxidase reagent substantiates the presence of type C cytochrome.

Microdase disk is placed on a glass slide and inoculated with several isolated colonies. The disk is examined for up to 2 min for development of a blue color (positive reaction). No color change or a white to gray color after 2 min is considered a negative reaction.

Microdase is not designed for routine testing for oxidase activity in organisms other than *Staphylococcu*s and *Micrococcus*. *Staphylococcus sciuri* is the only *Staphylococcus* species recognized to give a positive microdase reaction (Murray et al., 2003).

Bile Solubility Test

Gross morphology alone is often unreliable to differentiate between *Streptococcus pneumoniae* and the viridans streptococci. *S. pneumoniae* lyse when treated with a 10% solution of sodium desoxycholate, whereas other streptococci and Gram-positive cocci are not bile soluble. Lysis occurs because bile-soluble organisms contain autolytic amidase that when activated by bile salts cleaves the bond between alanine and muramic acid in the cell wall.

One drop of desoxycholate is placed on a well-isolated 18–24 h culture of an alpha-hemolytic colony on a BAP and incubated at room temperature, agar side down for 15 min. The area where the reagent was applied is examined for evidence of colony disintegration or lysis. Dissolving of the colony is a positive test for *S. pneumoniae*. Colonies remaining intact is a negative test for *S. pneumoniae*.

False-negatives may occur when testing isolates older than 18–24 h. Occasionally, alpha-hemolytic colonies do not dissolve but merely lift off the surface of the agar, float away, and settle elsewhere on the plate. The plate should be carefully examined for evidence of this (Isenberg, 1992; Pratt-Rippin and Pezzlo, 1992).

PYR

PYR is a chromogenic substrate (L-pyrrolidonyl-β-naphthylamide, or PYR) which when hydrolyzed by PYRase (L-pyrroglutamyl-peptide hydrolase) produces a red color upon the addition of a specific reagent. PYR is a substrate that is hydrolyzed by 100% of the enterococci and group A streptococci but not by any other streptococcal species.

Two to 4 drops of a buffer reagent is applied to the PYR test strip circle. The strip is then inoculated with 3–5 colonies of the organism and incubated at room temperature for 2 min. Two drops of a second reagent is applied to the to the test strip circle. An intense red color develops immediately around the colonies in the presence of hydrolyzed PYR. The PYR test is negative if no color, an orange color or a weak pink-color develops.

Staphylococci may cause a positive PYR reaction (Isenberg, 1992; Forbes et al., 2002; Murray et al., 2003).

Leucine Aminopeptidase (LAP) Test

The LAP test is a rapid assay for the detection of the enzyme leucine aminopeptidase in bacteria cultured on laboratory media. It is used as one of the tests for the presumptive identification of catalase negative Gram-positive cocci. Leucine-β-naphthylamide impregnated discs serve as a substrate for the detection of leucine aminopeptidase. Following hydrolysis of the substrate by the enzyme, the resulting

β-naphthylamine produces a red color upon the addition of cinnamaldehyde reagent.

Moistened LAP disk is placed on a glass slide or in a Petri dish and inoculated with isolated colonies of catalase negative, Gram-positive cocci. The disk is incubated at room temperature for 5 min before a drop of the color developer is added and examined for up to 1 min for a pink to red color development. Pink/red color indicates a positive reaction. No color change/slight yellow indicate a negative reaction.

The LAP test is only part of the overall scheme for identifying catalase-negative, Gram-positive cocci. Further biochemical characterization and serological grouping may be necessary for specific identification. False-negatives may result from using too small an inoculum (Coleman and Ball, 1984).

Indoxyl Butyrate Disk

Moraxella catarrhalis produces the enzyme butyrate esterase. This property can be used as a rapid test in the identification of *M. catarrhalis*. Indoxyl is liberated from indoxyl butyrate by the enzyme butyrate esterase, forming an indigo color in the presence of oxygen.

Smear several colonies of oxidase-positive, Gram-negative diplococci across the disk surface using a loop or wooden applicator and observe for a blue-green color development within 5 min where the colonies were applied indicating a positive test for butyrate esterase production. A negative reaction is indicated by no color change.

Interpretation of results is based on testing only oxidase-positive, Gram-negative diplococci. Some strains of *Moraxella* spp. other than *M. catarrhalis* may produce a positive or weak positive reaction. *Acinetobacter, Staphylococcus,* and *Pseudomonas* may also yield a positive reaction (Murray et al., 2003).

Neisseria Enzyme Test (NET)

The *Neisseria* enzyme test consists of three synthetic chromogenic substrates contained in a single tube to detect preformed enzymes associated with three pathogenic *Neisseria* species.

Oxidase-positive, Gram-negative diplococci growing on Martin–Lewis agar are transferred to the NET tube and incubated for 30 min. Specific color reactions confirm the identity of *N. lactamica* (blue) and *N. meningitidis* (yellow). If neither color develops, a drop of PRO reagent is added. Development of a pink-red color indicates the isolate is *N. gonorrhoeae*; absence of a colored product, or a pale yellow color, is presumptive for *Moraxella catarrhalis*. The identification of *M. catarrhalis* can be confirmed by a positive *M. catarrhalis* butyrate test. The active chemical ingredients used in the tube and the enzymatic reactions detected are

A. 5-bromo-4-chloro-indoyl-β-D-galactopyranoside. Hydrolysis of the β-D-galactoside bond by β-galactosidase yields a blue color from the colorless substrate.

B. Gamma-glutamyl-para-nitroanilide. Hydrolysis of this substrate by gamma-glutamyl aminopeptidase releases yellow *p*-nitroaniline from the colorless substrate.
C. L-proline-beta-naphthylamide. Hydrolysis of this substrate by prolyl-aminopeptidase releases colorless free beta-naphthylamine derivative. Coupling of the beta-naphthylamine derivative with a diazo dye coupler (*o*-aminoazotoluene diazonium salt—Fast Garnet, GBC Salt) by adding a drop of PRO reagent produces a pink to red color.

The NET should be used on Gram-negative diplococci isolated from media such as Martin–Lewis agar. Do not use on isolates only grown on nonselective media such as chocolate agar because other *Neisseria* species (*N. sicca*, *N. mucosa*) may grow and lead to incorrect results. Similarly, *Kingella* species may be found on Martin–Lewis medium. It is essential to perform a Gram stain prior to selecting organisms for identification by NET. If the morphology of the organism selected is questionable, it is suggested that a catalase test be performed. *Kingella* species are catalase negative, and *Neisseria* and *Moraxella* species are catalase positive. *N. cinerea* will be pink after the addition of PRO reagent (D'Amato et al., 1978).

Hippurate

The hippurate hydrolysis test may be used to identify *Campylobacter jejuni, Gardnerella vaginalis, Listeria monocytogenes*, or to differentiate *Streptococcus agalactiae* from other beta-hemolytic streptococci. The assay is based on hydrolysis of the sodium hippurate by the enzyme hippuricase to sodium benzoate and glycine. Glycine is detected by oxidation with ninhydrin reagent that results in production of a deep-purple color.

Hippurate tubes are inoculated with a heavy suspension of the organism and incubated at 35°C for 2 h. The tube is then inoculated with 0.2 mL of ninhydrin and reincubated for additional 10 to 15 min. The presence of a deep-purple color indicates a positive hippurate and no color change indicates a negative hippurate.

A light inoculum or use of an old culture may give false-negative results (Forbes et al., 2002; Murray et al., 2003).

Lysostaphin

The lysostaphin test is used to differentiate members of *Staphylococcus* spp. from Micrococcus spp. based on the activity of lysostaphin, which cleaves the interpeptidic pentaglycine bridges of peptidoglycan. These cross-bridges are found in all *Staphylococcus* spp. but not in *Micrococcus* spp. or *Stomatococcus* spp.

A suspension of the organism equivalent to a 3.0 McFarland is prepared and 0.2 mL of the working lysostaphin solution is added to the tube and mixed. The tube is allowed to stand undisturbed for 2 h at 35°C. Clearing of the solution indicates susceptibility to lysostaphin. Turbid solution indicates resistance to lysostaphin. *Micrococcus, Stomatococcus*, and *Streptococcus* spp. are resistant to lysostaphin.

Reading the test beyond the 2 h incubation may result in false-positive tests (Koneman et al., 1997).

CLO Test

The CLO test is a rapid test for identification of *Helicobacter pylori*. The test is a sealed plastic slide holding an agar gel that contains urea, phenol red, buffers, and bacteriostatic agents. If the urease enzyme of *H. pylori* is present in the inserted gastric tissue biopsy, the urea in the gel is degraded resulting in an increased pH, and the color of the gel changes from yellow to a bright magenta.

Inoculate the CLO test slide with the specimen and incubate at 37°C in the non-CO_2 incubator for 3 h. The slide is examined for color change from yellow to magenta pink after 1 h of incubation and again at 2 h and 3 h. A magenta pink color indicates a positive reaction. If the biopsy contains urease, the change first appears around the sample and eventually colors all of the gel. The pH change in a positive test is first seen at the interface of the gel and the biopsy. If a significant amount of urease is present, the visible change is rapid. Any color change of the whole gel to a shade other than yellow (i.e., red, magenta, pink, deep-orange) indicates the presence of *H. pylori.* The test is considered negative if the medium remains yellow 24 h after insertion of the biopsy.

False-negative CLO tests may occur when very low numbers of *H. pylori* are present or if the bacteria are focally distributed. False-positive CLO tests can occur in patients with achlorhydria. Commensal organisms such as *Proteus* spp. that also produce urease will grow in the absence of acid (Delta West Ply, 2001).

Overnight Biochemical Tests

The overnight biochemical tests are a group of tests that require inoculating one or more culture media containing specific substrates and chemical indicators that detect pH change or specific microbial by-product. Similar to rapid tests, the choice of overnight tests is based on Gram-stain morphology and the results of preliminary testing with rapid enzyme tests. These tests are also inexpensive and easy to perform and may be used in three different ways. They may be used to obtain important initial information with respect to the identity of an unknown organism, such as the MILS test, which is used to screen for the presence of enteric pathogens. They may be used to verify the results of a preliminary positive/negative test or they may be used to assess an indeterminate finding. For example, Taxo P is an overnight test that will demonstrate if an isolate with an equivocal bile solubility result is *S. pneumoniae.* Similarly, a tube coagulase test will substantiate if a suspicious isolate, that is slide coagulase negative, is truly a coagulase-negative staphylococci. Finally, these tests may be used as the sole identification system (classical biochemical identification) to identity an unknown organism. This is generally labor intensive and requires the technologist to inoculate, incubate, read, interpret, and chart a number of biochemical reactions over several days. This is then followed by using various identification schemes or flow charts to generate

a final identification. As a rule, the classical biochemical identification system is used to identify fastidious or slow-growing organisms in the reference laboratories. These isolates are by and large rare biotypes that are not part of the commercial identification system's database. Table 6.1 depicts the list of biochemical tests that are commonly used to identify Gram-negative bacilli (Weyant et al., 1996).

Tube Coagulase Test

Tube coagulase test detects free coagulase (liberated by the cell) that acts on prothrombin to produce a thrombin-like product that then acts as fibrinogen to form a fibrin clot.

Prepare a heavy suspension of the *Staphylococcus* colonies in 0.5 mL of water. Place the suspension into a tube containing rabbit plasma and incubate at 35°C for 4 h. Examine for the presence of a clot. If negative for a clot, reincubate the tube and reexamine at 24 h. Any degree of clot formation at 4 h or 24 h is considered a positive reaction. No clot formation at 24 h is considered negative coagulase reaction (Koneman et al., 1997; Forbes et al., 2002; Murray et al., 2003).

DNA Hydrolysis

The DNA hydrolysis test detects the presence of enzyme DNase in an organism. Using this media, DNase-positive coagulase-positive staphylococci are differentiated from other *Staphylococcus* spp. The media contains either toluidine blue or methyl green, which upon hydrolysis of the incorporated DNA turns colorless.

The media is inoculated with the organism and incubated overnight at 35°C. The plate is examined for evidence of growth and loss of color (positive reaction). No color change indicates a negative reaction (Murray et al., 2003).

Vancomycin Disk Test

The vancomycin disk test is performed as a susceptibility procedure to help differentiate the Gram-positive, catalase-negative cocci. *Aerococcus*, *Gemella*, *Lactococcus*, *Streptococcus*, and some enterococci are susceptible to vancomycin. *Leuconostoc, Pediococcus, Lactobacillus*, and some enterococci are resistant to vancomycin.

A 0.5 McFarland suspension of the organism is prepared in sterile saline. Using a sterile swab, the bacterial suspension is inoculated onto a BAP. A vancomycin disk is placed in the center of the inoculated plate and incubated at 35°C in a CO_2 incubator for 18–24 h. The plate is observed for the presence of a zone of inhibition around the vancomycin disk. *Leuconostoc* spp., *Pediococcus* spp., *Lactobacillus* spp., and some *Enterococcus* spp. are resistant to vancomycin with growth to the edge of the disk $\leq$9 mm. *Aerococcus* spp, *Gemella* spp., *Lactococcus* spp., *Streptococcus* spp., and some *Enterococcus* spp. are susceptible to vancomycin and produce a zone of inhibition $\geq$12 mm (Koneman et al., 1997; Murray et al., 2003).

TABLE 6.1. Commonly used biochemical tests for identification of a Gram-negative organism.

	Date						
Biochemicals	1	2	3	4	5	6	7
Motility							
OF glucose (oxid)							
OF glucose (Ferm)							
Xylose							
Mannitol							
Lactose							
Sucrose							
Maltose							
Catalase							
Oxidase							
MacConkey							
Citrate							
Sodium acetate							
Urea							
Nitrate							
Nitrate to gas							
Indole							
TSI slant							
TSI butt							
H_2S (TSI butt)							
H_2S (Pb ac paper)							
Gelatin							
Pigment							
Arginine							
Lysine							
Growth at 42°C							

Bacitracin Inhibition Test (Taxo A Disk)

The bacitracin inhibition test presumptively differentiates group A streptococci (GAS) from other beta-hemolytic streptococci. The bacitracin at concentration of 0.04 units will selectively inhibit growth of GAS. Although there are rare strains of GAS that are bacitracin resistant, approximately 5% to 10% of strains of non–group A beta hemolytic streptococci (b, C, and G) are bacitracin susceptible.

Using a pure culture of the test organism, inoculate a BAP with the bacterial suspension. Place a bacitracin disk in the center of the inoculated BAP and incubate at 35°C for 18–24 h. Any zone of inhibition around the bacitracin disk is considered a positive test. Uniform lawn of growth right up to the rim of the disk indicates a negative bacitracin inhibition test (Isenberg, 1992; Koneman et al., 1997; Murray et al., 2003).

Taxo P Disks (Optochin)

Hydrocupreine hydrochloride (optochin) at the concentration 5.0 μg inhibits the growth of *S. pneumoniae*, but not of other streptococci. *S. pneumoniae* may, therefore, be differentiated from other alpha-hemolytic streptococci by the formation of a zone of inhibition around a disk impregnated with this compound.

Inoculate a BAP with a pure culture of the alpha-hemolytic *Streptococcus* isolate. Place a Taxo P disk (optochin) onto the inoculated plate and incubate the plate aerobically at 35°C for 24 h or as needed to obtain good growth. Incubation in a CO_2 enriched atmosphere will enhance growth but reduce zone size. Zones of inhibition of 14 mm or more are formed with pure cultures of *S. pneumoniae.* Other organisms may show zone sizes less than 14 mm in diameter. A diameter between 6 and 14 mm is questionable for *S. pneumoniae* and the strain should be tested for bile solubility (Murray et al., 2003).

CAMP Test

The CAMP test is based on the fact that group B streptococci produce a protein-like compound known as the CAMP factor that acts synergistically with a staphylococcal beta-hemolysin (β-lysin) on sheep erythrocytes to produce an enhanced zone of hemolysis.

Streak a loopful of β toxin–producing *S. aureus* in a straight line across the center of a BAP. Streak a loopful of group B streptococci perpendicular to and nearly touching the streak line of the staphylococci (positive control). Streak a loopful of group A streptococci perpendicular to and nearly touching the streak line of the staphylococci (negative control). Streak a loopful of unknown isolate perpendicular to and nearly touching the streak line of the staphylococci and incubate the plate at 35°C for 24 h in the aerobic non–CO_2 incubator. Following the incubation, if the patient isolate demonstrates an arrowhead zone of enhanced hemolysis, the isolate is identified as group B streptococci. If the patient isolate does not demonstrate an arrowhead of enhanced hemolysis, the isolate is not group B streptococci.

Do not incubate the CAMP test plate in the presence of 5–10% CO_2 incubator. This may result in an incorrect interpretation (Wilkinson, 1977; Isenberg, 1992).

Reverse CAMP Test

The reverse CAMP test is based on the fact that some organisms such as *Arcanobacterium haemolyticum* completely inhibit the effect of *Staphylococcus aureus* B-hemolysin on sheep erythrocytes. The β-hemolysin inhibition zone in the form of a triangle is formed.

A loopful of β toxin–producing *Staphylococcus aureus* is streaked in a straight line across the center of a BAP. Group B streptococci and group A streptococci are streaked perpendicular to and nearly touching the streak line of the staphylococci. Similarly, *A. haemolyticum* and the test isolate are streaked perpendicular to and nearly touching the line of the staphylococci. The plate is incubated at 35°C for 24 h in the aerobic non-CO_2 incubator. Following the incubation, if the test isolate demonstrates a triangular-shaped inhibition of β-hemolysis, it is a reverse camp test positive. If the test isolate does not demonstrate a triangle-shaped inhibition of β-hemolysis, it is a reverse camp test negative.

Do not incubate the reverse CAMP test plate in the 5–10% CO_2 incubator. This may result in an incorrect interpretation (Wilkinson, 1977; Isenberg, 1992).

Bile Esculin Agar Slant

Group D streptococci (including *Enterococcus* spp.) and a few other bacteria, such as *Listeria* spp., can grow in the presence of 40% bile and also hydrolyze esculin to esculetin. Esculetin reacts with ferric ions, supplied by ferric citrate in the agar medium, to form a diffusible black complex. Most strains of viridans streptococci that are capable of hydrolyzing esculin will not grow in the presence of 40% bile.

Streak the surface of the bile esculin agar slant with several colonies of the organism to be tested. Incubate at 35°C in non-CO_2 for 24 to 48 h. A diffuse blackening of more than half of the slant within 24 to 48 h is considered positive. No growth or growth without blackening of the medium after 48 h is considered negative test.

If the inoculum is too heavy, viridans streptococci may give a false-positive test result. Approximately 3% of viridans streptococci are able to hydrolyze esculin in the presence of bile. Growth in the presence of 6.5% salt is used to differentiate enterococci from non-enterococcal group D streptococci (Isenberg, 1992; Koneman et al., 1997).

6.5% Salt Broth

Trypticase soy broth is a general-purpose medium for the cultivation of both fastidious and nonfastidious organisms. With the addition of 6.5% sodium chloride, the medium can be used to differentiate between salt-tolerant and salt-intolerant organisms. It is especially useful for distinguishing *Enterococcus* spp., which are

salt-tolerant, from non-enterococcal group D streptococci, such as *S. bovis* and *S. equinus*.

Inoculate the tube containing 6.5% sodium chloride with the organism and incubate at 35°C in non-CO_2 for 24–48 h. A visible growth (turbidity) is considered positive and no growth is considered negative.

If the medium is inoculated too heavily, the inoculum may be interpreted as growth, resulting in a false-positive reaction. *Aerococcus, Pediococcus, Staphylococcus,* and up to 80% of group B *Streptococcus* can grow in 6.5% salt broth. In addition, *Aerococcus* may also be bile esculin positive (Isenberg, 1992; Koneman et al., 1997).

Indole Test

Indole, a benzyl pyrrole, is one of the metabolic degradation products of the amino acid tryptophan. Bacteria that possess the enzyme tryptophanase are capable of hydrolyzing and deaminating tryptphan with the production of indole, pyruvic acid, and ammonia. The indole test is based on the formation of a red color complex when indole reacts with the aldehyde group of *p*-dimethylaminobenzaldehyde, the active chemical in Kovac's reagent. In order to perform this test, the organism must be grown on a medium rich in tryptophan such as indole nitrite broth.

Inoculate the indole nitrite broth medium with 2–3 colonies of the organism to be tested. Incubate the tubes at 35°C in a non-CO_2 incubator for 24–48 h. Examine the tubes for growth. When the broth is visibly turbid, use a sterile pipette to transfer 3 mL into a sterile tube. Add 1 mL of xylene to the contents of the tube, which extracts the indole, if present, from the broth into the xylene. Wait 1–2 min, and add 0.5 mL Kovac's reagent and observe for the production of a pink to red color in the xylene layer. A pink to red color at the interface the of the reagent and the broth within seconds after the addition of Kovac's reagent indicates a positive reaction. No color change indicates a negative reaction (Koneman et al., 1997).

Nitrite Test

Organisms that reduce nitrate have the ability to extract oxygen from nitrates to form nitrites and other reduction products. The presence of nitrites in the medium are detected by the formation of a red diazonium dye, *p*-sulfobenzeneazo-α-naphthylamine, following the addition of α-naphthylamine and sulfanilic acid. If no color develops after adding the reagents, this indicates that nitrates have not been reduced (a true negative reaction) or that they have been reduced beyond the oxidation level of nitrite to products such as ammonia, nitrogen gas (denitrification), nitric oxide (NO), or nitrous oxide (N_2O) and hydroxylamine. Because the test reagents detect only nitrites, the latter process would lead to a false-negative result. Therefore, it is necessary to add a small amount of zinc dust to all negative reactions. Because zinc ions reduce nitrates to nitrites, the development of a red color after adding zinc dust indicates the presence of nitrates and confirms a true negative reaction.

Using a sterile inoculating loop, an indole nitrite broth medium is inoculated with 2–3 colonies of the organism to be tested and incubated at 35°C in a non-CO_2 incubator for 24–48 h. When the broth is visibly turbid, 3 mL of the broth culture is transferred into a sterile tube and 5 drops of *N,N*-dimethyl-α-naphthylamine (nitrate reagent A) is added to the broth. Five drops of sulfanilic acid (nitrate reagent B) is then added to the broth and observed for the production of a pink to red color within 30 s. If no color change occurs within 30 s, a small amount of zinc dust is added and the production of a pink to red color within 10 min is looked for (Koneman et al., 1997).

ALA (*Haemophilus influenzae* Porphyrin Test)

The porphyrin test is used in the rapid speciation of *Haemophilus* by separating those species that require an exogenous source of X factor from those that do not. *Haemophilus* species (*H. parainfluenzae* and *H. parahemolyticus*) that produce the enzyme porphobilinogen synthase have the ability to synthesize heme (factor X) and therefore do not require an exogenous source of factor X for growth. Porphobilinogen and porphyrin, precursors in heme synthesis, can be detected in an enzyme substrate inoculated with a porphobilinogen synthase producing *Haemophilus* spp. by the addition of modified Ehrlich's (Kovac's) reagent or by examination with a Wood's lamp.

Suspend a loopful of organism in 0.5 mL of the enzyme substrate. Incubate at 35°C for 4 h if the suspension is heavy or 18–24 h if the suspension is light. After incubation add an equal volume of modified Ehrlich's (Kovac's) reagent and vortex the mixture. Allow the substrate and reagent to separate. After the addition of Kovac's reagent, a red (pink) color will form in the aqueous phase, indicating the presence of porphobilinogen, and therefore a positive test for *Haemophilus* spp. not requiring factor X. Alternatively, a Wood's lamp can be used to detect fluorescence in the reagent phase, indicating the presence of porphyrins, also a positive test. No coloration or fluorescence indicates a factor X dependent *Haemophilus* spp. and a negative test (Killian, 1974).

Motility Indole Lysine (MILS)

MILS medium is a semisolid medium useful in the identification of members of the Enterobacteriaceae, specifically for screening suspicious colonies from stool cultures for potential pathogens.

It is used to demonstrate motility, indole production, lysine decarboxylase and deaminase activity, and hydrogen sulfide production. A small amount of agar is added to the media for demonstration of motility along a stab line of inoculation. Growth of motile organisms extends out from the line of inoculation, whereas nonmotile organisms grow along the stab line.

The pH indicator bromcresol purple is used to facilitate detection of decarboxylase activity. When inoculated with an organism that ferments dextrose, acids are produced that lower the pH, causing the indicator in the medium to change from purple to yellow. The acidic pH also stimulates enzyme activity. Organisms that

possess a specific decarboxylase degrade the amino acid provided in the medium, yielding a corresponding amine. Lysine decarboxylation yields cadaverine. The production of these amines elevates the pH and causes the medium in the bottom portion of the tube to return to a purple color. The medium in the upper portion of the tube remains acidic because of the higher oxygen tension. Lysine deamination produces a color change in the upper portion of MILS medium. Oxidative deamination of lysine yields a compound that reacts with ferric ammonium citrate, producing a burgundy-red color in the top of the medium. (The bottom portion of the medium remains acidic.) This reaction can only be detected if lysine decarboxylation is not produced, which is the case with *Proteus*, *Morganella*, and *Providencia* species.

Indole is produced in MILS medium by organisms that possess the enzyme tryptophanase. Tryptophanase degrades the tryptophan present in the casein peptone, yielding indole. Indole can be detected in the medium by adding Kovac's reagent to the agar surface. MILS medium is also used in the demonstration of hydrogen sulfide production. Hydrogen sulfide, which is produced by some enteric organisms from sulfur compounds contained in the medium, reacts with ferric ion, producing a characteristic black precipitate (BD Microbiology Systems, 1999).

ONPG (*O*-Nithrophenyl-beta-D-Galactopyranoside) Test

In order for an organism to ferment lactose, it must have the enzymes permease to transport the lactose inside the cell and beta-galactosidase to cleave the transported sugar. Some organisms (delayed lactose fermenters) though possessing beta-galactosidase do not have the enzyme permease. These organisms can utilize the enzyme beta-galactosidase to hydrolyze ONPG. ONPG is a colorless compound similar to lactose. In the presence of beta-galactosidase, ONPG is hydrolyzed to galactose and a yellow compound *o*-nitrophenyl.

Inoculate an ONPG broth tube with the organism and add the ONPG disk and incubate at 35°C. Periodically examine the color change for up to 24 h. Yellow color indicates a positive reaction and no color change indicates a negative reaction (Murray et al., 2003).

Methyl Red (MR) Test

This assay determines if an organism metabolizing pyruvic acid utilizes mixed acid pathway and produces acid end products that are detected by the indicator methyl red.

A 5 mL MR-VP broth tube is inoculated with the organism and incubated at 35°C for 48 h, then 2.5 mL of the broth culture is transferred to a fresh tube and inoculated with 5 drops of methyl red indicator. Positive MR is indicated if the methyl red reagent remains red. Negative result is indicated if the reagent turns yellow-orange (Koneman et al., 1997; Murray et al., 2003).

Voges–Proskaure (VP) Test

Organisms such as *Klebsiella*, *Enterobacter*, and *Serratia* spp. that utilize the butylenes glycol fermentation pathway produce acetoin, an intermediate in the fermentation of butylenes glycol. The VP test detects the production of acetoin by these organisms. In the presence of air and potassium hydroxide, acetoin is oxidized to diacetyl, which produces a red-colored complex. The addition of alpha-naphtol increases the sensitivity of the test.

A 5 mL MR-VP broth tube is inoculated with the organism and incubated at 35°C for 18–24 h, then 2.5 mL of the broth culture is transferred to a fresh tube and inoculated with 6 drops of alpha naphtol followed by 3 drops of KOH. A positive result is indicated by the presence of a red color that develops within 15 min. No color change indicates negative VP (Koneman et al., 1997; Murray et al., 2003).

Pseudosel Agar Slant

Pseudosel agar is a medium used for the identification of *Pseudomonas aeruginosa*. Magnesium chloride and potassium sulfate in the medium enhance the production of pyocyanin, a blue-green, water-soluble, nonfluorescent phenazine pigment. *P. aeruginosa* is the only Gram-negative rod known to excrete pyocyanin. In addition to the promotion of pyocyanin production, pseudosel agar also enables the detection of fluorescent products by some *Pseudomonas* species other than *P. aeruginosa*. Streak the surface of the pseudosel agar slant, and incubate at 35°C in non-CO_2 for 18–24 h. A blue-green pigmentation surrounding the growth on the agar slant indicates a positive reaction. No pigmentation indicates a negative reaction.

Negative pseudosel slants should be examined under short wavelength (254 nm) ultraviolet light to check for fluorescent products produced by some *Pseudomonas* species. *Pseudomonas aeruginosa* typically produces fluorescein as well as pyocyanin (BD Microbiology Systems, 1992).

Urea Agar Slant

Microorganisms that possess the enzyme urease are capable of hydrolyzing urea, which releases ammonia. This reaction raises the pH of the medium and is detected by phenol red, which turns pink-red above pH 8.0. The color change first appears in the slant because the oxidative decarboxylation of amino acids in the air-exposed portion of the medium enhances the alkaline reaction. The color change eventually spreads deeper into the medium.

Streak the surface of the urea agar slant with a heavy inoculum of a pure culture. Incubate at 35°C in non-CO_2 for 18 to 24 h. Production of intense pink-red color on the slant, which may penetrate into the butt, is considered a positive reaction. No color change indicates negative a reaction.

The medium is not specific for urease. The utilization of peptones or other proteins in the medium by some urease-negative organisms may raise the pH due

to protein hydrolysis and release of amino acid residues, resulting in false-positive reactions (Koneman et al., 1997; BD Microbiology Systems, 1992).

Citrate Agar Slant

Some organisms have the ability to utilize citrate, an intermediate metabolite in the Krebs cycle, as the sole external source of carbon. These organisms also utilize inorganic ammonium salts in the medium as the sole source of nitrogen. The resulting production of ammonia creates an alkaline environment that turns the bromthymol blue indicator to an intense blue.

Using an inoculating loop, select a well-isolated colony with and streak the surface of the citrate slant (do not stab the agar) and incubate at 35°C in non-CO_2incubator and examine daily for up to 4 days. Growth with an intense blue color on the agar slant indicates a positive reaction and no growth and no color change (green) indicates a negative reaction.

Luxuriant growth on the slant without an accompanying color change may indicate a positive test. This should be confirmed by incubating the tube for an additional 24 h. The biochemical reaction requires oxygen. Therefore, the medium should not be stabbed, and the cap must be kept loose during incubation. Carry-over of protein and carbohydrate substrates from previous media may provide additional sources of carbon and therefore cause false-positive reactions (BD Microbiology Systems, 1992).

Cetrimide Agar

Cetrimide agar is a selective differential medium used for the identification of *P. aeruginosa*. The principle of the test is to determine the ability of an organism to grow in the presence of cetrimide. Cetrimide acts as a detergent and inhibits the growth of most other organisms. The iron content of the medium stimulates the production of pyocanin and fluorescent yellow-green pigment by this organism.

Using an inoculating loop, select a well-isolated colony with and streak the surface of the cetrimide slant (do not stab the agar) and incubate at 35°C in non-CO_2 incubator and examine daily for up to seven days. Growth on the agar slant indicates positive reaction and no growth indicates a negative reaction (BD Microbiology Systems, 1992; Forbes et al., 2002).

Gelatin

The gelatin test is used to identify bacteria that produce the proteolytic enzyme gelatinase. Organisms that produce gelatinase are capable of hydrolyzing gelatin and cause it to lose its gelling characteristics. A gelatin tube may be inoculated with the organism and incubated at 35°C in ambient air. The tubes are then removed daily and incubated at 4°C to check for liquefaction. Alternatively, strips of exposed but undeveloped x-ray film are placed in the bacterial suspension equivalent to at least 2 McFarland standard and incubated at 35°C in a non-CO_2 incubator for 48 h.

The strip is examined after 24 h and 48 h for loss of gelatin coating that leave the radiograph clear (Murray et al., 2003).

Acetate Utilization

Some organisms have the ability to utilize acetate as a sole external source of carbon. Acetate slants contain a mixture of salts and sodium acetate in a medium without organic nitrogen. Organisms that can utilize acetate as a sole carbon source break down sodium acetate causing the pH of the medium to shift toward the alkaline range, turning the bromthymol blue indicator blue. Organisms that cannot utilize acetate as a sole carbon source do not grow on the medium. Acetate differential agar is useful in the differentiation of *Neisseria* and *Moraxella* spp.

Streak the surface of the acetate differential agar slant (do not stab the agar), with a colony and cap the tube loosely. Incubate at 35°C in non-$CO_2$and examine daily for up to 7 days. Growth with an intense blue color on the agar slant indicates a positive test and no growth or no color change (green) indicates a negative test.

Luxuriant growth on the slant without an accompanying color change may indicate a positive test. This should be confirmed by incubating the tube for an additional 24 h. The biochemical reaction requires oxygen. Therefore, the medium should not be stabbed, and the cap must be kept loose during incubation. Carry-over of protein and carbohydrate substrates from previous media may provide additional sources of carbon and therefore cause false-positive reactions (BD Microbiology Systems, 1992).

Lead Acetate for Hydrogen Sulfide Detection

Some organisms are capable of enzymatically liberating sulfur from sulfur-containing amino acids or inorganic sulfur compounds. The released hydrogen sulfide reacts with lead acetate to yield lead sulfide, an insoluble black precipitate. Lead acetate is the most sensitive H_2S indicator reagent and is useful with organisms that produce trace amounts of H_2S, especially organisms that are not in the family Enterobacteriaceae. Inoculate a TSI medium with the isolate (stab once through the center of the butt of the tube to within 3 to 5 mm of the bottom, withdraw the inoculating needle, and streak the surface of the TSI agar slant). Place the lead acetate strip so that it hangs down approximately 1″ inside the TSI tube. Incubate at 35°C in non-CO_2 for 18 to 24 h. A brownish-black coloration of the paper strip indicates a positive reaction. No coloration of the strip indicates a negative reaction.

Lead acetate is toxic to bacterial growth. Do not allow the strip to touch the medium. The TSI medium must support the growth of the test organism for H_2S production to occur (Koneman et al., 1997; Murray et al., 2003).

Lysine Iron Agar (LIA)

Lysine iron agar is a differential medium used for the identification of enteric bacilli based on their ability to decarboxylate or deaminate lysine and produce

hydrogen sulfide. Dextrose serves as a source of fermentable carbohydrate. The pH indicator, bromcresol purple, is changed to a yellow color at or below pH 5.2 and is purple at or above pH 6.8. Ferric ammonium citrate and sodium thio sulfate are indicators of hydrogen sulfide formation. Lysine serves as the substrate for detecting the enzymes lysine decarboxylase and lysine deaminase. Lysine iron agar is designed for use with TSI (tripe sugar iron agar) for the identification of enteric pathogens.

Using a sterile inoculating needle, stab the butt of the LIA slant twice then streak back and forth along the surface of the agar with the organism. Incubate at 35°C ± 2°C in non-CO_2 for 18 to 24 h.

Alkaline (purple) reaction in the butt indicates lysine decarboxylation; red slant indicates lysine deamination, and black precipitate indicates H_2S production. H_2S may not be detected in this medium by organisms that are negative for lysine decarboxylase activity because acid production in the butt may suppress H_2S formation. For this reason, H_2S producing *Proteus species* do not blacken this medium (BD Microbiology Systems, 1992).

Triple Sugar Iron (TSI) Agar Slant

TSI agar is a medium that differentiates Gram-negative bacilli on the basis of the ability to ferment carbohydrates and liberate hydrogen sulfide (H_2S). The medium contains 1 part glucose to 10 parts each of lactose and sucrose. Phenol red serves as an indicator to detect pH change, and ferrous sulfate detects the formation of H_2S. If the organism ferments glucose, the butt and slant of the agar will become acidic and turn yellow. If the organism ferments lactose and/or sucrose, the slant will remain acidic (yellow). If the organism is unable to ferment lactose or sucrose, the slant will revert to alkaline (red) when the glucose is used up and alkaline amines are produced in the oxidative decarboxylation of peptides (derived from protein in the medium) near the surface of the agar. Organisms unable to ferment glucose will not change the pH of the medium or will produce alkaline products, and the TSI tube will remain red. Blackening of the medium indicates H_2S production. Gas production is indicated by splits or cracks in the butt of the agar. Gas may also push the agar up the tube.

Using a sterile inoculating needle, stab the butt of the LIA slant twice then streak back and forth along the surface of the agar with the organism. Incubate at 35°C ± 2°C in non-CO_2 for 18 to 24 h. If acid slant–acid butt (yellow–yellow): glucose and sucrose and/or lactose fermented. If alkaline slant–acid butt (red–yellow): glucose fermented only. If alkaline slant–alkaline butt (red–red): glucose not fermented. The presence of black precipitate (butt) indicates hydrogen sulfide production, and presence of splits or cracks with air bubbles indicates gas production.

Early readings may result in false acid–acid results, and delayed readings may result in false alkaline–alkaline results. Copious amounts of H_2S may mask the glucose reaction. If this occurs, glucose has been fermented even if it is not observable. The utilization of sucrose may suppress the enzyme mechanism that results in the production of H_2S. Trace amounts of H_2S may not be detectable with the

ferrous sulfate indicator in the agar (BD Microbiology Systems, 1992; Koneman et al., 1997).

Phenylalanine Deaminase

This assay is used to detect the ability of an organism to oxidatively deaminate phenylalanine to phenylpyrovic acid. The phenylpyrovic acid is detected by adding a few drops of 10% ferric chloride.

Inoculate a phenylalanine agar slant with the organism and incubate at 35°C in non-CO_2 incubator for 18–24 h. Following the incubation, add 4–5 drops of 10% ferric chloride solution to the slant. The development of green color on the surface of the slant indicates a positive reaction. No color change indicates a negative reaction (Isenberg, 1992; Murray et al., 2003).

Decarboxylase

Decarboxylases are a group of substrate-specific enzymes that are capable of reacting with the carboxyl (COOH) portion of amino acids, forming alkaline-reacting amines. Each decarboxylase enzyme is specific for an amino acid. Lysine, ornithine, and arginine are the three amino acids used routinely in the identification of Enterobacteriaceae, *Aeromonas*, *Plesiomonas*, and *Vibrio* species. The decarboxylation of lysine and ornithine yield cadaverine and putrescine, respectively. Arginine is converted to citrulline by a dihydrolase reaction. A control tube containing the base without an added amino acid to verify that the organism utilizes glucose must accompany all decarboxylase tests. Because decarboxylation is an anaerobic reaction, it must be overlaid with mineral oil prior to incubation. If the organism is viable, both the control and the test tube with amino acid should turn yellow because of fermentation of the small amount of glucose in the medium. If the amino acid is decarboxylated, the alkaline amines cause the indicator (bromcresol purple) in the acid medium to revert back to its original purple color.

Inoculate a Moeller decarboxylase broth containing ornithine, lysine, and/or arginine. Overlay the contents of all tubes with 1 mL of sterile mineral oil and incubate in a non-CO_2 incubator at 35°C for 18–24 h. Examine for a color change. Negative reactions are examined daily for no more than 4 days (BD Microbiology Systems, 1992).

OF Glucose Medium

Bacteria can utilize glucose and other carbohydrates by using various metabolic cascades. Some are fermentative routes; others are oxidative. Oxidation-fermentation (OF) medium permits classification of organisms by a simple method that differentiates aerobic and anaerobic degradation of carbohydrates. The low protein to carbohydrate ratio in the medium prevents neutralization of acids by the alkaline products of protein metabolism, thus allowing small quantities of weak acids to be detected. Acid production results in a pH shift that changes the color of the bromthymol blue indicator from green to yellow.

Using an inoculating needle, 2 tubes of OF glucose medium are stab-inoculated halfway to the bottom of the tubes. The content of one tube is overlaid with 1 mL of sterile mineral oil. Both tubes are incubated at 35°C in non-CO_2, and examine daily for 72 h or longer for slow-growing organisms. Yellow color indicates the production of acid. Acid production in the tube without oil overlay is considered oxidative reaction. Acid production in both tubes is considered fermentative. No acid production in either tube is considered nonsaccharolytic. Nonsaccharolytic organisms produce slight alkalinity (blue-green color) in the tube without oil overlay, but the tube with oil will not exhibit a color change and will remain green (BD Microbiology Systems, 1992).

OF Sugars

OF basal medium, when supplemented with an appropriate carbohydrate, is used to determine an organism's ability to utilize sugars such as lactose, xylose, sucrose, maltose, and mannitol. The low protein to carbohydrate ratio in OF basal medium prevents the neutralization of small quantities of weak acids by the alkaline products of protein metabolism, which makes this medium ideal for determining carbohydrate utilization. Acid production from carbohydrate metabolism results in a pH shift that changes the color of the bromthymol blue indicator from green to yellow. Yellow color indicates carbohydrate metabolism.

Using an inoculating needle, touch the center of one colony and stab-inoculate the OF medium with the appropriate carbohydrate once halfway to the bottom of the tube. Cap the tubes loosely and Incubate at 35°C in non-CO_2, and examine daily for 72 h or longer for slow-growing organisms. A yellow color indicates carbohydrate utilization and no color change (green) or blue color indicates no carbohydrate utilization. The acid reaction produced by oxidative organisms is detected first at the surface and gradually extends throughout the medium. When oxidation is weak or slow, it is common to observe an initial alkaline reaction at the surface of the tube that may persist for several days. This must not be mistaken for a negative test. If the organism is unable to grow in the OF medium, add either 2% serum or 0.1% yeast extract prior to inoculation (BD Microbiology Systems, 1992).

Commercial Microbial Identification Systems

Commercial microbial identification systems are the backbone of microbial identification in clinical microbiology laboratories. They provide an advantage over conventional identification systems by requiring little storage space, have an extended shelf life, rapid turn-around, low cost, standardized quality control, and ease of use. They range from manual to semiautomated to fully automated systems. These systems require simultaneous inoculation and incubation of a series of miniaturized biochemical reactions that are either based on detecting bacterial enzymes or cellular products that do not require microbial growth and have fairly rapid turn-around time (2–4 h) or are based on metabolic activity that requires

TABLE 6.2. Commercial systems commonly used in clinical laboratories.

Product	Manufacturer	TAT
API Systems	bioMerieux Inc.	2 h to overnight
BBL Crystal Systems	Becton Dickinson	4 h to overnight
BBL Phoenix Systems	Becton Dickinson	2 h to overnight
Vitek	bioMerieux Inc.	2 h to overnight
MicroScan	Dade International	2 h to overnight
MIDI Sherlock	MIDI	Overnight
Sensititre AP80	Trek	5 h to overnight
Biolog Micro Plate	Biolog	Overnight

TAT turn around time

microbial growth and require several hours to overnight incubation. In either case, the enzymatic or biochemical end results are combined, and using the Bayer's theorem with the aide of a computer program the identity of the test organism is determined. The majority of metabolic-based automated commercial identification systems also incorporate antimicrobial susceptibilities testing. In fact, over the years, the growing numbers of clinically significant pathogens and their rapidly emerging resistance to various antimicrobial agents have led to innovation of several commercial identification (ID) and antimicrobial susceptibility testing (AST) systems. For the most part, these systems have a fairly extensive database. They are fast, accurate, and have significantly improved the turn-around time for ID and AST of the common organisms. Despite their extensive database, they remain less than optimal in identifying fastidious slow-growing esoteric organisms. Table 6.2 present the list of the most commonly used commercial identification systems.

API Identification Systems

The API identification systems (bioMerieux Inc. Hazelwood MO) consists of series of microcupules on a plastic strip that contain dehydrated substrates for the demonstration of enzymatic activity or the fermentation of carbohydrates. Depending on type of the organism and the API strip utilized, it may or may not require microbial growth. API systems are manual and do not incorporate AST (Traunt, 2002; bioMerieux, 2004).

API Gram-Negative Identification

1. API 20E is a 24-h identification test for identification of Enterobacteriacae and group/species of non-fermenting Gram-negative rods.
2. API Rapid 20E is a 4-h identification test for identification of Enterobacteriaceae.
3. API 20NE is a 24–48 h identification test for identification of Gram-negative non-Enterobacteriaceae.
4. API NH is a 2-h test for of identification of *Neisseria*, *Haemophilus* and *Moraxella*.

API Gram-Positive Identification

1. API Staph is an overnight test for identification of clinical staphylococci and micrococci.
2. RAPIDEC Staph is a 2-h identification of the commonly occurring staphylococci.
3. API 20 Strep is a 4- or 24–h test for identification of streptococci and enterococci.
4. API Coryne is a 24-h test for identification of corynebacteria and coryne-like organisms.

API Anaerobe Identification

1. API 20A is a 24-h test for identification of anaerobic organisms.
2. Rapid ID 32 is a 4-h test for identification of anaerobes.

BBL Crystal Identification System

The BBL Crystal System (Becton Dickinson, Cockeysville, MD, USA) is a manual method that utilizes miniaturized fluorogen and/or chromogen linked substrates to detect enzymes that microbes use to metabolize a variety of substrates. These kits consist of BBL Crystal panel lids, bases, and inoculum fluid tubes. A suspension of the test organism is prepared in the inoculum fluid and then used to fill the reaction wells in the base. The substrates are rehydrated when the base and lid are aligned and snapped into place. Following the recommended incubation time, the wells are manually examined for color changes or the presence of fluorescence. The resulting pattern of positive and negative test scores is the basis for identification (Traunt, 2002; Becton Dickinson, 2004).

1. BBL Crystal Enteric/Nonfermenter (E/NF) Identification System is an overnight identification method utilizing modified conventional and chromogenic substrates. The E/NF identifies clinically significant aerobic Gram-negative Enterobacteriaceae isolates and non-fermenting Gram-negative rod.
2. The BBL Crystal Rapid Stool/Enteric (RS/E) Identification System is a miniaturized 3-h identification method employing modified conventional and chromogenic substrates. It is intended for the identification of clinically significant aerobic Gram-negative bacteria that belong to the family Enterobacteriaceae as well as most pathogens isolated from stool specimens.
3. The BBL Crystal *Neisseria/Haemophilus* (N/H) Identification System is a miniaturized 4-h identification method employing modified conventional, fluorogenic, and chromogenic substrates. It is intended for the identification of *Neisseria, Haemophilus, Moraxella, Gardnerella vaginalis*, as well as other fastidious bacteria.
4. The BBL Crystal Gram-Positive ID System is a miniaturized 18-h identification method employing modified conventional, fluorogenic, and chromogenic

substrates. It is intended for the identification of both Gram-positive cocci and bacilli.

5. The BBL Crystal Rapid Gram-Positive ID System is a miniaturized 4-h identification method employing modified conventional, fluorogenic, and chromogenic substrates. It intended for the identification of Gram-positive bacteria isolated from clinical specimens.
6. The BBL Crystal Anaerobe ID kit is a miniaturized 4-h identification method employing modified conventional, fluorogenic, and colorimetric substrates to identify clinically significant anaerobic organisms.

BBL Phoenix Identification and Susceptibility System

The BBL Phoenix (Becton Dickinson) is an automated identification and susceptibility system that can identify clinically significant Gram+/− microorganisms. The Phoenix ID panel utilizes a series of conventional, chromogenic, and fluorogenic biochemical tests to determine the identification of the organism. Both growth-based and enzymatic substrates are employed to cover the different types of reactivity. The tests are based on microbial utilization and degradation of specific substrates detected by various indicator systems. Acid production is indicated by a change in phenol red indicator when an isolate is able to utilize a carbohydrate substrate. Chromogenic substrates produce a yellow color upon enzymatic hydrolysis of either *p*-nitrophenyl or *p*-nitroanilide compounds. Enzymatic hydrolysis of fluorogenic substrates results in the release of a fluorescent coumarin derivative. Organisms that utilize a specific carbon source reduce the resazurin-based indicator. The AST method is a broth-based microdilution test. The system utilizes a redox indicator for the detection of organism growth in the presence of an antimicrobial agent. Continuous measurements of changes to the indicator as well as bacterial turbidity are used in the determination of bacterial growth. Each AST panel configuration contains several antimicrobial agents with a wide range of twofold doubling dilution concentrations. Organism identification is used in the interpretation of the MIC values of each antimicrobial agent.

The system includes an inoculation station for panel set-up and an incubator/reader carousel module. The carousel houses four horizontal tiers of 26 panel carriers to accommodate a tier-specific Normalizer and 25 Phoenix Panels. Phoenix Panel utilizes up to 51 microwells for ID and up to 85 microwells for AST. A bacterial inoculum concentration approximately equivalent to an 0.5 McFarland Standard is required for the identification of either Gram-negative or Gram-positive bacteria. Susceptibility testing is performed with an inoculum concentration of 3×10^5 to 7×10^5 CFU/mL. Kinetic measurements of bioreactivity within individual microwells via red, green, blue, and fluorescence readings are collected and comparatively analyzed with the Phoenix database (Becton Dickinson, 2004).

VITEK and VITEK 2 Identification System

The Vitek (bioMerieux Inc. Hazelwood, MO, USA) is an automated ID and AST system that utilizes identification cards with miniaturized wells. The system is fairly automated. It requires the user to prepare a suspension of the isolate in saline and verify the organism concentration with a densitometer. The inoculum tube is then placed into a rack, called the cassette. The sample identification number is entered into the carrier via barcode or keypad and electronically linked to the supplied barcode on each test card. ID and AST test cards can be mixed and matched in the cassette. All information entered at the bench is then transported to the instrument in a memory chip attached to the cassette.

VITEK 2 is the fully automated version, and all processing steps are completely autonomous including test set-up verification, AST inoculum dilution test inoculation, card sealing, incubator loading, optical reading and data transmission, and card disposal. The VITEK 2 optical system reads all the wells every 15 min. There are several cards that are designed for ID and susceptibility testing with these systems including Vitek GPI (Vitek 1), Vitek GPC (Vitek 2), Vitek EPS, GNI Plus, UID and UID, Vitek 2 ID-GNB, and Vitek NHI and AST panels for Gram-positive and Gram-negative organisms (bioMerieux, 2004).

Microscan WalkAway

The MicroScan WalkAway (Dade MicroScan Inc., West Sacramento CA, USA) is an automated ID and AST system that requires the ID and/or AST panels (96-well plates) be manually inoculated with bacteria isolated from clinical specimens and inserted into the WalkAway System. The panels are then incubated at 35°C for 16 to 42 h, depending on panel and organism type and results of readings. At the appropriate time, the WalkAway System automatically dispenses reagents into the appropriate biochemical wells and incubates the panels for an additional period of time (approximately 2–20 min, depending on the panel type). The WalkAway System then reads the panels. The identification of bacteria is based on measuring a series of biochemicals contained in panels designed for the speciation of most medically significant bacteria. The panels contain identification media consisting of substrates and/or growth inhibitors, which, depending on the species of the bacteria present, will exhibit color changes or increases in turbidity after incubation.

The panel may also contain series of antibiotics that are present in specified concentrations in the wells of applicable MicroScan panels. The WalkAway System reads the MICs and certain biochemicals and, if the criteria are met for adding reagents, reagents are added, and the panel is incubated for an additional period of time (approximately 5–30 min) depending on the panel type. The readings for the biochemicals needing no reagents and MIC wells (for combo panels) are stored prior to reagent addition. If additional incubation is necessary for the biochemicals, the susceptibilities and certain biochemicals will be read first and stored.

The reagents will not be added until after additional incubation, at which time biochemicals not previously read will be determined. Following is the list of commonly used MicroScan panels: MicroScan Gram Pos ID panel, MicroScan Rapid Gram Pos ID panel, MicroScan Neg Type 2, MicroScan Rapid Neg ID Types 2 and 3, and MicroScan NHID (Dade MicroScan, 1998).

Sensititre Microbiology Systems

The Sensititre ARIS 2X (TREK Diagnostic Systems, Inc. Cleveland, OH, USA) is an automated ID and AST system. The Sensititre ID and AST panels (96-well plates) may be inoculated manually or with an autoinoculator that is designed to automatically deliver inoculum in multiples of 50 μL to the 96-well sensititre plate. The Sensititre ID system is based on 32 biochenmical tests pre-dosed and dried in the Sensititre plate that are formulated to allow fluorometric reading along with unique fluorescent tests. The AST plate may be read manually or using the automated system. The automated system is fluorescent based and detects bacterial growth by monitoring the activity of specific surface enzyme produced by the test organism. Growth is determined by generating a fluorescent product from a non-fluorescent substrate. Presumptive ID of Gram-negative organisms can be obtained in 5 h; identification to species level for both Gram-negatives and Gram-positives can be obtained after overnight incubation. The Sensititre ARIS 2X is a combined incubation and reading system that fits onto an autoReader. Sensititre uses an internal barcode scanner to identify each plate type, assign the appropriate incubation time, and when this assigned time has elapsed, transport the plate to the autoReader for fluorescence measurement. The system has the capacity to accommodate up to 64 ID or AST plates. Following is the list of ID and AST plates with this system: GNID (AP80) for Gram-negative, GPID for Gram-positive identification, Gram-positive and Gram-negative MIC plates, Sensititre *Haemophilus influenzae* or *Streptococcus pneumoniae* susceptibility plates**,** Anaerobe MIC plate, EBSL Confirmatory MIC plate, and *S. pneumoniae* MIC plate (Traunt, 2002; TREK, 2004).

MIDI Sherlock

The MIDI Sherlock ID system (MIDI, Inc. Newark, DE, USA) is based on gas chromatographic (GC) analysis of the bacterial fatty acids. Branched-chain acids are known to predominate in most Gram-positive bacteria, while short chain hydroxy acids often characterize the lipopolysaccharides of the Gram-negative organisms. The system is fairly labor intensive and is designed for use in reference laboratories to identify isolates that are not easily identified by the routine identification systems. The Sherlock system detects the presence or absence of more than 300 fatty acids and related compounds (9–20 carbons in length) as well as the quantity of these compounds. The peaks are automatically named and quantified by the system.

Initially, the organism undergoes saponification, methylation, extraction, and base wash before GC analysis. A GC with phenyl methyl silicone fused silica capillary column is injected with the final prep. The temperature program in GC ramps from 170°C to 270°C at 5°C per minute. Following the analysis, a ballistic increase to 300°C allows cleaning of the column. The electronic signal from the GC detector is then passed to the computer where the integration of peaks is performed. The electronic data is stored on the hard disk, and the fatty acid methyl ester composition of the sample is compared with a stored database using the Sherlock pattern recognition software (BD Microbiology Systems, 1992; MIDI, 2004).

Biolog ID System

The Biolog Micro Plate ID Systems (Biolog, Inc. Hayward, CA, USA) relies on carbon source utilization test methodology in a 96-well format. The system is based on 95 reactions from 6 to 8 different classes of carbon sources with redox indicator (tetrazolium dye) and one negative control well with no carbon source. The isolates are inoculated to the microwell plate and incubated. If the isolate oxidizes any of the carbon sources, the net electron will reduce the tetrazolium to highly colored formazin (purple color). The carbon source utilization produces a characteristic pattern or "fingerprint" that is then compared to the Biolog database for identification. The system can identify environmental as well as fastidious organisms. In addition to the original Microlog manual, and a semiatomated version, the manufacturer has recently introduced a fully automated version (Omnilog) with a data base to identify more than 700 species of Gram-positive and Gram-negative organisms (Traunt, 2002; Biolog, 2004).

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7
Rapid Bacterial Characterization and Identification by MALDI-TOF Mass Spectrometry

DIANE DARE

Introduction

Bacterial infections account for a large proportion of people admitted to hospitals each year as well as some acquired by patients already in medical care. These can arise from the ingestion of contaminated food or exposure to nonsterile environments through wounds where opportunistic pathogenic bacteria are present. The symptoms and treatment of these illnesses vary and although some clues can be obtained from observing a patient's symptoms, the causative agent needs to be determined in order for a complete understanding of the nature of the infection, its origin, and the appropriate treatment. It is therefore of immense importance to characterize and identify bacteria wherever they are found in significant quantities, not only to aid clinicians with their diagnosis but also to prevent outbreaks of infections from potential medical, environmental, or terrorist sources. Furthermore, the identification of bacteria needs to be as rapid as possible. In this chapter, the use mass of matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) is discussed as an emerging technology for the rapid characterization and identification of bacteria.

General Principles of Mass Spectrometry Techniques

Mass spectrometry is a technique invented around the beginning of the 20th century and is generally used to ascertain knowledge of molecular structure, as the mass spectral pattern consists of a number of structurally related mass spectral peaks. The mass spectrometer first ionizes, then mass separates and finally detects ions associated with an analyte, thus producing a mass spectrum. The largest mass in the spectrum is generally that of the parent molecular ion, and its value corresponds to the molecular mass of the univalent molecular ion under analysis. Other ions in the spectrum are derived from the parent ion by sequential loss of smaller molecules (e.g., water or hydroxyl groups). Analysis of the mass differences between the fragmented ions in the mass spectrum can therefore yield information regarding the type of ions contained and the arrangement of the ions in the parent molecule.

Furthermore, the amount of material required to elicit this information is minute, of the order 10^{-12} g for a compound of mass/charge (m/z) ratio of 1000 Daltons (Da). This technique was initially limited to small easily ionized volatile organic molecules. However, with the introduction of new ionization techniques such as fast atom bombardment (FAB), plasma desorption (PD), electrospray (ES), and matrix-assisted laser desorption/ionization (MALDI), the limitation to volatile organic molecules no longer applies, enabling spectra of nonvolatile macromolecules of masses greater than 10,000 Da to be obtained. This makes mass spectrometry one of the most powerful analytical techniques available for analysis today and has resulted in many specialized biological applications; for example, fatty acid profiling (Ross et al., 1986), analysis of carbohydrate (Dell, 1987), nucleic acids (Nordhoff et al., 1996), and proteins (Andersen et al., 1996).

Currently, the most widely used ionization techniques for the characterization of bacteria are ES and MALDI. In ES ionization, the analyte is nebulized through a capillary as a fine spray into a high-voltage electric field at atmospheric pressure. This produces small charged droplets, which then disintegrate into smaller charged particles before reaching the detector, as collisions occur with the air molecules within the spectrometer. In general, ES ionization produces multiply charged ions of an analyte, and this more complex spectral pattern requires deconvolution into the simpler singly charged pattern prior to interpretation. An advantage of ES ionization, however, is that the production of multiply charged ions for large biomolecules results in a lowering of the mass range of the spectrum to typically 2000 Da, because for a doubly charged ion, where $z = 2$, the mass of the observed spectral peak (m/z) is halved. The mass range detected is then compatible with most types of detectors. Preparation of the sample, however, requires solubilization in a suitably volatile solvent together with the removal of salts/debris that might block the nebulizer. This sample format also has the advantage of using a tandem mass spectrometry technique employing liquid chromatographic or capillary electrophoresis to separate components of the analyte prior to mass spectral analysis. Because whole bacterial cells are not amenable to solubilization, ES ionization is more suitable for the analysis of cellular components, for example, fatty acids of lipids, or soluble components of cell lysates, such as base compositions of polymerase chain reactions (PCR) (Hofstadler et al., 2005), and information regarding identification of bacteria are based on differences in these individual components.

The preferred ionization technique for the analysis of whole bacterial cells is MALDI. It was initially developed around 1980 and involves the generation of ions by photon bombardment of the analyte deposited onto a target plate (Karas and Hillenkamp, 1988; Tanaka et al., 1988). The photons are normally generated by a pulsed nitrogen laser producing ultraviolet (UV) rays of 337-nm wavelength, and the mass separation of the ions is by time-of-flight (TOF). Time-of-flight detection is more suited to the high-mass singly charged ions produced by MALDI, thus giving the acronym MALDI-TOF MS. In order to successfully obtain ions from the analyte, however, a matrix of solid organic chemical is required. The co-crystallized sample of analyte and matrix allows successful absorption of the

photons followed by vaporization and ionization of the sample. A disadvantage of the technique is that the co-crystallized sample is heterogeneous, and this can lead to a variation of intensities in the spectral peaks from different spots on the target plate (Westman et al., 1995). Furthermore, the choice of matrix is empirical resulting in a mass spectrum that is protocol dependent (Marvin et al., 2003). Laser energy is also a factor, as is sample preparation, making comparison of spectral patterns of bacteria reported in the literature problematic, due to the different methods employed (Williams et al., 2003). Nevertheless the technique is proving a powerful tool for the characterization of whole bacteria. An added advantage is the comparative simplicity of the mass spectrum produced due to the soft laser ionization technique. This produces significantly less fragmentation of these large biomolecules compared with other ionization techniques such as pyrolysis and FAB. Furthermore, the spectral pattern is very reproducible for any given set of protocols and more importantly for bacterial identification is very rapid, requiring only minutes to acquire a spectrum. Extensive reviews regarding the characterization of bacteria are given in papers by van Baar (2000), Fenselau and Demirev (2001), Lay (2001), and Marvin et al. (2003).

Comparison of MALDI-TOF *MS Techniques for Bacterial Identification*

MALDI-TOF MS has proved the most suitable method for the analysis of whole bacterial cells, due to its capacity to rapidly produce reproducible, relatively simple spectral patterns over a wide mass range. Furthermore, these patterns contain unique characteristics capable of characterizing bacterial species directly without the need for extraction and separation of cellular components (Claydon et al., 1996; Holland et al., 1996; Krishnamurthy et al., 1996; Keys et al., 2004). The technique is, however, protocol dependent (Wang et al., 1998; Domin et al., 1999; Evason et al., 2000; Williams et al., 2003; Valentine et al., 2005). This section describes a selection of different MALDI-TOF MS techniques employed for bacterial identification. The conclusion in each case is that characterisation and hence identification of bacteria can be achieved by MALDI-TOF MS under controlled experimental conditions.

Sample Preparation

The choice of biological molecule for analysis will determine the type of sample preparation; these can involve DNA, RNA, lipids, fatty acids, and proteins either from whole cells or cell extracts. The most abundant of these, however, is protein, representing 50% of the dry weight of the cell; the least abundant is the DNA, with only one copy per cell. Lipids and fatty acids represent 5% to 8% and RNA 0.01%. In most cases, the analyte component is derived from a pure bacterial culture. To access the component of interest, generally the cell is lysed by exposing the cell to water, solvent, and/or strong acid and depending upon the molecule to be analyzed, further extraction, purification, and/or amplification steps may

be required. Currently, the most widely used MALDI-TOF MS techniques for bacterial identification are DNA and protein analysis, these are discussed in more detail below.

DNA/RNA *Based*

Identification techniques using DNA/RNA [e.g., single nucleotide polymorphism (SNP)] compare a limited DNA sequence of an unknown bacterium to a database of known sequences. Preparation is similar to other DNA techniques, although in this case the sequence analysis is achieved using MALDI-TOF MS. In this technique, the DNA is extracted and primers are chosen to select a target region (e.g., 16S rDNA), which is amplified and subjected to base-specific cleavage by polymerase chain reaction (PCR), transcription to RNA, followed by a clean-up procedure. The MALDI-TOF MS analysis of the DNA sequence is then determined and used for identification against a database of known sequences, for example, GenBank (Nordhoff et al., 1996; von Wintzingerode et al., 2002; Lefmann et al., 2004).

Protein Based

Proteins expressed by the bacterium can be obtained from the lysed cell and analyzed by depositing the lysate directly on the MALDI target plate. This is normally achieved by addition of a highly acidic MALDI matrix solution to bacterial cells harvested from a culture plate, followed by mixing, washing, deposition, and co-crystallization of the lysate on to the MALDI target plate. This technique allows proteins within the cell to be analyzed as well as the extracellular surface proteins. Comparison of the protein profile obtained experimentally with the annotated proteins of known microorganisms in a proteome database (e.g., SWISS PROT) provide an identification (Demirev et al., 1999; Jarman et al., 2000; Pineda et al., 2000; Bairoch et al., 2005). Accurate mass data is, however, required for database searching, because the masses of some proteins are very similar. Therefore, this technique is greatly enhanced if the protein identification is validated by MS/MS, where the protein peak of interest is fragmented by a second mass spectral analysis to provide more discriminatory amino acid sequence data and hence more conclusive identification of the protein. Alternatively, the expressed proteins from the bacterium can be extracted and digested prior to MS analysis and the subsequent amino acid data used to identify the protein of interest (Warscheid & Fenselau, 2004). For this type of identification, a particular unique biomarker protein or set of proteins leads to identification. It is assumed in these cases that unique biomarker protein(s) are always expressed by the DNA of the bacterial cell and are therefore independent of sample preparation. However, this is unlikely to be the case, as different MALDI-TOF spectral fingerprints can be obtained for *Escherichia coli* when cultured on two significantly different types of basal media (Arnold et al., 1999; Keys et al., 2004).

Recently, separation of the proteins within the lysate has been achieved using surface-enhanced MALDI target plates to select specific types of cell components

for analysis by MALDI-TOF MS and is referred to as SELDI-TOF MS. The target plates can be hydrophobic, hydrophilic, electrophilic, and so forth, depending on the separation required, and has proved useful in exploiting the difference of closely related organisms (Lancashire et al., 2005).

The simplest and most rapid preparation technique, however, is the direct deposition of the bacterial cells from the culture plate onto the MALDI target plate, followed by addition. Immediate co-crystallization of the sample enables the analysis of mainly surface proteins and produces a more selective spectral pattern. For all these preparation techniques, the unique spectral fingerprint can be used to identify a bacterium by comparison of unknown with known bacterial fingerprints. For this to be successfully achieved, however, the fingerprints must be derived using the same standardized protocol (van Baar, 2000; Keys et al., 2004).

Application of MALDI-TOF MS for Rapid Identification of Bacteria

All the above mass spectrometry techniques are capable of bacterial identification. Acquisition of the mass spectra in each case is very rapid, making all the candidate techniques feasible for rapid data acquisition and analysis. However, for the majority of techniques, the sample preparation from pure culture is complex and time consuming and can often involve the use of costly chemical kits specifically designed for the technique. Furthermore, interpretation of the mass spectral data often requires the specialist knowledge normally residing in research institutions or specialized contract laboratories. Consequently, some techniques are therefore unsuitable for many routine microbiology laboratories. One technique, however, offers a rapid, simple sample preparation and analysis, and because the mass spectrometer is fully automated, provides a strong candidate technique for rapid bacterial identification in the more routine microbiology laboratory. The use of whole bacterial cells for MALDI-TOF MS, in which the spectral fingerprint of an unknown bacterial cell is compared with a database of known library fingerprints, offers the most attractive solution for rapid bacterial identification (Table 7.1). Currently, there is only one system in which the mass spectral acquisition is fully automated and the data acquired searched seamlessly against a fully curated database from validated bacterial strains. This is the Microbelynx bacterial identification system (Waters Corporation, Manchester, UK). The following section therefore focuses upon the MicrobeLynx system with respect to its suitability for rapid routine bacterial identification.

MicrobeLynx System for Automatic Bacterial Identification

The MirobeLynx rapid bacterial identification system has been developed in collaboration between Manchester Metropolitan University (MMU; Manchester, UK), the Molecular Identification Service Unit of the Health Protection Agency (MISU; London, UK), and the Waters Corporation (Manchester, UK). The system has

TABLE 7.1. Comparison of MALDI-TOF MS techniques for bacterial identification.

Target molecules	Sample preparation from pure culture for transfer to MALDI target plate	Identification based on	General reference	Comments
DNA	Selection, amplification, PCR, transcription, cleanup.	DNA sequences	(Nordhoff et al., 1996)	Lengthy and specialized sample preparation and analysis requiring a high level of expertise.
Proteins	Add lysate, mix, wash, possibly separate.	Selective biomarkers or selective protein identification	(Demirev et al., 1999; Jarman et al., 2000)	High level of expertise required for analysis of proteins.
SELDI	Add lysate, mix, separate by inoculation on selected SELDI target plate, wash & apply MALDI matrix	Selective biomarkers	(Lancashire et al., 2005)	Useful comparison of very closely related organisms, employing specialized separation technology.
Cell surface proteins	Transfer of whole cell to target plate & application of MALDI matrix	Fingerprints	(Keys et al., 2004)	Rapid, simple, automated analysis with high throughput, suitable for routine analysis.

at its core a curated database of fingerprint spectra over the mass range 500 to 10,000 Da derived from quality-controlled BS EN ISO 9001:2000 freeze-dried bacterial strains supplied from the National Collection of Type Cultures (NCTC; London, UK). The database spectra are prepared using strict protocols, to ensure reproducibility. Initially, the freeze-dried quality-controlled ampoule is rehydrated and inoculated onto Columbia blood agar (CBA) (Oxoid Ltd, Basingstoke, UK) then incubated aerobically for 24 h at 37° C. In some cases, however, these conditions are altered to facilitate growth (e.g., for strict anaerobes). To ensure the organism has recovered fully from the stressed dehydrated state, two further subcultures are undertaken on CBA as above, prior to analysis. For analysis of "real" samples against the database, however, where the sample is unlikely to be stressed, one subculture is sufficient prior to analysis. Single colonies of the bacterium are then used to directly inoculate a minimum of 4 MALDI target wells, using a 1μL sample loop. For database preparation, however, a total or 12 target wells are used in order to (i) assess the reproducibility of the fingerprints prior to database addition and (ii) produce a statistical estimation of variance. The use of a single colony also has the advantage that different colonies on a mixed culture plate can be distinguished and identified separately. The time taken to inoculate

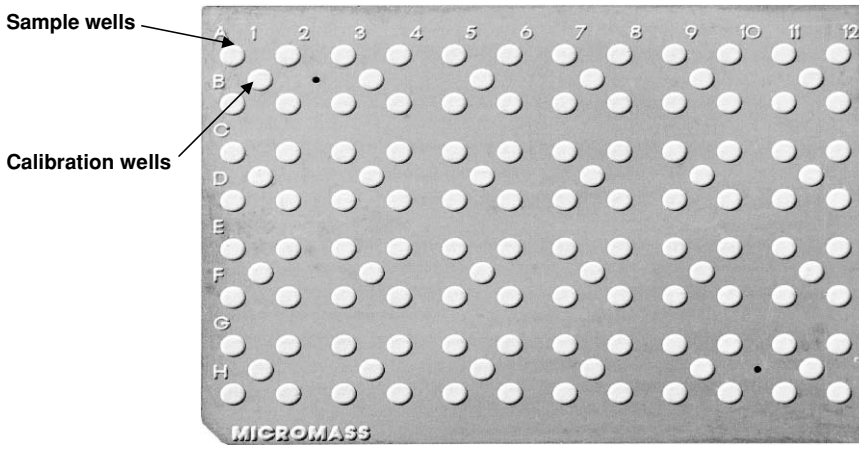

FIGURE 7.1. Ninety-six–well MALDI target plate, detailing sample rows A to H and the 24 external calibration wells between the sample rows.

the 96-well MALDI target plate is very short, of the order 5 to 10 min. After allowing the bacterial samples to dry for approximately 1 h, the MALDI target wells are overlaid with 1μL of the MALDI matrix solution to aid the ionization process. For Gram-positive organisms, this is a saturated solution of 3 Mg/mL of 5-chloro-2-mercaptobenzothiazole (CMBT) dissolved in acetonitrile, methanol, and water in the ratio 1:1:1 containing 0.1% formic acid and 0.01 M 18-crown-6-ether. For Gram-negative organisms, the CMBT is replaced by 14 Mg/mL α-cyano-4-hydroxycinnamic acid (αCHCA). The saturated CMBT and αCHCA solutions are freshly prepared prior to use; the acetonitrile, methanol, formic acid, and 18-crown-6-ether solvent can, however, be prepared and stored in a cool, dark glass bottle for up to 6 months. In order to calibrate the time of flight tube and correct for any variation in the flight length across the MALDI target plate, lock mass wells, positioned between the sample wells are inoculated with αCHCA matrix solution containing seven peptides of known mass (Fig. 7.1). Upon application of the appropriate MALDI matrix solution to each target well, the samples are left at room temperature for approximately 5 min to allow co-crystallization of the bacterial sample in the MADLI matrix before inserting the plate into the MALDI-TOF MS for automatic analysis.

Automatic acquisition of the mass spectral fingerprints is then achieved using the sample list, which details the well numbers of the bacterial samples, the corresponding data files, the wells containing replicate samples together with the experimental parameters used to collect the data and details of the database search. The experimental details are preselected by the operator after initially setting up the instrument to perform (i) a spatial calibration to automatically locate the well positions; (ii) optimization of the rennin substrate peak resolution, by adjustment of the pulse voltage to produce a sharp narrow peak at 1760 Da (i.e., $\leq$3 mass units

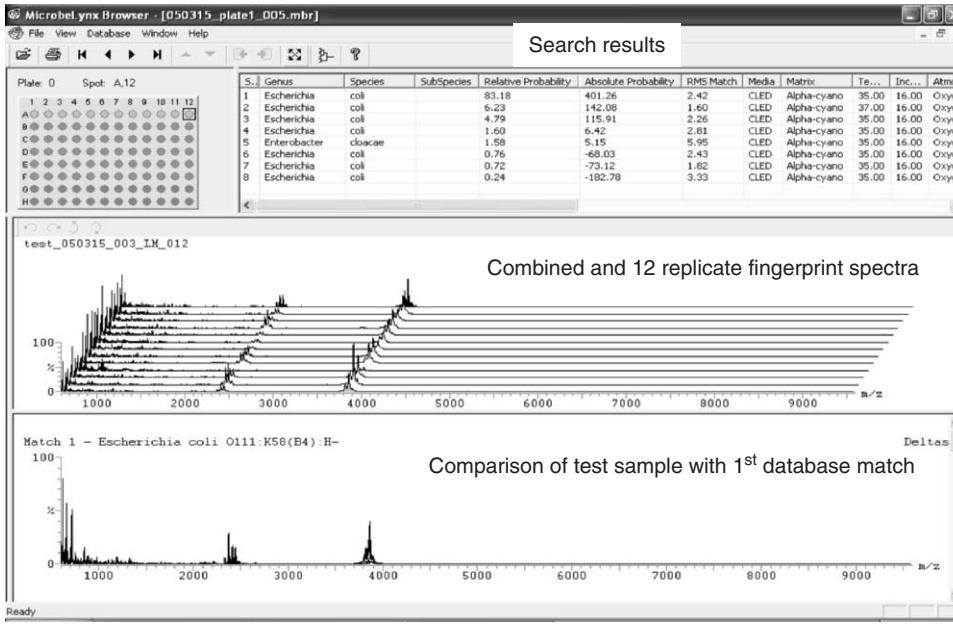

FIGURE 7.2. The browser details the wells searched (row A), the number of spectra collected for each replicate well (not shown), the fingerprint spectrum for each replicate well, the combined spectrum for all the replicate wells, and comparison of the test sample spectrum with up to 8 top spectral matches (only first match shown; second to eighth matches not presented). A list of the search results, together with information on database entry with respect to the probability of the match, the RMS value, basonyms or previous names (not shown), and the culture conditions used for the database spectra are also given.

at half peak height); and (iii) calibration of the time of flight tube from the known masses of the 7 peptide calibrants. These parameters are then automatically used in the experimental file together with criteria for rejecting any spectra that are either too intense or too noisy. This ensures that only quality data from good spots on the target well are selected. Furthermore, the sample well is sampled from a minimum of 3 different sites to produce a maximum of 15 spectral profiles, with each profile produced from the sum of 10 individual shots to maximize the signal to noise ratio and further optimize reproducibility. The experimental file also has the potential to ramp through a series of laser energies in order to acquire the optimized spectra, should this be required. The quality-controlled reproducible spectra from the replicate bacterial sample wells are then automatically combined and searched against the chosen database and the results presented in a browser format (Fig. 7.2). The browser details the wells searched, the number of spectra collected for each replicate well, the spectrum for each individual well, the combined spectrum for all the replicate wells, and comparison of the test sample spectrum with up to 8 top spectral matches. A list of the search results, together with information on the database entry with respect to the probability of the match, the root mean square (RMS) value, basonyms, and the culture conditions used for the database spectra are

also given. This information together with the comparison of the spectral profiles produces information as to the classification and identification of the bacterial sample. The mass spectral analysis requires approximately 1.5 h to acquire and analyze the data for a 96-well target plate. This means that in a normal working day (9 a.m. to 5 p.m.), the first MALDI target plate containing a maximum of 24 samples can be run approximately 1 h 15 min after culture, (10 min to inoculate the bacteria onto the plate + 1 h drying + 5 min for the addition of matrix and peptide solution and co-crystallization). The preparations of subsequent plates are then concurrent with analysis of the previous plate and result in the comfortable analysis of 5 MALDI target plates containing 120 samples during one working day. Because the sample preparation is simple and rapid and the MALDI-TOF MS analysis is automatic, minimal operator time is required for the instrument, leaving sufficient time for preparing further overnight cultures following the appropriate protocols. The high sample throughput, rapid analysis, and ease of acquiring the skills to prepare the target plates and run the instrument make this an attractive method for routine bacterial identification, where current microbiology staff can easily adapt to this new technique. Furthermore, because the cost of consumables is negligible due to the low concentrations of matrix and peptide solutions used, together with ability to reuse the MALDI target plates, the main cost factor is the instrument and database. Depending on the sample throughput, however, this cost can be offset against the high consumable costs currently associated with other identification techniques. This together with the speed of analysis now makes MALDI-TOF MS either an attractive complementary or an alternative to currently used identification techniques.

The system also allows for the production of an "in-house" database, which can be searched alone or together with the proprietary MMU database. Addition of spectral fingerprints to a database requires comparison of data for each replicate well using a root mean square (RMS) (Storms et al., 2004) function. Each replicate spectrum is compared in turn with the average of the other combined replicate spectra, and any spectrum found to be significantly different is automatically rejected. The total combined spectra for the acceptable replicate data is then added to the database, along with a statistical estimate of variance. The proprietary database generally uses 12 replicate wells to obtain a statistically representatative fingerprint spectrum for the database. Significant numbers of the spectral patterns in the proprietary MMU database are also checked for reproducibility using different operators and instruments prior to release.

Currently, the MMU database contains spectral fingerprints for the NCTC type strains, together with other representative strains of the same species and at present includes more than 4000 spectral fingerprints, covering more than 500 different species. The database is updated yearly with a minimum of 500 new spectral fingerprints (Fig. 7.3a). It is currently separated into 4 databases, which can be searched simultaneously, or separately; the core aerobic database, a database for Urinary Tract Infection (UTI) (Hofstadler et al., 2005) employing a more specialized media, a database of anaerobes, and latterly a database of clinical strains from well-recognized sources (Fig. 7.3b). Further details on the compilation of the MMU fingerprint databases are given by Keys et al. (2004).

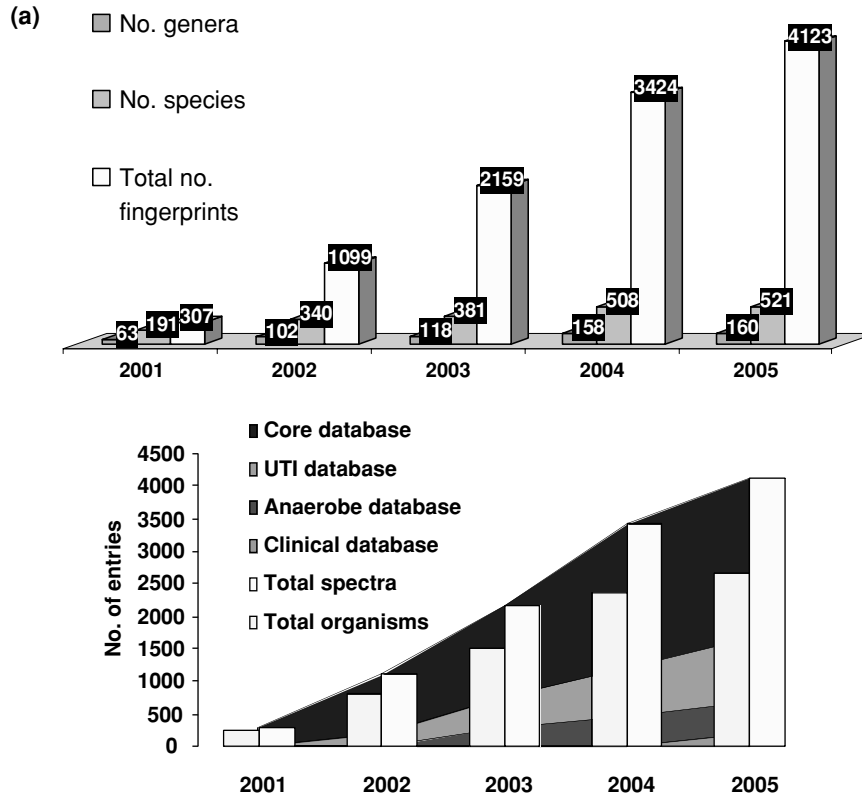

FIGURE 7.3. Yearly growth (a) and separation (b) of the proprietary MMU databases.

Limitiations of MALDI-TOF *MS for Bacterial Fingerprinting*

As with all identification techniques, there are limitations that need to be considered when interpreting the analytical results. For the MicrobeLynx system, these limitations are as follows:

1. **Protocol dependence**. The technique is protocol dependent, and therefore comparison of spectral data with database entries is only valid when the same protocols are adopted, especially in terms of the culture conditions. This applies to all protein-based MALDI-TOF MS analysis.
2. **Knowledge of Gram stain**. The Gram stain of the organism is required prior to analysis because for Gram-positive bacteria, the MALDI matrix contains CMBT and for Gram-negative bacteria αCHCA. Use of the incorrect matrix generally leads to poorly defined spectral fingerprints, especially for Gram-positive organisms.
3. **Database coverage**. Because any database is finite and bacteria are continuously evolving, the search results can only provide an indication of the

characteristics of the test organism compared with the database entries. Identification is therefore limited to the strains covered within the database, and interpretation of the results must be viewed in this context.

4. **Taxonomy**. The taxonomical classification of bacteria is by no means static, and many organisms have been reclassified as new techniques become available. MALDI-TOF MS is no exception. The spectral fingerprint patterns for some strains sometimes appear anomalous with other strains from the same species and suggest their classification, at least by MALDI-TOF MS, requires amending. Care must therefore be taken to compare the spectral pattern of all the top matches with the unknown before assigning identification. Adequate representation of the spectral fingerprint for a number of strains per species, however, supports more conclusive identification.
5. **Mixed cultures**. Although different colonies can be assigned to different MALDI target wells for analysis and identification, inadequate separation of mixtures produces a mixed fingerprint profile. Identification in this case may give misleading or anomalous results. Visual inspection of the spectral profiles is therefore required to aid interpretation, and further purification steps or alternative identification techniques may need to be used for confirmation.
6. **Operator/instrument variability across different microbiology laboratories**. Slight differences in technique can affect the spectral fingerprints. These can be limited, however, by using the same protocols to generate the spectral fingerprints in the database. The addition of a bacterial sample known to be represented in the database to each batch of test isolates also gives an assurance as to the standardization of the technique across operators and laboratories.
7. **Software limitations**. Some of the fingerprint patterns for some strains contain relatively small but distinctive high mass peaks. Currently, limitations in the software are unable to correctly discriminate on these peaks. In such cases, visual inspection of the fingerprint patterns above 3000 Da can assist in determining the most appropriate fingerprint match.
8. **Nondiscriminate spectral patterns**. In some cases where the organisms are very closely related (e.g., Enterobacteriaceae family), the spectral patterns are very similar. This together with poor taxonomy can lead to inconclusive or misleading identification. In these cases, better differentiation can be achieved by changing culture media and or conditions as in the case of UTI samples. Differentiation is improved using the more specialized UTI medium of cystine lactose electrolyte deficient (CLED) agar in place of the more universal Columbia blood agar (CBA) and gives more conclusive identification of this problematic family.
9. **Strain comparison**. Comparison of the fingerprint patterns to a database cannot produce strain identification due to (i) the finite number of strains within the database and (ii) the inherent experimental variability. For strain comparison to be achieved, the number of variables needs to be minimized. This can be largely achieved by culturing and analyzing the two strains simultaneously on the same MALDI target plate, thereby maintaining the same culture and experimental conditions. Differences in the spectral fingerprints can then be interpreted as having arisen from different strains. However, it must be noted

that similarity of the spectral fingerprints is only valid under the conditions on which the experiment is based.

Notwithstanding these limitations, MALDI-TOF MS is a powerful tool for comparing bacteria. Examples using the MicrobeLynx system for bacterial characterization and identification are presented in peer-reviewed papers (Du et al., 2002; English et al., 2003; Hindre et al., 2003; Dare et al., 2004a; Keys et al., 2004; Krader & Emerson, 2004; Uguen et al., 2005; Dare, 2006) and poster presentations at international scientific meetings (Keys et al., 2000; Bright et al., 2001; Dare et al., 2001; McKenna et al., 2001a; McKenna et al., 2001b; McKenna et al., 2001c; Bright et al., 2002a; Bright et al., 2002b; Carlson & McKenna, 2002; Coales et al., 2002; Dare et al., 2002a; Dare et al., 2002b; McKenna et al., 2002; Sutton et al., 2002; Thuy-Trang et al., 2002; Dare et al., 2003a; Dare et al., 2003b; Dare et al., 2003c; Dare et al., 2003d; Keys et al., 2003; Sutton et al., 2003; Dare et al., 2004b; Dare et al., 2005a; Dare et al., 2005b; Nielsen et al., 2005; Sutton et al., 2005).

Summary

In the past decade, there has been an explosion in the number of papers covering the application of mass spectrometry to biological macromolecules due to the introduction of new ionization techniques. The two most significant ionization techniques to emerge for bacterial characterization are ES and MALDI. Electrospray ionization has the advantage that it can be coupled to separation technologies and is therefore invaluable in the analysis and comparison of individual components of bacterial cells. In contrast, MALDI ionization has the advantage of characterizing the whole bacterial cell, without the need to separate individual components. The simple sample preparation also makes this technique a much more rapid technique for bacterial characterization. Furthermore, the characteristic spectral fingerprints generally associated with surface proteins of bacterial strains can be applied to bacterial identification provided the fingerprints are obtained using the same protocols. In addition, automatic fingerprint acquisition and searching against a quality-controlled database of validated strains as demonstrated by the MicrobeLynx system now extends the application of MALDI-TOF MS technique beyond the more specialized research laboratories and facilitates routine use.

Conclusion

Rapid bacterial characterization and identification by MALDI-TOF MS is emerging as a powerful new cost-effective tool suitable for application in routine microbiology laboratories. The low consumable costs, automation, high sample throughput, and ease of sample preparation also offers significant advantages in the field of microbiological characterization and identification.

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8
Probe-Based Microbial Detection and Identification

TAO HONG

Introduction

The most important property of nucleic acid is its nucleotide sequence, which carries the identity of unique organisms. The basic principle of nucleic acid probe-based assay is the intrinsic ability of single-stranded DNA or RNA to anneal specifically to a complementary sequence and form a double-stranded hybrid. Most microorganisms encountered in the clinical microbiology laboratory can be identified using conventional methods. However, the conventional/culture-based approach may take a long time to identify slow-growing or fastidious organisms. Nonviable or nonculturable organisms simply can not be identified by conventional/culture-based approach. The nucleic acid probe-based approach provides a rapid, specific way of detecting/identifying microorganisms. Nucleic acid probe-based microbial identification is widely used in clinical laboratories. The probes can be used for identification of microorganism directly from the specimen, from culture, or on formalin-fixed and paraffin-embedded tissue.

Target of Probe-Based Identification

Ribosomes are highly conserved and essential organelles responsible for protein synthesis. The rRNA possesses distinct features that make it a good marker for bacterial identification. As the backbone of the ribosome, rRNAs are found in all known living cells. In growing bacterial cell, as many as 10^4 to 10^5 copies of 5S, 16S, and 23S rRNAs can be found in the cell (Delong, 1989; Kemp, 1993); sufficient target, is present for direct detection with the need for further amplification, in contrast to DNA target, which usually has one or a few copies per cell. The nucleotide sequence of rRNA gene is well conserved within a species and quite variable between most different species of microorganisms, making rRNA an ideal target for species identification for medically important organisms. The 16S and 23S rRNA molecules consist of variable sequence motifs that reflect their phylogenetic origins. The sequence variability allows the design of species-specific

probes for an organism's identification. Other RNA targets have also been applied to identify/differentiate bacteria; tmRNA, a RNA molecule of 363 nucleotides that combines properties of tRNA and mRNA, has been successfully used for bacterial identification (Schonhuber, 2001); mRNA has also been used as target for *in situ* hybridization (Wagner, 1998).

Probes

The oligonucleotide probes used to identify bacteria are usually short DNA molecules, between 15 to 25 nucleotides long. The shorter the probe, the lower the probe can tolerate mismatches. The probes can be labeled with a variety of compounds (chemiluminescence, fluorescence dye, peroxidase, lectin, etc.) and be used in combination with corresponding detection methods. The most common probe labeling involves enzymatically linked reporter molecules like digoxigenin, alkaline phosphatase, or horseradish peroxidase. These probes need an additional step after the hybridization procedure with fluorescent anti-DIG or use tyramid signal amplification (TSA) detection kit. The TSA kit consists of a fluorescent tyramide, which would be radicalized by horseradish peroxidase and then bind intracellularly to arominatic amino acids (tyrosine, phenylalanine, and tryptophan). The signal intensity may be increased 10- to 20-fold by using the TSA kit (Schonhuber, 1997; Juretschko, 1999). For laboratory equipped with fluorescent microscope, the use of fluorescent labeled probe is ideal; it usually produces strong signal and less background. Probes can be designed on different phylogenetic levels, specific for domain, phylum, family, genus, or species.

Hybridization Formats

Nucleic acid hybridization can be performed in a few formats: in liquid, with both probe and target free to interact (solution hybridization) or with the probe free and the target nucleic acid bound to a solid surface (solid-support hybridization), and *in situ* hybridization, in which intact cells or tissue sections are fixed onto glass slides and the target nucleic acid is detected directly in cells. The controlled enzymatic digestion of cellular membranes and other proteins allows the probes to gain access to target sequences. Hybridization using metal beads combines solution and solid-phase hybridization; the labeled nucleic acid probe (the signal probe) hybridizes with the target nucleic acid in the solution. Metal beads coated with probes (the capture probe) hybridize to a different region of the target. Then a magnet is applied to the reaction tube, and the hybrids are separated from the rest of the reaction. Unbound probes and other unrelated molecules are removed by washing. This is the so-called sandwich hybridization: the signal probe will remain with the reaction only if the target is hybridized with both signal and capture probes.

Hybridization and Detection

A very important factor that influence the sensitivity and specificity of probe hybridization is hybridization stringency; this is the only condition that can be adjusted during the reaction, which defines the number of mismatches that can be tolerated in a hybrid molecule. Optimizing stringency is the key for successful hybridization assay. At high stringency, mismatches are rare. An overly stringent reaction may decrease the sensitivity of the assay due to well-matched hybrids may be disrupted. A less stringent reaction may detect unwanted, nonspecific reaction. Stringency can be easily adjusted by varying the washing conditions; stringency is increased by increasing temperature and formamide concentration or lowering salt concentration. Most commercial hybridization assays have been optimized, however, each laboratory may still need to work out its own hybridization/washing conditions.

The hybrid molecules formed during the reaction are usually detected by the reporter molecules directly or indirectly connected with the probe. The reporter molecules are usually enzymes, affinity labels, chemiluminescent or fluorescent moieties. For fluorescence *in situ* hybridization, a fluorescence microscope with appropriate filter(s) is essential for detection. For *in situ* hybridization probes labeled with alkaline phosphatase or horseradish peroxidase, the dark cellular staining can be visualized by using conventional light microscope. The most popular commercial probe-based assays, the Gen-Probe AccuProbe and PACE-2, use chemiluminescent labeled probes, and the hybrids are detected using a luminometer; the light intensity correlates with the amount of hybridized probe.

Gen-Probe Direct Nucleic Acid Detection Method

Gen-Probe (San Diego, CA) provides chemiluminescent labeled probes for detecting specific rRNA target for various organisms. The Gen-Probe Accuprobe System uses a single-stranded DNA probe with a chemiluminescent label that is complementary to the ribosomal RNA of the target organism. rRNA target has an advantage because there are many thousands of copies in each cell, increasing the test sensitivity. After the ribosomal RNA is released from the organism, the labeled DNA probe combines with the target organism's ribosomal RNA to form a stable DNA:RNA hybrid. The selection reagent allows for the differentiation of nonhybridized and hybridized probe. The labeled DNA:RNA hybrids are measured in a Gen-Probe luminometer. A positive result is a luminometer reading equal to or greater than the cutoff. A value below this cutoff is a negative result.

For mycobacterial identification, probes are available for *Mycobacterium tuberculosis* complex, *Mycobacterium kansasii*, *Mycobacterium gordonae*, *Mycobacterium avium* complex, *Mycobacterium intracellulare*, *and Mycobacterium avium.*

For fungal identification, probes are available for *Blastomyces dermatitidis, Coccidioides immitis*, and *Histoplasma capsulatum.* For bacterial identification,

TABLE 8.1. Gen-probe ACCUPROBE sensitivity and specificity.

	Sensitivity (%)	Specificity (%)
Mycobacterial identification		
Mycobacterium avium	99.3	100
Mycobacterium intracellulare	100	100
Mycobacterium avium complex	99.9	100
Mycobacterium gordonae	98.8	99.7
Mycobacterium kansasii	92.8	100
Mycobacterium tuberculosis complex	99.2	99.0
Fungal identification		
Blastomyces dermatitidis	98.1	99.7
Coccidioides immitis	98.8	100
Histoplasma capsulatum	100	100
Bacterial identification		
Campylobacter	100	99.7
Enterococcus	100	100
Group A *Streptococcus* (*Streptococcus pyogenes*)	99.0	99.7
Group B *Streptococcus* (*Streptococcus agalactiae*)	97.7	99.1
Haemophilus influenzae	97.1	100
Neisseria gonorrhoeae	100	100
Staphylococcus auresus	100	100
Listeria monocytogenes	100	99.7
Streptococcus pneumoniae	100	100

Information provided by Gen-Probe.

probes are available for *Campylobacter*, *Enterococcus*, Group A *Streptococcus* (*Streptococcus pyogenes*), Group B *Streptococcus* (*Streptococcus agalactiae*), *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Staphylococcus aureus*, *Listeria monocytogenes*, and *Streptococcus pneumoniae*. The advantage of these assays is they are nonisotopic, simple to use, and have high sensitivity (ranging from 92% to 100%) and specificity (ranging from 99% to 100%) (see Table 8.1).

Affirm DNA Probe

Becton Dickinson and Company (Sparks, MD, USA) provides a DNA probe-based test, the Affirm VPIII, which uses complementary sequences of DNA that hybridize with the targeted organisms and can detect and differentiate three major agents with cause vaginitis: *Candida*, *Gardnerella*, and *Trichomonas*. The test uses two distinct single-stranded nucleic acid probes for each organism, a capture probe and a color development probe, which are complementary to unique genetic sequences of target organsims. The capture probes are immobilized on a bead embedded in a probe analysis card, which contains a separate bead for each target organsim. The color development probes are contained in a multiwell reagent cassette.

The ability to multiplex three analytes into a single easy-to-perform test is the advantage of this system. The Affirm VPIII Microbial Identification Test for

Candida species can detect 1×10^4 CFU of *Candida* species in log phase per assay, for *G. vaginalis* can detect 2×10^5 CFU of *G. vaginalis* in log phase per assay, and for *T. vaginalis* can detect 5×10^3 trichomonads per assay (Affirm VPIII package insert). Because of its high specificity (97.1%), the Affirm VPIII test is an excellent tool for diagnosing the presence of bacterial vaginosis. The sensitivity for grade 3 (Nugent et al., 1991) is 89.5%. The Affirm VPIII test does not yield a positive result for germ counts below 2×10^5, which ensures that only clinically relevant cases of infection are detected, thereby avoiding overinterpretation and overtreatment. This test provides an excellent tool for the diagnosis or exclusion of bacterial vaginosis (Armin Witt et al., 2002). Another study conducted by Brown et al. (2004) has shown the Affirm assay was significantly more likely to identify *Gardnerella* and *Candida* than wet mount. Asymptomatic women were significantly more likely to be negative for Affirm and wet mount. Therefore, the Affirm VPIII test is a more sensitive diagnostic test for detection and identification of symptomatic vaginitis/vaginosis than conventional clinical examination and wet mount testing.

In Situ Hybridization (ISH) Probes for Virus Detection/Identification

ISH uses labeled nucleic acid probes to detect specific DNA or RNA targets in tissue sections and intact cells. ISH combines the specificity and sensitivity of nucleic acid hybridization with the ability to obtain histological and/or cytological information. Probes for ISH were originally labeled radioisotopically with ^{35}S, ^{32}P, or ^{125}I. Newer techniques using nonisotopic hapten digoxigenin are equally as sensitive and exhibit lower background and provide greater resolution than radiolabeled probes. The use of no isotopic labels eliminates the health hazards and disposal problems associated with radioactive probes. Digoxigenin-labeled probes are detected enzymatically with antigoxigenin antibodies conjugated with alkaline phosphatase or horseradish peroxidase. These enzymes convert soluble substrates into insoluble precipitates that appear as dark, localized cellular or subcellular staining. Biotin is another popular nonisotopic label that can be detected with enzyme conjugates of avidin, streptavidin, or antibiotin antibodies.

ISH is performed by transferring a small aliquot of a solution containing labeled probe (single-stranded or denatured double-stranded probes) to protease-digested tissue section. A coverslip is then placed over the specimen to prevent evaporation. Double-stranded targets must be denatured prior to hybridization, and denaturation may enhance hybridization in mRNA or rRNA by eliminating secondary structures. The stability of the hydrogen bonds between probe and target nucleic acid molecule is dependent on temperature, salt and formamide concentration, length, and GC (Guanine-Cytosine) content of the hybrid. Optimum conditions for a successful ISH should be developed by the laboratory performing the test; when a commercial ISH kit is used, the laboratory may still need to modify the procedure to obtain optimum results.

ISH is an important technique for identifying and localizing viral nucleic acids associated with infectious disease and cancer. ISH has been used to determine the intracellular localization of the hepatitis viruses, human papillomaviruses, and herpes simplex viruses, and to detect these viruses. ISH has also been used to detect adenovirus, cytomegalovirus (Wu et al., 1992), JC virus, Epstein–Barr virus (Prange et al., 1992), and HHV-8 (Li et al., 1996). Human papilloma virus (HPV) is accepted as the primary causative agent in the development of cervical cancer. Although there have been approximately 100 HPV genomic types identified, most of these are not oncogenic and therefore do not lead to the development of cervical cancer. Those HPV genotypes that have been identified as types that contribute to the development of cervical cancer are categorized into intermediate and high risk HPV. ISH has been widely used to detect and differentiate HPV in cervical specimens. Dako Corporation (Carpinteria, CA, USA) provides biotinylated DNA probes for HPV ISH, including probes for high-risk group or type-specific probe.

Peptide–Nucleic Acid Probe

Fluorescence *in situ* hybridization (FISH) using peptide–nucleic acid (PNA) probes (PNA FISH) is a novel diagnostic technique combining the simplicity of traditional staining procedures with the unique performance of PNA probes to provide rapid and accurate diagnosis of infectious diseases. Peptide nucleic acids are novel synthetic DNA-like compounds with nucleotide bases attached to a peptide backbone. PNA probes are DNA probe mimics with an uncharged, neutral backbone that provides the PNA probes with improved hybridization characteristics such as high degrees of specificity, strong affinities, and rapid kinetics, as well as an improved ability to hybridize to highly structured targets such as rRNA. In addition, the relatively hydrophobic character of PNA probes compared with the character of DNA enables PNA probes to penetrate the hydrophobic cell wall after preparation of a standard smear.

PNA FISH probes have been developed and evaluated for *S. aureus*, *C. albicans*, *E. faecalis*, *E. coli*, coagulase-negative staphylococci, *C. dubliniensis*, *Klebsiella pneumoniae*, and *Pseudomonas aeruginosa*. Among these probes, currently the *S. aureus* PNA FISH, *C. albicans* PNA FISH, and *E. faecalis* PNA FISH are FDA approved for *in vitro* diagnosis and are available from AdvanDx, (Woburn MA, USA). The PNA FISH procedures have been extensively evaluated for rapid diagnosis of positive blood cultures for *S. aureus* (Chapin and Musgnug, 2003; Oliveira et al., 2002, 2003), *E. coli*, and *C. albicans* (Oliveira et al., 2001, Rigby et al., 2002) with high sensitivity and specificity. A recent multicenter evaluation (Wilson et al., 2005) of the *C. albicans* PNA FISH assay (AdvanDx) demonstrated that this method is an accurate means of differentiating *C. albicans* from non–*C. albicans* species present in blood culture bottles. The overall sensitivity, specificity, positive predictive value, and negative predictive value of the combined routine screening methods used at the various institutions were 100%, 97.3%, 96.0%,

and 100%, respectively. PNA FISH is also a promising procedure for identifying *Mycobacterium tuberculosis* from liquid culture (Stender et al., 1999; Drobniewski et al., 2000).

PNA FISH procedures may also be applied to formalin-fixed, paraffin-embedded tissue for identifying bacterial pathogens. The procedure may be useful in identifying organisms in heart-valve tissue (for patients undergoing heart-valve replacement surgery). Infective endocarditis (IE) is usually diagnosed by clinical, histological, and/or microbiological parameters according to the Duke scheme. Approximately 2.5–31% of cases are culture negative. Without etiological identification, choosing an effective therapeutic regimen can be challenging. In fresh tissue, PCR for amplification of bacterial 16S rRNA combined with nucleotide sequencing has significantly improved the identification of bacterial agents in culture-negative IE. When the diagnosis is based on histological findings in the formalin-fixed paraffin-embedded heart-valve tissue only, species identification historically has been limited to routine Gram stain. In theory, the combination of PNA FISH probes for *Streptococcus* spp., *Staphylococcus aureus*/coagulase–negative staphylococci, and *Enterococcus faecalis*, will detect a majority of Gram-positive cocci identified in the heart valves from patients with infective endocarditis and facilitate antimicrobial selection.

Technically, the most critical part of a successful FISH procedure on a paraffin tissue section on a glass slide is to fully deparaffinize slides. The following procedure works well: immerse slides in xylene (or Safeclear II, tissue clearing agent) for 10 min at room temperature. Repeat twice using fresh xylene (or Safeclear II). Dehydrate slides in 100% EtOH for 5 min at room temperature. Repeat one more time and air-dry the slides. Then perform the PNA FISH procedure.

Because the target of the PNA FISH test is the rRNA, the success of the test depends on the amount of well-preserved rRNA present in the bacteria and the relative amount of bacteria present in the tissue section.

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9
Pulsed-Field Gel Electrophoresis

FANN WU AND PHYLLIS DELLA-LATTA

Introduction

The field of molecular diagnostics has rapidly expanded to include technology for accurate and timely determination of clonal relatedness of microorganisms of epidemiological interest as well as the detection of infectious agents in real-time. The predominant technique used for strain characterization has been pulsed-field gel electrophoresis (PFGE), first developed in the early 1980s, to genotype microorganisms by electrophoretic separation of chromosomal DNA by molecular weight (Van der Ploeg et al., 1984). Over the years, this technology has proved to be a powerful tool used alone or in conjunction with restriction endonuclease digestion of the DNA in order to understand the evolution of antimicrobial resistance generated within a single clone and to determine genetic relatedness among microbial strains in industrial and agricultural settings, as well as health care associated epidemiologic investigations. This chapter will discuss the principle characteristics and the clinical applications of PFGE technology, including examples that illustrate its successful application to epidemiology. The strengths and limitations of PFGE are discussed as well as alternative strain-typing methods.

Principle of the PFGE Technique

The principle of PFGE is to use a specially designed electrophoretic apparatus to separate large DNA fragments typically ranging in size from 40 kb to 2000 kb.

The Development of PFGE

Conventional Electrophoresis

DNA is negatively charged at a neutral pH, and its migration in an electrophoretic field is molecular weight dependent. When molecules <50 kb are subjected to an electric current in an agarose matrix, they migrate at a rate that is inversely proportional to its size (i.e., the larger the DNA fragments, the slower the rate of

migration). However, when the molecular size exceeds the threshold of >50 kb, all fragments exhibit size-independent mobilities (Carle et al., 1986). This poses a major limitation to the use of conventional electrophoresis for microbial analysis because bacterial chromosomes are several mega base pairs in size.

The Introduction of PFGE

The concept of subjecting chromosomal DNA of microorganisms to two alternating electric fields for separation of large DNA fragments (40 to 2000 kb) within agarose gels was introduced in 1984 by Schwartz and Cantor. Subsequently, a variety of alternative electrophoretic configurations, using currents "pulsed" in different directions over controlled time intervals, have been developed. These include orthogonal field alternation gel electrophoresis (Carle and Olson, 1984), vertical alternating field gradient gel electrophoresis (Gardiner et al., 1986), periodic field inversion gel electrophoresis (Carle et al., 1986), and contour-clamped homogeneous electric field electrophoresis (CHEF) (Chu et al., 1986).

CHEF coupled with a programmable autonomously controlled electrode gel electrophoresis (PACE) have become the most common pulsed field methods used for DNA fingerprinting (Clark et al., 1988). Both systems contain three major components: a power module to generate the electrode voltages and to store switching function parameters, a cooling module to keep the temperature at 14°C, and an electrophoresis chamber. The chamber contains 24 horizontal electrodes, some of which are clamped to eliminate DNA lane distortion. The electrodes are arranged in a hexagon that offers reorientation angles of 60 or 120 degrees, in contrast to traditional orthogonal field alternation gel systems with two perpendicular electrodes. The resolution of PFGE is dramatically affected by the number and configuration of the electrodes used, because these alter the shape of the applied electrical field. For high-resolution separation, the most effective electrode configurations yield angles of more than 110 degrees (Cantor et al., 1988). In PACE, each electrode's voltage is independently controlled and can generate an unlimited number of electric fields of different voltage gradients, orientations, and intervals sequentially in time, whereas the traditional CHEF systems are limited to two alternating electric fields at a fixed reorientation angle. Both CHEF and PACE technologies are best configured to offer a unique tool to distinguish large-molecular-weight DNA molecules.

The PFGE Procedure

The quality of PFGE results can be significantly influenced by the following key steps within the protocol:

- Cell lysis and release of intact chromosomal DNA
- Restriction endonuclease digestion of chromosomal DNA
- Separation of the DNA fragments
- Analysis of DNA fragment length polymorphism

Bacterial Cell Lysis and Release of Intact Chromosomal DNA

A pure culture of a bacterial isolate of known identity is incubated overnight in a nutrient broth, such as trypticase soy broth (BD Biosciences, Sparks, MD, USA) in order to achieve 10^9cells/mL, which is the concentration needed to obtain a visible band pattern. Standard DNA extraction procedures are inappropriate for the analysis of large chromosomal DNA molecules because of DNA shearing caused by the application of mechanical force in the protocol. In order to prevent DNA damage, it is important to mix intact bacterial cells with warmed, liquid-phase agarose, and this mixture can then be pipetted into plastic molds to form $10 \times 5 \times 1.5$ mm agarose plugs. The whole cells embedded in plugs are lysed and deproteinized by detergents and enzymes (e.g., lysostaphin, lysozyme, proteinase K, mutanolysin or lyticase) *in situ* (Table 9.1). Following cell lysis, the plugs are washed 4–5 times with wash buffer containing 20 mM Tris and 50 mM EDTA (pH 8.0) to remove cell debris and proteinase. The agarose gel matrix keeps chromosomal DNA intact while removing the rest of cellular components from the plug. This step, including the post-lysis washing, usually takes 2 days. In recent years, several investigators have reported improvements to the traditional DNA preparation process. The most notable time-saving approaches have included (i) directly using bacterial colonies grown on plates of clinical specimens, (ii) using a combination of lytic enzymes, (iii) adding lytic enzymes to the bacterial suspensions before preparing the agarose plugs, (iv) shortening the cell lysis time by reducing the size of agarose plug, and (v) expediting the wash steps by using a large volume (10 mL) of preheated (50°C) water and TE buffer (Gautom, 1997; Turabelidze et al., 2000; Lopez-Canovas et al., 2003). Of particular interest, a DNA purification system was designed to automate all steps involved in the preparation of DNA plugs for PFGE (Fiett et al., 2004).

Restriction Endonuclease Digestion of Chromosomal DNA

A large amount of clean, intact chromosomal DNA embedded in agarose plugs can be easily digested with a variety of restriction endonucleases. Each of those enzymes is found to cleave double-stranded DNA at a specific nucleotide sequence, known as the enzyme's recognition site. Once the recognition site is located, the enzyme catalyzes the digestion of DNA at that defined position either close to or within the targeted sequence, causing a break in the nucleic acid strand, and producing discrete restriction fragments. The choice of the restriction enzyme is dependent upon the bacterial species studied. The number and size of fragments generated by an endonuclease depends on the frequency of the specific restriction enzyme recognition sites located on a particular bacterial genome. This cutting frequency is a function of the number of base pairs required for enzyme recognition (longer sequences lead to less enzyme recognition), and the GC content of the genome. For example, the restriction enzyme, *Sma*I, recognizes the CCC/GGG sequence that cleaves the DNA of most Gram-positive bacteria, whereas *Xba*I recognizes the T/CTAGA sequence that cleaves that of many Gram-negative bacteria.

TABLE 9.1. Commonly typed organisms by PFGE method.

Organism	Recommended lysis enzyme	Restriction enzyme	Approximate no. of restriction fragments	Fragment size range (kb)
Gram-positive bacteria				
Enterococcus spp.	LZ, LS, PK	*Sma*I	15–20	5–400
Clostridium difficile	LZ, PK	*Sma*I, *Sac*II	10–15	10–900
Clostridium perfringens	LZ, PK	*Sma*I, *Sac*II	10, 12	15–1640
Staphylococcus aureus	LZ, LS, PK	*Sma*I, *Csp*I	10–15, 15–20	10–700, 30–500
Staphylococcus (coagulase negative)	LZ, LS, PK	*Sma*I	15–20	5–400
Streptococcus spp. (group A and B)	LZ, ML	*Sma*I	15–20	5–500
Streptococcus pneumoniae	LZ, ML	*Apa*I, *Sma*I	10–19	20–300, 20–250
Gram-negative bacteria				
Acinetobacter calcoaceticus	PK	*Sma*I	20–25	5–300
Acinetobacter baumannii	PK	*Sma*I, *Apa*I	20–40, 20–30	5–300, 10–300
Bacteriodes spp.	PK	*Not*I	8–10	200–1200
Bordetella pertussis	PK	*Xba*I	20–30	20–700
Borrelia burgdorferi	LZ, PK	*Sma*I	10–30	10–300
Burkholderia cepacia	PK	*Spe*I	20–25	40–700
Campylobacter jejuni	PK	*Sma*I	8–10	40–400
Campylobacter fetus	PK	*Sma*I, *Sal*I	10–15	40–400, 40–300
Chlamydia trachomatis	PK	*Sse*83871	17	9–220
Coxiella burnetii	PK	*Not*I	19	10–293
Enterobacter spp.	PK	*Xba*I	15–20	10–700
Escherichia coli	PK	*Xba*I, *Not*I, *Sfi*I	15–20, 12–15 15–20	10–500, 10–1000 10–700
Haemophilus influenzae	PK	*Sma*I, *Rsr*II	10–12	10–500
Klebsiella spp.	PK	*Xba*I	15–20	10–700
Legionella pneumophilia	PK	*Sfi*I, *Not*I	10–15, 5–10	50–700, 50–2000
Mycobacterium spp.	LZ, PK	*Ase*I	12–20	10–700
Neisseria gonorrhoeae	LZ, PK	*Spe*I	12–17	10–500
Neisseria meningitidis	LZ, PK	*Not*I, *Bgl*II	20–30	5–200
Proteus mirabilis	PK	*Sfi*I, *Not*I	7–10, 6–10	50–700, 75–700
Pseudomonas aeruginosa	PK	*Spe*I, *Xba*I	20–25, 40–50	10–700, 10–300
Salmonella spp.	PK	*Not*I	40–50	5–400
Shigella spp.	PK	*Xba*I, *Sfi*I	15–23, 15–20	10–700
Vibrio cholerae	LZ, PK	*Not*I	20–30	10–400
Stenotrophomonas maltophilia	PK	*Xba*I	15–20	10–700
Yersinia pestis	PK	*Xba*I	15–20	10–700
Yeast				
Candida albicans	LC, PK	*BssH* II	25–35	10–700
Candida glabrata	LC, PK	*Sfi*I, *BssH* II	25–35	300–800,100–600
Candida guillermondi	LC, PK	*Sfi*I, *BssH* II	25–40	300–800,100–600
Candida lusitaniae	LC, PK	*Not*I, *BssH* II	25–40	100–600
Candida parapsilosis	LC, PK	*BssH* II	25–40	10–700

LS, lysostaphin; LZ, lysozyme; LC, lyticase; ML, mutanolysin; PK, proteinase K.

*Sma*I is able to cleave DNA at rarely occurring sites due to the low GC content and AT-rich sequences in Gram-positive bacteria. In order to achieve best separation by PFGE technology, careful selection of low-frequency cleaving enzymes enables cutting the whole bacterial chromosome of any species into 10 to 30 fragments, typically 40 to 1000 kb in size (Maslow & Mulligan, 1996; Goering, 2003). The most common enzymes used to type specific microorganisms are summarized in Table 9.1.

The concentration of the restriction enzyme required for digesting DNA embedded in agarose is slightly higher than that needed to digest DNA in solution, because of the limited enzyme diffusion into the agarose plug. However, overnight digestion in agarose is usually unnecessary because genomic DNA can be completely digested in 2 to 4 h following manufacturers' instructions. After restriction enzyme digestion, the plugs are cut into appropriate sizes, loaded onto comb teeth, sealed with molten agarose, and placed in the electrophoresis chamber. This DNA plug loading procedure permits careful adjustment of each plug to ensure proper alignment and to achieve clear band patterns that facilitate analysis.

Separation of Large DNA Fragments

To achieve the best resolution across a broad range of DNA sizes by PFGE, there are several factors that must be considered: concentration and composition of the agarose gel and the buffer, the running temperature, pulsed-field conditions including switching times, electric field strength, pulse angle, and total electrophoresis duration. For example, the agarose concentration determines the size range of DNA molecules separated and the sharpness. DNA fragments can migrate at different rates in TAE (Tris-acetate-EDTA) and TBE (Tris-borate-EDTA) buffers due to differences in ionic strength. Raising the buffer temperature increases the DNA mobility, and changing PFGE system parameters can also affect the migration rate of DNA molecules. With careful selection of these conditions, PFGE can be applied to genotypic typing of most bacteria and yeast. As a result of PulseNet, the National Molecular Subtyping Network for Foodborne Disease Surveillance, PFGE conditions have been standardized for multilaboratory comparison. A standard protocol uses 0.8–1% agarose (molecular biology grade), 0.5X Tris-borate-EDTA buffer (45 mM Tris, 45 mM borate, 1.0 mM EDTA [pH 8.3]), orientation angle of 120°, and voltage of 6 V/cm for most bacterial typing. However, switching times and duration depend on the size of DNA fragments generated by various restriction enzymes. For example, to resolve 25–700 kb sized *Xba*I fragments of *K. pneumoniae* DNA by PFGE, switching times can be ramped from 2.2 to 54.2 s at 6 V/cm at 14°C for 22 h. To ensure the standardization between multiple gels performed in several laboratories, a marker of known molecular weight should be included in each gel to verify the sizes of unknown samples and optimize the electrophoresis conditions.

The most common problems encountered that influence the ability to detect the correct size of the bands of interest in PFGE analysis include:

- **DNA degradation in the gel**. DNA degradation, which is the most common problem yielding nonspecific fragments ranging from 40 to 150 kb, is due to the activity of nucleases present in some microorganisms. This problem can be prevented by the use of HEPES buffer instead of Tris buffer (Koort et al., 2002) or adding 50–75 μM thiourea in the running buffer to eliminate reactive Tris radicals (Romling and Tummler, 2000).
- **Incomplete digestion by restriction endonucleases**. On occasion, some enzyme recognition sites on chromosomal DNA are not cleaved during sample digestion. This partial digestion results in the production of DNA fragments that are too large to migrate and therefore remain near the top of the gel. To prevent enzyme degradation, the protease and detergent added during sample preparation should be completely removed before adding the restriction enzymes.
- **Incorrect electrophoresis conditions.** To permit microbial genotyping, optimal electrophoretic conditions for appropriate migration of DNA fragments can be modeled from previous studies or designed using a standardized marker of known molecular weight. The use of incorrect settings could cause chromosomal DNA fragments either to migrate too quickly for retention in the gel or they may be too close together to interpret.

Analysis of DNA Fragment Length Polymorphism

The DNA fragments in the agarose gel generated by PFGE are visualized by staining with ethidium bromide. Each lane on the gel represents the chromosomal pattern of one bacterial isolate. The migration of DNA fragments form patterns that determine chromosomal similarity and hence clonality of strains. The standardized recommendations (Tenover et al., 1995) for the interpretation of PFGE patterns of isolates linked to an epidemiological investigation are dependent upon the number of band differences on the gel. Those yielding the same pattern should be considered "indistinguishable," one to three band differences are "closely related," reflecting a single genetic change, four to six band differences are "possibly related," representing two independent genetic events, and six or more band differences represent three or more genetic changes and are considered "unrelated." However, comparisons of DNA fragment patterns present on multiple gels from large sets of isolates are technically difficult to interpret (Chung et al., 2000). There are variables that might alter fragment patterns and cause lack of interlaboratory reproducibility, such as type of PFGE instrumentation, protocols, or individual user techniques. Several commercially available software packages that provide computerized gel scanning and data analysis can compensate for these intra- and intergel variations (Duck et al., 2003). For example, the DNA patterns on the same or multiple gels can be more clearly represented as a dendrogram, showing the percent similarity obtained through Dice coefficients and the unweighted pair group method with arithmetic average. Through the use of computer-assisted analysis of DNA fragment polymorphism, investigators are able to create searchable databases of DNA patterns for multilaboratory comparison and for future strain comparisons.

PFGE Performance Characteristics

The performance of strain typing technology, including PFGE, is measured by the following criteria (Struelens, 1998; Pfaller et al., 2001):

- **Discriminatory power** describes the probability that indistinguishable or closely related strains are truly clonal and part of the same chain of transmission. This parameter can be calculated based on Simpson's index of diversity, using an index greater than 0.95 as acceptable (Simpson, 1949; Struelens, 1998).
- **Reproducibility** is the ability to obtain the same results upon repeat testing of the same strain.
- **Stability** is measured by the ability of clonal isolates to consistently express particular markers over time.
- **Typeability** measures the proportion of isolates within a bacterial species that can be designated a genotype by a molecular typing system.

PFGE has high discriminatory power and reproducibility. This performance feature is based on direct analysis of greater than 90% bacterial chromosomal polymorphism (Goering, 2000). It has significant advantages compared with other nonamplification methods, which include plasmid DNA analysis and restriction endonuclease analysis of chromosomal DNA (REA). First, PFGE digests large DNA chromosomal fragments with infrequent-cutting restriction endonucleases, yielding well-separated bands that are easy to read. Second, because conventional electrophoresis is limited to the separation of relatively small (<50 kb) DNA fragments, the chromosomal DNA must be digested with frequent-cutting restriction endonucleases, thus generating hundreds of uninterpretable bands.

In recent years, a number of PCR amplification-based methods have been developed for genotyping microbial pathogens, such as arbitrarily primed polymerase chain reaction (AP-PCR), random amplified polymorphic DNA (RAPD), multilocus primed PCR or repetitive chromosomal elements PCR (rep-PCR), and amplified restriction fragment length polymorphism (AFLP). Amplification-based technologies are less discriminatory but have the advantages of being less costly and labor intensive, taking approximately 2 days to obtain results, as compared with 4 to 5 days for PFGE (Wu and Della-Latta, 2002). In addition, DNA fragments smaller than 50 kb cannot be reliably separated by PFGE, because the system is not able to switch the field orientation quickly enough to separate these smaller molecules. Certain organisms such as *Clostridium difficile* and *Aspergillus* spp., which are difficult to type by PFGE because they are either uncultivable or their DNA cannot be isolated intact, can be analyzed using PCR-based typing methods. The typeability of PFGE may not be excellent for some bacterial species, such as *Acinetobacter* spp. because of DNA degradation challenges (Silbert et al., 2003). The comparison of the procedural features of PFGE and amplification-based typing methods are summarized in Table 9.2.

Many different PFGE protocols have been developed, and this has led to some variability in assay design and reproducibility among laboratories. It is important to

TABLE 9.2. Comparison of the procedural features of pulsed-field gel electrophoresis and PCR-based typing methods.

Procedural characteristics	Nonamplification typing PFGE	Amplification typing (RAPD, rep-PCR, MLST)
Genomic region	Entire chromosome	Selected region on the chromosome
Sample preparation	Intact cells embedded in agarose	DNA extraction
Fragment generation	Restriction endonuclease digestion	DNA polymerase amplification
Electrophoresis	Pulsed field	Single homogeneous
Fragment size	50–2000 kb	<10 kb
Time to results	3–4 days	2–3 days

establish standardized PFGE protocols, particularly with critical elements such as the DNA concentration, the effectiveness of restriction enzyme digestion, and the electrophoresis conditions including agarose gel volume and concentration, buffer volume, and ionic strength. The running conditions including voltage, switching times, reorientation angle, and total run times of electrophoresis are other variables to consider (Chung et al., 2000; Murchan et al., 2003). To insure good-quality gels and consistent reproducibility, a quality control strain should be included with each gel run for comparison. Using a standardized approach, and computer-assisted programs that demonstrate enhanced capability of comparing DNA fragment patterns present on multiple gels, investigators can create a searchable database of PFGE fragment patterns for interlaboratory comparison and facilitate cluster analyses.

General Guidelines for the Use of PFGE Technology

PFGE has been widely used in genetic and epidemiological analyses of at least 98 different pathogens, including Gram-positive and Gram-negative bacteria and fungi (Goering, 1998). In efforts to avoid nosocomial infections and to curtail outbreaks, epidemiologists rely on the microbiology laboratory to provide evidence for strain relatedness of these organisms as an aid in epidemiologic investigations to identify the point source of transmission. Although extensive genomic and phenotypic diversity exists within populations of microbial pathogens of the same species, the isolates of an organism that are part of the same chain of transmission are clonally related; that is, the progeny of the same ancestor cell. Some clinical applications of PFGE genotyping of bacterial pathogens that address hospital-related outbreak investigations and epidemiologic surveillance efforts are presented below.

Application to Gram-Positive Bacteria

PFGE is considered the accepted standard for molecular typing of nosocomial pathogens such as *Staphylococcus aureus* and vancomycin-resistant enterococci

(VRE). Nasal carriage of *S. aureus* occurs in 20–60% of the general population, and methicillin-resistant S. aureus (MRSA) pose a particular risk for nosocomial transmission (Kluytmans et al., 1997). It is clear that MRSA can be transferred between patients in the hospital setting and cause nosocomial infections. Rapidly assessing the clonal relatedness of these isolates is critical in determining the extent of transmission during an outbreak and in measuring the strategies for its containment. PFGE is often used to examine the genetic identity of MRSA isolates. In a recent publication from our medical center, PFGE results showed that vertical transmission of one clone of MRSA occurred from a mother to her preterm infants, followed by horizontal spread to other infants in the same neonatal intensive care unit (Morel et al., 2002). We demonstrated that the most likely reservoir of the MRSA clone were the colonized infants (Graham et al., 2002). It has been reported that some of the MRSA clones, endemic in hospitals in different countries, may have a high degree of genetic variation, leading to the appearance of multiple subtype variations (Aires de Sousa and de Lencastre, 2004).

In recent years, the increased prevalence of community-associated (CA)-MRSA has become a major public health concern (Saiman et al., 2003). In contrast to hospital-associated (HA)-MRSA, CA-MRSA strains are commonly susceptible to many antibiotics. Also CA-MRSA appears to have a distinct exotoxin *Panton–Valentine Leukocidin* (*PVL*), which has been associated with severe infections (Centers for Disease Control, 1999; Baba et al., 2002; Kazakova et al., 2005). Using PFGE, investigators have found that the typing patterns of CA-MRSA were distinct from those of HA-MRSA, indicating that different clonal populations have been successfully propagated in the community. Investigators also reported that MSSA strain 476 shared an identical PFGE pattern with a CA-MRSA strain, termed MW2. The only significant difference between the chromosomes of these two strains was the presence of type IV SCC*mec* in MW2, suggesting that the progenitor was the MSSA strain from which MW2 was generated by acquiring type IV SCC*mec* (Okuma et al., 2002).

Application to Gram-Negative Bacteria

PFGE has been successfully used as a tool to investigate the epidemiological relatedness of strains of Gram-negative bacteria including *Acinetobacter, Enterobacter, E. coli, Klebsiella, Pseudomonas, Salmonella*, and *Serratia* within a hospital, a community, or school or daycare center (Durmaz et al., 2003; Pavlopoulou et al., 2004). We have reported an outbreak of extended-spectrum beta-lactamase (ESBL)-producing *Klebsiella pneumoniae* infections in our neonatal intensive care unit (NICU) (Gupta et al., 2004). A total of 19 infants were either infected or colonized with *K. pneumoniae*, in which 9 of 19 infants developed invasive disease. Surveillance cultures revealed that two health care workers carried *K. pneumoniae* on their hands. The PFGE patterns of isolates from health care workers were compared with 19 isolates recovered from the infants. Results indicated that the one clone was shared by both the infants and the hands of health care workers. One

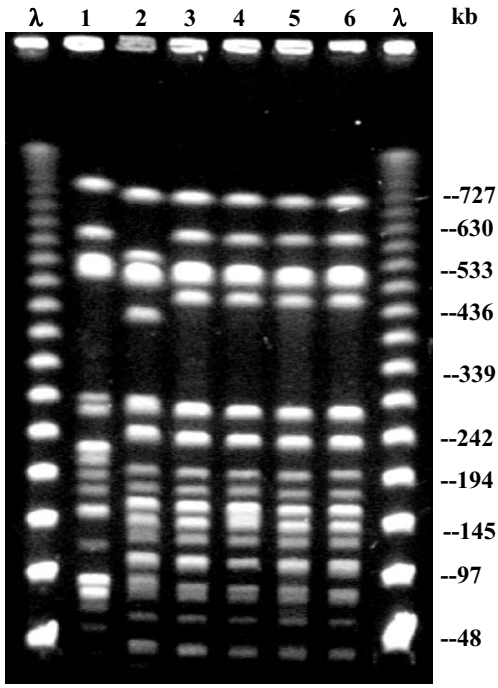

FIGURE 9.1. PFGE of *K. pneumoniae* isolates. Genetic profiles were obtained by digestion of chromosomal DNA with *Xba*I restriction endonuclease. Lanes 2 to 6 represent chromosomal DNA patterns of *K. pneumoniae* isolates recovered from five patients during an outbreak. Lane 1 shows chromosomal DNA from unrelated isolates obtained from other hospital units during the course of the outbreak. Lane λ shows the molecular weight standard.

health care worker wore artificial nails, and epidemiological evidence suggested that wearing artificial fingernails was a high risk factor for acquisition and transmission of the pathogen among infants. Recently, nosocomial multidrug-resistant *K. pneumoniae* infections are increasing in prevalence, highlighting the growing concern for heightened infection control precautions and surveillance efforts. Figure 9.1 shows the PFGE patterns that were generated when multidrug-resistant *K. pneumoniae* chromosomal DNA was digested with *Xba*I restriction enzyme. A dendrogram that presents the percent similarity of the DNA fragment patterns of the isolates is illustrated in Fig. 9.2.

PFGE has been adapted for use in epidemiological investigations by national and international surveillance networks. Multicommunity outbreaks caused by specific pathogens, such as *Shigella* and *Legionella*, often cause food- or water-associated infections and require comprehensive public health measures (Centers for Disease Control, 2004; Decludt et al., 2004). Appropriate surveillance and timely detection of the outbreak sources is necessary for interruption of pathogen transmission. The strategy involves submitting selected isolates from ongoing outbreak sources to a designated center laboratory where PFGE genotyping is routinely performed. When a cluster of identical genomic profiles of isolates is determined, the local health authorities are immediately notified and an environmental investigation is

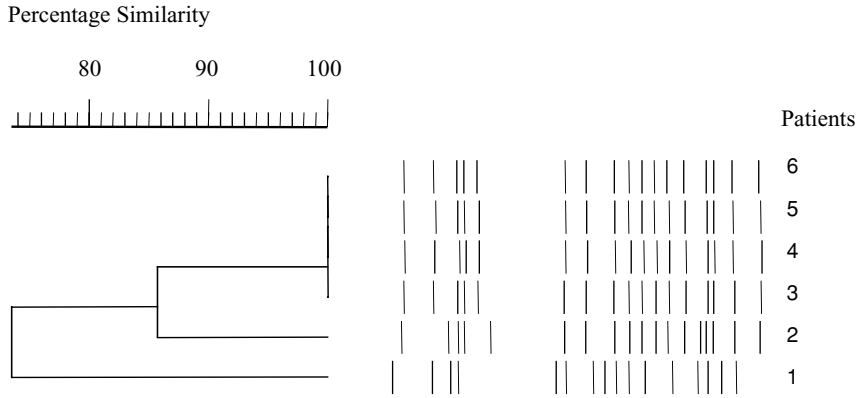

FIGURE 9.2. Percent similarity of PFGE patterns of *K. pneumoniae* strains. The dendrogram was constructed by the unweighted pair group method with arithmetic mean clustering by using the Dice correlation coefficient (Wu and Della-Latta, 2002).

conducted. Thus, PFGE typing enables reliable tracking of epidemic clones and assists in determining the extent of outbreaks.

Application to Yeast

PFGE genotyping plays a major role in the prevention and control of nosocomial candidiasis. It has been estimated that 10–20% of nosocomial bloodstream infections are due to *Candida* species (Jarvis, 1995) In addition to *Candida albicans*, *Candida parapsilosis* is emerging as a prominent bloodstream pathogen in the NICU. A multicenter cohort study demonstrated that NICU patients acquire *C. parapsilosis* from the hands of health care workers (Saiman et al., 2000, 2001). The use of PFGE technology to characterize isolates of *Candida* spp. has been successful in establishing that the gastrointestinal tract should be considered as a major endogenous reservoir for these organisms and that gastrointestinal colonization of infants has been strongly associated with sepsis (el-Mohandes et al., 1994) Another application of PFGE is in examining the DNA profiles of sequential yeast isolates recovered from the same infected patient over time to determine if one strain or several strains are involved in the infectious process. Although the antifungal susceptibility pattern of the isolates may vary, this might represent the emergence of resistance to current therapy in the same strain and not the acquisition of new strains (Bennett et al., 2004).

PFGE genotyping of *Candida* spp. can be performed with or without the restriction enzyme digestion. However, electrophoretic karyotyping analysis without enzyme digestion has been reported to provide sufficient discrimination for epidemiologic investigations (Espinel-Ingroff et al., 1999). When restriction enzymes are used for additional subtyping, *BssH*II, *Not*I, and *Sfi* are recommended for digestion of chromosomal DNA of *Candida* species (Table 9.1).

Summary

PFGE is widely used for genotypic characterization of microorganisms. It has been considered the method of choice for the analysis of most bacterial pathogens because of its high discriminatory power and reproducibility. Although PFGE is an important tool for outbreak investigations, it is not indicated for population analysis of microorganisms. In addition, PFGE is marginally valuable in epidemiological settings of long-term duration encompassing years or decades or at a country to continent level (Blanc et al., 2002). Its limitations also include substantial measurements of time and resources, and the non-typeability of some strains in some situations. Therefore, a combination of PFGE and PCR-based techniques may be necessary for genotyping a wide range of microorganisms under these circumstances.

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10
In Vitro Nucleic Acid Amplification: An Introduction

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Introduction

Over the past decade, the development of a series of *in vitro* nucleic acid amplification (NAA) technologies has opened new avenues for the detection, identification, and characterization of pathogenic organisms in diagnostic microbiology (Tang et al., 1997; Jungkind and Kessler, 2002; Yolken, 2002). The promise of these techniques is the replacement of traditional biological amplification of live pathogens by enzymatic amplification of specific nucleic acid sequences. These techniques have reduced the dependency of the clinical microbiology laboratory on culture-based methods and created new opportunities for the field to enhance patient care. According to the theoretical basis for each methods, *in vitro* nucleic acid amplification techniques can be placed into one of three broad categories, which all share certain advantages over traditional methods, particularly for the detection of fastidious, unculturable, and/or highly contagious organisms (Table 10.1). Application of NAA techniques enhances the speed, sensitivity, and sometimes the specificity of an etiologic diagnosis (Tang et al., 1999; Yolken, 2002; Hayden, 2004).

Target Amplification Systems

Target amplification systems are defined as nucleic acid amplification procedures in which many copies of the nucleic acid targets are made, which include PCR, nucleic acid sequence–based amplification (NASBA), transcription-mediated amplification (TMA), or strand displacement amplification (SDA). Among them, PCR and PCR-derived techniques are the best-developed and most widely used methods of nucleic acid amplification (Saiki et al., 1988; Eisenstein, 1990; Mullis, 1990). Commercial products as well as user-developed PCR-based NAA techniques are available for the detection of microbial pathogens, identification of clinical isolates, and strain subtyping (Tang et al., 1999; Hayden, 2004). PCR-derived techniques, such as reverse transcription PCR, nested PCR, multiplex PCR, arbitrary primed PCR, and broad-range PCR, have collectively expanded the flexibility and power

TABLE 10.1. Nucleic acid amplification methods.

Amplification method	Amplification category	Manufacturer/license (trade mark)	Enzymes used	Temperature requirement	Nucleic acid target	Main references
Polymerase chain reaction (PCR)	Target	Roche Molecular System, Inc., Branchburg, NJ, USA (Amplicor)	*Taq* DNA polymerase	Thermal cycler	DNA or RNA	(Saiki et al., 1988; Mullis, 1990)
Transcription-mediated amplification (TMA)	Target	Gen-Probe, Inc., San Diego, CA, USA (APTIMA)	Reverse transcriptase, RNA polymerase, RNase H	Isothermal	RNA or DNA	(Kwoh et al., 1989; La Rocco et al., 1994)
Nucleic acid sequence–based amplification (NASBA)	Target	Organon-Teknika, Corp., Durham, NC, USA (Nuclisens)	Reverse transcriptase, RNA polymerase, RNase H	Isothermal	RNA or DNA	(Compton et al., 1991; Revets et al., 1996)
Strand displacement amplification (SDA)	Target	Becton-Dickinson, Sparks, MD, USA (ProbTec)	Restrictive endonucleonase, DNA polymerase	Isothermal	DNA or RNA	(Walker et al., 1992; Hellyer et al., 1996)
Invader technology	Probe	Third Wave, Madison, WI, USA	Cleavase	Isothermal	DNA or RNA	(Brow et al., 1996; Rossetti et al., 1997)
Cycling probe technology (CPT)	Probe	ID Biomedical Corp., Vancouver, Canada	Rnase H	Isothermal	DNA	(Duck et al., 1990; Cloney et al., 1999)
Ligase chain reaction (LCR)	Probe	Abbott Laboratories, Abbott Park, IL, USA (LCx)	DNA ligase	Thermal cycler	DNA or RNA	(Wu and Wallace, 1989; Cecil et al., 2001)
Hybrid capture system	Signal	Digene Diagnostics, Inc., Silver Spring, MD, USA	None	Isothermal	DNA	(Brown et al., 1993; Mazzulli et al., 1999)
Branched DNA (bDNA)	Signal	Chiron Corp., Emeryville, CA, USA	None	Isothermal	DNA or RNA	(Urdea et al., 1991; Lau et al., 1993)

of these methods in diagnostic laboratories across the world. Roche Molecular System, the current holder of the PCR patents, has several PCR-based diagnostic products available for infectious disease pathogen detection and quantitation (Tang et al., 1999; Jungkind et al., 2002).

Given the patent restrictions on PCR and the expanding interest in nucleic acid–based diagnosis, alternative amplification methods have been sought. Another target amplification system, transcription-mediated amplification or nucleic acid sequence–based amplification, involves several enzymes and a complex series of reactions that all take place simultaneously at the same temperature and in the same buffer (Kwoh et al., 1989; Compton, 1991). The advantages include very rapid kinetics and the lack of requirement for a thermocycler. Isothermal conditions in a single tube with a rapidly degradable product (RNA) help minimize (but may not eliminate) contamination risks. Amplification of RNA not only makes it possible to detect RNA viruses but also increases the sensitivity of detecting bacterial and fungal pathogens by targeting high copy number RNA templates. A TMA-based system manufactured by GenProbe Inc. has been used to detect *Mycobacterium tuberculosis* in smear-positive sputum specimens, to confirm *Chlamydia trachomatis* and *Neisseria gonorrhoeae* infection, as well as to screen human immunodeficiency virus (HIV)-1 RNA in donor blood specimens (La Rocco et al., 1994; Revets et al., 1996; Gaydos et al., 2003). NASBA system–based products are commercially available from bioMérieux and have been used for the detection of enteroviruses in cerebrospinal fluid and for the quantitation of hepatitis C virus (HCV) levels in serum (Hollingsworth et al., 1996; Landry et al., 2003).

Another isothermal, non-PCR target amplification technique is SDA, which uses specific primers, a DNA polymerase, and restriction endonuclease to achieve exponential amplification of the target (Walker et al., 1992). The key technology behind SDA is the generation of site-specific nicks by the restriction endonuclease. Since its initial description, it has evolved into a highly versatile tool that is technically simple to perform but conceptually complex. Commercial kits have been available from Becton Dickinson for diagnosis and monitoring of *C. trachomatis, N. gonorrhoeae*, and *M. tuberculosis* infections (Hellyer et al., 1996; Spears et al., 1997). The ProbeTec ET system combines amplification of nucleic acids by SDA and real-time identification by using fluorescence resonance energy transfer (Little et al., 1999).

Probe Amplification Systems

In probe amplification systems, many copies of the probe that hybridizes the target nucleic acid are made (Birkenmeyer and Mushahwar, 1991). The ligase chain reaction (LCR), cleavase-invader assay, and cycling probe technology (CPT) have been successfully applied in diagnostic microbiology. A gapped LCR procedure, which is designed following a target amplification method, such as PCR, can be sensitive and useful for the detection of point mutations (Osioway, 2002). Although

convenient and readily automated, one potential drawback of LCR is the difficult inactivation of postamplification products. The nature of the technique does not allow for the most widely used contamination control methods to be applied. A combination LCR kit for the detection of both *C. trachomatis* and *N. gonorrhoeae* is now commercially available from Abbott Laboratories (Carroll et al., 1998). The inclusion of a real-time identification system within the same reaction tube (closed reaction systems) would significantly decrease the possibility of contamination that is associated with the opening of reaction tubes (Harden et al., 2004).

Another similar system, cycling probe technology, uses a unique chimeric DNA-RNA-DNA probe sequence that provides an RNase H sensitive scissile link when hybridized to a complementary target DNA sequence (Duck et al., 1990). The CPT reaction occurs at a constant temperature, which allows the probe to anneal to the target DNA. RNase H cuts the RNA portion of the probes, allowing the cleaved fragments to dissociate from the target DNA. A cycling probe has been designed for detection of a specific sequence with the *mecA* and *vanA/B* genes, and the former one has been cleared by the Food and Drug Administration for *in vitro* diagnostic use as a culture confirmation assay for methicillin-resistant *Staphylococcus aureus* (Beggs et al., 1996; Cloney et al., 1999; Fong et al., 2000; Modrusan et al., 2000).

The homogenous invader technology relies on cleavase enzymes, which cleave the 5′ end single-stranded flap of a branched base-pair duplex (Brown et al., 1993). The characteristics of the technique make it a powerful tool for genetic analysis of single nucleotide polymorphisms in both microorganisms and hosts that are associated with specific diseases. Detection is accomplished through a fluorescence resonance energy transfer mechanism (Lyamichev et al., 1999). In addition to its wide application in molecular genetics, the technology has been used in diagnostic microbiology to genotype HCV and to test for drug resistance mutation in *S. aureus* and *M. tuberculosis* (Sreevatsan et al., 1998; Cooksey et al., 2000).

Signal Amplification Systems

Signal amplification is a nucleic acid amplification procedure in which a signal or reporter molecule attached to the probe is detected, and the signal is amplified enormously. Signal amplification methods are designed to strengthen a signal by increasing the concentration of label attached to the target nucleic acid. Unlike procedures that increase the concentration of the probe or target, signal amplification increases the signal generated by a fixed amount of probe hybridized to a fixed amount of specific target. The fact that signal amplification procedures do not involve a nucleic acid target or probe amplification is a theoretical advantage because of lower susceptibility to contamination problems inherent in enzyme-catalyzed nucleic acid amplification. Sensitivity, however, compared with target nucleic acid amplification techniques may be a limiting factor. Another limitation of signal amplification is background noise due to the nonspecific binding of reporter probes.

Currently, two diagnostic companies have their signal amplification products available for diagnostic microbiology purposes. The Digene hybrid capture system is widely used to determine human papillomavirus (HPV) infection and viral types in cervical swabs or fresh cervical biopsy specimens as well as other diagnostic targets (Brown et al., 1993). Persistent high-risk human papillomavirus infection detected by the System represents a reliable tool to select populations at risk for the development of high-grade cervical lesions (Brown et al., 1993; Schiffman et al., 1995). Besides HPV, Hybrid capture assays for the detection of hepatitis B virus, cytomegalovirus, *C. trachomatis*, and *N. gonorrhoeae* in clinical specimens are commercially available (Ho et al., 1999; Mazzulli et al., 1999; Schachter et al., 1999).

Another signal amplification-based product is the branched DNA (bDNA) probe developed and manufactured by Chiron Corp., which uses multiple specific synthetic oligonucleotides hybridize to the target and capture the target onto a solid surface (Urdea et al., 1991). Synthetic bDNA amplifier molecules, which are enzyme conjugated, branched oligonucleotide probes, are added. Hybridization proceeds between the amplifier and the immobilized hybrids. After addition of a chemiluminescent substrate, light emission is measured and may be quantified. This technique represents an excellent method for quantitation and therapeutic response monitoring of HCV and HIV-1 (Lau et al., 1993; Revets et al., 1996).

Current commonly used *in vitro* NAA techniques are categorized and summarized in Table 10.1. Each of the three categories is discussed in the following several chapters of this book, and the discussion is followed by a closer look at individual techniques including principles and applications in diagnostic microbiology.

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11
PCR and Its Variations

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PCR: The Archetypal Nucleic Acid Amplification Method

The polymerase chain reaction (PCR) is an *in vitro* technique used to replicate, or amplify, a specific region of DNA billions-fold in just a few hours (Saiki et al., 1985, 1988; Mullis and Faloona, 1987). The amplification is primer directed—oligonucleotide primers anneal to and flank the DNA region to be amplified. PCR is used in diagnostic and research laboratories to generate sufficient quantities of DNA to be adequately tested, analyzed, or manipulated. Because of the exquisite sensitivity it offers, PCR has rapidly become a standard method in diagnostic microbiology. More recently, reagent kits and various instrument platforms have added speed, flexibility, and simplicity (Tang et al., 1997; Fredricks and Relman, 1999; Tang and Persing, 1999). How significant is the contribution of PCR to the field of biomedicine? This question is perhaps best answered by the results of a PubMed search using the key word "PCR" (214,352 hits) or a search using the key words "PCR" and "diagnosis" (74,447 hits).

PCR was conceived in 1983 by Kary B. Mullis (Mullis, 1990), an achievement that earned him the Nobel Prize in chemistry in 1993. The first practical application of PCR was described by Saiki and colleagues in 1985 (Saiki et al., 1985), and less than 10 years later the U.S. Food and Drug Administration cleared the first PCR-based test for diagnosis of an infectious disease (Tang et al., 1997). The 1990s saw the birth of a number of alternative nucleic acid amplification methods, including Qβ replicase, ligase chain reaction, strand-displacement amplification, transcription-mediated amplification, and others. Some of these methods are discussed elsewhere in this text. Research and diagnostic applications of PCR continued to be developed during the 1990s. In an incredibly short period of time, PCR revolutionized the field and became a staple on the clinical microbiologist's menu of tests. Indeed, molecular diagnostics is now a recognized subspecialty within clinical microbiology.

Principles of PCR

Enzymatic Amplification of DNA: Components of the PCR Reaction

Two early innovations responsible for making PCR a practical research and diagnostic tool are thermal stable DNA polymerase and the thermal cycler. The thermal cycler will be discussed later in this chapter. PCR was first performed using heat-labile DNA polymerase. This necessitated manual replenishment of enzyme that was destroyed after every cycle. Heat-stable DNA polymerase was isolated from the bacterium *Thermus aquaticus,* which inhabits hot springs where temperatures exceed 90°C. This enzyme, called *Taq* DNA polymerase, remains active despite repeated heating during many cycles of amplification.

The basic procedure used in PCR is depicted in Fig. 11.1.

In addition to DNA polymerase, essential components of the PCR reaction include oligonucleotide primers, deoxynucleotide triphosphates (dNTPs), a divalent cation such as magnesium chloride, template or target DNA, and buffer (usually Tris). Primers are oligonucleotides, generally 20 to 25 bases long. They are designed to recognize specific sequences of the intended target and define the amplified region. At temperatures appropriate for annealing, the two primers bind

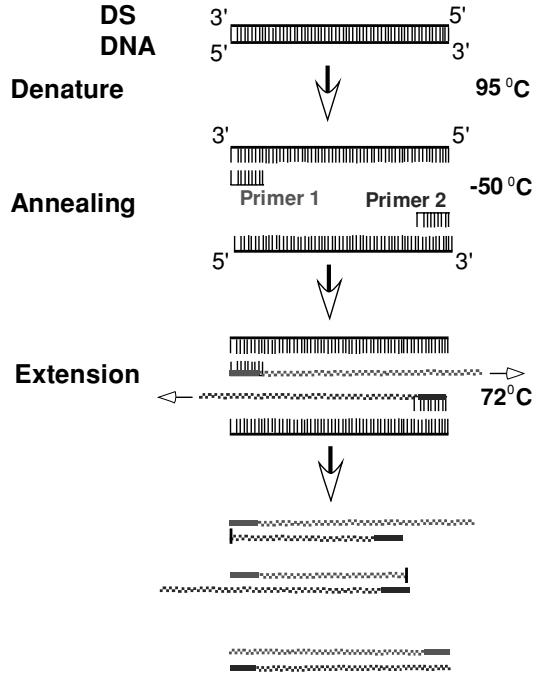

FIGURE 11.1. PCR cycling steps.

to opposite ends of this region, each to a complementary strand of target DNA. Primers must be designed carefully to avoid self-annealing or dimerization. The length and sequence of the primer determine its melting temperature and hence annealing temperature. Once annealed to target DNA, primers create a binding site for DNA polymerase, which requires a double-stranded DNA template. This short double-stranded section primes the DNA replication or amplification process. As stated at the beginning of the chapter, PCR is a primer-directed amplification of DNA. *Taq* DNA polymerase is the enzyme responsible for synthesizing or extending the new DNA strand. Complementary base pairing creates a new strand, which is in essence the mirror image of the template strand. dNTPs are the building blocks for the new DNA strands, or amplicons. The dNTP mixture includes dATP, dCTP, dGTP, and dTTP, generally at equimolar concentrations. If the enzyme uracil *N*-glycosylase (UNG) is used in the PCR reaction to prevent carry-over contamination, dUTP is added in place of or in combination with dTTP. Magnesium is the cofactor most commonly used in PCR reactions and is required for *Taq* DNA polymerase activity. Magnesium concentration must be carefully optimized, as the window of optimal activity is rather narrow.

The PCR Cycle

PCR consists of three steps: denaturation, primer annealing, and extension. One round of these three steps is referred to as a PCR cycle. These processes require different temperatures. This is accomplished using an automated thermal cycler, which can heat and cool tubes rapidly. Although most PCR protocols use three different temperatures for each step, two-temperature PCR cycles, where primer annealing and extension occur at the same temperature, have been described. Generally, 30 to 40 rounds of temperature cycling are required to generate a sufficient amount of amplicon.

Denaturation

At a temperature of 93°C to 94°C, the two strands of the DNA target are separated, or denatured. At this temperature, all enzymatic reactions, such as the extension from a previous cycle, stop.

Annealing

Following denaturation, the temperature of the reaction is reduced to allow strands of DNA with complementary sequence to anneal. The annealing temperature varies, depending on the sequence and hence melting temperature of the oligonucleotide primers, but is often between 50°C and 60°C. At annealing temperature, the primers are in movement, caused by Brownian motion. Ionic bonds are constantly formed and broken between the single-stranded primer and DNA target. When primers come in contact with a perfectly complementary target sequence, the bond that forms is sufficiently stable to allow DNA polymerase to sit and initiate DNA synthesis at the 3′ end of each primer.

Extension

Extension of the primers (Mullis, 1990) generally occurs at 72°C. *Taq* DNA polymerase is most active at this temperature. As bases are added to the 3′ end of the primer and the double-stranded section lengthens, the resulting ionic bond is greater than the forces that break these attractions.

Each round of temperature cycling theoretically doubles the amount of DNA. After several rounds of temperature cycling, the amount of short double-stranded DNA product (flanked by sequence complementary to the primers) vastly exceeds the amount of the original target DNA. As a result, short DNA product (amplicons) accumulates geometrically (Fig. 11.2). After the first PCR cycle, a single starting piece of double-stranded DNA becomes two, after two cycles there are four copies, after three cycles, eight copies, and so on. As stated, 30 to 40 rounds of PCR are generally required to produce detectable amounts of amplicon. Due to the presence of inhibitory substances in the PCR reaction and other factors, amplification efficiency probably never reaches 100%. Although the analytical sensitivity of PCR is theoretically at the single copy level (White et al., 1992; Fredricks and Relman, 1999), sampling error and lower amplification efficiency generally prevent reliable detection of less than 10–20 target copies per PCR reaction.

The entire procedure is carried out in a programmable thermal cycler—a computer-controlled cycling system with heating and cooling parameters. Many new techniques for thermoregulation are used in the designs of thermal cyclers. One common design uses thermal engines that are based on the Peltier effect (Collasius, et al., 1989), the heated and chilled air-streams (Wittwer et al., 1995, 1989), or in a continuous flow manner as described most recently (Martin, 1998). In this design, heat from one side of a semiconductor is transfered to another, heating or cooling the overall temperature of the system. This design is much more effective than traditional designs of thermoregulation, which requires the use of refrigerants and compressors (Upadhyay, 1995). Other approaches for thermoregulation include the use of continually circulating air-streams, water baths, or a combination of Peltier and convective technologies. When choosing a new thermal cycler, functions that should be considered include gradient functionality, ability to upgrade to real-time PCR, and availability of interchangeable blocks or modules.

Detection and Analysis of the PCR Product

The PCR product should be a fragment or fragments of DNA of defined length. Before the PCR product is used in further applications, it should be analyzed. For diagnostic applications, this analysis can be performed on an ongoing basis for every patient specimen or during the initial method development and verification. First, reactions should be examined to ensure product is actually formed. This seems intuitive, but when amplicon is detected with a probe, unexpectedly negative results could be due to either lack of amplification or probe hybridization/detection problems. Although biochemistry is an exact science, not every PCR reaction is successful. Causes are many and include poor quality of target DNA, too much

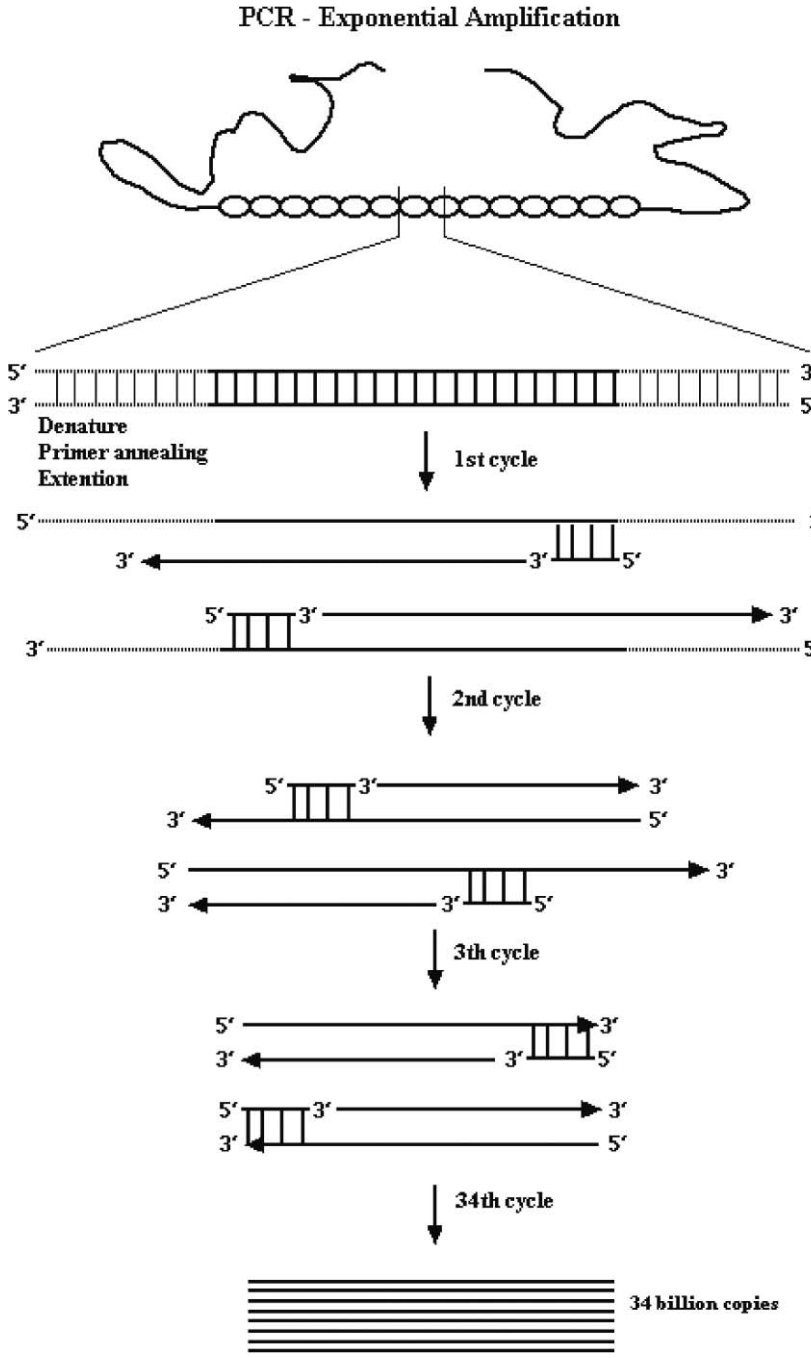

FIGURE 11.2. Exponential amplification of DNA by PCR.

target DNA, lack of sequence homology between primers and the intended target, and failure to optimize PCR conditions. PCR product must also be the correct size. Unexpected amplicon size indicates that the target region itself is different than expected, that the target sequence is shared, or that amplification conditions are suboptimal and allow nonspecific annealing. PCR product should also be evaluated to ensure that the correct number of distinct products are produced. In most diagnostic applications, a single amplicon is generated by one primer pair. Additional, unintended product is usually produced as a result of suboptimal amplification conditions (poor primer design, *Taq* or $MgCl_2$ concentration too high, annealing temperature not optimized). PCR product is analyzed by electrophoresis in agarose gels and visualization with ethidium bromide. DNA fragment size is determined by comparison with known molecular weight markers. Agarose gel electrophoresis is also a PCR detection format but is not recommended as a stand-alone method, as amplicon sequence cannot be confirmed. PCR detection formats are discussed in detail in another chapter of this text.

PCR-Derived *In Vitro* Nucleic Acid Amplification Techniques

Hot-Start PCR

Hot-start PCR was first described in the literature in 1991 by Kary Mullis (Mullis, 1991), and practical applications were demonstrated in 1992 (Chou et al., 1992). Hot-start PCR techniques focus on the inhibition of DNA polymerase activity during reaction setup. By limiting polymerase activity prior to the elevated temperatures of PCR, nonspecific amplification is reduced and target yield is increased. This is accomplished by physically separating or chemically inactivating one or more of the reaction components until high temperature triggers mixing or reactivation to give a complete reaction mixture.

In manual hot-start PCR, reactions lacking one essential component (usually DNA polymerase) are prepared and held at a temperature above the threshold of nonspecific binding of primer to template. Just prior to cycling, the missing component is added to allow the reaction to take place at higher temperature. This procedure limits nonspecific annealing of the primers and generally improves yield of the desired amplicon. This manual method is tedious and ungainly, as the tubes must be kept at 95–100°C. At this temperature, tubes are uncomfortable to handle. The additional opening of tubes to add the final reagent increases the chances of introducing contamination or cross-contaminating tubes. To simplify the process, tubes can be placed in the prewarmed thermal cycler just before adding the last component.

Hot-start PCR is also accomplished by creating a physical barrier between the essential components, such as primers and template or enzyme and magnesium chloride. This barrier can be created by adding wax over an incomplete PCR reaction mixture in a tube (Bassam and Caetano-Anolles, 1993; Horton et al., 1994; Riol et al., 1994). The wax can be preformulated for PCR reactions or can

be in bulk form, such as paraffin. The remaining PCR component(s) is placed on top of the wax layer. During the first denaturation step, the wax barrier melts and convection currents mix the essential PCR components.

Additional hot-start methods include chemically modified *Taq* DNA polymerase (Birch, 1996; Kebelmann-Betzing et al., 1998) and an antibody-inhibited *Taq* DNA polymerase. The antibody is directed against the active site of the enzyme, preventing DNA replication until the high temperature of the denaturation step disassociates the antibody (Kellog, 1994). These modified enzyme preparations require a longer initial denaturation step than standard *Taq* DNA polymerase. Wax preparations and modified *Taq* DNA polymerase are commercially available.

Chemical "Hot-Start" PCR

Similar to controlled temperature and physical separation of PCR reaction components, cosolvents and enzymes have also been used to reduce or eliminate nonspecific annealing of primers. Cosolvents such as dimethyl sulfoxide (DMSO) and formamide increase stringency by changing the melting temperature of the primer–template hybrid. Glycerol is believed to function similar to cosolvents. Cosolvents have various effects on the polymerase enzyme. Glycerol increases the temperature stability of *Taq* DNA polymerase, whereas formamide lowers it. The enzyme uracil *N*-glycosylase (UNG) is used in PCR as part of a system to degrade dUTP-containing product carried over from previous PCR reactions (Pang et al., 1992; Udaykumar et al., 1993). Another benefit of UNG is that it degrades PCR product formed during the PCR setup process, prior to the high temperatures of cycling that provide specificity. In this role, UNG essentially provides an enzymatic hot-start PCR.

Touchdown PCR

Unlike a standard PCR program that uses a constant annealing temperature, touchdown PCR incorporates a range of annealing temperatures. The earliest cycles of touchdown PCR have high annealing temperatures. In subsequent cycles, the annealing temperature is decreased by small increments (usually 1°C) every several cycles to a final "touchdown" annealing temperature, which is then used for the remaining 10 or so cycles. This gradual decrease in annealing temperature selects for the most complementary primer–target binding in early cycles. This is most likely the sequence of interest. As the annealing temperature decreases, primers will anneal to nonspecific sequences; however, amplification of these products will lag behind that of the specific product. This favors synthesis of intended product over any nonspecific products (Don et al., 1991). Touchdown PCR was originally used to simplify the process of determining optimal PCR annealing temperatures.

Degenerate PCR

Degenerate PCR is a procedure that intentionally lowers analytical specificity to allow divergent sequences to be detected in spite of sequence variation in the primer

binding region. Rather than using a single primer pair with a specific sequence, degenerate primer sets may contain several primers that vary at one or more nucleotide positions, or a primer containing a nonspecific base, such as inosine, at a divergent position. There are circumstances in diagnostic microbiology when greater inclusivity is useful. For example, the genus *Norovirus* is composed of dozens of distinct strains with relatively high genetic diversity. A single, standard primer pair would lack enough complementarity with most strains and have little diagnostic value. Degenerate RT-PCR has been used successfully to detect a broad range of Noroviruses (Moe et al., 1994). Because viruses often lack highly conserved sequences such as ribosomal DNA genes, degenerate PCR generally has been applied to diagnosis of these pathogens.

Nested and Heminested PCR

Nested and heminested PCR are designed to increase the sensitivity of PCR by directly reamplifying the product from a primary PCR with a second PCR. Nested PCR uses two sets of amplification primers and two separate rounds of PCR (Haqqi et al., 1988; Schmidt et al., 1996). The second (nested) set of primers anneal to a sequence internal to the region flanked by the first set. In heminested PCR, the second round of PCR uses one of the first-round primers and one new, internal primer. The amplicon from the second round of PCR is shorter than that of the first (Fig. 11.3). The advantage of nested PCR is increased sensitivity and specificity of the reaction, because the internal primers anneal only if

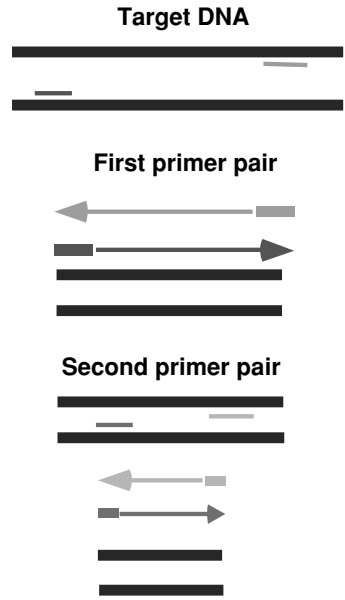

FIGURE 11.3. Nested PCR.

the amplicon has the corresponding, expected sequence. Disadvantages of nested PCR include extra time and cost associated with two rounds of PCR and the increased risk of contamination incurred during transfer of first-round amplification products to a second tube. The physical separation of amplification mixtures with wax or oil (Whelen et al., 1995) and designing the second primer set with a higher annealing temperature are two variations used to reduce the potential for contamination.

Multiplex PCR

In multiplex PCR, two or more unique DNA sequences in the same specimen are amplified simultaneously (Chamberlain et al., 1988; Tang et al., 1999). Primers used in multiplex reactions must be designed carefully to have similar annealing temperatures and to lack complementarity to avoid dimerization. Multiplex PCR requires careful optimization of annealing conditions for maximal amplification efficiency. Some commercial kits have been shown to efficiently amplify different sequences with little or no need for optimization of annealing conditions (Rossister, 1991). This is due to the buffer composition, which widens the temperature window for optimal annealing. Multiplex PCR diagnostic assays are used in our laboratory most frequently to amplify an internal control with one set of primers and the target DNA sequence of interest with a second set of primers. The internal control is included to verify the integrity of the PCR. A positive result with the internal control primers demonstrates that conditions favorable for PCR were present, and when included in the specimen processing step, confirms the integrity and availability of target nucleic acid. Multiplex PCR is also used in clinical laboratories to simultaneously detect DNA sequences of two or more several different organisms in a single PCR reaction (Bej et al., 1990; Geha et al., 1994; Roberts and Storch, 1997). Primers are designed so that each amplification product is a unique size, has a unique melting temperature, or unique probe binding sequence. This allows the detection and identification of different microorganisms in the same specimen. As stated, multiplex PCR assays must be developed carefully to avoid dimerization or competition among primer sets. Failure to prevent this can result in lower sensitivity, typically for one target.

Reverse Transcription PCR

Reverse transcription (RT)-PCR is a technique used to amplify RNA targets. Because DNA polymerase requires a double-stranded DNA template, RNA must be transcribed into complementary (c) DNA prior to PCR by the enzyme reverse transcriptase. The cDNA then serves as the template for the first PCR temperature cycle (Fig. 11.4). The combined use of RT and PCR to amplify RNA targets was first described in 1987. Early studies coined the terms RT-PCR, RNA-PCR, RNA phenotyping, and message amplification phenotyping. Reviews describing the numerous applications of RT-PCR are available (Larrik, 1992). RT-PCR is

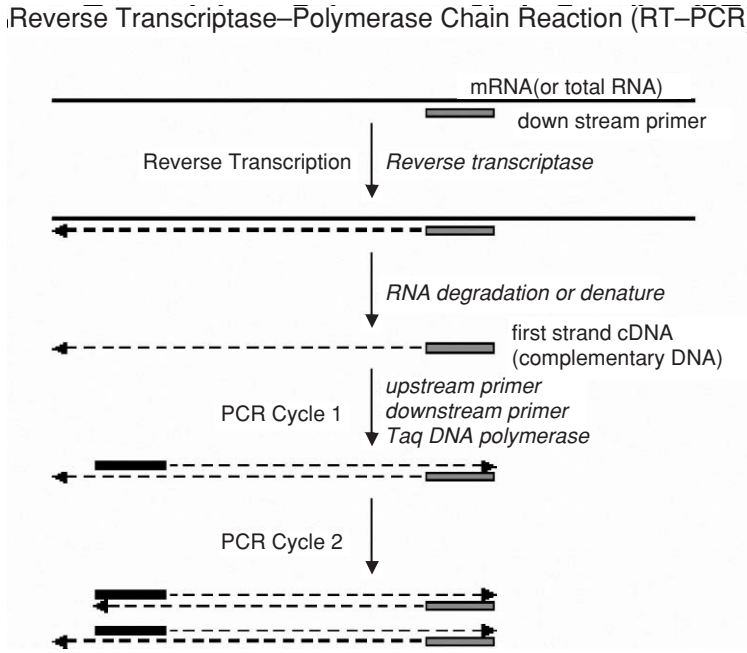

FIGURE 11.4. Amplification of RNA.

an important technique in the diagnosis of infectious and genetic diseases and is the key procedure used to detect and quantify RNA as a measure of gene expression.

Two reverse transcriptase enzymes commonly used are Moloney murine leukemia virus (M-MuLV) reverse transcriptase and avian myeloblastosis virus (AMV) reverse transcriptase. Both enzymes have the same fundamental activities but differ in some characteristics, including temperature and pH optima. In addition to M-MuLV and AMV, other variants of this enzyme are available for use in the molecular diagnostic laboratory. These enzymes are available in preoptimized RT-PCR kits.

In vitro reverse transcription is primer directed. A single primer is used to generate cDNA and can be one of the primers used in the subsequent PCR reaction (sequence-specific) or a random oligonucleotide. Specificity is not required of reverse transcription. Random oligonucleotides are convenient in that one RT kit or reaction can be used for all RNA targets.

Traditionally, the RT step has been performed in a separate tube containing only components necessary for reverse transcription. After RT, an aliquot is removed, added to a PCR reaction tube, and subjected to amplification. Drawbacks of the separate tube method include inconvenience and cross-contamination risk. More recently, single-tube RT-PCR assays, either two-enzyme or single-enzyme, have been described.

RT-PCR is often used interchangeably to describe reverse transcription PCR and real-time PCR. To avoid confusion, "real-time" will not be abbreviated in this chapter.

Quantitative PCR

A variety of quantitative PCR assays have been developed to accurately quantify nucleic acid targets in clinical specimens (Clarke and McClure, 1999; Boyer and Marcellin, 2000; Mylonakis et al., 2001). In addition to PCR, other molecular techniques such as branched (b) DNA provide accurate quantification of nucleic acids. Although these methods determine the amount of DNA or RNA template in a clinical specimen, the results can be easily extrapolated to organism equivalents, hence the use of terms bacterial load, viral load, and so forth. Quantitative PCR results have become a valuable tool for guiding antiviral therapy, monitoring clinical course, and predicting outcome from a variety of infectious diseases (Hodinka, 1998; Orlando et al., 1998; Jung et al., 2000). The value of quantitative PCR has led to commercialization of tests for such viruses as human immunodeficiency virus (HIV), cytomegalovirus, hepatitis C virus, and hepatitis B virus.

Nucleic acids can be quantified using an absolute standard in order to generate concrete numbers or a relative standard to give comparative data. Absolute standards can be used whenever definite numbers are needed. Relative standards are useful when absolute quantities are less important than knowing how a sample differs from a control. Fundamental RT-PCR quantification strategies are relative, competitive, and comparative.

Relative quantitative PCR compares nucleic acid amount across a number of serial dilutions of a sample, using a coamplified internal control for sample normalization. Results are expressed as ratios of the sequence-specific signal to the internal control signal. This yields a corrected relative value for the sequence-specific product in each sample. Relative PCR uses primers for an internal control that are multiplexed in the same PCR reaction with the target-specific primers. Internal control and target-specific primers must be compatible—that is, they must not produce additional bands or hybridize to each other. The signal from the internal control is used to normalize sample data to account for variation in RT or amplification efficiency. Common internal controls include the housekeeping genes (or their mRNAs) β-actin and GAPDH and 18S rDNA.

Competitive RT-PCR provides absolute quantification of a nucleic acid target in a sample. An internal control or quantification standard is added at a known concentration to samples and coamplified with the target sequence. Addition of the internal control to the sample prior to processing monitors for nucleic acid recovery during this step. The internal control is often a synthetic RNA or DNA with the same primer binding sequence (hence the term competitive PCR) but designed to produce an amplicon slightly different in size than the target amplicon or with a unique internal sequence allowing detection with a different probe. After

amplification and detection, the amount of product or signal generated by the internal control is equated to its known input copy number. This relationship is then used to determine the copy number of the target sequence.

The availability of real-time PCR has allowed comparative quantification comparing PCR results to an external standard curve to determine target copy number. Internal controls are not required when performing real-time PCR. Because measurements are taken at each cycle, during the exponential phase of PCR, efficiency is consistent between samples. Conventional PCR measures product only at the end point, when the effects of inhibitors are significant. Because PCR product is measured during the exponential cycles, quantification is more accurate and precise over a greater range than conventional PCR. These characteristics allow the use of external standards—well-characterized control nucleic acid at known copy numbers—while obviating the need for an internal control. External standards are used to create a standard curve across the dynamic range of the PCR assay. Real-time PCR generates a threshold (C_T) or crossing point (C_P) cycle for each sample. This is the point where product (fluorescence) crosses a predetermined threshold. The higher the amount of starting target, the lower the C_T. The C_T for an unknown patient sample is analyzed against a standard curve to yield a target DNA or RNA copy number.

PCR-Based Strain Typing Techniques

PCR-based strain typing techniques are designed to generate multiple bands that provide a unique fingerprint for a particular species or strain of microorganism (Olive and Bean, 1999; Fernandez-Cuenca, 2004). Unlike diagnostic tests that determine presence or absence of a microorganism (or its nucleic acid) in a specimen, these procedures are used to differentiate epidemiologically unrelated organisms at the species or subspecies level. They must generally produce multiple DNA bands to provide sufficient discrimination power, and these banding patterns must be reproducible run-to-run and among isolates of the same predefined group while clearly distinguishing isolates that epidemiologically or phenotypically fall outside of that group.

AP-PCR and RAPD

Arbitrarily primed PCR (AP-PCR) or random amplified polymorphic DNA (RAPD) are methods of creating genomic fingerprints from species, even if little is known about the target sequence to be amplified (MacGowan et al., 1993; Welsh et al., 1994; Woods et al., 1994; van Belkum et al., 1995; Grattard et al., 1996; Matsui et al., 1998). Strain-specific arrays of amplicons (fingerprints) are generated by PCR amplification using arbitrary, or random sequence oligonucleotides that are often less than 10 nucleotides in length, and low-temperature annealing. A single primer is often used, because it will anneal in both orientations.

Detectable PCR product is generated when the primers anneal at the proper orientation and within a reasonable distance of one another. In spite of the arbitrary nature of the assay and amplification conditions that are relatively nonspecific, these methods have been shown to generate reproducible DNA banding patterns. These same characteristics make these methods suitable for a wide range of bacteria.

AFLP

Amplified fragment-length polymorphism (AFLP) involves the restriction of genomic DNA, followed by ligation of adapters or linkers containing the restriction sites to the ends of the DNA fragments. The linkers and the adjacent restriction site serve as primer binding sites for subsequent amplification of the restriction fragments by PCR. Selective nucleotides extending into the restriction fragments are added to the 3′ ends of the PCR primers such that only a subset of the restriction fragments are recognized. Only restriction fragments in which the nucleotides flanking the restriction site match the selective nucleotides will be amplified. The amplified fragments are visualized by means of autoradiography, phosphoimaging, or other methods. Like AP-PCR and RAPD, AFLP can be applied to organisms without previous knowledge of genomic sequence.

ERIC-PCR, Rep-PCR, BOX-PCR, IS-PCR, and VNTR-PCR

Enterobacterial repetitive intergenic consensus (ERIC)-PCR, repetitive element (Rep)-PCR, insertion sequence (IS)-PCR, and variable number tandem repeat (VNTR)-PCR are examples of PCR-based typing methods that target repetitive, conserved sequences found in bacteria and, in some cases, fungi. In a seminal 1991 paper, Versalovic and colleagues (Versalovic et al., 1991) described the

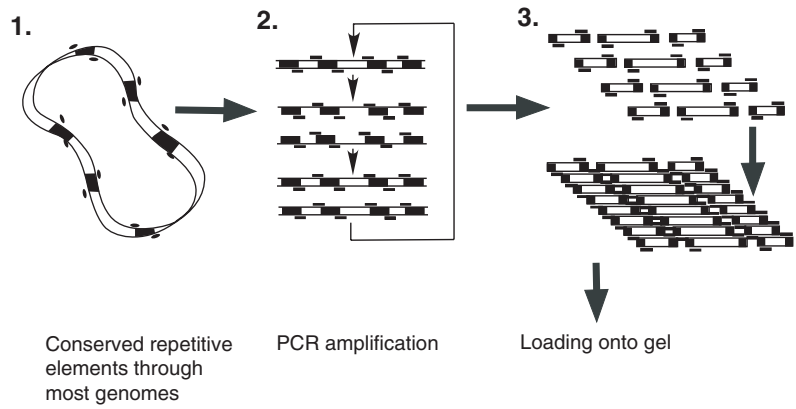

FIGURE 11.5. Repetitive element (Rep)-PCR.

presence of repetitive sequences in a wide range of bacterial species and demonstrated their use to directly fingerprint bacterial genomes (Fig. 11.5). Specific repetitive sequences include the 124–127 base-pair ERIC sequence, the 154 base-pair BOX sequence, and the 35–40 base-pair repetitive extragenic palindromic sequence. These sequences are located intergenically throughout the chromosome. Some repetitive sequences translocate to new locations in the genome and are called transposons or insertion sequences. Some ISs are species-specific, whereas others have no species restriction. VNTR's are repeated sequences of non-coding DNA. Whether ERIC, IS, VNTR, or other repetitive element or sequence, the basis of the strain typing is the same. The ability of repetitive element–based PCR methods to distinguish unrelated strains or species is based on the random distribution of elements within the genome and the time required for these to become established. That is, all bacteria associated with a common source outbreak are highly unlikely to have any differences in the number or location of repetitive elements, whereas bacteria that are geographically, temporally, and epidemiologically unrelated are more likely to have experienced mutational events. Repetitive element–based PCR assays are designed so that primers anneal to the specific sequence in an outward orientation, so that DNA between the repeated elements is amplified. Variability between unrelated organisms is due to the random number and location of the elements on the genome.

Appendix 1

Preparation of PCR Reaction

The PCR master mix contains all of the components necessary to make new strands of DNA in the PCR process. The master mix reagents include:

Final Conc.	Component	Purpose
1X	Buffer	Maintains proper pH of the PCR reaction.
200 μM	Deoxynucleotides	Provide both the energy and nucleosides for the synthesis of DNA. It is important to add equal amounts of each nucleotide (dATP, dTTP, dCTP, dGTP) to the master mix to prevent mismatches of bases.
0.2–1.0 μM	Primers	Short pieces of DNA (20–30 bases) that bind to the DNA template allowing Taq DNA polymerase enzyme to initiate incorporation of the deoxynucleotides. Both specific and universal (arbitrary) primers can be used.
1.5–2.5 μU	Taq polymerase	A heat-stable enzyme that adds the deoxynucleotides to the DNA template.
<1.0 μg	Template DNA	The DNA amplified by the PCR reaction.

Master mix buffer is often stored as a 10X stock solution (100 mM Tris-HCl, pH 8.3, 500 mM KCL, 1.5 mM MgCl2) and diluted to 1X for use. Both the master mix

buffer and the purified water can be stored at room temperature. Oligonucleotides (primers and probes), enzyme, and dNTPS should be stored at −20°C or according to manufacturers', recommendations.

Depending on the PCR platform and the configuration of the reaction tubes, the PCR reaction volume can vary greatly. Regardless of the reaction volume, the concentration of the individual components should remain constant.

Master mix reagents can be obtained from a number of vendors. Initial concentrations vary, and it is important to read the specifications carefully and make appropriate dilutions. The formula used to determine volume of a stock reagent is as follows:

$$(\text{initial concentration}) \times (\text{volume needed}) = (\text{final concentration})$$

Master mix kits containing ready to use reagents are available from a number of vendors. These kits benefit the molecular diagnostic laboratory by providing standardization, decreased preparation time, and reduced risk of contamination.

Appendix 2

Primer Design Resources

The world wide web has literally put primer design resources at our fingertips. Below is a sampling of Web sites that provide tutorials, lectures, papers, and tips on primer design.

For information on	Web address
Primer design software	http://www.ebi.ac.uk/biocat/biocat.html OR http://www.chemie.uni-marburg.de/~becker/prim-gen.html OR http://norp5424b.hsc.usc.edu/genetools.html
General hints on primer design	http://alces.med.umn.edu/VGC.html
Designing primers for cycle sequencing	http://www.biotech.iastate.edu/Facilities/DSSF/
Designing primers for detecting unknown sequences (degenerate primer design strategies)	http://www.blocks.fhcrc.org/blocks/help/CODEHOP OR http://www.dartmouth.edu/artsci/bio/ambros/protocols/other/koelle/degenerate_PCR.html

Computer programs simplify the complex task of designing PCR primers. In addition to the many commercial primer design programs available, there are a number of free programs available on the world wide web. Below is a sampling of primer design programs available at no cost, found by searching the Web for "primer design".

Software	Operating systems	Available froma
CODEHOP	On-line	http://blocks.fhcrc.org/blocks/codehop.html
DegenDesigner	UNIX	ftp anonymous evolution.bchs.uh.edu (Directory/pub/gene-server/unix)
Primer Design	DOS	ftp anonymous ftp.chemie.uni-marburg.de (Directory/pub/PrimerDesign)
Primer-Master	DOS	ftp anonymous ftp.ebi.ac.uk (Directory/pub/software/dos)
PRIMER-MIT	UNIX, Mac, DOS	ftp anonymous genome.wi.mit.edu (Directory/pub/software/primer)
Primers!	Mac	ftp anonymous wuarchive.wustl.edu (Directory/systems/mac/info-mac/sci/primers-10-installer.hqx)
Primer Selection (VGC)	On-line	http://alces.med.umn.edu/vgc.html
Primer Selection (USC)	On-line	http://norp5424b.hsc.usc.edu/genetools.html

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12
Non–Polymerase Chain Reaction Mediated Target Amplification Techniques

MICHAEL L. PENDRAK AND S. STEVE YAN

Introduction

Today's development of target-specific and molecular-based therapies relies more than ever upon accurate and sensitive molecular diagnostic technologies. The assessments of therapeutic regimens in areas such as cancer chemotherapy and gene replacement therapy, as well as identification of infectious agents in the diagnostic microbiology laboratory are essential for the success of molecular medicine. The polymerase chain reaction (PCR) (Saiki et al., 1985) has been the workhorse in these areas, and ample evidence can be found throughout this volume. However, the ability of the PCR to amplify as few as a single copy of double-stranded DNA (dsDNA) targets has presented additional challenges in the diagnostic laboratory. The potential for contamination from carry-over product and amplification of DNA in contaminated matrices, as well as tight patent restrictions concerning the use of PCR, have been some factors in the search for non-PCR–based target amplification methods. Currently, the alternatives to PCR for nucleic acid amplification involve mainly isothermal transcription-based amplification (ITA) techniques that are based on either bacteriophage RNA polymerases or a group of highly processive DNA polymerases.

The idea of nucleic acid amplification without thermal cycling is not new. The predominant technologies in use today are based on mimicking cellular or viral DNA or RNA replication "machines" (Guatelli et al., 1990; Walker et al., 1992a), and two primary amplification methodologies have emerged: (1) transcription-based systems mediated through reverse transcription for RNA replication (Guatelli et al., 1990), and (2) DNA amplification through a rolling-circle type mechanism (Lizardi et al., 1998; Walker et al., 1992a; Dean et al., 2001).

Transcription-based systems are used for RNA detection and produce an RNA amplicon rather than DNA amplicon (Deiman et al., 2002). Because RNA amplicons are more labile than DNA, this helps reduce the possibility of carry-over contamination (Fahy et al., 1994). A single round of transcription can produce 10–1000 RNA copies *per* cycle as compared with PCR that produces only two copies per cycle (Saiki et al., 1985; Kwoh et al., 1989; Guatelli et al., 1990). The result is million-fold increase of copies within a short period of time (Kwoh et al.,

1989; Guatelli et al., 1990). This amplification strategy depends on each RNA amplicon serving as a template for additional rounds of transcription.

DNA polymerase-based non-PCR amplification systems are used for the detection of dsDNA molecules and require an initial denaturation step prior to isothermal amplification. Rolling-circle amplification (RCA) (Fire and Xu, 1995; Lizardi et al., 1998) and strand displacement amplification (SDA) (Walker et al., 1992a) rely on the activity of DNA polymerase to invade a nicked double-stranded molecule and displace the downstream DNA strand during elongation. Both strategies resemble rolling circle replication used by small plasmids, viroids, and some bacteriophage (Doermann, 1973; Diener, 1991; del Solar et al., 1998). A key in the adaptation of this technology to the laboratory is the development of a method to permit the cleavage of only one strand of a double-stranded DNA molecule, and this will be described in more detail below. In contrast to PCR, all steps in the isothermal reactions occur simultaneously and exponential amplification rates can be reached within 10 to 15 min (Kwoh et al., 1989; Guatelli et al., 1990; Brink et al., 1998; Heim et al., 1998). Isothermal transcription-based amplification methods have been shown to be versatile and robust in a variety of detection systems. They have been developed in multiplex formats (Westin et al., 2000), adapted to real-time detection systems (Leone et al., 1998; de Baar et al., 2001), used in high-throughput formats (Westin et al., 2000), *in situ* DNA or RNA amplification in fixed or unfixed tissues (Singer et al., 1996, Nuovo, 2000), as well as used with chip-based systems (de Baar et al., 2001; Huang et al., 2004). The rolling circle type assays are also particularly suited for mutation and SNP (single nucleotide polymorphism) detection (Wang et al., 2003). Additionally, methods have recently been developed for whole genome analysis (Lage et al., 2003; Barker et al., 2004) including preimplantation genetic diagnosis (Lasken and Egholm, 2003).

Varieties of ITA technologies have been used in the clinical diagnostics laboratory and include both transcription-based RNA amplifications and isothermal DNA amplifications. Traditional methods of culturing the organisms can be slow, hazardous in the case of infectious agents, or even nonexistent. Applications of these non-PCR–mediated target amplification technologies are promising for diagnostic microbiology.

Isothermal Transcription-Based RNA Amplification

Transcription-based ITA methods have been developed that generate amplified RNA from RNA templates (Deiman et al., 2002), although an initial denaturation step must be included when using dsDNA targets (Guatelli et al., 1990). The RNA amplicons produced in the reactions serve as targets for the production of additional dsDNA templates, and this cycling gives exponential amplification kinetics to the reaction. The basic concept for the exponential amplification of a target nucleic acid sequence is modeled on the strategy of retroviral replication (Guatelli et al., 1990). Transcription-based assays have been developed using different names including NASBA (nucleic acid sequence–based amplification) (Deiman et al., 2002),

TMA (transcription-mediated amplification), TAS (transcription-based amplification system) (Kwoh et al., 1989), and 3SR (self-sustained sequence replication) (Fahy et al., 1991, Guilfoyle et al., 1997). All these methods use the same basic concept for exponential amplification of a target nucleic acid sequence and are modeled on the strategy of retroviral replication. For purposes of simplifying the terminology, we will refer to the general method as a 3SR reaction in the following text.

The Basic Concept

Retroviral replication relies on the use of reverse transcriptase to make a complementary DNA (cDNA) copy from its RNA target(s) (Varmus, 1988). Reverse transcriptase has two enzymatic activities that are important for cDNA formation; that is, a DNA polymerase activity and an RNAse H activity. The retroviral cDNA synthesis reaction relies on the presence of a virion-packaged cellular tRNA molecule that serves as a primer for first-strand DNA synthesis. The RNAse H activity degrades the initial RNA template so the first-strand cDNA can then be used as a template for an additional round of reverse transcription to make the second-strand DNA. The dsDNA "genome" now serves as a transcriptional template for the synthesis of viral proteins as well as new genome copies that will be packaged in viral particles (Varmus 1988). Adaptation of this reaction sequence to the laboratory was attractive because it could be used to target RNA directly (Guatelli et al., 1990; Compton, 1991). Target amplification would rely on primers that substitute for the retroviral tRNA molecules (Guatelli et al., 1990; Compton 1991).

The original publication describing the transcription-based amplification reaction used AMV RT (avian myeloblastosis virus reverse transcriptase), bacteriophage T7 RNA polymerase, and *E. coli* RNAse H (Guatelli et al., 1990). The first step or "noncyclic" phase of the reaction begins with first-strand cDNA synthesis initiated from a primer (P1) containing a T7 RNA polymerase promoter sequence at its 5′ end and a target-specific sequence at the 3′ end (Fig. 12.1). Reverse transcriptase extends the primer to yield a first-strand "anti-sense" cDNA contained within an RNA:DNA hybrid. RNAse H hydrolysis of the RNA template enables the second-primer (P2) to anneal and prime second-strand cDNA synthesis using the first cDNA strand as a template. This results in the production of a dsDNA template for the DNA-dependent T7 RNA polymerase (Fig. 12.1). Transcription from a dsDNA template containing the T7 promoter can produce 10–1000 copies of antisense RNA (Dunn and Studier, 1983), and they themselves serve as templates for additional rounds of replication. Primer P2 can also incorporate a T7 promoter sequence to enable the generation of transcripts from both ends of the dsDNA molecule (Compton, 1991). The T7 promoter sequences may be substituted by others such as SP6 (Brown et al., 1986) and T3 (Bailey et al., 1983).

The self-sustaining nature of the reaction occurs through successive cycles as primers P1 and P2 are used for first-strand cDNA synthesis from anti-sense and sense transcripts, respectively. The resultant cDNA copies are then primed for

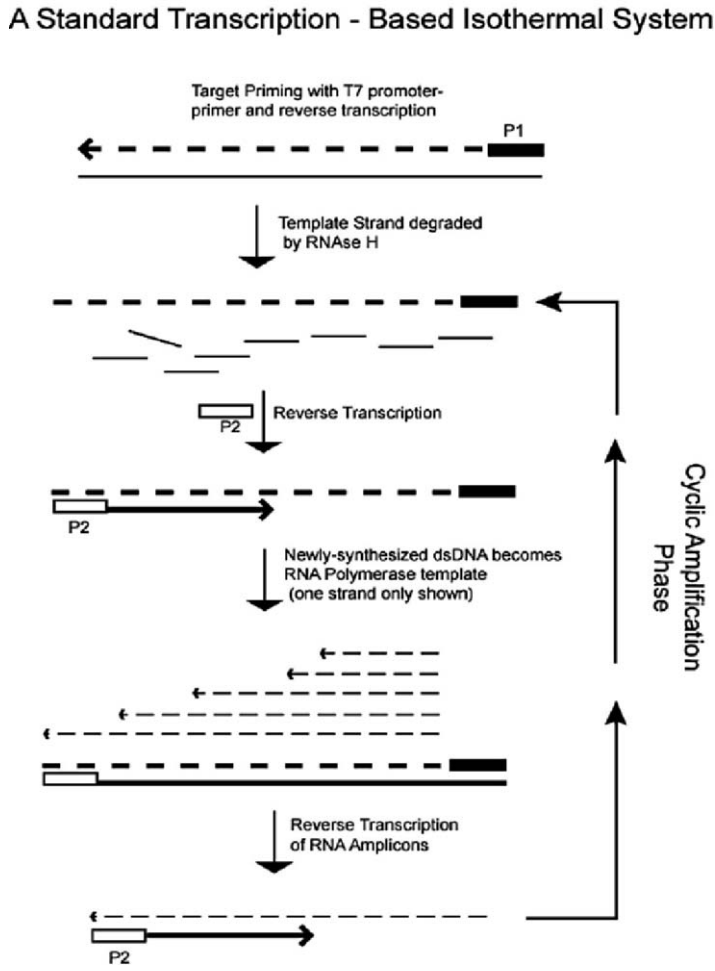

FIGURE 12.1. A standard transcription-based isothermal system. The basic principle of this technique is the introduction of a bacteriophage RNA polymerase promoter to end of the cDNA generated by reverse transcription. The first-strand cDNA is reverse-transcribed from primer P1 and then replicated through the DNA polymerase activity of RT using a second primer (P2). This results in a dsDNA molecule that is a substrate for RNA polymerase. This sequence of events generates a self-sustained reaction consisting of simultaneous rounds of transcription, reverse transcription, and DNA polymerization to yield an exponential amplification of the target RNA within 10–15 min. This assay has been given numerous designations including self-sustained sequence replication (3SR), nucleic acid sequence–based amplification (NASBA), transcription-mediated amplification (TMA), and transcription-based amplification system (TAS). See text for details.

dsDNA synthesis using the complementary primer resulting in another template for T7 RNA polymerase transcription (Fig. 12.1). Product accumulation beginning at this stage is exponential and cycles of transcription and cDNA synthesis enable the reaction to enter the "cyclic" phase of amplification (Guatelli et al., 1990). The rapid kinetics of the 3SR reaction can yield a 10^6-fold amplification in 10 min, whereas a PCR reaction to reach similar magnitude would require about 20 cycles (Guatelli et al., 1990). Background DNA does not interfere with the reaction because single-stranded RNA sequences are specifically targeted. Additionally, because the reaction is carried out at a relatively low temperature, near 40°C (Compton, 1991), this makes it attractive for *in situ* assays where cell and tissue integrity are important (Mueller, 1997). The latter is a concern especially when the 3SR technique is combined with histochemical staining procedures.

The ability of the 3SR reaction to specifically amplify ssRNA (single-stranded RNA) makes the assay particularly attractive for the detection of viral genomes, mRNA, and rRNA. This extends the range of nucleic acid amplification methods for both diagnostics and research. However, the reaction conditions are isothermal, and this offers unique challenges in terms of probe design and target choice due largely to the potential for RNA to form stable secondary structures. For the development of high-throughput assays, development of real-time amplification methods such as molecular beacons has been particularly challenging (Leone et al., 1998; Szemes and Schoen, 2003).

Fahy and co-workers experimented to optimize standard reaction conditions of substrate concentrations, temperature, pH, and ionic strength (Fahy et al., 1991, 1994). In such a complex reaction mixture, the optimal rNTP and dNTP concentrations of 4 and 0.05 mM, respectively, were much higher than K_m values reported for single enzyme reactions (Fahy et al., 1991, 1994; Cline et al., 1996). However, the standard reaction conditions of 0.1 μM each primer, 20 mM KCl, 30 mM MgCl2, and 40 mM Tris pH 8 were unremarkable. Interestingly, the RNAse H activity of AMV RT could be enhanced by the addition of 15% DMSO and 15% sorbitol or 10% glycerol to the reaction, and this allowed *E. coli* RNAse H to be omitted (Fahy et al., 1991). With the further omission of chloride, the temperature of the reaction could be increased from 42°C to 50°C (Fahy et al., 1994). This two-component method was further modified with the substitution of HIV reverse transcriptase. In this case, RNAse H activity was considerably slower, but this led to more homogeneous reaction products and a higher RNA to DNA ratio (Gebinoga, 1996). Significant efforts at the design of primers have been documented including the optimization of the T7 sequence in the context of the reaction, as well as guidelines for the choice of length and composition of the target-specific sequences (Fahy et al., 1991). A general guide to probe design is given in Deiman et al., (2002).

An important consideration in all nucleic acid amplification procedures is to ensure that replication fidelity is maintained, and this criterion is met by the 3SR reaction. Transcription-based systems were demonstrated to give an error frequency of less than 0.3% in cloned DNA products from two different segments of the HIV-1 gag gene (Sooknanan et al., 1994). An overall error rate of 2×10^{-4} was calculated for the combined effects of both polymerases (Sooknanan et al., 1994).

This approximates the error rate of thermostable DNA polymerases, which range from approximately 0.7×10^{-4} for *Taq* polymerase to 1.6×10^{-6} for PFU and other "proof-reading" polymerases (Tindall and Kunkel, 1988; Brail et al., 1993; Cline et al., 1996). This is especially important for the use of 3SR systems in SNP or mutation detection procedures (Berard et al., 2004).

Applications and Variations of Isothermal RNA Amplifications

The transcription-based ITA techniques have been given various names especially during the early stages of development. As described above, names that have appeared in the literature include NASBA, TMA, TAS, and 3SR (Table 12.1) (Kwoh et al., 1989; Fahy et al., 1991; Guilfoyle et al., 1997; Deiman et al., 2002). The major differences between these procedures are found in the detection systems, and this flexibility is achieved by the modification of the 5′ end of the P2 primer to bind a detection probe (Fig. 12.1). Kwoh et al. used oligo-coated Sephacryl beads to bind the amplicon (Kwoh et al., 1989), but a number of modifications have emerged. Electrochemiluminescent (ECL)-labeled probes (van Gemen et al., 1994) have been incorporated into a basic system design that allows extensive flexibility in assay development. Enzyme-linking technologies have been incorporated into a gel assay (ELGA) that uses a horseradish peroxidase–labeled probe to detect the amplicon. In this case, bound and free probe are separated on a polyacrylamide gel, and the products are visualized using the peroxidase substrate

TABLE 12.1. Selected applications of commercially available non-PCR–mediated target amplification techniques for detection of microorganisms.

Test name	Type	Use	Manufacturer	URL
NucliSens HIV-1 QT	NASBA	HIV-1 viral load	bioMerieux, Inc. Durham, NC, USA	http://www.biomerieux.com
HIV-1/HCV Assay (Procleix)	TMA	Plasma donor screen	Gen-Probe San Diego, CA, USA	http://www.gen-probe.com
NucliSens	NASBA	CMV pp67 mRNA	bioMerieux, Inc. Durham, NC, USA	http://www.biomerieux.com
BDProbeTecET DNA Amplified Assay	SDA	*C. trachomatis, N. gonorrhoeae*	Becton Dickinson Sparks, MD USA	http://www.bd.com
Amplified MTD	TMA	*Mycobacterium tuberculosis*	Gen-Probe San Diego, CA, USA	http://www.gen-probe.com
BD ProbeTec ET DNA Amplified Assay	SDA	*Legionella pneumophila*	Becton Dickinson Sparks, MD, USA	http://www.bd.com

(van der Vliet et al., 1993). Samuelson et al. designed a capture probe to bind amplified products to a streptavidin-coated plate that is followed by the application of a digoxigenin-labeled detection probe (Samuelson et al., 1998). The TMA reaction uses an acridinium ester–labeled DNA probe to detect the amplicon via luminescence (also called a hybridization protection assay; HPA) (Arnold et al., 1989).

Initial studies using transcription-based ITA methods focused upon detection of HIV RNA as an important example of RNA targeting (Guatelli et al., 1990; Bush et al., 1992; van Gemen et al., 1993a; 1993b; Sherefa et al., 1998). Recently, it has been incorporated into a real-time format (de Baar et al., 2001), and the use of molecular beacons and other fluorescent detection systems are enabling development of high-throughput and quantitative assays (Arens, 1993; Romano et al., 1997; Kamisango et al., 1999; Greijer et al., 2001; Yates et al., 2001). Targeting RNA has also been used as an indicator of cell viability (Simpkins et al., 2000; Keer and Birch, 2003) and to assess antimicrobial treatment regimens where problems may be caused by the presence of nonviable organisms (Morre et al., 1998). Because RNA is relatively unstable compared with DNA, detection of RNA is a better indicator of viability as in the case of cytomegalovirus infection (Amorim et al., 2001) and helps to facilitate differentiation of reactivation or acute infection from latent presence of the virus (Hodinka, 1998; Preiser et al., 2001; Caliendo et al., 2002; Hebart et al., 2002).

The transcription-based ITA technology is also well suited to distinguish between viral and proviral sequences. This technology has been applied to detection of HIV after anti-retroviral therapy (Bruisten et al., 1993), as well as after therapies of CMV (Greijer et al., 2001; Goossens et al., 2004) human herpes virus 8 (Polstra et al., 2003), and Epstein–Barr virus (Brink et al., 1998), respectively. The direct detection of RNA has been applied to cancer diagnostics, for instance, in transcription detection from bcr3-abl2 and bcr2-abl2 junctions to diagnose chronic myeloid leukemia (Sooknanan et al., 1993; Langabeer, 2002). The detection of telomerase activity is expected to be a new diagnostic and prognostic marker of human cancer (Hirose et al., 1998). A sensitive TMA assay coupled with the hybrid protection assay was developed that could measure the addition of telomeric repeats with a sensitivity and reproducibility equal to or greater than that of PCR-based telomeric repeat amplification assay (Hirose et al., 1998). With the recent discoveries of small regulatory RNA molecules (Novina and Sharp, 2004), perhaps an entirely new field of direct RNA detection will develop using self-sustained transcription-based ITA principles.

Isothermal DNA Amplification Systems

The second non-PCR–mediated isothermal amplification technology is based on the rolling circle replication (RCR) strategy used by small plasmids, viroids, and a variety of bacteriophage (Doermann, 1973; Diener, 1991; del Solar et al., 1998). The minimalist view presented in Fig. 12.2 illustrates the salient points of this technology: (i) extension of a primer by DNA polymerase around a circular or closed

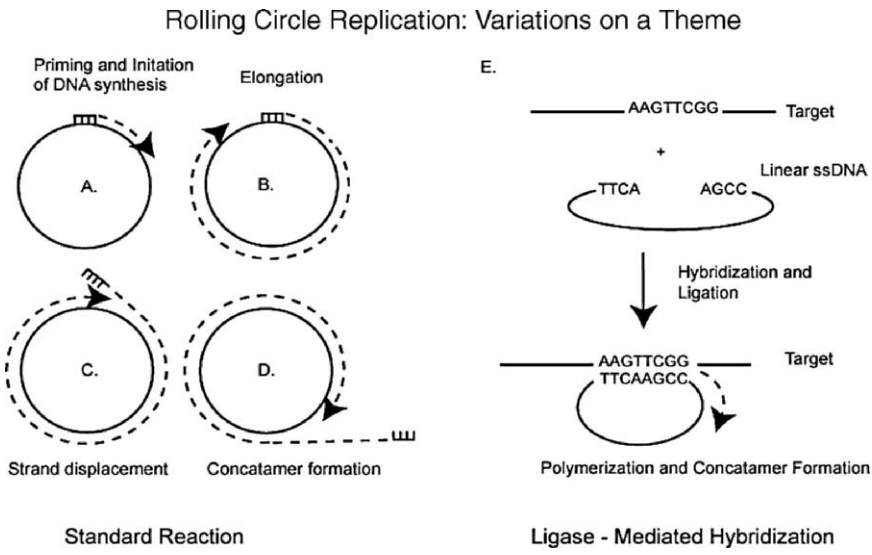

FIGURE 12.2. Rolling circle replification: variations on a theme. (A–D) Rolling circle amplification uses the strand displacement activity of an exonuclease-deficient DNA polymerase to synthesize concatemeric DNA molecules. (E) An example of a "padlock" probe that can be used for allelic and mutation discrimination. A linear ssDNA containing either a perfect or imperfect complement at the ligation site is added to the enzyme–target mixture. Ligation and subsequent amplification will only occur if the target hybridization and ligation is successful. This will only occur when a perfectly complementary ssDNA probe sequence is used.

template, (ii) strand displacement upon reaching a double-stranded area, and (iii) accumulation of concatameric molecules upon continued synthesis of the circular strand (Figs. 12.2A–12.2D). These concatamers can then serve as detection targets. The simplicity and robustness of this system has spawned the development of strategies for the detection of nucleic acids as well as proteins, as will be discussed below. Rolling circle amplification (RCA) and strand displacement amplification (SDA) are the major two designs used with RCR technologies. The ability of DNA polymerase to carry out strand displacement lies at the heart of these technologies.

Rolling Circle Amplification

Overview of the Technique

As the name implies, RCA is a derivation of rolling circle replication adapted to use small single-stranded DNA minicircles as templates for strand displacement synthesis by DNA polymerase. Synthesis using a single primer hybridized to the circle will generate concatamers in a linear amplification mode. The addition of a second primer specific for a newly synthesized copy results in geometric

amplification (Fire and Xu, 1995; Lizardi et al., 1998). The simplicity of RCA has enabled the development of new technologies in target detection and includes enhanced sensitivity in DNA quantification (Nallur et al., 2001), DNA mutation detection (Lizardi et al., 1998; Ladner et al., 2001), SNP detection (Qi, et al., 2001; Pickering et al., 2002), and array-based sandwich immunoassays (Schweitzer et al., 2000; Schweitzer et al., 2002). Major advancements in SNP and mutation detection, whole genome amplification and analysis, and amplification using immobilized oligonucleotides have made RCA one of the most versatile new technologies.

Applications of RCA Techniques

Detection of Single Nucleotide Changes

Identifying genomic mutations and polymorphisms has both current and future implications in disease detection and prevention. RCA is emerging as a fundamental technology due to its ability to be adapted to real-time, high-throughput, and immobilized probe platforms. The main adaptation of RCA in this area is illustrated in Fig. 12.2E. If a linear ssDNA molecule is added to a denatured template, the ends of the molecule will be brought together at the target. If properly hybridized, the two ends can be joined by ligation thus creating an entrance primer for DNA polymerase replication of the incoming molecule. This has been termed a padlock probe (Nilsson et al., 1994; Baner et al., 2001; Nilsson et al., 2002). Using a padlock probe, DNA ligase can accurately discriminate between matched and mismatched substrates in this region such as for allelic discrimination, SNP detection, or mutation detection (Luo et al., 1996; Landegren et al., 1988; Faruqi et al., 2001). By introducing mismatches at the hybridization site, it allows the system to discriminate between single nucleotide changes between samples for accurate genotyping (Pickering et al., 2002).

RCA has also been applied to whole genome amplification from small numbers of cells and is especially useful when dealing with precious clinical specimens. For instance, ramification amplification (Zhang et al., 2001) uses a circular probe (C-probe) in which the 3′ and 5′ ends are brought together in juxtaposition by hybridization to a target. The two ends are then covalently linked by a T4 DNA ligase in a target-dependent manner, producing a closed DNA circle (e.g., see Fig. 12.2E). Upon addition of forward and reverse primers, DNA polymerase extends the bound forward primer along the C-probe and displaces the downstream strand generating a multimeric ssDNA. This multimeric ssDNA can then serve as a template for reverse priming to extend and displace downstream DNA, generating a large ramified (branching) DNA complex. This process continues until all ssDNAs become double-stranded, resulting in an exponential amplification (Zhang et al., 2001).

A similar procedure has also been applied to the amplification of whole genomes for high-throughput genomic analysis (Detter et al., 2002). These procedures, however, were inefficient in the amplification of fragmented DNA (Lage et al., 2003). A recent adaptation termed restriction and circularization-aided RCA (RCA-RCA) was developed for the need to amplify partially degraded DNA present in complex

and contaminated matrices including formalin-fixed tissues (Wang et al., 2004b). The basic method involves restriction endonuclease digestion of total DNA and circularization of fragments with DNA ligase. After elimination of noncircularized DNA by exonuclease digestion, the mixture is amplified using random primers and Phi 29 Polymerase (Dean et al., 2001). Examination of the products showed a balanced genome coverage exceeding that of balanced PCR amplification (Wang et al., 2004a, 2004b).

An even greater lever of amplification of circular DNA probes can be achieved using circle-to-circle amplification (C2CA), which provides a means for more than a 10^8-fold amplification (Dahl et al., 2004). DNA circles are first generated in a basic RCA reaction using padlock probes (Nilsson et al., 1994) (Fig. 12.2E) to generate single-stranded concatenated products (Fig. 12.3A). After heat inactivation of the polymerase, an excess of the complementary replication primer is hybridized and the concatenated products can be monomerized using an restriction enzyme that cuts in the dsDNA region formed by the binding of the complementary primer to the linear strand (Fig. 12.3B). The restriction enzyme is then heat inactivated, which allows both primers to dissociate and re-anneal at each end of the linear template. DNA ligase is then added to reform the circular template that can be used for additional rounds of replication (Fig. 12.3C) (Dahl et al., 2004).

Each round of a C2CA reaction is a linear amplification cycle so the reaction can be precisely quantified. Because the circles are of a defined polarity, this can facilitate hybridization-based downstream processing or quantified in real-time (Lizardi et al., 1998). Dahl et al. also demonstrated multiplexed genotyping using the C2CA reaction (Dahl et al., 2004). This system is robust in its design, and the accuracy and fidelity of replication is maximized using the Phi 29 DNA Polymerase (Blanco et al., 1989).

Anchored and Ligation-Mediated RCA Technologies

The use of immobilized nucleic acids and proteins is now commonplace in both basic research and diagnostics. The general methodology used for signal detection involves the passive hybridization of a detector probe and can limit the sensitivity of the assay depending on target abundance. Accordingly, RCA has been successfully applied to immobilize oligonucleotide targets to increase sensitivity and signal intensity. Immobilized RCA also takes advantage of the nondiffusible nature of the RCA concatameric product. RCA assays have been developed for SNP and mutation analysis (Christian et al., 2001; Pickering et al., 2002; Alsmadi et al., 2003), and the amplification mechanism is useful for protein microarrays (Schweitzer et al., 2000, 2002; Zhou et al., 2004).

The basic methodology of immobilized RCA was initially developed using a biotinylated oligonucleotide primer that could anneal to the ends of a circular probe. This formed a double-stranded complex that could be attached to streptavidin beads (Hatch et al., 1999). If the hybridization of the ends was perfect, this would allow DNA ligase to covalently join the two ends; therefore; polymerization from an external circle primer would occur. If the ends were not perfectly complementary,

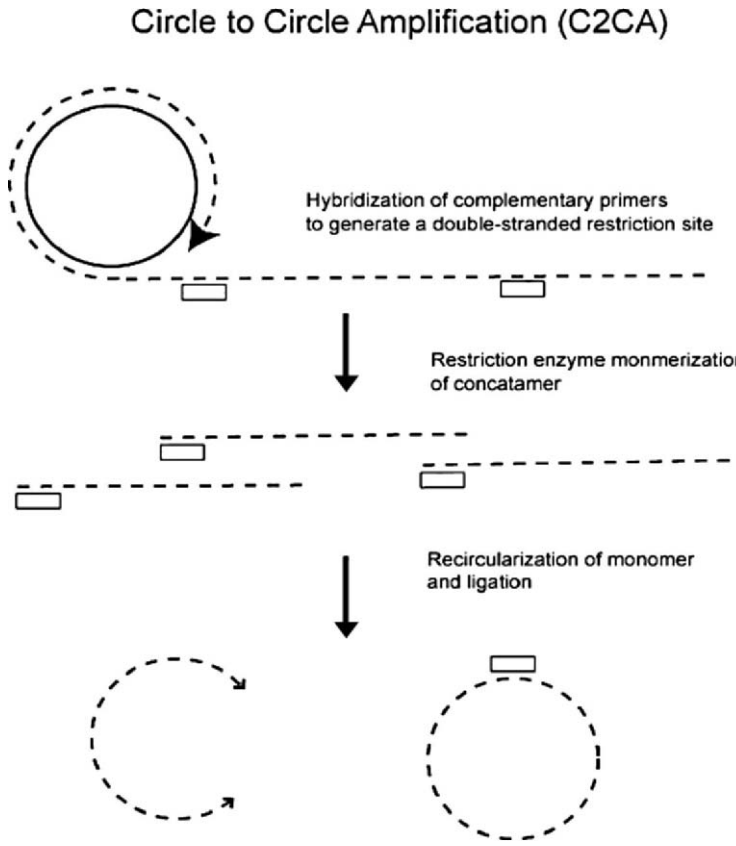

FIGURE 12.3. Circle-to-circle replication (C2CA). C2CR is an adaptation of rolling circle amplification wherein the concatamers are generated using a primer containing a restriction endonuclease site. A primer complementary to the replication primer is hybridized to generate a dsDNA restriction endonuclease site. Cleavage at this site resolves the concatamers and then can be recircularized to repeat the process.

ligation would fail (Fig. 12.2E). Hatch et al. modified their initial assay to detect polymorphisms by adding an additional template to the system (Hatch et al., 1999). In this case, if the new template is a match, ligase will complete circularization, and the added template will prime the RCA reaction. With a mutated target sequence, and in the case of a genomic mutation, there will be only a minimal amplification of the linear template. This technology is promising for large-scale mutation screening because different groups of probes can be fixed at known locations and used to generate an array capable of screening many unique sequences in parallel (Hatch et al., 1999).

Methods using RCA for SNP detection in a high-throughput format was recently developed for microtiter plates using universal fluorescent energy transfer primers (Thomas et al., 1999; Faruqi et al., 2001; Myakishev et al., 2001). The investigators

used two linear allele-specific probes each labeled with a unique fluorophore at one end and a quencher at the other (Thomas et al., 1999; Faruqi et al., 2001; Myakishev et al., 2001). The probe backbone contained a common binding site for amplification-specific primer. When both primers were used in the same reaction tube, the relative amount of each polymorphism could be determined from the sample. This system showed a greater sensitivity than PCR SNP methods, which was demonstrated by genotyping using 1 ng of genomic DNA (Faruqi et al., 2001). Pickering et al. modified probe design that overcame background problems seen by probe–probe interactions in previous studies (Faruqi et al., 2001; Pickering et al., 2002). Thus, an end-point fluorescent assay was developed that used a high-throughput format and did not require the use of real-time fluorescent detectors, although the assay has been developed for real-time measurements (Christian et al., 2001; Alsmadi et al., 2003).

Immobilized RCA has also been adapted as a signal enhancer for antibody microarrays, or "immunoRCA" (Schweitzer et al., 2000, 2002). This assay has been carried out in either the "sandwich" format where a matched pair of antibodies capture are used to "sandwich" the antigen between the two antibodies (Schweitzer et al., 2002) or in a format where the antigen itself is immobilized and a single ligand-specific antibody is used for detection (Schweitzer et al., 2000; Zhou et al., 2004). Secondary biotinylated antibodies, each specific for their cognate ligand, are allowed to bind. This is followed by a third antibody specific for biotin, coupled to an RCA primer. The RCA proceeded as in a typical amplification reaction using fluorescent detection with Cy3 and Cy5 dyes in a microarray analysis format (Schweitzer et al., 2002). In these initial developmental studies, up to 75 cytokines were measured simultaneously with femtomolar sensitivity and a three log quantitative range (Schweitzer et al., 2002).

This type of assay illustrates a number of salient points about immobilized RCA technologies: (1) the amplicon is not diffusible, and end product inhibition typical of PCR is not observed; therefore, sensitivity can be increased; (2) the sole reliance on passive hybridization is eliminated; (3) universal amplification primers can be used because the selectivity is determined by the antibody or, in the case of SNP analysis, by the discrimination between matched and unmatched bases by DNA ligase; (4) the technology for spotting and coupling to slides, as well as slide reading and data analysis, has already been developed; and (5) these are end-point assays so real-time analysis is not necessary.

RCA and In Situ Hybridization

RCA methods have been adapted to *in situ* hybridization, allowing an increase in sensitivity and the ability to identify single nucleotide changes. The limits of sensitivity of the standard fluorescence *in situ* hybridization (FISH) methods are in the several kilobase range except for signal amplification using tyramide that can lower this range to the hundreds of nucleotides (Van Tine et al., 2004). Thus, FISH is not able to detect single nucleotide changes either in DNA within a cytological context or in single DNA molecules (Qian and Lloyd, 2003). The application of

RCA methods to *in situ* analysis was initiated by Nilsson et al. (Nilsson et al., 1994). However, the efficiency of amplification was low and thought to be a complication of polymerase accessibility of the template (Nilsson et al., 1994). The method was modified by the use of energy-transfer primers and resulted in an increased sensitivity with the ability to detect as few as 10 ligated padlock probes (Thomas et al., 1999). This method was tested for *in situ* analyses to detect single nucleotide changes in DNA in fixed cells and tissues and was able to detect nuclear targets as small as 50 nucleotides in interphase nuclei (Zhong et al., 2001). The issue of polymerization efficiency was addressed by Christian et al. by pretreating cells with a combination of endo- and exonucleases. They achieved an efficiency of polymorphism detection of more than 90% in fixed cells (Christian et al., 2001). These indicated that RCA provided a breakthrough and a means for direct physical haplotyping and the analysis of somatic mutations on a cell-by-cell basis. Additionally, methods have recently been developed for whole-genome analysis and amplification SNP analysis (Bergmann et al., 2000; Lage et al., 2003) including preimplantation genetic diagnosis (Lasken and Egholm 2003; Handyside et al., 2004). Isothermal whole-genome amplification from single and small numbers of cells and may represent a new era for preimplantation genetic diagnosis of inherited disease (Dean et al., 2001, 2002; Handyside et al., 2004).

Multiple Displacement Amplification and Whole-Genome Analysis

Recently, a rolling circle amplification method was developed for amplifying large circular DNA templates such as plasmid and bacteriophage DNA (Dean et al., 2002; Pask et al., 2004). Using Phi 29 DNA polymerase and random exonuclease-resistant primers, DNA was amplified in a 30 °C reaction not requiring thermal cycling. This is made possible in part by the great processivity of Phi 29 DNA polymerase, which synthesizes DNA strands 70 kb in length. The amplification is surprisingly uniform across the genomic target, with the relative representation of test loci differing by less than three-fold, compared with PCR-based whole-genome amplification methods that exhibited strong amplification bias ranging from 4 to 6 orders of magnitude. Multiple displacement amplification (MDA)-generated DNA product is >10 kb, and its performance has been demonstrated for a variety of applications, including SNP analysis, RFLP, and comparative genome hybridization. MDA was capable of accurate whole-genome amplification from <10 human cells. This simple and robust method also uniformly amplified the human genome directly from whole blood without a requirement for DNA purification (Smirnov et al., 2004).

Strand Displacement Amplification

Overview of SDA Technique

Strand displacement amplification is another variation of the rolling circle theme but differs from RCA in that the amplicons are displaced from a linear template and

do not generate concatamers (Walker et al., 1992a). The SDA reaction occurs in two stages: (i) duplication of the target sequence by DNA polymerase resulting in the addition of restriction endonuclease sites at each end of the amplified target and (ii) exponential amplification consisting of multiple rounds of restriction endonuclease nicking, extension of the nick by DNA polymerase, and strand displacement (Walker et al., 1992a). In practice, the basic exponential reaction components are an exonuclease-deficient (exo-) DNA polymerase, for example, Klenow fragment (Klenow and Henningsen, 1970), a restriction endonuclease, and three unmodified dNTPs (dGTP, dCTP, TTP) with the fourth containing 2′-deoxyadenosine 5′-*O*-(l-thiotriphosphate) (dATPS) (Walker et al., 1992a).

In the first stage of the reaction, target duplication is carried out using four primers, two for each strand, similar to the design of primers in nested PCR (Albert and Fenyo, 1990). The internal (Int) primers contain a target binding sequence at their 3′ end and a RE-specific sequence at their 5′ end. The external primer (Ext) (also called a "bumper") is complementary only to target sequences immediately upstream of the internal primer (Fig. 12.4A). DNA polymerase extends the first internal primer and, in doing so, a restriction site is incorporated into the first strand. Due to the presence of the phosphothiolated dNTP species, the newly synthesized strand will be resistant to restriction endonuclease cleavage with the exception of the primer region. Duplication of this strand using the second primer set generates hemiphosphothiolated restriction endonclease sites at each end of the double-stranded molecule (Fig. 12.4B). Therefore, there will always be one end of a double-stranded molecule that is hemiphosphothiolated because the nonmodified base will originate in the primer. These hemimodified RE sites will allow nicking by the RE and allow the exo-DNA polymerase to extend the nick and synthesize a complementary strand followed by strand displacement (Fig. 12.4C).

The second or amplification stage of the reaction relies on multiple cycles of nicking, polymerization, and strand displacement. These steps repeated continuously produce exponential growth in the number of target sequences such that a 10^7-fold amplification can be achieved (Walker et al., 1992a). The use of only one SDA primer set renders the reaction linear and is important for some applications such as precise quantization. However, SDA is not a synchronous process, and different steps can occur simultaneously.

The SDA blueprint has been modified and adapted to a variety of applications. Enzyme combinations differing from the original exo-Klenow and *Hinc*II pair have been examined for their more robust activity and increased thermal stability; for example, combinations of *Bso*BI or *Ava*I with exo-*Bca* or exo-*Bst* although a *Bso*BI/exo-*Bst* combination has been favored (Spargo et al., 1996; Milla et al., 1998). The optimal length for amplification is in the range 50–100 nucleotides (Hellyer et al., 1996). However, the use of highly processive enzymes such as bacteriophage Phi 29 polymerase or exo-*Bst* DNA polymerase to generate amplicons of 10–20 kb allows hyperbranched propagation for whole-genome amplification. Primer choice follows all the rules for good primer design as it does in PCR,

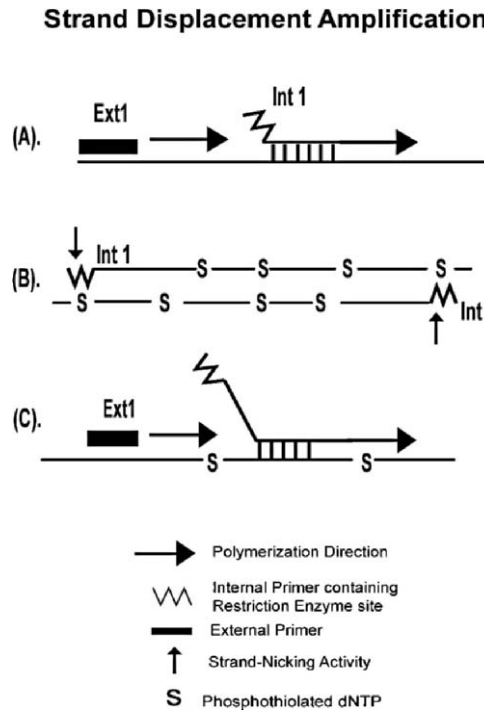

FIGURE 12.4. Strand displacement amplification (SDA). SDA is a variation of rolling circle amplifiction but differs in that the amplicons are displaced from a linear template and do not form concatamers. An internal primer containing a restriction endonuclease site (Int1) primes the first round of amplification. An externally located "bumper" primer (Ext1) is used to prime DNA polymerase, and synthesis from this location displaces the Int1-containing strand. A second round of priming and synthesis from primer ITS-2 completes a dsDNA molecule that becomes a substrate for the nicking activity of the restriction endonuclease (step A). The reaction is carried out in the presence of a phosphothiolated deoxynucleotide (S) that is represented in the restriction endonuclease site. Because the primer is not modified, the restriction endonuclease will nick only the hemi-thiophosphorylated strand of the newly synthesized dsDNA molecule. The polymerase can prime from the nick to start the amplification phase of the reaction (step B). DNA polymerase can then begin a new round of polymerization beginning at the nicked site and polymerizing in a strand-displacement mode (step C).

including minimizing fold-back loops, primer-dimers, and others (Walker et al., 1992a, 1992b). This is especially important in the design of real-time reporter assays such as molecular beacons and SNP analysis (Wang et al., 2003). SDA has also been adapted to RNA amplification with the inclusion of a reverse transcription step (Spargo et al., 1996; Nycz et al., 1998) and to a number of formats including real-time fluorescence detection (Wang et al., 2003; Nadeau et al., 1999), SNP analysis, mutation detection (Lage et al., 2003; Wang et al., 2003), and microarray analysis (Westin et al., 2000; Lage et al., 2003; Huang et al., 2004).

Application of the SDA Technique

Real-Time SDA

The use of real-time detection systems has become an important tool in both the research and diagnostic laboratory. These systems enable accurate quantization of both RNA and DNA templates and are stable components of the clinical diagnostic laboratory (Wang et al., 2003, Hellyer et al., 2004). Because the amplicons are confined in sealed wells, this also minimizes cross-contamination between samples. SDA has been adapted to this technology using a modification of fluorescence resonance energy transfer (FRET) (Little et al., 1999; Nadeau et al., 1999). However, due to the strand displacement nature of SDA, probes such as molecular beacons that rely on passive hybridization to single-stranded products cannot be used for real-time monitoring (Tyagi and Kramer, 1996). This is because SDA single-stranded amplicons are produced only transiently and are used as templates for additional rounds of amplification (Fig. 12.5). The solution to this problem was the improvement of fluorogenic probe design for use with SDA.

Nadeau et al. designed a dual-labeled hairpin probe containing rhodamine and a target-specific sequence at the 3′ end followed by a restriction endonuclease site in the loop and fluorescein attached at the 5′ end (Fig. 12.5A) (Nadeau et al., 1999). This molecule forms a hairpin loop that juxtaposes the labels, and the probe acts as the typical SDA internal primer to prime DNA synthesis (Fig. 12.5A). The external (bumper) primer functions in strand displacement as in the standard SDA reaction, and the displaced strand acts as a template for the second primer set (Fig. 12.5B). This second polymerization step results in the production of a double-stranded restriction endonuclease site that is flanked by both the fluorescein and rhodamine labels (Fig. 12.5C). Up to this point, both labels are in close proximity such that fluorescein emission is transferred to rhodamine, and fluorescein emission is effectively quenched. Cleavage at the restriction endonuclease site causes the physical separation of the labels so the net effect is that emission is detected from an excited fluorescein label (Nadeau et al., 1999) (Fig. 12.5D).

This system, for example, was developed into a commercial robust assay for *Mycobacterium tuberculosis* detection in clinical specimens (Bergmann and Woods, 1998; Little et al., 1999; Bergmann et al., 2000; Barrett et al., 2002; Wang et al., 2004c) as well as formalin-fixed and paraffin embedded tissues (Johansen et al., 2004). The assay sensitivity was equal or superior when directly compared with nested RT- PCR for the detection of *M. tuberculosis* and *Chlamydia trachomatis* (Verkooyen et al., 2003; Gaydos et al., 2004). These results demonstrate that SDA can be adapted to rapid and accurate clinical diagnostic procedures and ensure high sensitivity and reproducibility.

Real-time SDA system has also been applied to SNP and mutation detection (Little et al., 1999; Nadeau et al., 1999; Wang et al., 2003). There are two basic modifications applied to distinguish between two or more polymorphisms in the same reaction tube. First, dabcyl was substituted for rhodamine as the quencher in the stem-loop primer and enabled the use of alternative acceptor–donor pairs

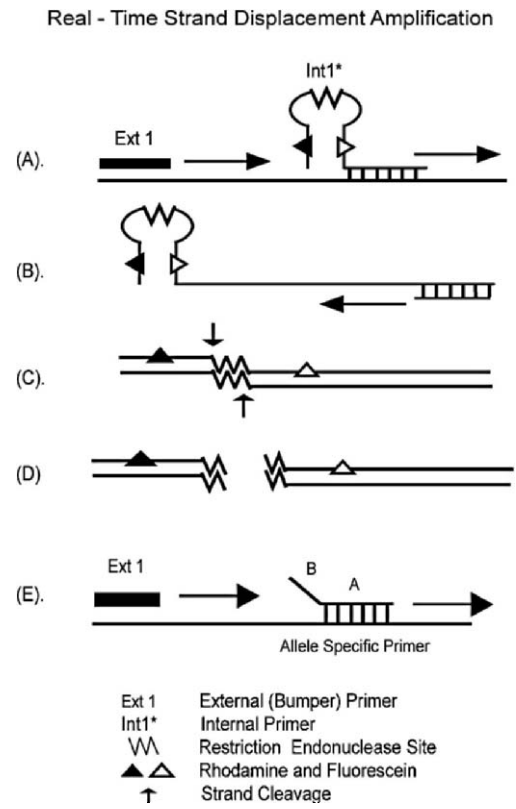

FIGURE 12.5. Real-time strand displacement amplification. Real-time detection using SDA employs a stem-loop primer containing a gene-specific region at its 5′ end. The 3′ end contains a stem-loop structure with the stem juxtaposing and quenching two fluorescent labels and the loop structure containing a restriction endonuclease site. Upon incorporation into a dsDNA molecule through the standard SDA reaction, the labels are still in close proximity and quenching still occurs. A fluorescent signal is generated when endonuclease cleavage occurs (steps A–D). Step E shows an adaptation using a gene-specific primer "A" and a generic "detector" sequence "B".

for FRET. The second modification was the inclusion of an unlabeled adaptor primer to generate allele specificity (Fig. 12.5E). The 3′ end of the adaptor is allele-specific and the 5′ end contains a generic detector sequence (Fig. 12.5E, A and B). The new primer is amplified through the basic SDA reaction and the newly synthesized strand is displaced with a bumper (Figs. 12.5A and 12.5E). The reaction course is that of the standard reaction. These amplicons possess the generic primer sequence that then can react with primer to enable amplification and restriction enzyme cleavage to generate the fluorescent signal (Wang et al., 2003).

Anchored SDA and Electronic Microarrays

Immobilized SDA has been applied to microarray technology to provide a platform for high-throughput and high-sensitivity detection of nucleic acid sequences. Thus far, the major outlet for anchored SDA has been the electronic microarray. Detailed discussion of this topic is beyond the scope of this chapter, and readers are referred to other references (Heller et al., 2000; Gurtner et al., 2002). The basic underlying principle of this technology is the anchoring of SDA primers onto an immobilized surface. This first step is accomplished by attaching biotin-tagged primers to an electronic array chip permeation layer containing streptavidin. The primers are placed in discrete locations through electronic biasing, each with a unique address. The template for the reaction is electronically hybridized, and SDA is performed *in situ* on the chip (Westin et al., 2001). The SDA reaction is stopped by removal of the supernatant, double-stranded DNA products are denatured on the microchip, and internal reporters are hybridized to the amplicon products remaining on the chip. However, because the target strands are displaced into solution and each microarray shares a common solution, amplicon capture was necessary to increase assay sensitivity.

The approach of Huang et al. was to use a nonamplifiable primer (NAP) that would be replicated in a linear mode and remain attached due to the lack of a restriction endonuclease site (Huang et al., 2004). The NAP is extended but not cleaved during the SDA reaction and in essence becomes an anchor for amplicons that are generated through the use of amplified primers (APs). The anchoring of NAPs on the chip in a 4:1 to 20:1 (NAP:AP) ratio resulted in a 20-fold increase in signal intensity when compared with the use of AP alone (Huang et al., 2004). This assay was successful in genotyping nine different alleles on the same microarray without sample cross-contamination.

Summary

In recent years, development of non-PCR–based target amplification techniques has gained ground in the detection of microbial and viral pathogens, among many other uses in the diagnostic laboratory. Table 12.2 provides a summary of comparison of PCR and non-PCR isothermal target amplification methods. Numerous commercial products have emerged using either isothermal transcription-based RNA amplification or isothermal DNA template amplification systems. The basic methodologies that have emerged are variations of the theme of (i) transcription-based systems for RNA amplification and (ii) strand displacement technologies based on rolling circle replication. Though both systems have their primary template targets as either RNA or DNA, in reality both RNA and DNA can serve as templates under the appropriate experimental circumstances making the non-PCR amplification techniques more versatile. In addition, the relatively mild conditions employed in isothermal amplification makes it ideal for the detection of microorganisms *in situ* in tissues. The non-PCR target amplification techniques will compensate the shortcomings of PCR-based target amplification and will find

TABLE 12.2. A comparison of PCR and isothermal amplification methods.

	PCR	Isothermal amplification
Temperature restrictions	Machine cycling	Isothermal (machine independent)
Enzyme requirements	Thermostable DNA polymerase	Reverse transcriptase, RNA polymerase
Technique	Alternating cycles of amplicon denaturation, primer annealing, and polymerization	Isothermal; dependent on characteristics of enzyme system used
Amplifications per cycle	2	50–1000
Patent restrictions	Yes	No
In situ applications	Limited; destructive	Yes; nondestructive and can be used in mounted specimens
Single nucleotide polymorphism/mutation detection	No (although yes with primary sequence analysis)	Yes (as a stand-alone method)
Real-time detection	Yes	Yes
Target amplification on immobilized support	No	Yes
Contamination control	DNA amplicon; carry-over contamination problem	RNA amplicon; labile so minimizes carry-over

their niche in today's diagnostic microbiology arena to allow greater sensitivity and specificity of microbial pathogen detection.

Acknowledgment. The opinions and information in this chapter are those of the authors and do not represent the views and/or policies of the agencies to which the authors are affiliated.

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Recent Advances in Probe Amplification Technologies

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Introduction

Oligonucleotide probes provide a useful tool for the detection of target nucleic acids by the formation of a double helical structure between complementary sequences. The stringent requirements of Watson–Crick base pairing make hybridization extremely specific. However, the detection of target sequence by hybridization is often insensitive due to the limited number of signal molecules that can be labeled on the probe. In general, the analytical sensitivity of probe hybridization is of the order 10^6 molecules. Therefore, it cannot meet the needs of most clinical diagnostic applications. Many technologies have been developed to improve the detection sensitivity by amplifying the probe sequence bound to the target. All probe amplification technologies are developed based on the recent advancement in molecular biology and the understanding of *in vivo* nucleic acid synthesis (i.e., ligation, polymerization, transcription, digestion/cleavage, etc.).

A fundamental advantage of probe amplification technologies ascribes to their isothermal nature, (i.e., accomplishing amplification at a constant temperature with the exception of LCR, which requires temperature cycling). Isothermal amplification allows the test to be done using a simple instrument and makes quality control of the instrument easier. In order for the probe to be amplified, the probes have to be specially designed or synthesized. For example, in rolling circle amplification (RCA), a circularized probe is used, whereas the Invader assay employs an overlapping structure within the probes. Finally, maximum amplification is achieved by generating new DNA products (RCA, RAM, SMART, Q-beta replicase, etc.), although some of the technologies (i.e., LCR and CPT) use existing DNA primers without a net increase of DNA products.

In addition to amplification of probe sequence to achieve a desired sensitivity, each technology has its unique features, thus unique clinical applications. For example, Invader technology is very useful for single nucleotide polymorphism (SNP) scoring due to specific recognition by the enzyme cleavase to the overlapping structure of two probes. On the other hand, RCA is probably the only technology that can be used for on-chip amplification due to the attachment of product to the primer sequence linked on the chip surface. Therefore, in order to select a

technology for a particular application, one has to understand the principle of the technology and address the need of the clinical problem accordingly.

This chapter will review the most common probe amplification technologies and present some of their applications with primary focus on microorganism diagnosis in clinical laboratory. For more in-depth discussion of clinical applications, the readers should refer to other excellent chapters in this book.

Rolling Circle Amplification

Circularizable probe (C-probe or padlock probe) is a uniquely designed oligonucleotide probe that contains three regions: two target complementary sequences located at the 5′ and 3′ termini and an interposed generic linker region (Nilsson et al., 1994; Zhang et al., 1998). Once the C-probe hybridizes to its target, the 5′ and 3′ ends are juxtaposed (Fig. 13.1A). A closed circular molecule is then generated after incubation of the C-probe-target complex with a DNA ligase. The resulting

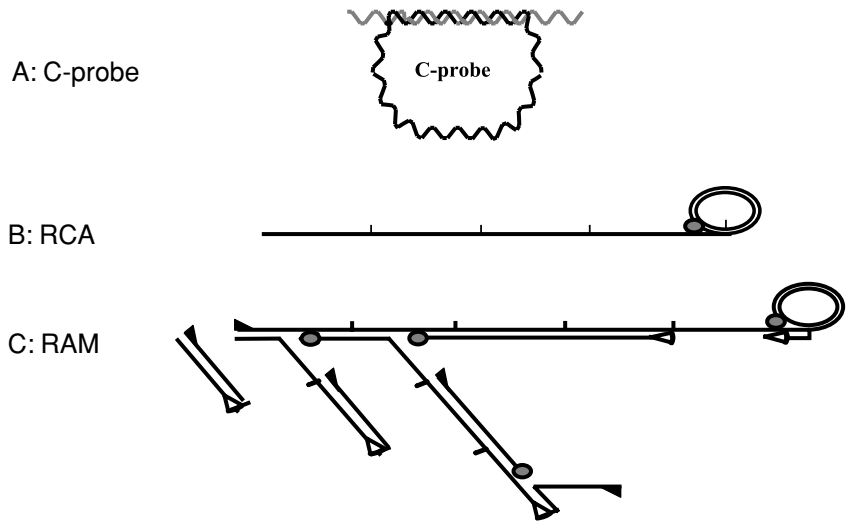

FIGURE 13.1. Schematic representation of C-probe, RCA, and RAM. (A) A C-probe hybridizes to its target through its complementary regions and helical turns formed between C-probe and target results in the locking of C-probe onto the target. The sequence between the target-binding regions is generic for the binding of primers. (B) A DNA polymerase (●) extends a bound primer along a closed C-probe for 5 rounds through rolling circle amplification (RCA). (C) A forward primer (►) bound to a C-probe is extended by DNA polymerase (●), generating a long ssDNA. Multiple reverse primers (◅) bind to the nascent ssDNA as their binding sites become available. Each bound reverse primer extends and displaces the upstream primers and their extended products. The forward primer binding sites of the displaced ssDNA are then available for the forward primers to bind and extend similarly, thus forming a large ramifying DNA complex (RAM).

TABLE 13.1. Comparison of probe amplification technologies.

Property	RCA	RAM	Q-beta	SMART	Invader	LCR	CPT
Amplification capability	1U	2^u	2^n	2^n	1U	2^n	1U
Temperature alteration	−	−	−	−	−	+	−
Detection of DNA target	+	+	+	+	+	+	+
Detection of RNA target	+	+	+	+	±	+	−
Detection of protein target	+	±	−	−	−	±	−
Real-time	+	+	+	+	+	±	+
Enzyme used	DNA pol	DNA pol	RNA-RNA pol	DNA-RNA pol	cleavase	ligase	RNase H
On-surface amplification	+	±	−	−	−	−	−
Multiplexing	+	+	±	±	+	±	+
SNP detection	+	+	+	±	+	±	±

RCA, rolling circle amplification; RAM, ramification amplification; SMART, signal-mediated amplification of RNA technology; LCR, ligase chain reaction; CPT, cycling probe technology; u, number of rounds accomplished by DNA polymerase along a C-probe; n, number of cycle; SNP, single nucleotide polymorphism; DNA pol, DNA polymerase; RNA-RNA pol, RNA directed RNA polymerase; DNA-RNA pol, DNA directed RNA polymerase.

closed circular molecule is helically twisted around the target strand (Nilsson et al., 1994). The permanently locked C-probe permits stringent washing for the removal of unbound components, thereby enhancing assay signal to noise ratios.

The unique design of the C-probe allows its amplification by a rolling circle (RCA) mechanism as observed in *in vivo* bacteriophage replication (Fig. 13.1B) (Fire and Xu, 1995; Baner et al., 1998; Zhang et al., 2001). In this scheme, a single forward primer complementary to the linker region of the C-probe and a DNA polymerase bearing strand displacement activity are employed. The polymerase extends the bound primer along the closed C-probe for many revolutions and displaces upstream sequences, producing a long single-stranded DNA (ssDNA) of multiple repeats of the C-probe sequence that can be as long as 0.5 megabase (Baner et al., 1998). This type of amplification, however, only results in linear growth of the products with up to several thousand-fold amplification (Baner et al., 1998). Some of the properties of RCA are summarized in Table 13.1.

Because the product of RCA remains attached to the primer, RCA is amenable to an on-chip probe amplification system (Fig. 13.2A). In this way, the target molecule can be recognized, amplified, and detected directly on a solid support, such as a microarray platform. With RCA, Nallur et al. (2001) were able to detect 480 fmol (150 molecules) of spotted primers, corresponding to an 8000-fold increase in detection sensitivity over hybridization under the same conditions. This level of amplification by RCA on microarray was comparable to that achieved in solution phase format, indicating that RCA can function with virtually 100% efficacy when

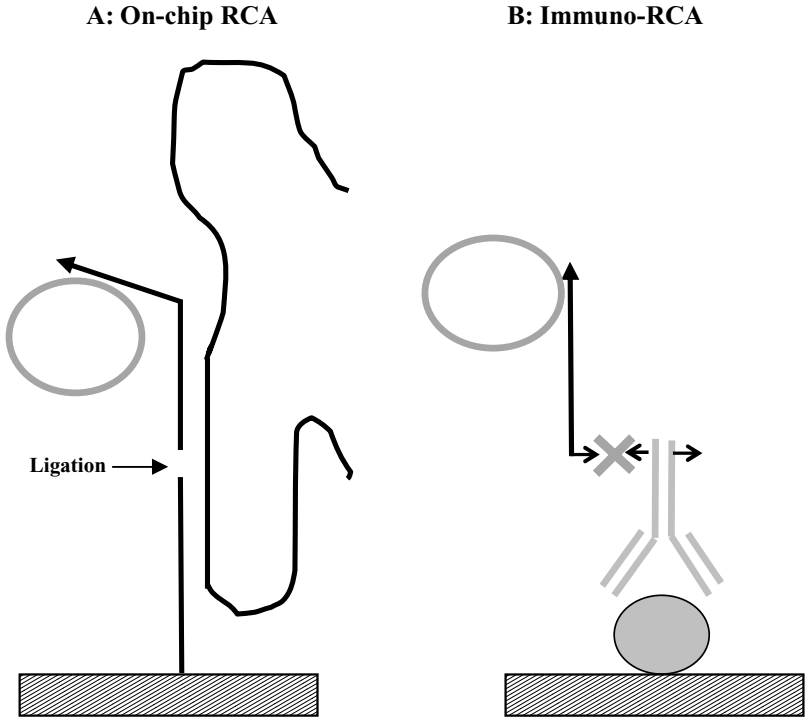

FIGURE 13.2. Schematic representation of on-chip RCA and immuno-RCA. (A) A probe with a portion of the sequence complementary to a target is spotted onto a support. An RCA primer contains a 5′ region complementary to the target sequence adjacent to the spotted probe and a 3′ region complementary to C-probe. In the presence of target, the RCA primer links to the spotted probe by ligation. The C-probe is amplified by RCA, and the resulting single-stranded DNA is linked to the probe spotted on the support. (B) An antibody tagged with an RCA primer binds to a protein spotted onto a support. The RCA primer links to an antibody through the interaction of biotin–avidin–biotin. The bound C-probe is amplified by RCA, and the resulting single-stranded DNA remains linked to the antibody.

used on microarray. Thus, combination of RCA and DNA microarray allows the real-time detection of multiple targets with great sensitivity and specificity.

Recently, an RCA-based protein detection method, referred to as immuno-RCA, has been developed (Schweitzer et al., 2000, 2001). In this scheme, a primer is linked to an antibody and the signal is amplified by RCA (Fig. 13.2B). Detection of allergen-specific IgE in blood samples using this approach was demonstrated in a microarray format (Wiltshire et al., 2000; Kim et al., 2002). Wiltshire et al. (2000) printed several allergen extracts, including cat dander, house dust mites, and peanuts onto a glass slide, which was then incubated with 10 μL of patient's serum to allow anti-allergen antibody to bind. After washing, an anti-IgE antibody tagged with an RCA primer complexed with its complementary precircularized C-probe was added to the slide. The RCA products were visualized with a microarray

scanner after hybridization with fluorescence-labeled probe complementary to RCA products. With this system, the authors tested 30 patients whose allergen status has been confirmed by a skin-prick test. The authors compared the assay with a commercially available kit, autoCAP (Pharmacia, Kalamazoo, Michigan) and found that immuno-RCA was more sensitive than autoCAP for peanuts and cat dander but not house dust mites. The specificity of immuno-RCA was above 90%, which was superior to that of autoCAP. Although a relatively small group of allergens were tested on a small number of patients, the study showed that immuno-RCA on microarray holds great promise for allergen testing.

Ramification Amplification

Ramification amplification (RAM) (Zhang et al., 1998, 2001), also referred to as hyperbranched rolling circle amplification (Lizardi et al., 1998) or cascade rolling circle amplification (Thomas et al., 1999), is a novel, isothermal DNA amplification that amplifies a C-probe exponentially through the mechanism of primer extension, strand displacement, and ramification. In contrast with RCA, the RAM assay uses two primers, one complementary to the C-probe (forward), and the other identical in sequence to a second binding site in the C-probe (reverse). As with RCA, the initial rolling circle primer extension process generates a long ssDNA. However, as the ssDNA molecule expands, multiple reverse primers are able to bind to the growing ssDNA and initiate a second "round" of primer extension templated by the initial "rolling circle" products. Once a downstream primer encounters a bound upstream primer, the polymerase displaces the upstream bound primer along with any extended sequence that may be attached to it. The displaced ssDNAs serve as templates for further primer extension and amplification (Fig. 13.1C). Like the constant unfurling of streamers, multiple primer extensions take place simultaneously, resulting in a large ramified complex. Because the displaced DNAs are single-stranded, the binding of primers occurs at a constant temperature, thus obviating the need for thermocycling to generate single-stranded DNA, as in the case for LCR primers. Some of the properties of RAM are summarized in Table 13.1.

The practical use of RAM has been shown in several studies for detecting target nucleic acids in clinical samples. Zhang et al. (2002) were able to detect *Chlamydia trachomatis* in cervical specimens collected in PreservCyt cytological solution. Thirty clinical specimens were tested using the RAM assay, and the assay conferred accurate detection of all the positive samples that were confirmed by PCR and LCx. The RAM assay can detect as few as 10 *C. trachomatis* elementary bodies in less than 2 hrs. similar to the lower limit of detection for Amplicor PCR and LCx. Therefore, the RAM assay can serve as a feasible alternative to PCR and LCx for the detection of sexually transmitted infectious agents owing to its simplicity and isothermal amplification conditions.

The RAM assay was also used in the identification of *Escherichia coli* O157:H7 and other Shiga toxin–producing *E. coli* (STEC) in food and human samples.

Combining magnetic bead–based DNA isolation, amplification of a *stx2*-specific C-probe by RAM and real-time fluorescence detection, Li and colleagues (Li et al., 2005) accurately identified all 27 pathogenic *E. coli* isolates producing Shiga toxin 2 from food and human samples, as previously confirmed by PCR using primers specific for the *stx2* gene. One *Shigella dysenteriae* and three nonpathogenic *E. coli* were found negative by RAM assay. With respect to such application, the RAM assay provides a simple yet sensitive method that can be readily employed in clinical laboratories for the detection of food-borne pathogens and in meat product inspections.

Q-beta Replicase Amplification

Q-beta replicase is an RNA-dependent RNA polymerase derived from the bacteriophage Q-beta (Haruna and Spiegelman, 1965). It comprises four different subunits with only one polypeptide (i.e., subunit II) encoded in the Q-beta phage genome. The other subunits are generated by the host protein synthesizing apparatus (30S ribosomal protein S1, elongation factor EF-Tu and EF-Ts) (Blumenthal and Landers, 1976). Q-beta replicase has stringent specificity for its templates (Wu et al., 1992). Only a few naturally occurring RNAs can serve as Q-beta replicase templates, including plus and minus Q-beta RNAs and several smaller "variant" RNAs from *in vitro* replication reactions (Wu et al., 1992). Such replicase recognizes the specific structure within the template and initiates new strand synthesis from the 3′ end of the template without the need of primers. Because the daughter strands also serve as templates for the enzyme, RNA production proceeds exponentially. A single probe molecule can yield a detectable amount of product RNA in a 30-min amplification reaction.

Midvariant-1 (MDV) RNA is a 220-nucleotide-long variant that can be recognized and replicated by Q-beta replicase (Wu et al., 1992). Within the RNA sequence, Kramer and his colleagues inserted a link sequence to which additional probe sequences can be inserted. These recombinant RNAs can then serve as vehicles for amplifying probe sequences to million-folds to allow easy detection of the products by conventional methods such as dot blot and fluorescence. To eliminate nonspecific amplification of the probe, two RNA fragments were made, each containing only half of the probe sequence, and none of them were amplifiable (Fig. 13.3). Upon hybridization to the target, two fragments of the probe sequence were brought together and were subsequently ligated to yield a fully replicable RNA (Tyagi et al., 1996). Some of the properties of Q-beta replicase amplification are summarized in Table 13.1.

Q-beta replicase-based assay has been successfully used to detect various microorganisms such as *Chlamydia trachomatis*, *Mycobacterium tuberculosis*, and HIV (Tyagi et al., 1996). Shah et al. described a "dual capture" method to detect *C. trachomatis* in urogenital samples (Shah et al., 1994). In this method, the hybrids between chlamydial-specific MDV RNAs and chlamydial rRNA targets were captured onto magnetic beads via a separate capture probe. After washing, these

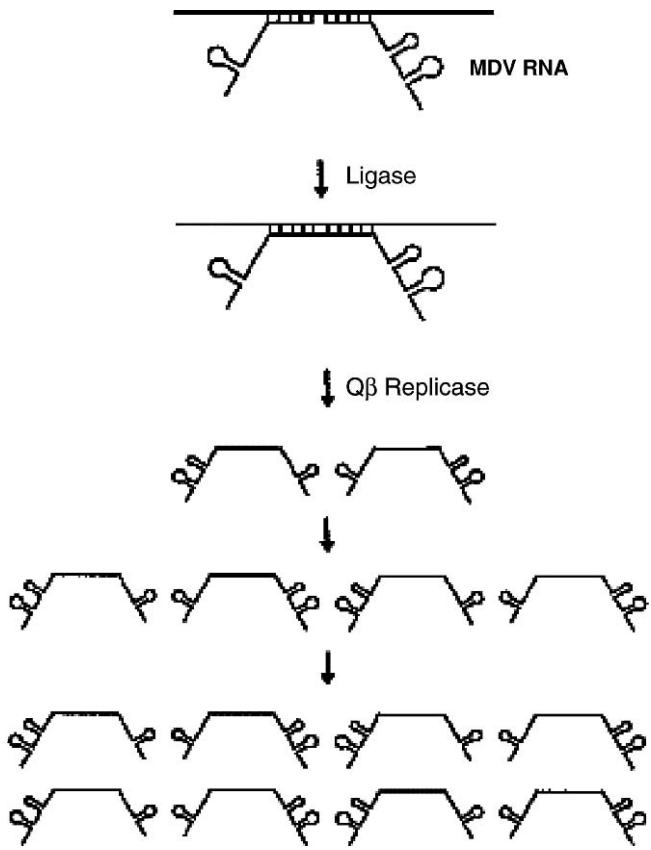

FIGURE 13.3. Schematic representation of the Q-beta replicase assay. The two fragments of a recombinant MDV RNA probe hybridize to a target, bringing the two ends in close proximity. After removal of unbound probes, the RNA probes are linked together by a T4 DNA ligase to form a fully replicable RNA, which is then amplified exponentially by Q-beta replicase.

hybrids were released and recaptured to eliminate nonspecific binding of the MDV RNAs to the beads. The chlamydial-specific MDV RNAs were then amplified by Q-beta replicase in the presence of propidium iodide, and detection was carried out in a real-time fashion using a kinetic fluorescence reader. The analytical sensitivity of the assay was 1000 molecules. In their study of 94 urogenital samples, the assay detected 5 of the 6 culture-positive samples and did not detect *C. trachomatis* target in 85 of the 88 culture-negative samples.

An automatic instrument (Galileo) was developed to process the samples and detect amplification products in a closed disposable test pack to reduce contamination (Smith et al., 1997). In a clinical trial, Smith et al. (1997) designed a recombinant MDV-1 RNA containing a probe sequence specific for 23S rRNA of *Mycobacterium tuberculosis*. Seven hundred eighty respiratory tract samples

(sputum or bronchoalveolar lavage specimens) were tested using this assay, and the results were compared with those of culture and microscopic examination of acid-fast staining bacillus. Seventy-one out of the 90 (78.9%) culture-positive samples were found positive when tested in the assay, while 7% of the culture-negative samples were assay positive, corresponding to a sensitivity of 79% and a specificity of 93%. After discrepancy analysis, the sensitivity and specificity for the assay were 84% and 97%, respectively. A total of 69.2% of smear-negative (culture-positive) samples were detected by the assay. Although relatively good sensitivity and specificity were demonstrated in this study, the assay and the instrument have not yet been implemented for routine use in clinical laboratory settings.

Signal-Mediated Amplification of RNA Technology

Signal-mediated amplification of RNA technology (SMART) is a novel isothermal amplification technology that uses a three-way junction (3WJ) structure to facilitate target-dependent production of multiple copies of a RNA product (Wharam et al., 2001). The 3WJ structure is composed of two target-specific single-stranded DNA probes (the "template" probe and the "extension" probe) and a target sequence. Both probes have a longer region that hybridizes to the target at adjacent sites and a shorter region that only hybridizes to each other in the presence of the target, thus forming the three-way junction (3WJ) structure (Fig. 13.4A). In addition, the template probe also contains a nonfunctional single-stranded T7 RNA polymerase promoter sequence. After 3WJ formation and addition of *Bst* DNA polymerase, the polymerase extends the short probe (extension probe) along the single-stranded template probe to form a functional double-stranded promoter for T7 RNA polymerase. In the presence of T7 RNA polymerase, multiple copies of RNA can be synthesized (Fig. 13.4B). Both *Bst* DNA polymerase and T7 RNA polymerase can function under the same reaction condition, hence the reaction can be performed in a single tube. In order to further improve the signal, a second template oligonucleotide (probe for RNA amplification) containing a second T7 promoter sequence can be added to the reaction to allow the RNAs generated from 3WJ to bind, which, in turn, allows its extension by *Bst* DNA polymerase and generation of secondary RNAs by T7 RNA polymerase, ultimately leading to a further increase in RNA yield (Fig. 13.4B). The RNA product can be measured by an enzyme-linked oligosorbent assay. This assay is capable of generating a detectable signal from 50 nmol single-stranded synthetic target, 10 ng bacterial genomic DNA, or 0.1 ng total bacterial RNA (or 10^4 bacteria) (Wharam et al., 2001). Some of the properties of SMART are summarized in Table 13.1.

Levi et al. evaluated the SMART assay (CytAMP assay kit, Cytocell Ltd., Adderbury, Oxford, UK) for the rapid detection of methicillin (oxacillin)-resistant *Staphylococcus aureus* (MRSA) (Levi et al., 2003). Two sets of probes were designed against the *coa* (coagulase) and *mecA* (methicillin resistance) genes, respectively, hence, simultaneous identification of *S. aureus* and methicillin

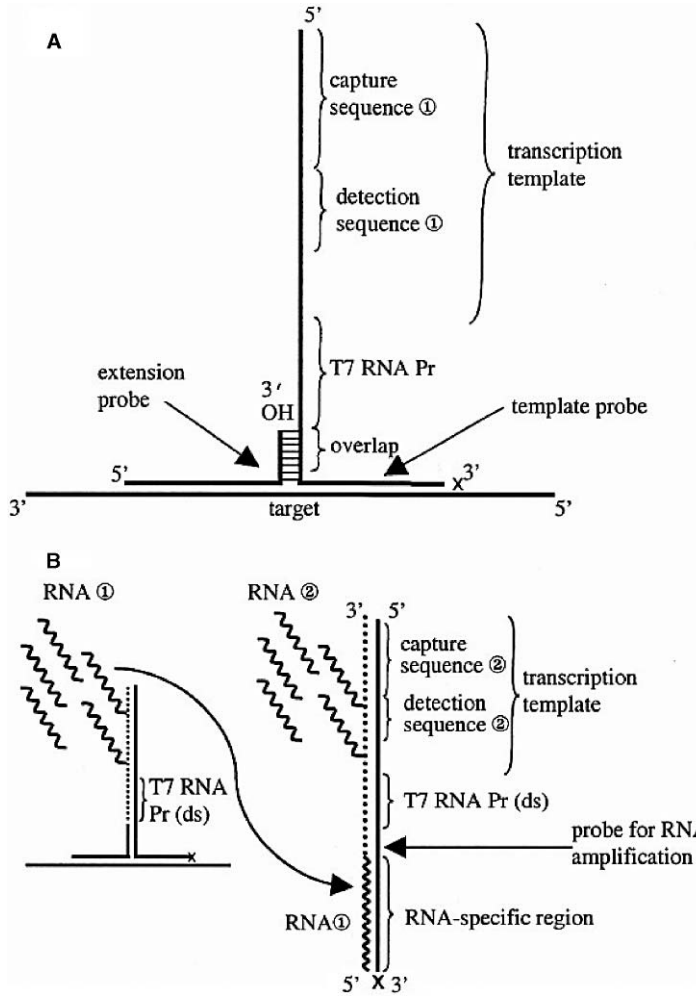

FIGURE 13.4. Schematic representation of the SMART assay. (A) Formation of a 3WJ. Extension and template probes anneal to the target, and only then can they hybridize with each other. The short extension probe has a free 3′-OH to allow extension. The template probe includes a single-stranded (nonfunctional) T7 RNA polymerase promoter (Pr) and sequences to allow the capture and detection of the RNA signal. The 3′ end of the template probe is blocked (x) by phosphorylation to prevent extension. (B) Extension and transcription generate an RNA signal. *Bst* DNA polymerase extension of the extension probe generates a double-stranded (ds), hence functional, T7 RNA polymerase promoter (Pr), allowing transcription of multiple copies of an RNA signal (RNA1) by T7 RNA polymerase. If required, RNA ① anneals to a second template (probe for RNA amplification), leading to further extension and transcription by the DNA and RNA polymerases to generate increased amounts of a second RNA signal (RNA ②).

(oxacillin) resistance is possible. The detection limit of the assay was 2×10^5 and 10^6 CFU/assay for *mecA* and *coa*, respectively. When tested with *S. aureus* isolates, the assay detected 113 MRSA among 396 *S. aureus* with 100% sensitivity and specificity, compared with a *mecA-femB* PCR assay. When 100 enrichment broths containing sets of screening swabs from individual patients were tested, the presence of MRSA was detected in 19, 24, and 31 enrichment broths by SMART assay, conventional culture, and *mecA-femB* PCR, respectively. Six enrichment broths were found negative by SMART assay but positive by both PCR and culture. Five of these contained an equivalence of 10^2 to 10^5 CFU/assay (below the predicted detection limit of 2×10^5 CFU/assay for SMART assay), and the sixth contained an equivalence of 10^6 CFU/assay. Overall, culture and SMART had similar sensitivities and specificities relative to those of PCR.

Invader Assay

The Invader assay is a unique, isothermal amplification technology that can detect DNA or RNA with high specificity and sensitivity. The basis for the Invader assay is the cleavage of a unique secondary structure formed by two partially overlapping oligonucleotides (an allele-specific primary probe and an invader probe) that hybridize to a target sequence to create a "flap" (Lyamichev et al., 2000) (Fig. 13.5). Cleavase VIII (flap endonuclease I from *Archaeoglobus fulgidus*) recognizes this three-dimensional structure as a specific substrate and cleaves the 5′ flap of the primary probe. The flap initiates a secondary reaction in which the released 5′-flap serves as an invader probe on a fluorescence resonance energy transfer (FRET) cassette to create another overlapping tertiary structure that is, in turn, recognized and cleaved by the Cleavase enzyme (Fig 13.5A). The Invader assay is optimal with a high concentration of primary probe and at temperatures near its melting temperature (60°C) at which the primary probe can easily cycle on and off the target for cleavage. When the FRET cassette is cleaved, a fluorophore dissociates from the quencher labeled on the FRET cassette, emitting a detectable fluorescence signal proportional to the target sequence.

Wong et al. (2004) utilized the Invader assay to detect hepatitis B virus (HBV) in patients' serum and liver biopsies. Three different viral DNA structures occur in HBV life cycle: linear double-stranded DNA (nonreplicative), relaxed circle DNA, and covalently closed circular DNA (cccDNA), which serves as the template for the production of viral and pregenomic messenger RNA. Because the specific three-dimensional structure is required for cleavase, the Invader assay is an ideal method to detect various forms of HBV as well as HBV viral load. Wong et al. (2004) designed two sets of Invader probes targeting direct repeat 2 region, in which the negative and positive strands anneal together to bring both ends of the linear form of HBV DNA together to form a relaxed circle. Both Invader probe signals should be detected if cccDNA is present, one probe signal for relaxed circle DNA and one for linear DNA. In their study, the lower limit

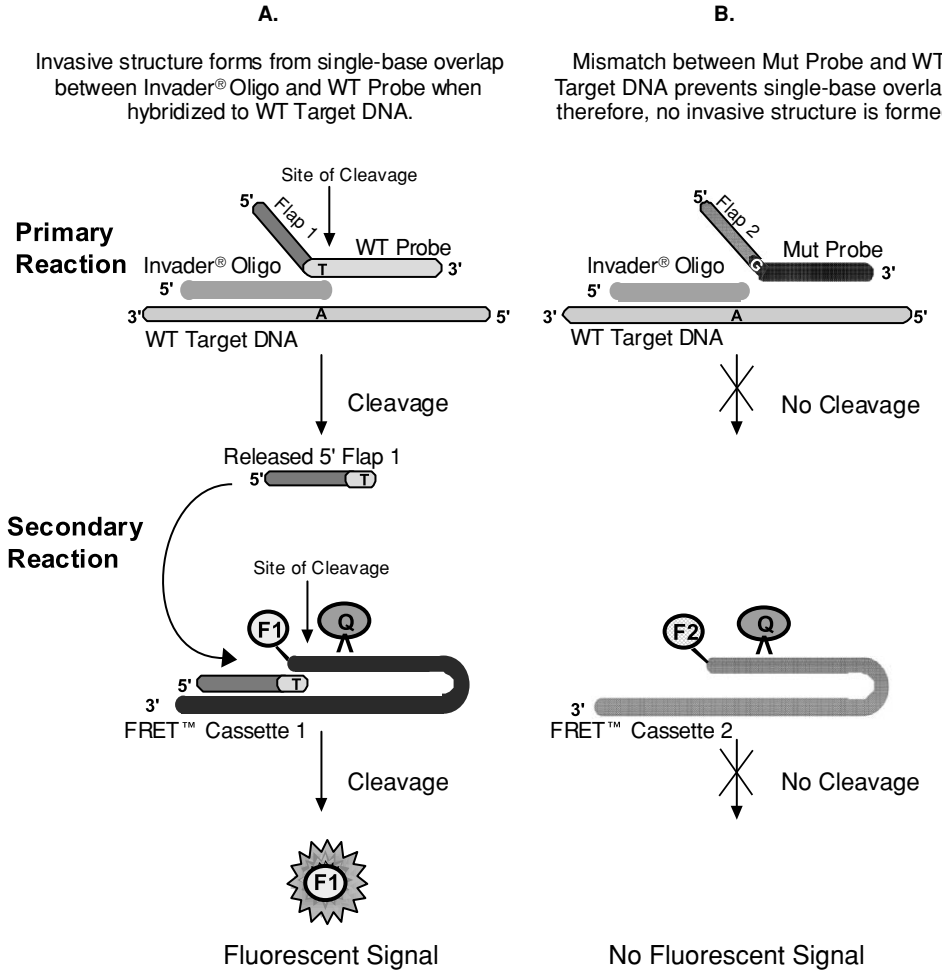

FIGURE 13.5. Schematic representation of the Invader assay. During an initial reaction, a discriminatory primary probe and an invader oligo hybridize to the target, overlapping at the SNP position and forming a three-dimensional flap structure that is recognizable by the cleavase enzyme at this site. The flap subsequently anneals to a FRET cassette in a separate reaction and initiates secondary cleavage that releases a fluorescent dye detectable by a fluorometer. Fluorescence is detectable only when a match occurs; if the primary probe is mismatched, cleavase remains inactive and no fluorescence is detected.

of detection was 50 copies/assay or 0.0002 copies/cell for hepatic tissue or 10^4 copies/mL for serum with a dynamic range of 5 orders of magnitude. cccDNA was detected in liver biopsy tissue in 16 hepatitis B e-antigen (HBeAg)-positive and 36 antibody-to-HBeAg-positive (anti–HBe-positive) chronic hepatitis B patients, and these results correlated positively with the total intrahepatic HBV DNA. Anti–HBe-positive patients had lower median total intrahepatic HBV DNA and

intrahepatic cccDNA levels than HBeAg-positive patients. However, the proportion of intrahepatic HBV DNA in the form of cccDNA was inversely related to the amount of total intrahepatic HBV DNA. A small amount of cccDNA was detected in 39 of 52 (75%) serum samples. Anti–HBe-positive patients had lower median serum cccDNA levels than HBeAg-positive patients. Serum HBV DNA correlated positively with intrahepatic total HBV DNA and intrahepatic cccDNA. Serum and intrahepatic total HBV DNA and cccDNA levels diminish as the disease progresses from HBeAg positive to anti–HBe-positive phase, with cccDNA becoming the predominant form of intrahepatic HBV DNA.

The Invader assay could be a sensitive method for detecting certain mutations associated with drug resistance in microbial pathogens. Cooksey et al. (2000) applied the Invader assay to detect mutations associated with resistance to rifampicin (RIF) and isoniazid (INH) in *M. tuberculosis*. Nine pairs of probes, five for mutations in *rpoB* gene (resistance to RIF) and *katG* gene (resistance to INH) and four for the corresponding wild-type (drug-susceptible) alleles, were synthesized. Each allele-specific primary probe had a different length of 5′ flap (from 4 to 13 nucleotides) and was labeled with different fluorophores. The PCR-amplified DNA fragments were tested and the fluorescence-labeled cleavage products were resolved by denaturing polyacrylamide (20% to 24%) gel electrophoresis. All nine alleles could be identified and differentiated on the basis of product size. Multiple mutations of the *rpoB* gene in PCR products could be identified, as could mutants that were present at $\geq$0.5% of the total population of PCR products.

Ligase Chain Reaction

Ligase chain reaction (LCR) is a probe amplification technique that requires temperature cycling. LCR uses two sets of probes that hybridize to the target DNA strand at adjacent location (Barany, 1991) (Fig. 13.6). The initial steps of LCR consist of denaturation of the double-stranded DNA by increasing the temperature to 94°C, followed by annealing of probes to their target DNA adjacent to each other by reducing the temperature below the melting temperature of the probes (45–55°C). The third step is to the joining of the 3′ end of one probe with the 5′ end of the other probe by a thermostable DNA ligase at a higher temperature (72°C). At second round of temperature cycling, the ligated probes dissociate from the target DNA strand and are available to serve as templates for another set of probes to hybridize and ligate. These temperature cycles can proceed up to 20–30 rounds, resulting in an exponential amplification of the full-sized probe. In order to reduce nonspecific ligation, two probes can be designed in such a way that upon hybridization to the target DNA, a one- or two-nucleotide gap remains between the two probes. After perfect hybridization to the target sequence, a DNA polymerase readily fills the gap while the ligase covalently joins the two probes together, forming a single contiguous DNA strand. The addition of a biotin on the first probe and a suitable non-isotopic reporter group tagged on the second probe allows for accurate product capture and detection in a manner that is readily

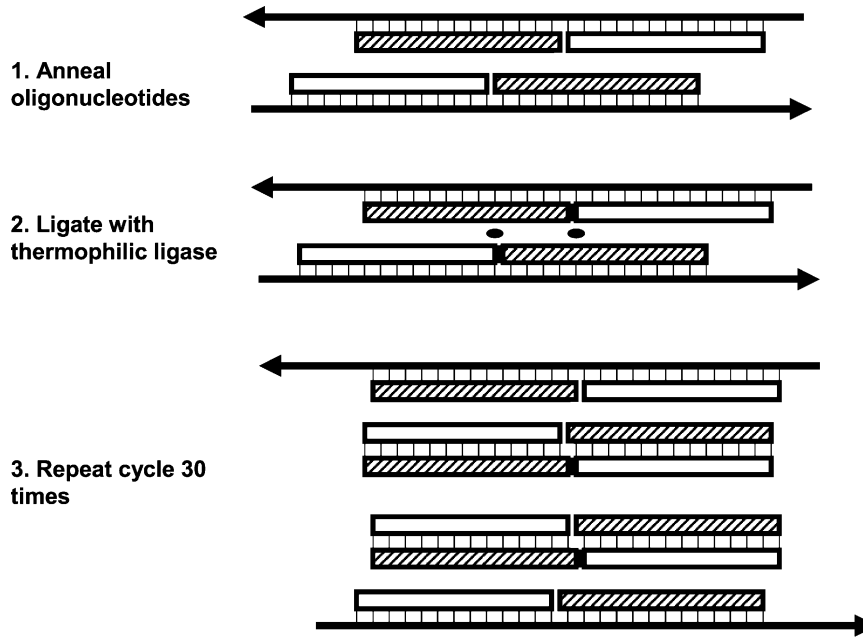

FIGURE 13.6. Process of ligase chain reaction (LCR). In LCR, two pairs of oligonucleotide probes that are complementary to the entire target sequence hybridize to the denatured DNA strands, such that the 3′ end of the first probe is immediately adjacent to the 5′ end of the second probe. A thermostable DNA ligase covalently links the two probes together, provided that the nucleotides at the junction are perfectly base-paired to the target strand. The newly ligated probes can then serve as templates for subsequent cycles, leading to exponential amplification of the DNA target.

amenable to automation (Landegren et al., 1988). One of the advantages of LCR is that ligation cannot occur unless both probes perfectly hybridize to the target and no gap between the 5′ end of one probe and 3′ end of the other probe. Therefore, it offers better allele specificity for genotyping point mutations and single nucleotide polymorphisms (SNPs) (Tong et al., 1999).

LCR has been employed for the detection of many microorganisms, such as HIV (de Mendoza et al., 2002), HBV (Osiowy, 2002), *Mycobacterium tuberculosis* (Lumb et al., 1999; Rajo et al., 2002), *Chlamydia trachomatis*, and *Neisseria gonorrhoeae*, in clinical specimens. The LCR assay kits (LCx) for *C. trachomatis* and *N. gonorrhoeae* is marketed by Abbott Laboratories (Abbott Park, IL, USA). The lower detection limit of the assay for *C. trachomatis* was revealed to be 32 EB/mL of urine (Blocker et al., 2002). The sensitivity conferred by LCR assay for *C. trachomatis* in first-void urine sample is found to be 10–15% higher than that of urethral or endocervical culture and 15–35% higher compared with non-culture assays done on urethral or cervical secretions. The overall specificity of LCR assay is typically over 99% (Lee et al., 1995; Schachter et al., 1995; Ridgway

et al.,1996). However, significant reproducibility variations between batches often occur during routine use of the LCx assay for *C. trachomatis* and *N. gonorrhoeae* (Gronowski et al., 2000). These problems can go undetected by the quality-control procedures outlined in the manufacturer's package insert.

Cycling Probe Technology

Cycling probe technology (CPT) is an isothermal probe amplification method (Bekkaoui et al., 1996) (Fig. 13.7). The probe is a single-stranded oligonucleotide, approximately 25–30 bases in length, containing a short run of four to six ribonucleotides flanked by deoxynucleotides (i.e., chimeric DNA-RNA-DNA). The CPT reaction is carried out at a single elevated temperature (55–65°C) in the presence of thermostable RNase H, an enzyme that degrades RNA portion of the probe–target hybrid. The DNA portions of the probe have lower thermal stability (melting temperature) than that of the intact probe. At the reaction temperature, the probe fragments dissociate from the target sequence, leaving the target free to hybridize to another probe molecule. The cleaved products can be observed using a variety of methods, most commonly by gel electrophoresis. The assay is a linear reaction with analytical sensitivity of 6×10^5 copies/reaction (Modrusan et al., 1998). Although the scale of amplification is limited, this assay does provide an easy means of quantitating target DNA with the aid of fluorescence labeling.

CPT assay in combination with a lateral-flow strip was used to detect the *mecA* gene from methicillin-resistant *S. aureus* (MRSA) in cultures (Fong et al., 2000).

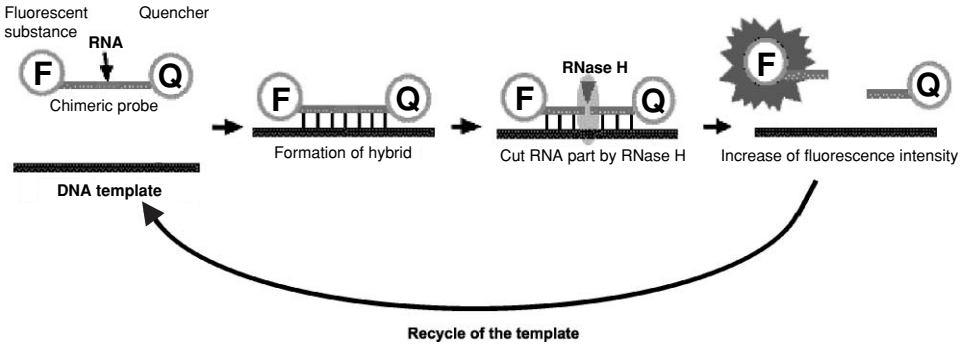

FIGURE 13.7. Cycling probe technology. A sequence-specific single-stranded probe (approx. 25–30 nucleotides) contains an internal stretch of 4–6 ribonucleotides (RNA) flanked by deoxyribonucleotides (DNA). The probe is labeled with a fluorophore and a quencher, which hybridizes to the target sequence. Thermostable RNase H binds to the RNA/DNA duplex region and cleaves the RNA segment. Because thermal stability of the resulting cleaved products is lower than the intact probe, the products dissociate from the target sequence, and the target sequence then becomes available to hybridize with another intact CPT probe. The cleaved probe emits fluorescence and is detected by a fluorometer.

The *mecA* probe was labeled with fluorescein at the 5′ terminus and biotin at the 3′ terminus. The nitrocellulose was impregnated with streptavidin and immunoglobulin G antibody. In the absence of the *mecA* gene, the uncut probe is bound to an antifluorescein-gold conjugate and subsequently captured by streptavidin to form a test line. In the presence of the *mecA* gene, the probe is cut and no test line is formed on the strip. A screen of 324 *S. aureus* clinical isolates by CPT-strip assay revealed a 99.4% sensitivity and a 100% specificity compared with the results of PCR for the detection of the *mecA* gene. The assay takes 1.5 h, starting from a primary culture to the time of detection of the *mecA* gene in *S. aureus* isolates.

Summary and Future Direction

In the past decade, probe amplification technologies have advanced significantly, from the initial description of Q-beta replicase amplification in 1986 (Chu et al., 1986) to the most recently introduced RAM (Zhang et al., 1998). It is expected that more probe amplification methods will be invented in the next 10 years, and the applications of the current probe amplification methods will become more diversified. Homogeneous and real-time monitoring of amplification will be devised to probe amplification technologies to reduce detection time and improve quantification capability of the assay. Additional technologies will be developed to be used for the detection of RNA, DNA, and protein (antigen/antibody) on a single platform, which will further enhance the detection sensitivity and specificity. Finally, the applications of these technologies will become broader as the fields of genomics, proteomics, and pharmacogenomics advance. Therefore, a technology that offers *in situ* detection and amplification, microarray, immunoassay, real-time monitoring, whole-genome amplification, and SNP detection will be more favorable. However, no single technology can meet all of these requirements, and possible combination of these technologies may be the answer. Also, PCR, the dominant amplification technology, cannot fulfill all these needs, and ample room is available for probe amplification technology to grow.

On the other hand, the instrumentation for probe amplification will change significantly in the next 10 years. Fluorescence-based real-time detection instrument will be widely used in the diagnostic laboratory, which will certainly improve throughput. Miniaturized microfluidic assay format will soon be available in the clinical laboratory, which will significantly reduce sample volume. Automation and miniaturization of the instrument will make molecular diagnosis at a doctor's office and at the bedside possible. It is expected that the array-based assay and instrument will be significantly improved, and the cost will be reduced to an affordable level. Given the advantages of probe amplification (isothermal, multiplex, on-chip amplification, etc.), probe-based amplification could be easily adapted in these formats and will become the dominant technologies in clinical diagnostic applications.

However, most described probe amplification technologies are still at the early stage of development. Most publications only demonstrated the feasibility in

clinical diagnosis, and their clinical performance has not yet been demonstrated in large clinical trials. It is anticipated that some of these technologies may not meet the clinical diagnostic requirements and will consequently be lost in market competition. For example, Q-beta replicase technology did not reach the clinical laboratory even after an initial favorable clinical trial, and the LCx assay (LCR technology) for *Chlamydia* was voluntarily withdrawn by Abbott in 2003 due to significant reproducibility problems (Gronowski et al., 2000). Therefore, it is expected that more changes (exciting or disappointing) will happen in the field of probe-based amplification technologies in the next 10 years.

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14
Signal Amplification Techniques: bDNA, Hybrid Capture

YUN F. (WAYNE) WANG

Introduction

Several molecular technologies are designed to avoid target amplification so to minimize the possibility of contamination by target amplification products. One of the alternatives to enzymatic amplification of target nucleic acid such as polymerase chain reaction (PCR) is to increase or amplify the signal generated from the probe molecule hybridized to the target nucleic acid sequence, which is referred to as signal amplification. Commonly used signal amplification technologies include branched DNA (bDNA) and hybrid capture (HC) assays. The bDNA method was initially developed by Chiron (Emeryville, CA, USA) and marketed by Bayer Diagnostics (Emeryville, CA, USA), and the hybrid capture method was developed and marketed by Digene Corporation (Gaithersburg, MD, USA).

Signal amplification methods including both bDNA and HC DNA technologies do not rely on enzymes for the amplification and also meet the challenges for better molecular assay other than by target amplification: specific detection, dynamic range, ease-of-use, standardization, and reproducibility. Both methods for certain assays have been used in clinical laboratories.

Principles and Characteristics of Techniques

Branched DNA Technology

bDNA, in contrast with PCR (which amplifies a portion of the gene sequence), is a signal amplification technology that detects the presence of specific nucleic acids by measuring the signal generated by specific hybridization of many branched, labeled DNA probes on an immobilized target nucleic acid. Signal amplification is achieved by sequential (or simultaneous) hybridization of synthetic oligonucleotides, assembling a branched complex structure on the immobilized target nucleic acid. In general, one end of bDNA binds to a specific target and the other end has many branches of DNA. The branches amplify detection signals. Each target molecule will have several hundred labels on it. The final detection step does use

alkaline phosphatase (AP) to generate chemiluminescence. The amplified signal on the target molecules is related to the number of target molecules. The signal amplification is linear. Thus, the standard curve in each assay allows calculation of the number of targets in the samples and therefore bDNA is a quantitative technology and is used in the determination of viral load (Cao, 1995; Kern, 1996; Collins, 1997).

In general, there are seven steps of the assay which can be completed in 2 days. The first two steps can be done on day 1 and the rest on day 2. The first step in the assay is to release the nucleic acid from the target, such as virus, and is called the target nucleic acid release. The release occurs through viral lysis buffer to disrupt the virus, degrade nucleases (RNases), and release viral target RNA or DNA (DNA targets require additional denaturation to yield single-stranded target). A detergent such as proteinase K disrupts the viral coat to release the nucleic acid from the virus and also inactivates RNases.

The released nucleic acid is captured to a solid surface by multiple capture probes either in a microwell plate or in solution. The second step is target probe hybridization and capture, or so-called target capture. Capture probes hybridize target nucleic acid to the capture probe–coated microwell, and target probes hybridize to the target. The oligonucleotides called capture probes (in solution) hybridize to multiple sites on the target viral RNA as well as the capture probes that are coated on the microwells. The target probes also hybridize to multiple sites on the target. They will hybridize the next oligonucleotide added to the samples. The target viral RNA is thus "captured" to the microwell through the hybridization of the two types of capture probes in solution and on the microwell.

The next step, called preamplification probe hybridization (to target probes and thus to the microwell), can be performed on the second day. After the overnight incubation, the microwells are washed to remove unbound capture probes, target probes, lysis reagent, and cellular debris. Preamplifier probes are added to the microwells. Each preamplifier probe hybridizes to two adjacent target probes in a cruciform configuration or cruciform design. One leader binds target probes at 5′ end. There are 14 preamplifier sites with 7 linker sites for ligation. After preamplification is the amplifier probe hybridization. Amplifier probes are added to the microwells. They then hybridize and bind to preamplifiers. There are multiple amplifier-binding sites present on each preamplifier for the amplifier probe to hybridize to the preamplifier and form a bDNA complex or so-called signal amplification multimer for amplification. Thus, the amplifier molecule is the key to bDNA technology (Horn and Ureda, 1989).

The next step is the alkaline phosphatase (AP) labeled probe hybridization. AP-conjugated probes called label probes are added to the microwells and hybridize to immobilized amplifier complex. There are multiple label probe-binding sites present on each amplifier. Dioxetane substrate (Lumi-Phos Plus, Lumigen, Detroit, MI, USA) is added to the microwell for signal generation. The dioxetane substrate chemically reacts with the alkaline phosphatase from the label probes, which excites an electron, resulting in emission of a photon of light producing chemiluminescence (Beck, 1990).

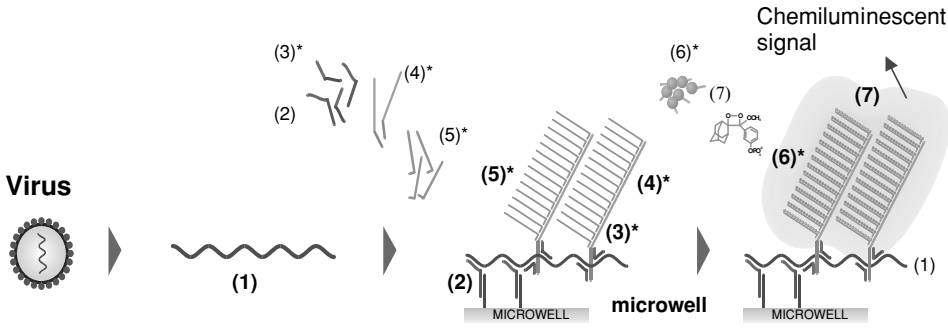

FIGURE 14.1. Branched DNA (bDNA) technology. Nucleic acid target (1) release by lysis buffer to disrupt virus and degrade RNases. Capture probes (2) on microwell and in solution hybridize to target nucleic acid. Preamplifiers probes (4) hybridize to target probes (3) Amplifier probe (5) hybridization. Alkaline phosphatase (AP) conjugated label probe (6) hybridizes to amplifier. Dioxetane substrate (7) reaction with AP generates signal of chemiluminescence that can be read by System 340 Analyzer. * Note: Interaction between oligonucleotides is minimized by incorporating non-natural bases (Iso5MeC and IsoG) in the sequences of the target probes (3), preamplifiers (4), amplifiers (5), and alkaline phosphatase conjugated label probes (6).

The final step is the amplified signal generated from the chemiluminescence being detected and read by a photomultiplier tube in the System 340 analyzer. The amount of light produced by dioxetane substrate will be measured in step seven and is proportional to the initial target RNA concentration. Results are recorded as relative light units (RLUs) by the analyzer. The data management software takes standards of known concentrations assayed in the same run and creates a standard curve (Collins et al., 1995). The concentration of viral material in specimens is determined by comparing the RLU of each sample with this standard curve. Photomultiplier tube calibration can be performed before and after reading the wells.

The first generation of bDNA was first introduced in the early 1990s. The current bDNA 3.0 version is modified with the following probe design features to increase sensitivity. Two of the probe design features for the bDNA assay are cruciform target probes or binding design and Iso5MeC and IsoG. Two target probes are required to stabilize binding of the preamplifier probe (Fig. 14.1). This reduces background by minimizing hybridization of amplification molecules to nonspecifically bound target probes. Isocytosine (Iso5MeC) and isoguanosine (IsoG) are isomers of cytosine (C) and guanosine (G), which are non-natural bases. Iso5MeC and IsoG participate in Watson–Crick base pairing with each other but have unstable interactions with DNA sequences containing natural bases (C and G). Approximately

every fourth nucleotide in selected probes is Iso5MeC or IsoG. Use of a six-base code allows the design of amplification sequences that do not interact with target sequences or other bDNA components (Collins, 1997).

Interaction between oligonucleotides is minimized by incorporating non-natural bases (Iso5MeC and IsoG) in the sequences of the amplification complex (target probes, preamplifiers, amplifiers, and label probes). The non-natural bases do not hybridize effectively with their natural bases (Switzer, 1993; Collins, 1997). Thus, the capture probes (on microwell or in solution) do not hybridize with the amplification complex, therefore reducing nonspecific probe interactions. Using probes made with IsoC and IsoG increases specificity and sensitivity because higher concentrations of probes can be employed. With the amplification complex (preamplifier, amplifier, and AP-conjugated label probes), potential hybridization to nontarget nucleic acids are reduced, signal to noise ratio is increased 30 times (Collins, 1997), and thus signal amplification is improved with equivalent sensitivity to some target amplification technologies like PCR.

Hybrid Capture Technology

The HC system is a signal amplification assay using antibody capture and chemiluminescent signal detection. The HC technology combines nucleic acid technology such as RNA probes for RNA:DNA hybridization with the simplicity of an immunoassay using monoclonal antibody RNA:DNA hybrids for rapid gene detection. HC technology detects nucleic acid targets directly and uses signal amplification to provide sensitivity that is comparable with target amplification methods.

The hybrid capture assay 1 (HC1) was first introduced by Digene in 1995 (Clavel, 1999). Since then, the second generation of hybrid capture assay (HC2) uses a microtiter plate instead of tubes and has been approved by the FDA for the detection of high-risk HPV types on thin preparation, liquid-based cervical specimens.

The entire process takes approximately $3\frac{1}{2}$ h. Same-day results can be achieved in a chemiluminescent microplate format. The HC technology also uses DNA probes to detect RNA targets. Minimal specimen preparation facilitates processing samples quickly and efficiently. The following is an example of the principle of hybrid capture technology using an RNA probe to detect DNA targets in five sequential steps (Fig. 14.2).

The first step is to release DNA from cells and the denaturation of nucleic acids. An alkali such as sodium hydroxide is added to the specimen to disrupt the virus or bacteria, release target DNA, and make the target DNA molecules single-stranded and accessible for hybridization.

The second step is the hybridization of target DNA with RNA probe. The specimen is transferred to a container, and a single-stranded RNA probe that is complementary to the target DNA sequence is added to the solution and heated. The RNA probe finds its complementary DNA target sequence and attaches or binds (hybridizes) to it, forming a double-stranded RNA:DNA hybrid complex.

The capture of RNA:DNA hybrids onto a solid phase is the third step. The sample is then transferred to a second container that has been coated with antibodies

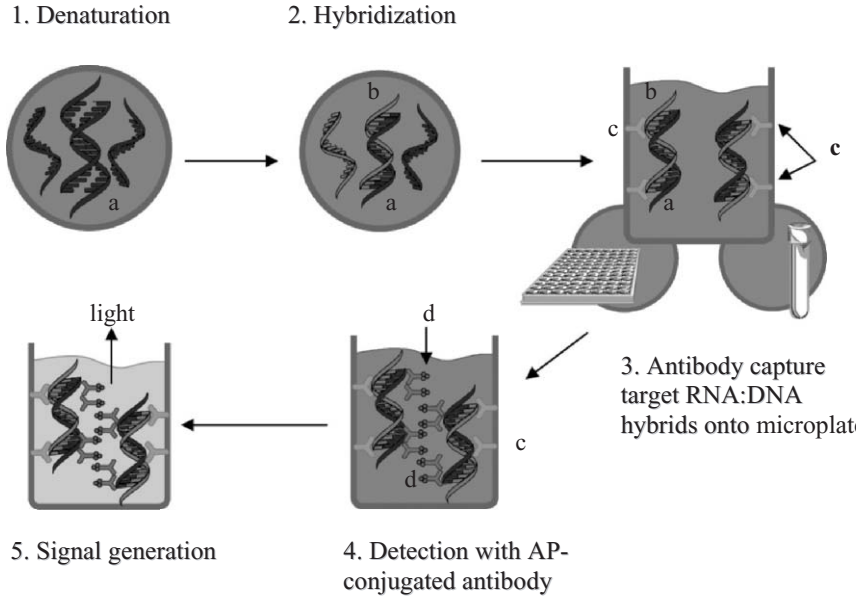

FIGURE 14.2. Hybrid capture 2 (HC2) technology. (1) Release target DNA from cells and denature nucleic acids (a). (2) Hybridize RNA probe (b) with target DNA. (3) Capture RNA:DNA hybrids by anti-RNA:DNA hybrid antibody (c) onto a solid phase in microplate format. (4) React captured hybrids with multiple alkaline phosphatase (AP) conjugated anti-RNA:DNA hybrid antibody (d). (5) Detect amplified chemiluminescent signal.

(i.e., goat anti-RNA:DNA hybrid antibody) that specifically recognize and bind to RNA:DNA hybrids. During this process, multiple RNA:DNA hybrids are captured or bound onto the microplate surface by the coated antibodies specific for RNA:DNA hybrids.

The next step is the reaction of captured hybrids with multiple antibody conjugates and label for detection. A second antibody is added to the solution, which recognizes and binds to the RNA:DNA hybrids that are captured onto the surface of the container. This anti-RNA:DNA antibody is conjugated with alkaline phosphatase (AP), an enzyme, that, in the presence of chemiluminescent substrate [i.e., CDP-Star Emerald II by Applied BioSystem (Forest City, CA, USA) or LumiPhos 530 by Lumigen (Detroit, MI, USA)], produces light and acts as a signal amplification. Several AP molecules are conjugated to each antibody, and multiple conjugated antibodies bind to each captured hybrid, which in turn results in substantial (about 3000-fold) signal amplification.

The final step is detection of amplified chemiluminescent signal. The container is washed to remove all of the unbound or free components while the RNA:DNA hybrids and the labeled antibody remain bound to the container. Chemiluminescent dioxetane substrate is added, which is cleaved by the bound alkaline phosphatase to produce light (Beck, 1990), and the light is emitted, which is detected and measured

TABLE 14.1. Comparison of bDNA and HC2 technologies.

	Test	
	Branched DNA (bDNA)	Hybrid capture II (HC2)
Manufacturer	Bayer	Digene
Signal amplification	Many probes including capture, target, preamplifier, amplifier probes	Anti-RNA:DNA hybrid antibody
Detection	AP-conjugated label probe	AP-conjugated anti-RNA:DNA hybrid antibody
Chemiluminescent substrate	Dioxetane Lumin-Phos Plus, Lumi Phos 530	Dioxetane Lumi Phos 530 CMV; CDP-Star Emerald II
Common features	One-room technology No enzymes involved for target amplification, less contamination concern No DNA or RNA extraction is needed Microplate, immunoassay-like format	One-room technology No enzymes involved for target amplification, less contamination concern No DNA or RNA extraction is needed Microplate, immunoassay-like format
Semiautomation application	System 340, <168 samples in 2 days HIV-1, 75 (or 50) to 5×10^5 copies/mL HCV, 615 (or 520) to 769×10^6 IU/mL HBV, 2000 to 1×10^8 copies/mL	Rapid capture system, <352 samples (4 plates) in 8 h HPV, qualitative CMV, qualitative *C. trachomatis*, qualitative *N. gonorrhoeae*, qualitative HBV, 1.42×10^5 to 1.7×10^9 copies/mL

as relative light units (RLUs) on a luminometer (Microplate Luminometer DML 2000 Instrument, Digene, Gaithersburg, MD). The intensity of the light emitted can detect target DNA in the specimen.

Contrast of These Techniques

As shown in Table 14.1, both technologies do not use enzymes for target amplification, thus there is less concern of contamination and enzyme inhibition. Both technologies use the dioxetane chemiluminescent method for detection. No DNA or RNA extraction is needed in both technologies. Microwell plate immunoassay-like format can be performed in one room and by semiautomated systems, making both technologies easy to be implemented in the clinical laboratory setting.

The difference is that many synthetic oligonucleotides (probes) are used in bDNA for signal amplification and two anti-RNA:DNA hybrid antibodies are used in HC2 for signal amplification. The bDNA technology is mainly used for quantitative analysis of viruses such as human immunodeficiency virus (HIV) and hepatitis C virus (HCV). The HC2 technology is mainly used for qualitative detection of viral or bacterial infection, with the exception of hepatitis B virus (HBV) and cytomegalovirus (CMV).

Application of the Techniques in Diagnostic Microbiology

bDNA Assays

The bDNA assays are quantitative signal amplification methods for the measurement of viral load and are commercially available from Bayer (Versant HIV RNA 3.0 Assay, HCV RNA 3.0 Assay, and HBV DNA Assay). Target region is the polymerase (*pol*) gene of the HIV-1 viral RNA, the 5′-untranslated (UTR) and core regions of HCV, and the genome of HBV.

The first- and second-generation bDNA assays lacked sensitivity compared with the target amplifications systems. The changes incorporated into the third-generation (3.0 version) assays have increased the sample volume and the signal-to-noise ratio to such a high level that the analytical sensitivity of system bDNA approaches that of PCR. Nonspecific hybridization can be further reduced by finding more effective blockers for the solid phase or by redesigning the amplifier molecule or the solid phase itself (for reviews, see Kern, 1996; Wilber, 1997; Nolte, 1998).

The Versant HIV-1 RNA 3.0 Assay is a sandwich nucleic acid hybridization procedure for the quantitation of human immunodeficiency virus type 1 (HIV-1) RNA in plasma over the range 75–500,000 HIV-1 RNA copies/mL. Lower limit of 50 copies/mL and dynamic range can reduce repeat testing. In addition, broad plasma samples containing Group M subtypes A–G has been validated for quantitation by the assay (Cao, 1995; Pachl, 1995; Collins, 1997; Erice, 2000; Murphy, 2000; Elbeik, 2000, 2002). The bDNA shows the least variation [the mean coefficient of variation (CV) was 12%] in HIV-1 RNA for the values within dynamic ranges of the assays in repeated study of assays including other target amplification methods (Lin, 1998).

The test's broad dynamic range eliminates the need for reflex testing and does not require viral RNA extraction steps. HIV is denser than HCV and HBV and therefore can be concentrated by centrifugation. Beads are added to the sample before centrifugation to make the HIV pellet more visible. In addition, a set of target probes hybridizes to both the viral RNA and the preamplifier probes. The capture probes, composed of 17 individual capture extenders, and the target probes, composed of 81 individual target extenders, bind to different regions of viral RNA. A standard curve is defined by light emission by incubating the complex with chemiluminescent substrate from standards containing known concentration of beta-propiolactone (BPL)-treated virus. The high level of precision afforded by bDNA allows threefold changes in viral load to be distinguished.

The HCV RNA 3.0 Assay is for the quantitation of human hepatitis C viral RNA (HCV RNA) in the serum or plasma (EDTA and ACD) of HCV-infected individuals. It is the only FDA-approved quantitative viral load assay. The assay measures HCV RNA levels at baseline and during therapy and is useful in predicting non-sustained response to HCV therapy. HCV RNA 3.0 Assay (bDNA) quantitates all HCV RNA genotypes (genotypes 1–6). Like the HIV test, the broad dynamic range (615 to 7,690,000 IU/mL) of HCV dramatically reduces repeat testing; that is the need to dilute and re-run test due to out-of-range samples (Jacob, 1997; Beld, 2002; Germer, 2002; Konnick, 2002; Veillon, 2003; Elbeik, 2004).

Because HBV is a double-stranded DNA virus, it must be denatured (single-stranded) prior to hybridization of the DNA to the capture probes. The HBV DNA Assay is designed to quantitate all HBV genotypes. Six different HBV genotypes (A through F) are prevalent in different parts of the world. With global migration, different forms of HBV disease appear in different regions. HBV viral loads rarely exceed the upper limit of the assay due to the broad dynamic range (2000 to 1.0×10^8 copies/mL) of HBV assay, thus reducing repeat testing. In addition, it eliminates tedious nucleic acid extraction or virus concentration steps (required with PCR assays) that increase risk of cross-contamination. (Hendricks, 1995; Yao, 2004).

Assays are run in a 96-microwell format using the System 340 Analyzer (Bayer Diagnostic, Tarrytown, NY), a semiautomated instrument that performs incubations, washes, and detection. It has two 96-well microtiter plate capability and can have 12–168 samples per run. The combination of bDNA technology and the System 340 can provide better precision, accuracy, and tolerance limit.

Hybrid Capture Technology

The hybrid capture 2 (HC2) technology is the platform for signal-amplified, nucleic acid tests (for review, see Lorincz and Anthony, 2001). HC2 systems are available to detect human papillomavirus (HPV), cytomegalovirus (CMV), *Chlamydia trachomatis* (CT), *Neisseria gonorrhoeae* (GC), and hepatitis B virus (HBV). An assay for herpes simplex virus (HSV) is in development.

Cervical cancer is one of the few malignancies for which the cause has been identified: the human papillomavirus, a small DNA tumor virus that belongs to the family Papovaviridae and is sexually transmitted (Schiffman, 2000; Munoz, 2003). There are more than 100 types of HPV. Low-risk types of HPV may cause genital warts. High-risk types have been shown to cause most cases of cervical cancer. The HC2 HPV test uses two RNA probe cocktails to differentiate between carcinogenic and low-risk HPV types. Thirteen types are implicated in the pathogenesis of High-Grade squamous intraepithelial lesion (HSIL) and invasive cancer: 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68. Five probes detect low-risk viral types associated with Low-Grade squamous intraepithelial lesion (LSIL): 6, 11, 42, 43, and 44. This test is used to screen patients with ASCUS (atypical squamous cells of undermined significance). Pap smear results determine the need for referral to colposcopy. HC2 HR HPV DNA test was initially approved for follow-up evaluation in women with inconclusive Pap-test results. It has a proven 99% negative predictive value (NPV) (Manos, 1999; Solomon, 2001). The negative predictive value of HPV DNA testing would be of particular significance in excluding HPV-associated dysplasias in postmenopausal women diagnosed with ASCUS (Manos, 1999).

The test was approved in 2003 by the U.S. Food and Drug Administration (FDA) for cervical cancer screening, in conjunction with a Pap test, in women age 30 and older. The HC2 HR HPV DNA test (marketed as the DNA*with*PAP) became Digene's flagship product. Specimens containing the target DNA hybridize with a specific HPV RNA probe cocktail. An RLU measurement equal to or greater than the cutoff value (CO) indicates the presence of HR HPV DNA sequences in the

specimen. A study (Kulmala, 2004) comparing performance of the hybrid capture 2 assay and polymerase chain reaction (PCR) in screening HPV did not find much difference between the two. The results of PCR and the HC2 assay were concordant for 85% of samples, resulting in substantial reproducibility. Hybrid capture 2 has been shown to have similar analytic sensitivity to some PCR methods for HPV DNA detection (Clavel, 1998; Peyton, 1998).

Rapidly emerging as a standard of practice in cervical cancer screening, the HPV test helps clinical diagnosis of women who are most at risk of having or developing cervical cancer. In addition, one sample collected and rinsed in Cytyc's ThinPrep Pap Test vial Cytyo Corp., Marlborough, MA can be used for both the Pap test and the HPV test. Another liquid-based method, AutoCyte PREP TriPath Imaging, Inc., Burlington, NC, is expected to be used for the same purpose. HPV, chlamydia, and gonorrhea testing can be performed using one sample. Cervical specimens are collected with a broom collection device and rinsed in the ThinPrep System PreservCyt solution with the Digene Cervical Sampler. The Digene Sample Conversion Kit is used to allow the HPV DNA test to be performed on the same specimen that the ThinPrep Pap Test is performed on. In addition, cervical biopsies are collected in Digene Specimen Transport Medium.

Chlamydia trachomatis (CT) and *Neisseria gonorrhoeae* (GC) are the most common bacterial infections of the lower genital tract. The CT/GC Probe Cocktail contains a probe mixture specifically chosen to eliminate or minimize cross-reactivity with DNA sequences from human cells, other bacterial species, *Chlamydia* species other than CT, or GC. The CT/GC Probe Cocktail supplied with the hc2 CT/GC DNA test is complementary to approximately 4% of the CT genomic DNA (1×106 bp) and 7500 bp or 100% of the cryptic plasmid; and 0.5% of the GC genomic DNA. A specimen positive by the HC2 CT/GC DNA test must be tested by HC2 CT-ID DNA test or HC2 GC-ID DNA test or another method to verify organism detection. The Digene CT/GC, CT-ID, and GC-ID tests are designed for the detection of CT and GC from cervical specimens collected using the Digene Cervical Sampler or from urine specimens processed using the Digene Urine Prep Kit for male specimens. The Digene Urine Preparation Kit for male specimens is required to process male urine specimens for use with any of the Digene CT/GC tests (Girdner, 1999; Schachter, 1999; Dawin, 2002). It is recommended that positive results be confirmed by another method if the likelihood of *N. gonorrhoeae* or *C. trachomatis* infection is uncertain or questioned. Analytical sensitivity of the HC2 CT/GC DNA test to detect *Chlamydia* ranges from 50 to 2500 CFUs/assay (1000 to 50,000 CFUs/mL). The lower limit of detection for GC isolates ranges from 25 to 5000 CFUs/assay (500 to 100,000 CFUs/mL).

HC2 CMV DNA test is the first molecular diagnostic test to be FDA cleared for the qualitative detection of human cytomegalovirus DNA in peripheral white blood cells isolated from whole blood (Mazzulli, 1999). Active CMV infection in immunosuppressed and immunocompromised patients, such as solid organ transplant, bone marrow transplant, and HIV-positive/AIDS patients, can be detected more accurately. Analytical studies using cloned HPV plasmid DNA demonstrated that the assay using high-risk probe could detect these types at levels ranging from 0.62 pg/mL to 1.39 pg/mL. Analytic sensitivity for CMV is

0.48 pg/mL. The RNA probe for CMV is about 40,000 bp, about 17% of the CMV genome.

The HBV DNA test is a signal amplification test to quantify hepatitis B viral DNA in human serum. The test detects HBV *ad* and *ay* subtypes. Standard test dynamic range is 1.42×10^5 to 1.7×10^9 copies/mL (0.5 to 6000 pg/mL) using a sample volume of 30 mL. Ultrasensitive dynamic range is 4.7×10^3 to 5.6×10^7 copies/mL (0.017 to 200 pg/mL) using a sample volume of 1 mL, which is concentrated by centrifugation. The Digene HC2 assay and the PCR assay had similar intra-assay and inter-assay variabilities. For the patients with HBV DNA levels detectable by the HC2 assay, the HBV DNA levels obtained by the HC2 assay and by the PCR assay showed an excellent correlation. The PCR assay was more sensitive than the HC2 assay and more suitable for monitoring low levels of HBV viremia (Yuan, 2004; Konnick, 2005).

Rapid capture system (RCS), a semiautomated pipetting and dilution system, provides high-volume labs with the ability to run high-risk HPV, CT and GC, and HBV HC2 tests. Automation of RCS does not include sample denaturation, as well as the chemiluminescent signal detection and result reporting that are performed using the microplate luminometer system (DML 2000 Instrument, Digene, Gaithersburg, MD). This system handles up to 352 specimens (4 microplates) in 8 h. Thus, the HC2 system offers multiple testing using a single platform. A single sample can be tested for HPV, CT and GC, and, in the future, HIV-1 and HSV, a test currently under development (Cullen, 1997). This system provides a comprehensive risk-screening approach based on one patient visit.

Future Direction and Summary

bDNA Technology

Automation of specimen preparation (Versant 440) is expected to provide rapid, cost-effective, and consistent results. *In situ* hybridization (ISH) allows for the histologic and cytologic localization of DNA and RNA targets. Current approaches involving signal amplification (branched DNA amplification). Application of some of these techniques has extended the utility of ISH in diagnostic pathology and in research because of the ability to detect targets with low copy numbers of DNA and RNA (Qian, 2003). A bDNA ISH method for detection of DNA and mRNA in whole cells was developed. Using normal and HPV-infected cervical biopsy specimens, cell type–specific distribution of HPV DNA and mRNA was analyzed by bDNA-ISH, which may improve diagnosis of cancers and infectious agents (Player, 2001; Kennedy, 2002). The modified bDNA technology can also be used for multiplexed, particle-based detection of DNA using flow cytometry with bDNA dendrimers for signal amplification (Lowe, 2004).

HC Technology

Signal amplification system (Digene's Sharp Detection) provides rapid nonisotopic detection of PCR products in a convenient microplate format. The signal

amplification technology has made HPV DNA testing possible to reduce the incidence of cervical carcinoma substantially, especially in patients diagnosed with ambiguous low-grade lesions, such as ASCUS, or on Pap smears. Reflex HPV DNA testing of thin-layer preparations diagnosed as ASCUS will play a major role in the management of abnormal cervical cytology. Other applications, such as primary screening, in the future may play a substantial role in cervical cancer screening. In addition, a new way of processing liquid-based cervical cytology specimens by filtration-based processing method (NPM) exists for HPV DNA testing by HC2 (Castle, 2005). NPM reduces specimen handling and decreases total testing time by approximately 33% without significant losses in HC2 test performance.

The hybrid capture 3 (HC3) is being developed and compared between prototype HC3 and HC2 HPV DNA assays for detection of high-grade cervical intraepithelial neoplasia and cancer (Lorincz and Anthony, 2001; Castle, 2003). HC3, like HC2 test, relies on the formation of target DNA:RNA probe hybrid and the chemiluminescent detection of these hybrids with an AP-conjugated antibody to DNA:RNA complexes with dioxetane substrate in a 96-well immunosorbent assay format. A primary technical distinction between HC3 and HC2 is that HC3 employs a biotinylated DNA specific for selected HPV DNA sequences for the capture of the DNA:RNA complexes on streptavidin-coated wells, whereas HC2 uses wells coated with polyclonal antibody against DNA:RNA hybrid for hybrid capture (Lorincz and Anthony, 2001). The use of capture oligonucleotide instead of an immobilized antibody also diminishes the possibility of nonspecific RNA:DNA hybrids, present as the result of improperly alkali-denatured specimens, from binding to the microplate well, and consequently may reduce false positivity for HC3 compared with HC2 (Peyton, 1998; Castle, 2002). HC3 is expected to provide a highly selective and sensitive method for identifying closely related nucleic acid targets or mutations. In general, these potential applications will require more in-depth study and research and will ultimately have to stand the test of time.

Summary

Signal amplification technology has unique features and even some advantages over target amplification systems for direct detection or quantification of target nucleic acid sequences. Reliable detection is achieved without the need for dedicated or isolated lab space or concern regarding inhibition and contamination that could be found in target amplification assays. The bDNA and hybrid capture technologies provide uncomplicated assay procedures, high throughput, and reliable signal amplification tests for diagnosis of viral or bacterial infection in routine clinical laboratories.

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Detection and Characterization of Molecular Amplification Products: Agarose Gel Electrophoresis, Southern Blot Hybridization, Restriction Enzyme Digest Analysis, and Enzyme-Linked Immunoassay

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Introduction

The need for accurate detection and characterization of nucleic acid targets has prompted the development of a range of methodologies. Highly complex and often expensive techniques, such as oligonucleotide arrays, are being used increasingly. Such methods can be extremely valuable, but issues such as cost, the need for specialized equipment, and a high level of expertise for both the technical and analytical aspects of implementation may limit their use in a clinical setting (Chee et al., 1996; Cheung et al., 1999). Although such systems are certainly effective for gathering large amounts of information and can be extremely useful in the research arena (Khan et al., 1999), their use may be unnecessary if only single PCR target detection is required. The use of real-time molecular product detection methods, largely relying on the principle of fluorescent resonance energy transfer (Chen et al., 1997) (FRET), has also become quite commonplace. These methods are useful for high-throughput diagnostic assays and are amenable to automation.

Some may think that these recently developed techniques have completely supplanted more traditional procedures, such as agarose gel electrophoresis, Southern blot, and restriction fragment length polymorphism (RFLP) analysis and even somewhat newer techniques, such as enzyme immunoassay (EIA). However, such methods remain useful, often critical tools for research and assay development, and sometimes still have advantages for clinical testing. All are easy to perform, require relatively inexpensive equipment, and can be mastered in a short period of time. Few methods of detecting a PCR product are as easy and inexpensive as pouring an agarose gel, electrophoresing the PCR product through the gel, and visualizing the PCR product with a UV light source. The advantages of such direct product visualization are irreplaceable in assay development and troubleshooting. In cases of frequent target variation, such methods may offer the only practical means of positive, reproducible detection and characterization. EIA-based methods remain

simple, inexpensive to develop and use, rely on commonly available equipment, and offer excellent analytical performance characteristics. This chapter will discuss the use of agarose gel electrophoresis, Southern blot hybridization, RFLP analysis, and PCR-EIA as methods for the detection and characterization of PCR products.

Agarose Gel Electrophoresis

Principles

Agarose is a polysaccharide composed of long chains of cross-linked galactopyranose residues (Sambrook et al. 2005) substituted with pyruvate, sulfate, and methyl esters. The pore size of agarose (determined by agarose concentration in the gel) is responsible for much of its DNA separation properties. DNA molecules migrate at a rate that is primarily size-dependent. The rate of such movement is inversely proportional to the $\log_{10}$ of the length of the DNA strand, such that smaller molecules of nucleic acid move more quickly than large ones. DNA molecules of approximately 20,000 bp are the largest molecules that can be resolved using continuous-field (electrical current) agarose gel electrophoresis. Larger DNA molecules require methods such as pulsed-field gel electrophoresis (Arshad et al., 1993; Finney, 2000; Sambrook et al., 2005). One factor that can slow DNA migration and hinder separation, particularly of larger DNA fragments, involves electroendosmosis (EEO). EEO is dependent on the number of sulfate and pyruvate residues present in a given agarose gel (Upcroft and Upcroft, 1993; Sambrook et al., 2005). It results from positive ions moving toward the cathode, pulling water molecules with them in opposition to the migration of negatively charged DNA toward the anode. The effects of EEO are seen mostly in resolution of fragments >10 kb.

The supplies and equipment needed to perform agarose gel electrophoresis make it one of the most readily performed, widely available, and inexpensive molecular methods. Horizontal slab gels, loading and running buffers are often prepared in-house but can be purchased from commercial suppliers, with precast gels available in a range of sizes, using various concentrations of agarose. Hardware required for agarose gel electrophoresis consists of an electrophoresis gel box and gel casting tray, gel combs (used to make loading wells or slots), a microwave oven or hot plate, and an electrophoresis power supply (Sambrook et al., 2005). UV light–transparent material should be used to make the casting tray so it can be placed directly on a UV transilluminator. A fitted cover on the gel box is necessary to prevent contact with running buffer during electrophoresis. Although gel boxes, casting trays, and combs are relatively simple to construct, commercially available hardware is widely available.

Technical Considerations: Gel Performance

Factors effecting gel performance and the ability to resolve DNA fragments include both characteristics of the gel itself (agarose concentration, class, and grade),

conditions under which the electrophoresis is run (voltage applied to the gel, loading and running buffers used, and duration of the electrophoretic run), and characteristics of the nucleic acid fragments being separated (quantity, size, and conformation) (Sambrook et al., 2005).

The concentration and type of agarose best used in a gel can be assessed based on DNA fragement sizes to be resolved and on the need for subsequent analysis, such as the need to recover nucleic acid from the gel after electrophoresis (Upcroft et al., 1993; Sambrook et al., 2005). Higher concentrations of agarose increase resistance to the movement of DNA molecules, slowing their migration. For this reason, the concentration of agarose used to separate shorter segments of DNA is typically higher than that used for larger pieces. Five percent agarose is used for the separation of the smallest linear molecules (5–100 base-pairs; bp), and 1% agarose is used for the separation of the largest molecules (300–5000 bp). Agarose is commercially available in many grades with the more expensive grades containing lower levels of contaminating polysaccharides, salts, and proteins, all potentially affecting gel performance. Depending on application, different preparations, such as standard (high melting point), low melting point (LMP) preparative grade for large fragments ($<65^{\circ}$C melting point, good for fragments >1000 bp), and LMP preparative grade for small fragments (10 to 1000 bp) can be obtained (Upcroft et al., 1993; Sambrook et al., 2005). The major use for LMP agaroses is for the recovery of nucleic acid after size separation by electrophoresis. In such cases, the DNA of interest can be removed for further analysis by remelting at a temperature that will not denature the nucleic acid.

The two most frequently used electrophoresis buffers (running buffers) for agarose gel electrophoresis are Tris-acetate with EDTA (TAE) and Tris-borate with EDTA (TBE) (Voytas, 2000). The pH of both buffers is greater than 7.0, meaning that the phosphate backbone of DNA has a net negative charge and migrates toward the anode during electrophoresis. TAE has less buffering capacity but greater ability to resolve high-molecular-weight DNA fragments. It is often used when DNA is to be isolated from the gel or for resolution of larger DNA fragments (>12 kb). The interaction of TBE with agarose results in a smaller apparent pore size producing better resolution of small DNA molecules (<1 kb) and reducing the tendency of DNA bands to broaden due to dispersion and diffusion.

Before adding DNA samples to a gel, loading buffer is added in order to increase the density of the sample so it sinks to the bottom of the well and to add color to the sample. In turn, this color serves as a marker to simplify the loading process and to allow the progress electrophoresis to be monitored, based on the movement of dye(s) through the gel (Sambrook et al., 2005). There are several different commonly used sample loading buffers, including bromophenol blue/xylene cyanol FF in glycerol, bromophenol blue/xylene cyanol FF in sucrose, bromophenol blue/xylene cyanol FF in Ficoll, bromophenol blue-orange G/xylene cyanoll FF in Ficoll, bromocresol green/xylene cyanol FF in NaOH and Ficoll, and bromophenol blue in sucrose. Selection of a loading buffer is largely related to personal preference. The two primary dyes used, xylene cyanol and bromphenol blue, migrate at different rates, the former comigrating with slower fragments

TABLE 15.1. DNA stains for use with agarose gels.

Stain	Visualized by UV light?	Sensitivity[a]	Comments
Ethidium bromide (Le Pecq et al., 1966, 1971; Singer et al., 1999)	Yes	3	Widely used
SYBR Green I (Singer et al., 1999; Cambrex Bio Science Rockland, 2005; Singer et al., 2005)	Yes	1	Expensive
GelStar (White et al., 1999)	Yes	2	
Methylene blue	No	5	Nontoxic, poor sensitivity
Silver stain	No	3	Relatively complicated

[a] Sensitivity: 1, most sensitive; 5, least sensitive.

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(approximately 5 kb) and the latter with faster fragments (approximately 0.5 kb). These can be used to monitor shorter and longer runs, respectively. However, it is important to remember that these dyes may interfere with the ability to see comigrating fragments (Voytas, 2000). As loading buffers are usually made up in 6X stock solutions, only a small amount needs to be added to each DNA sample.

The amount of DNA that can be loaded into the well of an agarose gel depends on well or slot volume, number, size, and the size distribution of DNA molecules (Voytas, 2000; Sambrook et al., 2005). The DNA capacity of an agarose gel decreases as the size of the DNA molecules increases. DNA migrates through the gel at a speed proportional to the applied voltage. This is a nonlinear relationship, however, with longer length nucleic acids affected more than smaller ones by increased voltage (making large fragment bands harder to separate at high voltages). This means that higher voltages are better suited to DNA molecules <1000 bp, whereas lower voltages are better suited to DNA molecules >1000 bp.

Technical Considerations: Target Detection

Detection of DNA in an agarose gel is typically accomplished through the use of stains (Table 15.1) or nucleic acid probes applied post-electrophoresis or incorporated into the gel before current is applied. Ethidium bromide (EtBr), the most commonly used agent, is an intercalating dye that binds to double-stranded DNA and gives a fluorescent emission (Le Pecq and Paoletti, 1966; Le Pecq, 1971). The fluorescence of ethidium bromide is enhanced 20- to 30-fold after binding to nucleic acid. UV light (300 nm), transmitted through the gel from below, is the most frequently used method for visualizing stained bands. Using EtBr with this lighting method, a band containing 1–5 ng of double-stranded DNA can be detected (Sharp et al., 1973; Voytas, 2000). EtBr can be added to the gel before electrophoresis or staining can be accomplished after the run is complete (Sambrook et al., 2005).

Importantly, EtBr added before electrophoresis can affect DNA fragment mobility, with stained DNA fragments moving approximately 15% slower compared with migration rates in an unstained gel. Post-electrophoresis gel staining should be considered if altering the rate of mobility is a concern. Pre-electrophoresis staining with EtBr can also result in high background staining, sometime requiring destaining of the gel before fragments can be resolved.

SYBR Green I is a highly sensitive, cyanine-based, proprietary fluorescent gel stain for nucleic acid. SYBR Green I has very strong affinity for DNA with a large fluorescence enhancement after DNA binding (Singer and Haughland, 1999; Cambrex Bio Science Rockland, 2005). DNA/SYBR Green I complexes produce a fluorescence quantum yield more than 5 times greater than that of DNA/EtBr complexes (Singer et al., 1999). Similar to EtBr, SYBR Green I can be used to stain agarose gels either before or after electrophoresis. However, sensitivity is higher when post-electrophoresis staining is employed. Unlike ethidium bromide, SYBR Green I affects DNA mobility in a nonlinear fashion. Large fragments (>4000 bp) move faster and small fragments move slower than anticipated. Nonlinear DNA migration kinetics can be avoided with post-electrophoresis staining. As little as 60 pg of double-stranded DNA (ds DNA) per band can be detected in an agarose gel with SYBR Green I, using 300-nm transillumination. The sensitivity of SYBR Green I therefore is 15 to 80 times greater than can be achieved with ethidium bromide using similar methods. Some additional advantages associated with this dye are low background staining, lack of interference with many common restriction endonucleases, and compatibility with Southern blotting. Potential weaknesses of this product (and of SYBR Green II) is a lack of sensitivity for single-stranded DNA (ssDNA), a lack of photostability, and an inability to penetrate thick (>4 mm) gels or gels made with high percentage agarose (Tuma et al., 1999).

SYBR Gold is also an unsymmetrical cyanine dye that appears to address many of the short-comings of the SYBR Green products. With a fluorescence excitation peak at 300 nm, it has an even higher sensitivity for dsDNA, ssDNA, and RNA than does SYBR Green I, using typical transillumination, with a detection limit of <20 pg of dsDNA. It has a higher degree of photostability and can penetrate thick, high percentage agarose gels (Tuma et al., 1999). The use of both SYBR Gold and the SYBR Green dyes for routine, size separation agarose gel electrophoresis are limited by their very high cost, relative to that of ethidium bromide (Sambrook et al. 2005).

Another proprietary dye, GelStar (Cambrex Bio Science Rockland, 2003) is a fluorescent stain for detecting nucleic acid in agarose gels (White et al., 1999). Similar to EtBr, SYBR Green, and SYBR Gold, GelStar can be applied before or after electrophoresis. GelStar has been demonstrated to be 4 to 16 times more sensitive than EtBr for detection of double-stranded DNA. Again, the cost of GelStar is considerably greater than that of ethidium bromide. As with EtBr-stained gels, GelStar-stained preparations can be visualized and photographed using 300-nm transillumination.

Methylene blue–stained DNA in agarose gels produces bands with a deep-blue color without the need for examination under UV light. DNA stained with

methylene blue does not fade, and agarose gels can be air-dried and kept as a permanent record, without the need for photography or electronic documentation. This dye is nontoxic, inexpensive, and readily available. The drawback is that it is approximately 40 times less sensitive compared with EtBr, limiting its use to instances when large amounts of DNA (>40 ng/band) are present.

Silver staining, commonly used to stain protein bands in polyacrylamide gels, can also be used for DNA staining in agarose gels (Andrews, 1991). As with methylene blue, it has the advantages of visualization without the use of UV light, and gels stained in this way can be dried and kept as a permanent record of the experiment. Drawbacks include a complex staining process with multiple solutions, requirements for precise timing at each step, and the need for high-quality (often expensive) reagents required to achieve consistent results. Sensitivity of this method is comparable to that of EtBr, at 1–5 ng of double-stranded DNA per band. Sensitivity of silver staining for DNA improves when used on very thin (1 mm or less) polyacrylamide gels.

Despite its disadvantages of toxicity and somewhat reduced sensitivity compared with other dyes, fluorescent illumination of ethidium bromide–stained DNA remains the simplest, least costly, and most commonly used method for detecting size-separated nucleic acid bands after agarose gel electrophoresis (Sambrook et al., 2005). Permanent records of band patterns can be made with a variety of methods. The least costly involve the use of a small portable camera with a fixed focal length and an attached darkroom hood. This can be done in just a few seconds right on a laboratory bench without the need for a photographic darkroom at a fraction of the cost. Limits in framing the shot due to the fixed focal length are a drawback, with variable focal length cameras required to frame images more tightly. These systems require more space and are preferably used with a darkroom setup. Digital gel documentation systems have become an increasingly common option, available for a significantly larger investment, but offering numerous advantages over the older, film-based systems. Such packages typically include a small housing for the gel, a UV illumination source, a peltier-cooled CCD (charge-coupled device) camera, and a computer to operate the system and to store and manipulate the images. Some of these systems can be used for fluorescent, nonfluorescent, and chemiluminescence image documentation. The digital images can be duplicated with ease, the strength of signal can be readily quantified, and the images easily databased, compared over time, and transmitted electronically.

Southern Blot Hybridization

Principles

Southern hybridization detection after PCR amplification can markedly increase sensitivity and specificity of clinical PCR tests in comparison to the use of traditional ethidium bromide staining of agarose gels for product detection. The increased sensitivity realized (up to a log or more) can be of particular import in assays designed for the direct detection of infectious agents. In clinical specimens,

target DNA may be present in only very low concentration, and the amount of amplified product may not reach concentrations necessary for visualization in ethidium bromide–stained gels. The increased sensitivity associated with Southern blotting can therefore have significant clinical impact, sometimes leading to a substantial increase in the positive result rate in a given assay. The other primary advantage of this method is its specificity. Size separation techniques can sometimes give misleading results due to nonspecific amplicons producing bands of the same (or approximately the same) size as would be expected from the intended PCR target. This may be particularly problematic in tests using samples that contain large amounts of host DNA, such as in highly cellular samples used for many viral detection assays. By incorporating probe-based identification of size-separated DNA fragments, Southern blotting allows clear differentiation between specific and nonspecific amplification products, substantially increasing the specificity of target amplification–based assays compared with those depending solely on detection and characterization of amplicon by size.

Southern blotting, as first described by E.M. Southern in 1975 (Southern, 1975), consists of the transfer of DNA from an electrophoresis gel to a solid support (membrane). Once DNA fragments are immobilized, they can be identified using probe-based hybridization. The term "Southern blot" is now typically used to describe the entire process including DNA transfer from gel to membrane and subsequent hybridization with nucleic acid probe. Since its original description, Southern blotting has been widely applied for the detection and identification of molecular amplification products in both research and clinical settings. Southern blot–based detection of PCR products is labor intensive and time-consuming, limiting its use in higher volume clinical labs, particularly as newer, real-time detection methods have come into use. However, Southern blotting remains a valuable laboratory tool. When testing is infrequent, the ease of developing a Southern blot–based test may be more cost and time-effective than implementing assays using ELISA or real-time detection formats. Another advantage is that once DNA is immobilized on a nylon membrane, amplified product can be reassayed as many as a dozen times with different DNA probes, without significant loss of signal strength (Brown, 1999; Perandin et al., 2001).

Technical Considerations

Several factors can affect the outcome of a Southern blot procedure, including the type of membrane, transfer buffer, transfer method, hybridization conditions, probe system, and probe sequence used (Brown, 1999; Kroczek, 1993). Nylon membranes, either charged or uncharged, are most commonly used. Although nitrocellulose membranes found common early use, with lower background compared with nylon (especially when chemiluminescent detection systems are used), nitrocellulose is more fragile than nylon, making handling difficult and often complicating the process of reprobing (Brown, 1999). With greater strength and ease of manipulation, nylon is now the membrane material of choice. Although some manufacturers of nylon membranes also note that nylon binds up to five times

more DNA per cm^2 than nitrocellulose, this increased capacity is of little practical significance, as the maximum binding capacity of the membrane is not reached during DNA transfers (Brown, 1999).

The buffers most often used for Southern blotting are 10–20 SSC (20X SSC = 1.5 M sodium chloride, 0.15M sodium citrate), SSPE (sodium chloride, sodium phosphate, EDTA), and 0.4 M sodium hydroxide (Brown, 1999). While alkaline buffers seem most effective when blotting to a positively charged membrane, SSC and SSPE (high-salt buffers) show excellent transfer results irrespective of membrane charge (positive or uncharged). The value of using an alkaline buffered transfer is that it allows covalent binding of DNA to the membrane, without necessitating posttransfer UV cross-linking (required after high-salt buffer transfers). A drawback to the use of alkaline buffer is the higher signal background that may occur, particularly when chemiluminescent detection methods are employed.

Blotting times, or the time needed for DNA transfer from gel to membrane, depend on gel thickness, agarose concentration, nucleic acid fragment size, and transfer methodology (Kroczek, 1993; Brown, 1999;). The three most commonly used transfer methods include upward capillary transfer, downward capillary transfer, and vacuum transfer. Capillary transfer devices can be constructed using a glass or plastic dish, a sponge, blotting paper, paper towels, a glass plate, and a weight; vacuum transfer devices are commercially available. Upward capillary transfer is the most frequently used technique (Fig. 15.1A). It begins with a sponge placed

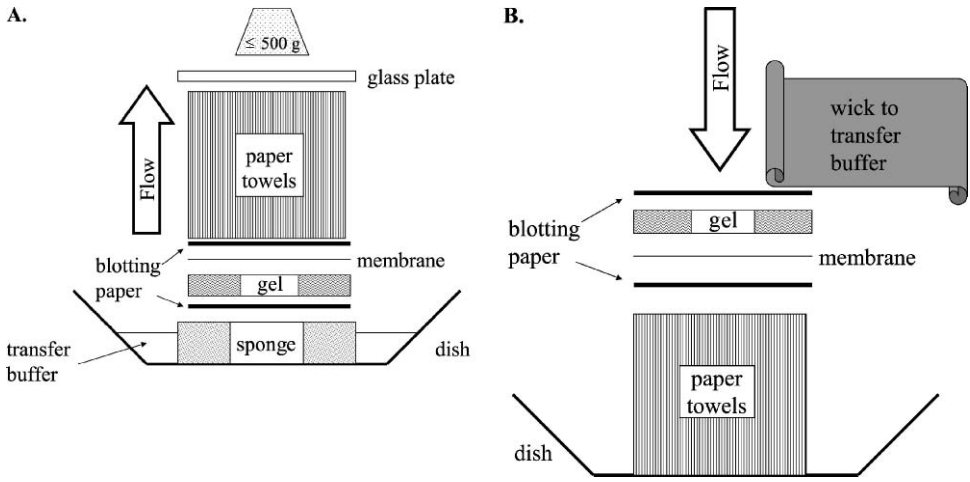

FIGURE 15.1. Two common types of home-made Southern blot transfer apparatus. (A) Upward capillary transfer apparatus. (B) Downward capillary transfer apparatus. Reprinted with permission from: Podzorski, RP. Gel electrophoresis, southern hybridization, and restriction fragment length polymorphism analysis. In *Molecular Microbiology: Diagnostic Principles and Practice*, D. Persing, F. Tenover, J. Versalovic, Yi Tang, E. Unger, D. Relman, and T. White (Editors), ASM Press, Washington, DC, 2004.

in a dish of transfer buffer. On top of the sponge, consecutively, are placed the following: blotting paper, gel, membrane, blotting paper, a stack of paper towels, a glass plate, and a weight. The weight serves to compress the paper towel stack, ensuring good capillary flow throughout. The weight must be only heavy enough to achieve good contact among the paper towels, as excessive weight can impede capillary flow and potentially crush the gel. Once the blotting process has begun, transfer buffer is drawn up through the sponge and gel by capillary action, pulling DNA fragments from the gel to the membrane. Upward capillary transfer using high-salt buffer requires 16–24 h to transfer small DNA fragments (<1000 bp) from a 3% agarose gel. Transfer times can reduced if alkaline transfer buffer is used; transfer onto a positively charged membrane under these conditions can be completed in 3 to 4 h. One potential problem associated with upward capillary transfer is that the downward pressure of the weighted stack of paper towels can impede the upward flow of transfer buffer. Downward capillary transfer can be accomplished more quickly (Kroczek, 1993; Brown, 1999). This is because it does not involve the use of a heavy weight pressing down on the gel. In downward capillary transfer systems (Fig. 15.1B), a blotting paper wick moves transfer buffer from a reservoir to the top of the gel. Because the gel sits on top of the stack of towels, transfer buffer flows downward. Downward transfer can be used with all membrane types and with either high-salt or alkaline transfer buffer. Vacuum transfer devices are the fastest of the three methods, requiring only about 30 min for transfer of PCR products less than 1500 bp, with larger oligonucleotides taking 90 min. Exact times depend, as with other methods, on gel thickness, agarose concentration, and fragment size. Any of the available membrane types and commonly used transfer buffers can be used with vacuum transfer methods. Although there are up-front expenditures for purchase of the equipment used for this technique, improved speed may be a sufficient advantage to justify these costs.

Membrane-bound DNA in Southern blots can be probed using an oligonucleotide probe coupled to a reporter system (Brown, 1993), with many probe-labeling systems available as commercially prepared kits. Hybridization of probe to bound target can take place in a sealed plastic bag immersed in a water bath. Alternatively, bottles in a specialized rotisserie oven can be used for incubation of membrane and probe solution. The latter are easier to handle than plastic bags, requiring less hybridization buffer, and producing much more even probing. Radioisotopic labels (^{32}P, ^{35}S, and ^{125}I), once the most commonly used reporter molecules (Kroczek, 1993), have been supplanted by nonradioactive reporter systems due to numerous drawbacks associated with handling of radioisotopes, particularly in the clinical laboratory setting. Nonradioactive systems now available include affinity labels, such as biotin and digoxigenin, incorporated into nucleic acid probes by either enzymatic or nonenzymatic methods (Cook et al., 1988; Nelson and Kacian, 1990; Pollard-Knight et al., 1990; Diamandis and Christopoulos, 1991; Boyle and Perry-O'Keefe, 1992; Perry-O'Keefe and Kissinger, 1994). Such affinity labels can be detected with avidin or enzyme–antibody conjugates, respectively (enzyme conjugates usually consisting of peroxidase or alkaline phosphatase).

Either colorimetric or chemiluminescent methods can be used for probe visualization. Chemiluminescent reporter enzymes release light when exposed to substrate (Nelson et al., 1990; Pollard-Knight et al., 1990; Boyle et al., 1992; Perry-O'Keefe et al., 1994). After hybridization has taken place (often requiring overnight incubation) and a washing step is used to remove unbound probe material, peracid salt is added together with substrate (such as Lumigen) and an enhancer. Light production is catalyzed by probe-bound enzyme, and the signal is detected by a short exposure of x-ray film (1 h or less). The high sensitivity and specificity of chemiluminescent assays provide accuracy equal to or greater than those of radiolabeled probes (Nelson et al., 1990; Boyle et al., 1992).

Restriction Fragment Length Polymorphism Analysis

Principles

Restriction fragment length polymorphism (RFLP) analysis is used to characterize PCR products based on sequence-specific enzymatic cleavage (Pourzand and Cerutti, 1993). Each restriction endonuclease recognizes and cleaves a specific double-stranded DNA sequence, usually 4, 5, 6, or 8 nucleotides long. Even single base-pair changes within a target will prevent enzymatic recognition, and cleavage will not take place. The process of RFLP analysis includes digestion or cleavage of a PCR product, typically followed by agarose gel electrophoresis. After electrophoresis, restriction products are visualized by one of several methods, including DNA staining of the gel or, after Southern blotting, using probe-based hybridization. Sequence changes in the target nucleic acid (RFLPs) can result in either the disappearance or creation of cutting sites. In turn, the number and size of DNA fragments produced is affected, resulting in characteristic banding patterns on electrophoresis. Situations well-suited to RFLP analysis are those that demand the interrogation of only a few different nucleotides in a PCR product. When a complete amplicon sequence analysis is required, nucleic acid sequencing, or oligonucleotide arrays, may be required.

Technical Considerations

With hundreds of commercially available restriction endonucleases available, selection of appropriate enzyme(s) for use in a particular RFLP analysis depends on the target nucleotide acid sequence of interest (Rogers et al., 1991; Pourzand et al., 1993; Bloch and Grossmann, 1995; Thiers et al., 1997; Buoro et al., 1999; Prix et al., 1999). In many such assays, PCR product can be used directly after amplification, without any intervening manipulation of the sample. No specialized equipment is required for RFLP analysis beyond that normally used for agarose gel electrophoresis (and Southern blot hybridization, if this is used). Only a water bath or incubator is needed for the enzyme digestion process. Time requirements for restriction enzyme digestion depend on the cutting efficiency of the enzyme

and the amount of DNA used in the digestion, with incubation times ranging from 1 h to overnight.

Conditions for the cleavage reaction, including concentration of enzyme, buffer, and target nucleic acid, as well as temperature (usually 37°C) and duration of reaction all vary from enzyme to enzyme. Multiple enzymes may be used together if buffer and temperature requirements are similar. If needed, potassium acetate or potassium glutamate buffers may be used, as they tend to accommodate a wide range of restriction enzymes (see more detailed references for specifics on the use of multiple enzymes). Purity of target nucleic acid can be essential for efficient cleavage reactions. Impurities, such as protein, high salt concentrations, anticoagulants, and solvents remaining from DNA extraction or purification processes can all inhibit the function of restriction endonucleases (Bloch et al., 1995). The effects of such contaminants can be mitigated through the use of higher concentrations of enzyme, increased reaction volume, increased time of incubation with the enzyme, or by repurification of the target nucleic acid. Other factors, such as secondary DNA structure (e.g., supercoiling), present in plasmid or some viral preparations, may also inhibit enzyme activity, requiring many times the normal concentration for cleavage.

Applications

RFLP analysis has found widespread application in clinical microbiology. It has been used to differentiate between HSV type I and HSV type II (Rogers et al., 1991; Podzorski et al., 2000). In this case, HSV-specific primers (Rogers et al., 1991) target a 476-bp region of HSV polymerase gene. The PCR product from HSV I and HSV II differ in that HSV I contains two *Ava*II restriction sites in the amplified region, and HSV II contains one *Ava*II site in this stretch. After *Ava*II restriction digestion of the PCR product, agarose gel electrophoresis demonstrates three bands (87, 183, and 296 bp) from the HSV I product, while two bands (87 bp and 389 bp) result from HSV II. Mutations in cytomegalovirus (CMV) genes, including the UL97 protein kinase gene, the UL64 DNA polymerase gene, and the UL54 polymerase gene have been associated with resistance to ganciclovir, cidofovir, and foscarnet (Rogers et al., 1991; Prix et al., 1999; Emery, 2001). The latter mutations have all been targeted and detected using PCR with subsequent RFLP analysis. HCV genotypes 1a and 1b can also be differentiated by a PCR-RFLP (Thiers et al., 1997; Buoro et al., 1999). Finally, RFLP has been used in many settings as a molecular epidemiological tool for strain identification. In the case of varicella-zoster virus (VZV) in cerebrospinal fluid (or in other sites), this method can be used to determine if the source of varicella-zoster virus amplified DNA was from wild-type or vaccine strain virus (LaRussa et al., 1992; Salzman et al., 1997). RFLP in combination with other size separation techniques [most commonly pulse-field gel electrophoresis (Arshad et al., 1993)] has been used for molecular epidemiologic studies in a wide range of organisms, including viral, bacterial, and fungal pathogens (Weber et al., 1997; Lipuma, 1998; Soll, 2000; Erdman et al., 2002).

Solid-Phase Enzyme-Linked Immunoassay (EIA)

Probe-based methods for the detection and identification of PCR products have been developed for several different solid phases, including nylon membranes, microwell plates, microparticles, and oligonucleotide microarrays (Inouye and Hondo, 1990; Bobo et al., 1991; White et al., 1992; Schachter et al., 1994; Chee et al., 1996; DiDomenico et al., 1996; Cheung et al., 1999; Loeffelholz et al., 1999; Tang et al., 1999). Microtiter plate assays have a number of advantages, including the convenience of nonisotopic chemistry, rapid turnaround time, ease of use, and high throughput. This format is amenable to automation, with comparable sensitivity to that of Southern blot hybridization. It has many similarities with enzyme-linked immunosorbent assays (ELISAs) and is sometimes referred to as ELOSA (enzyme-linked oligosorbent assay) (Mallet et al., 1993).

Microtiter plate formats for detection of amplicon were originally described in the late 1980s and early 1990s (Keller et al., 1989, 1990, 1991; Kawai et al., 1993; Rapier et al., 1993) and had their foundations with hybridization-based identification of synthetic oligonucleotides (Nagata et al., 1985; Cook et al., 1988). Early procedures used sandwich hybridization, in which at least two probes (a capture probe and secondary, labeled probes) were used for amplicon detection (Keller et al., 1989, 1990; Rapier et al., 1993) (Fig. 15.2A). Typically, the secondary probe consisted of a single biotin-labeled oligonucleotide complementary to the captured amplicon (Keller et al., 1990). In a variation of this procedure, two secondary probes are used to detect hybridized amplicon; one complementary probe containing amplicon-specific sequence at the 5′ end and a 3′ poly-T tail, and a second probe (not sequence-specific) having a 5′ poly-A tail and biotin (Rapier et al., 1993). Some have simplified the sandwich, creating a direct hybridization procedure, in which amplicon is biotin-labeled during PCR extension phase, obviating the need for a secondary probe (Keller et al., 1991; Kawai et al., 1993) (Fig. 15.2B).

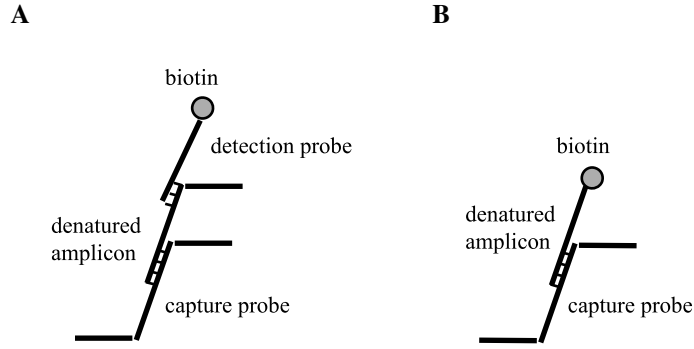

FIGURE 15.2. Schematic diagrams of microwell plate detection using sandwich (A) and direct (B) hybridization formats. Reprinted with permission from: Loeffelholz, MJ. Microwell plate detection systems for amplicon detection and characterization. In *Molecular Microbiology: Diagnostic Principles and Practice*, D. Persing, F. Tenover, J. Versalovic, Yi Tang, E. Unger, D. Relman, and T. White (Editors), ASM Press, Washington, DC, 2004.

The latter direct hybridization method is simpler to perform than the sandwich method, with lower resulting background signal levels (Keller et al., 1991).

Microwell plate detection systems can be classified in two ways based on the capture molecule used (Lazar, 1994): oligonucleotide probe (sequence-specific capture) or avidin (non–sequence specific capture). Formats relying on immobilized probe for target capture (Keller et al., 1991) rely on sequence complementary hybridization of amplicon to probe. Formats using avidin (or streptavidin) for target capture agent depend on its strong affinity for biotin (Diamandis et al., 1991), incorporated into target during PCR (Cook et al., 1988; Boyle et al., 1992).

Technical Considerations

There are several important factors to consider when developing a microtiter plate detection assay, including hybridization probe design (probe sequence and labeling method), choice of microtiter plate, technique for affixing probe to the microwell surface, hybridization and wash conditions, and means of detecting hybridized amplicon (Boyle et al., 1992; Kawai et al., 1993; Perry-O'Keefe et al., 1994). Biotinylated PCR product is often used to facilitate detection in microtiter plate systems (Nagata et al., 1985; Inouye et al., 1990). This requires biotin-labeled primer (Cook et al., 1988; Boyle et al., 1992) to be used in the PCR reaction. Following amplification, avidin or streptavidin binding to the target is used as a means either of amplicon capture or detection, depending on the specific assay design. Oligonucleotides that are 5′ end-labeled with biotin are commercially available and can be used as primers for such assays.

Polystyrene microtiter plates are most often used for amplicon detection, due to their high DNA binding affinity. An 8-well, removable strip-well format is also available, offering scalability, convenience, flexibility, and sometimes more cost-effective use of materials. DNA can be affixed to the polystyrene well surface by covalent binding of DNA probe (Kawai et al., 1993). Coating buffer is used to dilute probe to an appropriate concentration; it is then added to microwells, followed by an incubation step. Various probe-coating parameters are detailed in Table 15.2. Probe-coated plates stored with desiccant at 4°C have a shelf-life of weeks to months, although stability must be verified by individual users. Microwell plates precoated with covalently bound streptavidin are also commercially available. These can be used to bind biotinylated oligonucleotide (either amplicon or probe). The resultant biotin/streptavidin complexes are stable at salt concentrations of 500 mM NaCl and detergent concentrations of 1% SDS.

Hybridization conditions for binding of denatured amplicon to immobilized DNA probe vary substantially; over time, these methods have been modified, becoming increasingly simplified and rapid with fewer steps and shorter incubation times. Several different such procedures have been published, with some shown in Table 15.3. The hybridization step must allow efficient and stable binding of only sequence-specific probe and amplicon sequence. Stringency of the hybridization reaction is critical. As with other hybridization reactions, appropriate binding may be prevented if conditions are too stringent, with subsequent reduced sensitivity.

TABLE 15.2. Conditions for immobilization of DNA probes in microwell plates.

Coating buffer	Incubation conditions	Additional steps	Wash buffer	Reference
1 M ammonium acetate	37°C, O/N	None	PBS, 0.1% Tween-20	Loeffelholz et al., 1999
1 M ammonium acetate	37°C, 2 h	None	2X SSC, 1% Tween-20	Cook et al., 1988
PBS, 0.1 M $MgCl_2$	Room temp, O/N	Irradiation	None	Nagata et al., 1985
25 mM KH_2PO_4, 25 mM $MgCl_2$	Room temp, 2 h, on rotator	Blocking buffer	25 mM KH_2PO_4, 100 mM $MgCl_2$	Keller et al., 1990
1.5 M NaCl, 0.3 M Tris (pH 8.0), 0.3 M $MgCl_2$	37°C, O/N	Irradiation	1 M NaCl; 0.1 M Tris (pH 9.3); 2 mM $MgCl_2$, 0.1% Tween-20	Kawai et al., 1993

O/N, overnight; PBS, phosphate-buffered saline.
Reprinted with permission from: Loeffelholz, MJ. Microwell plate detection systems for amplicon detection and characterization. In *Molecular Microbiology: Diagnostic Principles and Practice*, Persing, Tenover, Versalovic, Tang, Unger, Relman, and White (Editors), ASM Press, Washington, DC, 2004.

TABLE 15.3. Microwell plate hybridization conditions.

Prehybridization step	Hybridization buffer	Time, temperature, other conditions	Wash buffer[a]	Reference
No	30% formamide; 2X or 4X SSPE[b]; 1% Triton X-100; 5% dextran sulfate	30–90 min; room temp.	0.2X SSC,[c] 0.1% Triton X-100	Cook et al., 1988
No	0.15 M NaCl; 0.12 M HEPES (pH 8.0); 25% dextran sulfate; 33% formamide	90 min; room temp.; shaking	2X SSC, 0.1% Tween-20	Rapier et al., 1993
Yes (2 h, 65°C)	4X SSC; 3.2X Denhardt's[d]; 10% dextran sulfate; 10 μg Salmon sperm DNA/mL	Overnight; 65°C	2X SSC (30 min at 65°C)	Nagata et al., 1985
No	50% formamide; 5X SSC; 1X FPG[e]; 25 mM KH_2PO_4 (pH 7.0); 0.2% SDS; 5% dextran sulfate; 200 μg salmon sperm DNA/mL	4 h; 42°C	2X SSC; 0.1% SDS	Keller et al., 1990
No	5X SSC; 5X Denhardt's; 0.2% SDS; 200 μg herring sperm DNA/mL	30 min; 50°C	2X SSC	Kawai et al., 1993

[a] Unless otherwise stated, plate washing did not include prolonged incubation times.
[b] 1X SSPE is 0.18 M NaCl; 10 mM sodium phosphate buffer (pH 7.0); 1 mM EDTA.
[c] 1X SSC is 0.15 M NaCl; 15 mM sodium citrate.
[d] 1X Denhardt's is 0.02% Ficoll 400; 0.02% polyvinylpyrrolidone; 0.02% bovine serum albumin.
[e] 1X FPG is 0.2% Ficoll 400; 0.02% polyvinylpyrrolidone 360; 0.02% glycine.
Reprinted with permission from: Loeffelholz, MJ. Microwell plate detection systems for amplicon detection and characterization. In *Molecular Microbiology: Diagnostic Principles and Practice*, Persing, Tenover, Versalovic, Tang, Unger, Relman, and White (Editors), ASM Press, Washington, DC, 2004.

However, low stringency binding can result in a less specific test, with poor positive predictive value. The wash step immediately after hybridization removes unbound probe that would otherwise react with detection reagents, potentially resulting in false-positive test results. Again, stringency of the wash step is critical, as bound amplicon must not be removed. Table 15.3 lists several wash buffers. Wash buffer is usually added to reaction wells and aspirated either immediately or after several seconds. This wash step is often repeated several times.

Detection of hybridized amplicon can be accomplished in several ways. Biotin-labeled target can be identified with avidin/enzyme conjugate and addition of enzyme substrate. Potential enzyme conjugates for solid-phase assays include alkaline phosphatase and horseradish peroxidase. Substrates for these enzymes are soluble in the aqueous buffers that are used in microtiter plate assays. Alkaline phosphatase substrates include *p*-nitrophenylphosphate and 5-bromo-4 chloro-3 indolyl phosphate. Peroxidase substrates include 3,3,5,5′-tetramethylbenzidine (TMB) and 2, 2′-azino-di (3-ethylbenzthiazoline-6-sulfonic acid). Weak acid can be added to some of the enzyme/substrate systems, stopping color development. An ELISA plate reader set at 405–450 nm (depending on enzyme substrate) can be used for colorimetric end-point reading.

As with other user-developed tests, each laboratory must define and optimize PCR assay performance characteristics, including sensitivity, specificity, accuracy, and precision (Perry-O'Keefe et al., 1994). Components that must be titrated include probe-coating concentration and hybridization stringency. Low probe concentrations can result in low or variable optical density readings. Hybridization stringency must be optimized (as noted above) to achieve the desired balance between sensitivity and specificity. When these assays are used for diagnostic purposes, verification of performance characteristics is required (Clinical and Laboratory Standards Institute, 1995). For colorimetric microwell plate assays, an optical density value distinguishing positive and negative results (cutoff) must be established and verified using a panel of well-characterized clinical specimens. In addition, the stability of probe-coated microwell plates and other in-house–prepared reagents must be determined and expiration dates applied. As with other clinical tests, the laboratory must develop and implement appropriate quality control testing of in-house–prepared reagents. The entire microwell plate detection system (plate, hybridization and wash buffers, enzyme conjugate, substrate) may be QC tested as a complete system, using stock amplicon. References for optimization, verification, and validation of PCR diagnostic tests are available (Clinical and Laboratory Standards Institute, 1995, 2005).

Applications

Microwell plate detection systems have been developed as user-defined assays (Keller et al., 1989, 1990, 1991; Bobo et al., 1991; Kawai et al., 1993; Rapier et al., 1993; Buck, 1996; Loeffelholz et al., 1999) and as commercially available kits and analyte specific reagents (ASRs). Widely used commercial products include those marketed by Chemicon (Temecula, CA, USA), Roche Diagnostics (Indianapolis,

IN, USA), Argene, Inc (North Massapequa, NY, USA), and Prodesse, Inc. (Waukesha, WI, USA). Reagents from these companies are packaged for research use only (RUO), as ASRs, and as U.S. FDA–approved diagnostic kits. Some, such as the ChemFLASH reagents from Chemicon, are universal detection systems, marketed as RUO, which can be adapted for the detection of a wide range of PCR products. Most, however, include specific primers and probes, directed at clinically relevant pathogens. Reagents are available for the detection of Epstein–Barr virus, herpes simplex virus, human immunodeficiency virus, adenovirus, *Bartonella* species, *Bordetella pertussis*, calicivirus, *Chlamydia pneumoniae*, cytomegalovirus, *Cryptosporidium*, hepatitis B and C, and numerous other bacterial, viral, and parasitic pathogens. Those that are packaged as complete kits include reagents for specimen processing and PCR amplification, as well as for product detection. These commercially available systems have the advantage of preoptimized hybridization conditions and reagent formulations, as well as offering consistency of quality and the ability to compare assay performance among many users.

Cross-Method Comparisons

The methods discussed above each have their own strengths and weaknesses. Each is best suited to its own spectrum of applications, sometimes uniquely well-suited and sometimes one of many possible means to an end. Table 15.4 depicts some of the advantages and disadvantages of these techniques and reiterates some applications of each. Common to all four is that they are relatively simple, inexpensive, and adaptable to a wide variety of applications and targets. Agarose gel electrophoresis is perhaps the simplest of all and can be thought of as a basic tool that is integral to work in a molecular biology laboratory. It is often used extensively in research and in assay development and in tandem with Southern blot for discrimination and identification of nucleic acid targets. This combination of methods was used extensively as molecular techniques first made their entry into the clinical diagnostic laboratory, although it has now been supplanted in many settings by EIA and real-time detection formats. RFLP is most often used either for discrimination of a small number of similar targets (e.g., discriminating viral subtypes) or for epidemiologic studies. RFLP has been a mainstay of molecular epidemiology and phylogenetic studies. It is an easily adopted method that can be interpreted in a straightforward manner, usually with little ambiguity. The large number of enzymes available and the extent to which this method has been used worldwide has meant that its use is well defined for many organism groups. The numerous publications related to such studies have provided a common language for outbreak investigations and other such applications. Although sequencing has been used increasingly for such purposes, the simplicity of RFLP analysis has made it a persistent favorite for these types of analyses. PCR-EIA is perhaps the best suited of the methods discussed here for implementation in the setting of a high-throughput clinical diagnostic laboratory. It offers the high degree of target

TABLE 15.4. Comparison of techniques for detection and characterization of molecular amplification products.

Method	Advantages	Disadvantages	Applications
Agarose gel electrophoresis	Simple, minimal technical requirements Insensitive to novel polymorphisms in target Rapid, flexible Can retrieve nucleic acid for further characterization	No probe or sequence-based positive ID of target Susceptible to artifacts, poor gel performance Agarose mix, gel size, and voltage must match target size	Used primarily for assay development, troubleshooting, and for user-defined assays
Southern blot transfer	Simple, minimal technical requirements Can retrieve nucleic acid for further characterization Can use sequence-specific probes for target identification	Time-consuming; not amenable to rapid-throughput diagnostics Susceptible to artifacts, poor gel performance, background	Used primarily for assay development, troubleshooting, and for user-defined assays
RFLP	Simple, minimal technical requirements Large numbers of well-characterized enzymes available Good technique for polymorphisms in short, specific regions	Variable buffering requirements for different enzymes May not detect novel polymorphisms outside of enzyme target region Not amenable to automated applications	Primary applications in user-defined assays Viral subtyping Detection of polymorphisms correlated to antimicrobial and antiviral susceptibility Molecular epidemiology studies
PCR-EIA	Simple, widely available technology Adaptable to any target sequence Adaptable to different signal chemistries, signal amplification methods Amenable to automation	May require postamplification product manipulation May be insensitive to polymorphisms in target May show diminished sensitivity in the presence of target polymorphisms	Applications in user-defined and in commercially available assays Qualitative and quantitative detection assays Hematogenous targets Respiratory targets

identification specificity intrinsic to probe-based systems while taking advantage of technology already available in most clinical labs to allow rapid, unambiguous signal detection and the potential for automation. The advent of EIA-based molecular detection systems and the widespread availability of commercially prepared assays has finally helped propel molecular diagnostics into common use, beyond the formerly exclusive province of academic and reference laboratories.

Conclusion

Agarose gel electrophoresis, Southern blotting, RFLP, and EIA analysis remain useful laboratory procedures for detection and characterization of nucleic acid amplicon. EIA now plays a prominent role as a detection methodology for molecular diagnostic testing in the clinical lab. Although the other procedures listed are often not the methods of choice for use in high-volume clinical settings, their continued value in assay development or in some aspects of clinical testing is indisputable. In situations where sample throughput is small, amplification targets are changed frequently, or cost of new molecular diagnostic methods is prohibitive, these procedures are very practical. Their simplicity, ease of implementation, relative low cost, and widespread applicability to many nucleic acid detection and/or characterization problems allow them to maintain a niche even in today's highly complex molecular diagnostic laboratory.

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16
Direct Nucleotide Sequencing for Amplification Product Identification

TAO HONG

Introduction

The advances of technology to determine the nucleotide sequence of DNA have fundamentally changed the field of biological research and medicine. For diagnostic molecular microbiology, the most precise method of identification of a PCR product (amplicon) is to determine its nucleotide sequence. Although it is not always necessary to sequence the entire amplicon for routine diagnostic procedures, DNA sequence has been used to analyze a broad range of PCR products for bacterial identification; for gene mutations related to antimicrobial resistance; for bacterial strain typing and viral genotyping; and so forth. Most of the amplicons of these applications are large (range approximately from 300 base pairs to 1500 base pairs), and the exact nucleotide sequence of the amplicoms are crucial for the results.

Two basic methods are created for DNA sequencing: the ddNTP-mediated chain termination method of Sanger et al. (1977) and the chemical cleavage method of Maxam and Gilbert (1977). The Sanger method has been widely performed in most research laboratories using radioisotope-labeled nucleotide (e.g., ^{32}P or ^{35}S) and standard manual method. The method relies on enzymatic DNA synthesis from a specific oligonucleotide primer. The primer is annealed to the complementary sequence adjacent to the DNA of interest on a genetic element (Sambrook, 1989). The method of DNA sequencing developed by Maxam and Gilbert is based on the specific cleavage of DNA at specific nucleotide. A homogeneous sample of DNA radiolabeled at one end is treated with four separate chemical reactions, each of which modifies a particular type of base. Conditions of the subsequent cleavage reactions are set such that cleavage occurs an average of only once for each DNA molecule.

Not long ago, DNA sequence–based analyses were laborious and time consuming. These methods were available only in the research setting. Recent advances in the use of fluorescent dye terminator chemistry and laser scanning in polyacrylamide gel electrophoresis (PAGE), and application of capillary electrophoresis technique combined with fluorescent dye terminator, combined with base-calling software, has made DNA sequencing much less labor intensive.

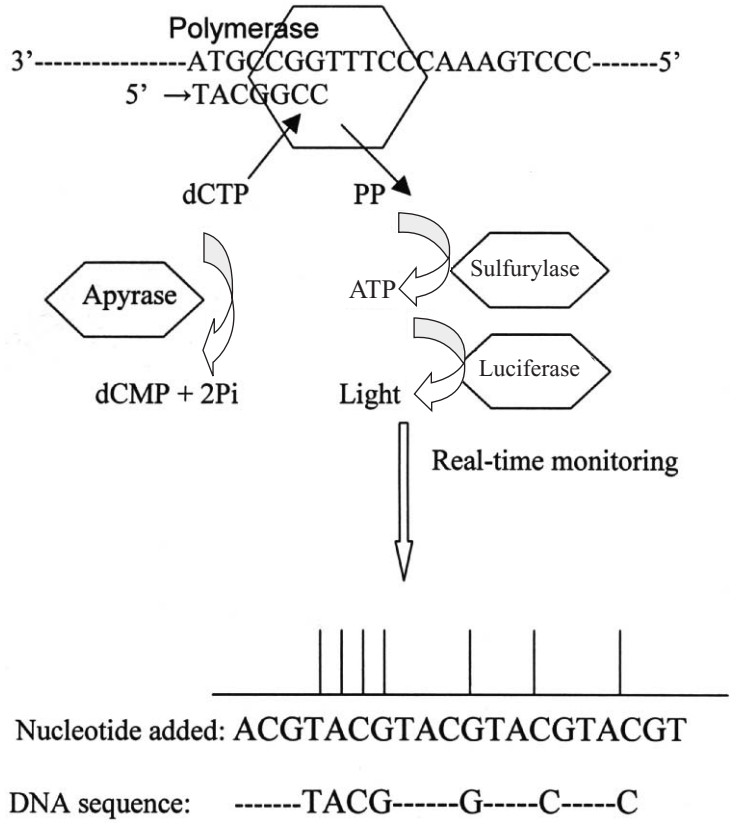

FIGURE 16.1. Schematic diagram of pyrosequencing. The reaction mixture consists of single-stranded DNA with an annealed primer, DNA polymerase, ATP sulfurylase, luciferase, and apyrase. The four nucleotide bases are added to the reaction mixture in a particular order (e.g., A, C, G, and T). If the added nucleotide forms a base pair (in this case, two Cs base pair to the template), the DNA polymerase incorporates the nucleotide and a pyrophosphate (PPi) is released. The released pyrophosphate is converted to ATP by ATP sulfurylase, and luciferase uses this ATP to generate detectable light. This light is proportional to the number of nucleotides incorporated and is detected in real-time. The pyrosequencing raw data are displayed simultaneously, and in this example the sequence generated reads TACGGCC. Excess quantities of the added nucleotide are degraded by apyrase. If the nucleotide does not form a base pair with the DNA template, it is not incorporated by the polymerase and no light is produced. Apyrase then rapidly degrades the nucleotide.

Pyrosequencing is a non–gel-based DNA sequencing technique that is based on the detection of the pyrophosphate (PPi) released during DNA synthesis. In a cascade of enzymatic reactions, visible light is generated at a level that is proportional to the number of incorporated nucleotides (Fig. 16.1). This method generates 30 to 40 base sequences with each primer, and the throughput is 96 samples in

approximately 10 min (i.e., the throughput is much higher than that which can be achieved by conventional Sanger sequencing on gel or capillary-based automated sequencing machines). The limitation of pyrosequencing is that the sequence is only accurate within the first 30–40 bases; beyond that, the data is unreliable.

Methodology

Four steps are required to obtain the DNA sequence of a PCR product: nucleic acid extraction (either RNA or DNA), PCR amplification (or RT-PCR for RNA target), nucleotide sequencing, and database homology search/analysis and reporting.

Nucleic Acid Extraction

Depends on the PCR primers; for a broad range of primers, pure culture of a bacterial/viral agent is generally required for its identification. If PCR primer is designed specifically for a particular microbial agent, clinical specimens may be used directly for nucleic acid extraction. Various DNA extraction methods can be used, such as traditional phenol chloroform method, commercial DNA extraction kits, and so forth. Pure culture and relatively large quantity of target DNA makes contamination by background DNA from reagents and other sources negligible. In our experience, for most bacterial target, no DNA purification is necessary. Two colonies or the pellet of 1 mL positive liquid medium are resuspended in 200 μL sterile saline; 2 μL of the suspension is used directly in the subsequent PCR reaction. Alternatively, the bacterial suspension can be boiled for 10 min and centrifuged for 5 min at 8000× and the supernatant (2 μL) can be used for PCR.

PCR

Depends on the target and primer set; the PCR condition varies. It is important to verify the purity of PCR product by visualizing the amplified DNA on an agarose gel before starting DNA sequencing, especially in the assay validation stage. Once the procedure is validated and a single PCR product is routinely obtained, the agarose gel step may not be necessary. Usually, PCR amplicon amplified from a pure target produces a large amount of DNA and is sufficient for nucleotide sequencing.

For PCR reaction that generates multiple products, a gel purification procedure is necessary to purify the amplicon of interest.

Nucleotide Sequencing

The PCR amplicon can be sequenced directly after removal of unpolymerized primers and 4-deoxynucleoside triphosphates that can be achieved by enzymatic digestion with exonuclease and shrimp alkaline phosphatase. No further

purification or concentration of the amplicon is generally necessary. Automated sequencing can be performed according to sequencing chemistry and the sequencing instrument of a laboratory. Usually, one of the PCR primers is used as the primer for sequencing reaction. If both strands of the amplicon are to be sequenced, two separate reactions are needed. For clinical microbiology laboratories with no DNA sequencing equipment, this final step can usually be achieved by sending the purified amplicon with one of the PCR primers to an in-house core sequencing facility or a commercial laboratory providing DNA sequencing service.

Ruano and Kidd (1991) have developed a method called coupled amplification and sequencing; it is a method for sequencing both strands of template as they are amplified. The procedure is biphasic: stage I selects and amplifies a single target from the genomic DNA, and stage II accomplishes the sequencing as well as additional amplification of the target using aliquots from the stage I reaction mixed with end-labeled primer and dideoxynucleotides. A modified procedure (CLIP) has been developed using Clipper sequencer (Yager et al. 1999). Two characteristics of the CLIP reaction as a modification of the original coupled amplification and sequencing method by Ruano and Kidd are (i) An engineered mutant of thermostable DNA polymerase is used that lacks 5′–3′ exonuclease activity and therefore produces uniform band intensities. (ii) Different far-red fluorescent dyes are linked to the two inward-facing CLIP primers, allowing a template to be sequenced in both directions in a single run.

Homology Search and Reporting

For sequence analysis, the sequence is compared with the data in a nucleotide sequences database, whether an in-house developed, commercial, or public database (such as GenBank). The match (sometimes multiple matches) need to be interpreted cautiously; specifically, consensus of the matches and/or the match with type strain should be sought. Preferably, sequences are from type strains with good quality (no unresolved nucleotides or artificial gaps) and from a reputable laboratory. One should be aware that the nucleotide sequence data in the public database have not been peer-reviewed. Early sequencing data generated by a manual method may not be very accurate.

Application of DNA Sequencing in Molecular Diagnosis

Sequencing of hsp65 for Identification of Mycobacterial Species

Clinical microbiology laboratories usually use a combined molecular/conventional approach for *Mycobacterium* identification. Commercial probes are available for *M. tuberculosis* complex, *M. avium-intracellulare* complex, *M. kansasii*, and *M. gornodae*. For other mycobacteria, conventional methods are applied for identification, which is very time consuming. Atypical biochemical reactions have

frequently caused problems for accurate species identification. Many molecular methods have been developed for identifying mycobacteria; the 16S rRNA gene sequencing is the most frequently used approach for sequence-based identification of mycobacteria. The 65-kDa heat shock protein gene (*hsp65*), present in all mycobacteria, is more variable than the 16S rRNA gene sequence and is useful for the identification of genetically related species. Sequence variations in the *hsp65* gene have been exploited to identify both slowly growing mycobacteria and rapidly growing mycobacteria (RGM) to the species level. Hance et al. (1989) reported amplifying a fragment of the 65-kDa heat shock protein gene (*hsp65*) to detect and, coupled with species-specific probes, identify mycobacteria from clinical samples. After this, Plikaytis et al. (1992) and Telenti et al. (1993) described, using separate gene regions, the successful identification of mycobacteria by using restriction digest analysis of amplified *hsp65* fragments (*hsp65* PRA). *hsp65* PRA has been widely used for identification, and an algorithm based on this approach has recently been developed for differentiating 34 mycobacterial species, including members of the RGM group. A sequence-based strategy has several potential advantages. It generates direct, unambiguous data and can distinguish medically relevant subspecific phylogenetic lineages. Recent advances in automated DNA sequencing have also made this approach much easier. To overcome the limitations of *hsp65* PRA and the potential advantage of generating direct unambiguous data, Kapur et al. (1995) developed a procedure for sequencing the *hsp65* amplicon generated by the Telenti primers as a means for identifying mycobacteria. This technique has since been used by many investigators to identify species, as well as characterize and define groups within a number of mycobacteria. A study by McNabb et al. (2004) assessed the viability of using *hsp65* sequencing to identify all mycobacteria routinely isolated by a clinical mycobacteriology laboratory and the ability of an in-house database, consisting of 111 *hsp65* sequences from putative and valid mycobacteria species or described groups, to identify 689 mycobacterial clinical isolates from 35 species or groups. The overall agreement between *hsp65* sequencing and the other identification methods is 85.2%. The study indicates that for *hsp65* sequencing to be an effective means for identifying mycobacteria, a comprehensive database must be constructed. *hsp65* sequencing has the advantage of being more rapid and less expensive than biochemical test panels, uses a single set of reagents to identify both rapid- and slow-growing mycobacteria, and can provide a more definitive identification. Due to limitations of appropriate database, the best approach for sequence identification of mycobacteria is to have both16S rRNA gene and the *hsp65* gene methods available in the laboratory.

Internal Transcribed Spacer

The internal transcribed spacer (ITS) region, a stretch of DNA that lies between the 16S and 23S rRNA subunit genes, has proved to show a high degree of variability in both sequence and size at the genus and species level (Barry et al. 1991; Gürtler, and Stanisich 1996). Hence, this region may allow efficient identification of species due to its enhanced variability within a genus (Garcia-Martinez et al., 1996).

The diversity of the intergenic spacer regions is due in part to variations in the number and type of tRNA sequences found among these spacers. Sequence of ITS region has been used for identification of mycobacterial species, for staphylococcal species (Couto et al., 2001), streptococcal species (Chen et al., 2004), and for rapid identification of medically important yeast (Chen et al., 2001).

For molecular identification of mycobacteria, the most frequently used DNA sequence-based method is the 16S rRNA gene. However, there are instances in which the sequences of 16S rDNA genes have been found to be very similar, if not identical, between different species in a genus, making it necessary to find alternative specific sequences. The intergenic 16S–23S internal transcribed spacer (ITS) region is considered to be less prone to selective pressure and consequently can be expected to have accumulated a higher percentage of mutations than the corresponding rDNA. Sequencing of the ITS regions of diverse bacteria indicates that considerable length and primary sequence variation occurs, and this variability has been successfully used to distinguish between closely related mycobacteria, such as the *M avium intracellulare* complex (Frothingham and Wilson, 1993; De Smet et al., 1995), *M. gastri*, and *M. kansasii*, both of them share a identical 16S rDNA sequence (Rogall et al., 1990; Roth et al., 1998); *M. farcinogenes* and *M. senegalense* (Hamid et al., 2002) and *M. chelonae* complex.

Streptococci are a very diverse group of microorganism; many molecular techniques have been used for identification of streptococci. Chen et al. (2004) have evaluated the feasibility of sequence analysis of the 16S–23S ribosomal DNA (rDNA) intergenic spacer (ITS) for the identification of clinically relevant viridans group streptococci (VS). The ITS regions of 29 reference strains (11 species) of VS were amplified by PCR and sequenced. The ITS lengths (246 to 391 bp) and sequences were highly conserved among strains within a species. The intraspecies similarity scores for the ITS sequences ranged from 0.98 to 1.0, except for the score for *S. gordonii* strains. The interspecies similarity scores for the ITS sequences varied from 0.31 to 0.93. Phylogenetic analysis of the ITS regions revealed that evolution of the regions of some species of VS is not parallel to that of the 16S rRNA genes. The accuracy of using ITS sequencing for identification of VS was verified by 16S rDNA sequencing for all strains except strains of *S. oralis* and *S. mitis*, which were difficult to differentiate by their 16S rDNA sequences. It was concluded that identification of species of VS by ITS sequencing is reliable and could be used as an alternative accurate method for identification of VS.

In staphylococci, there are several copies of the *rrn* operon. Gürtler and Barrie (1995) characterized the spacer sequences of *S. aureus* strains, including methicillin-resistant *S. aureus* (MRSA) isolates, and identified nine *rrn* operons whose 16S–23S spacer region varied from 303 to 551 bp. Three of these spacers contain the tRNAIle gene and two contain both the tRNAIle and the tRNAAla genes, while the remaining four 16S–23S spacers have no tRNA gene. Forsman et al. (Forsman et al., 1997) sequenced the 16S–23S spacer of five staphylococcal species (*S. aureus*, *S. epidermidis*, *S. hyicus*, *S. simulans*, and *S. xylosus*) and found that in addition to *S. aureus*, *S. hyicus* and *S. simulans* also had a tRNAIle gene in some of their *rrn* operons. The sequence conservation of the *rrn* operons argues

for the use of the 16S–23S spacer region as a stable and direct indicator of the evolutionary divergence of *S. aureus* strains.

Fungi are an incredibly diverse and ubiquitous group of eukaryotes, and traditional identification depends on morphological differences in their sexual or asexual reproductive structures. A PCR/sequencing-based approach may provide a rapid and more accurate identification method. Coding regions of the 18S, 5.8S, and 28S nuclear rRNA genes evolve slowly, are relatively conserved among fungi, and provide a molecular basis of establishing phylogenetic relationships (White, et al., 1990). Between coding regions are the internal transcribed spacer 1 (ITS1) and regions 2 (ITS2), respectively. The ITS region evolved more rapidly and varies among different species within a genus. The ITS regions are located between the 18S and 28S rRNA genes and offer distinct advantages over other molecular targets including increased sensitivity due to the existence of approximately 100 copies per genome (*Aspergillus* species). PCR amplification may facilitate the identification of ITS region DNA sequences with sufficient polymorphism to be useful for identifying medically important fungal species (Chen et al., 2000, 2001; Travis et al., 2000). The sequence variation of ITS regions has led to their use in phylogenetic studies of many different organisms (Guarro and Stchigel, 1999).

HCV Genotyping by Nucleotide Sequencing

There are nearly 4 million persons infected with HCV in the United States, and it is estimated that 30,000 acute new infections will occur annually. Progression to chronic disease occurs in approximately 85% of individuals. Chronic HCV infection is known to progress to cirrhosis and hepatocellular carcinoma. Interferon alfa and ribavirin are used to treat this infecion. HCV genotyping is recommended after diagnosis of HCV. HCV is classified on the basis of the similarity of nucleotide sequence into major genetic groups designated genotypes. Recent studies have shown that HCV viral genotyping may be able to help in selecting therapeutic regimen and outcome of therapy. The reference standard and most definitive method for HCV genotyping is sequencing of a specific PCR-amplified portion of the HCV genome obtained from the patient, followed by phylogenetic analysis. Although all these methods are able to identify correctly the major genotypic groups, only direct nucleotide sequencing is efficient in discriminating among subtypes (Bukh et al., 1995; Simmonds, 1995).

The TruGene HCV 5′NC Genotyping Kit (Bayer Health Care, Berkeley, CA) has provided a method to obtain the sequence of the 5′ noncoding region of HCV viral RNA in plasma. RNA is extracted from plasma and a 244 base-pair sequence in the 5′NC region is amplified by reverse transcription and polymerase chain reaction (RT-PCR) in a single tube, one-step amplification technique. Sequencing reactions are generated from the amplified cDNA by CLIP sequencing. CLIP allows both directions of the cDNA to be sequenced simultaneously in the same tube using two different dye-labeled primers for each of the sequencing reactions. The CLIP sequencing ladders (each direction being labeled with one dye) are then detected on the OpenGene automated DNA sequencing system. The forward and reverse

sequences are combined and compared with the sequences of several genotypes of HCV strains with the GeneLibrarian software in order to determine the genotype of HCV.

Sequence-Based Bacterial Genome Typing

Spa Typing of *Staphylococcus aureus*

Many techniques are available to differentiate *S. aureus*, and specifically MRSA, isolates. Conventionally, isolates were distinguished by phenotypic methods, including antibiotic susceptibility testing and bacteriophage typing. Both methods have limitations, as genetically unrelated isolates commonly have the same antibiogram, and many *S. aureus* isolates are nontypeable by phage typing. With the advancement of molecular biology, strain typing focused on DNA-based methods: restriction endonuclease patterns of chromosomal or plasmid DNA, Southern blot hybridization using gene-specific probes, ribotyping, polymerase chain reaction (PCR)-based approaches, and pulsed-field gel electrophoresis (PFGE). These methods require subjective interpretation and comparison of patterns and fingerprint images. Nucleotide sequence analysis is an objective genotyping method; sequencing data can be easily stored and analyzed in a relational database. Recent advances in DNA sequencing technology have made it possible for sequencing to be considered as a viable typing method. Two different strategies have been used to provide genotyping data: multilocus sequence typing (MLST), which compares sequence variation in numerous housekeeping gene targets, and single-locus sequence typing, which compares sequence variation of a single target. MLST has been developed for *Neisseria gonorrhoeae*, *Streptococcus pneumoniae*, and *Staphylococcus aureus*, based on the classic multilocus enzyme electrophoresis (MLEE) method used to study the genetic variability of a species. Sequence analysis of five to seven housekeeping genes provides a database from which to infer relationships in somewhat distantly related isolates that have had substantial time to diversify. The MLST approach is not practical to be used in a clinical laboratory because it is labor intensive, time consuming, and costly. A single-locus target, if discriminating, provides an inexpensive, rapid, objective, genotyping method to subspeciate bacteria. Two *S. aureus* genes are conserved within the species, and Shopsin and Colleagues have developed the protein A (*spa*) (Shopsin 2001) and coagulase (*coa*) (Shopsin et al., 2000) procedures for sequence-based staphylococci strain typing. DNA sequencing of the short sequence repeat (SSR) region of the protein A gene (*spa*) has been used as an alternative to current techniques for the typing of *S. aureus*. The SSR consists of a variable number of 24-bp repeats and is located immediately upstream of the region encoding the C-terminal cell wall attachment sequence. The sequencing of the *spa* SSR region combines many of the advantages of a sequencing-based system such as MLST but may be more rapid and convenient for outbreak investigation in the hospital setting because *spa* typing involves a single locus. The coagulase gene (*coa*) variable region has been evaluated for use in conjunction with *spa* sequencing for the strain typing

of MRSA. The coagulase protein is an important virulence factor of *S. aureus*. Like *spa*, *coa* has a polymorphic repeat region that can be used for differentiating *S. aureus* isolates. The variable region of *coa* is composed of 81-bp tandem SSRs that are variable in both number and sequence, as determined by restriction fragment length polymorphism analysis of PCR products. Coagulase gene (*coa*) short sequence repeat region sequencing was used to measure relatedness among a collection of temporally and geographically diverse methicillin-resistant *S. aureus* isolates. The results show that *coa* typing is a useful addition to *spa* typing for analysis of *S. aureus*, including methicillin-resistant strains.

Pyrosequencing

Pyrosequencing, with its ability to rapidly sequence a short piece of DNA, has been evaluated for applications in many areas: GC strain typing (*porB* gene sequencing) (Magnus et al., 2004); linezolid resistance in enterococci (Sinclair et al., 2003); lamivudine-resistance in HBV (Lindström et al., 2004); monitoring HIV protease inhibitor resistance (O'meara et al., 2001); rapid identification of bacteria from positive blood culture (Jordan et al., 2005); and detection of HSV-1 and 2 (Adelson et al., 2005).

For 16S rRNA gene-based bacterial identification, a minimum of 200 bp or more is needed for any meaningful identification. However, many investigators have tried to use short representative regions for rapid identification, notably identification for mycobacteria and rapid ID for sepsis-related bacteria. Jordan et al., (2005) have studied the possibility of using pyrosequencing to identify a 15-base hypervariable region within the 16S rRNA gene for bacterial ID, as compared with 380-bp 16S rRNA fragment sequencing. The results were not very encouraging; the 380-bp sequencing can give species-level identification while the 15-bp pyrosequencing can only give semi-genus level identification, such as Staphylococcus, Streptococcus, or enteric Gram-negative rods. The 15-bp pyrosequencing did not do much better than a simple Gram-stain smear reviewed by an experienced clinical microbiologist.

A 30-bp pyrosequencing method was evaluated for *Mycobacterium* identification. When blasted against GenBank, 179 of 189 sequences (94.7%) were assigned isolates to the correct molecular genus or group. Pyrosequencing of this hypervariable region afforded rapid and acceptable characterization of common, routinely isolated clinical *Mycobacterium* spp. However, additional sequencing primer or additional biochemical tests may be needed for more accurate identification (Tuohy et al., 2005).

Pyrosequencing did very well to identify mutant genes associated with drug resistance. A pyrosequencing assay for the rapid characterization of resistance to HIV-1 protease inhibitors (O'meara et al., 2001) allows parallel analysis of 96 reactions in 1 h, facilitating the monitoring of drug resistance in eight patients simultaneously. Twelve pyrosequencing primers were designed and were evaluated on the MN strain and on viral DNA from peripheral blood mononuclear cells from

eight untreated HIV-1–infected individuals. The method had a limit of detection of 20% to 25% for minor sequence variants. Pattern recognition (i.e., comparing actual sequence data with expected wild-type and mutant sequence patterns) simplified the identification of minor sequence variants. This real-time pyrosequencing method was applied in a longitudinal study monitoring the development of PI resistance in plasma samples obtained from four patients over a $2\frac{1}{2}$-year period. Pyrosequencing identified eight primary protease inhibitor resistance mutations as well as several secondary mutations.

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17
Microarray-Based Microbial Identification and Characterization

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Introduction

The development of molecular-based methodologies over the past two decades has dramatically improved our ability to detect microorganisms in clinical and environmental samples—enabling detection and identification within hours in many cases. However, most of these methods are only capable of monitoring individual or small groups of organisms at a time. Due to the extreme microbial diversity in many environments, such as the human intestine (Eckburg et al., 2005), it is necessary to monitor hundreds to thousands of different microbial populations simultaneously in order to detect all of the organisms of interest as a whole and understand these communities more comprehensively. Microarrays have the unprecedented potential to achieve this objective as specific, sensitive, quantitative, and high-throughput tools for microbial detection, identification, and characterization. Advances in printing technology have enabled the production of microarrays containing thousands to hundreds of thousands of probes. Although microarrays have been primarily developed and used for gene expression profiling of pure cultures of individual organisms, major advances have recently been made in their application to complex environmental samples. This chapter discusses the basis of different microarray formats and their application to issues of clinical interest. Several reviews on microarray technology have recently been published and may provide additional information of interest (Ye et al., 2001; Zhou and Thompson, 2002; Cook and Sayler, 2003; Zhou, 2003; Bodrossy and Sessitsch, 2004; Schadt and Zhou, 2005; Schadt et al., 2005).

Principles and Types of Arrays

Conceptually, microarrays are an extension of traditional membrane-based Northern and Southern blots where a labeled probe molecule is hybridized to target DNA or RNA attached to a membrane. However, this process is reversed in microarray analysis with the probe attached to the support substrate, usually a nonporous solid surface, and the labeled DNA or RNA then hybridized to the probe. The major

TABLE 17.1. Selected properties of microarrays for microbial detection and characterization.

	Type of array			
Property	POA	FGA	CGA	MGA
Probe template	Ribosomal rRNA genes	Functional genes	Whole genome	Environmental DNA
Probe length	~18–25 nt	~ 50–70 nt oligos or ~200–1000 nt PCR products	Whole genome	~1000+ nt
Targeted microorganisms	Cultured & uncultured	Cultured & uncultured	Cultured	Cultured & uncultured
Information provided	Phylogenetic	Functional	Phylogenetic	Functional
Specificity	Species level or single nucleotide difference	< 80–90% sequence homology	Species – strain	≥Strain
Sensitivity (ng of pure genomic DNA)	~ 500[a]	~1–8	~0.2	Undetermined
Quantitative	Depends on array design[a]	Yes	Yes	Undetermined

POA, phylogenetic oligonucleotide array; FGA, functional gene array; CGA, community genome array; MGA, metagenomic array.
[a]Undetermined for POAs based on perfectly matched and mismatched probe pairs.
Adapted from Zhou (2003).

advantage of this approach is that the sample can be screened with thousands of probes simultaneously.

Arrays with potential application to diagnostic clinical research can be divided into at least four major categories based on what genes are represented on the array: (1) phylogenetic oligonucleotide arrays (POAs), which are designed based on a conserved marker such as the 16S rRNA gene and are used to detect specific organisms and compare the relatedness of microbial communities; (2) functional gene arrays (FGAs), which are designed for key functional genes involved in various physiological processes, such as antibiotic resistance, and provide information on the genes and microbial populations involved with these processes; (3) community genome arrays (CGAs), which contain the whole genomic DNA of cultured microorganisms and can describe an isolate or community based on its relationship to these cultivated organisms; and (4) metagenomic arrays (MGA), which contain probes produced directly from environmental DNA itself and can be a potentially powerful technique because, unlike the other arrays, they can applied with no prior sequence knowledge of the community (Table 17.1).

Phylogenetic Oligonucleotide Arrays.

Most POAs contain short oligonucleotide (oligo) probes representing genes with diagnostic value, usually the small-subunit ribosomal RNA (16S rRNA) gene.

These arrays are commonly used to detect the presence of specific bacteria in complex samples based on unique sequence regions. Several factors make 16S rRNA ideal for microbial identification and differentiation including (1) 16S rRNAs genes are found in all bacteria; (2) there is no evidence of horizontal transfer of these genes between organisms; and (3) the genes contain both conserved and variable regions, either of which can be used for probe selection depending on the objective of the study (Olsen et al., 1986). Additionally, there is a vast amount (>100,000 sequences) of rRNA sequence data available via the Ribosomal Database Project (RDP) (Cole et al., 2005).

Because some regions of rRNA genes are highly conserved, it is often necessary to use short oligos (~20-mers) for POAs in order for the probes to be specific to individual organisms. Using shorter probes, it is possible to discriminate a single mismatch in a probe-target hybridization (Zhou et al., 2004). A commonly used POA design strategy consists of arraying several probes that perfectly match a given target along with corresponding probes containing a single mismatch (usually at the central position) relative to the target (Wilson et al., 2002a; El Fantroussi et al., 2003; Peplies et al., 2003; Urakawa et al., 2003). Detection of the target sequence is indicated by greater signal intensity for the perfectly matched probes compared with the mismatched probes. Although this strategy enables very specific detection of target sequences, it does have some potential disadvantages, which we discuss in a later section on specificity.

One of the challenges for 16S rRNA-based analysis is the innate propensity of these molecules to form stable secondary structures that may interfere with hybridization and lead to false-negative results. For example, one study (Peplies et al., 2003) reported that 17 out of 41 expected hybridization events were not detected. This possibility can be reduced by incorporating one of numerous available software programs, such as Mfold (Zuker, 2003), which can identify self-complementarity in oligo probes into the probe design process. This difficulty can also be addressed by either fragmenting the target prior to hybridization or by the inclusion of helper-probes in the hybridization mixture. The helper-probes are designed to disrupt the local secondary structure by binding to the target molecule adjacent to the actual probe binding site. However, there is a risk that the helper-probe could cause nonspecific binding if it binds too closely to the actual probe binding site (Chandler et al., 2003). Furthermore, disruption of secondary structure in one region may result in the formation of secondary structures in other regions that could possibly affect the binding sites of other probes.

Functional Gene Arrays

In contrast with POAs, which are primarily used for the detection of specific microorganisms, FGAs target genes involved in some process of interest. FGAs can also be used to determine the expression of these genes by measuring mRNA, but only a limited number of studies have used FGAs for mRNA analysis (Dennis et al., 2003; Rhee et al., 2004) due to the technical challenges of mRNA isolation (SalehLakha et al., 2005). Thus, FGAs not only provide a degree of phylogenetic

classification but they also can give information on the genetic capacity for, or activity of, a given process in the specific environment being studied.

The initial and one of the most critical steps for FGA analysis is the selection of the genes to be targeted by the array. This will depend on the specific research objectives and characteristics of the sample to be analyzed. In contrast with POAs, there is a limited sequence data available for many functional genes. This is an important consideration when selecting a gene(s) for inclusion on a FGA. This may also necessitate the generation of clone libraries for the gene and environment under study in order to obtain the requisite sequence information for probe design. Characteristics of an ideal target gene for an FGA include (1) it encodes a critical enzyme or protein in the process of interest; (2) its sequence is evolutionarily conserved but with sufficient divergence in different microorganisms to allow the design of species-specific probes; and (3) it has a wide spectrum of published sequence data from isolated organisms and environmental samples.

Once the target genes are selected, it is necessary to decide what type of probes will be used on the array. The molecules most commonly used as probes are PCR amplification products (∼200–1000 bp) and shorter, synthesized oligos (∼20–70 nt). The PCR amplicon probes have the advantage of being amplifiable from their source organisms via conserved primers without the need for specific sequence knowledge. These probes tend to be more sensitive than the shorter oligo probes (He et al., 2005), but they also have higher potential for cross-hybridization. Furthermore, depending on the number of organisms or genes to be represented on the array, it can be virtually impossible to acquire all of the necessary isolates and clones from their various sources in order to produce a comprehensive PCR amplicon-based array. Perhaps the greatest advantage of the oligo probes is that they can be designed and synthesized directly from available sequence data. This also enables greater control and flexibility in the design process, such as the ability to avoid highly conserved regions of genes.

Several factors that can affect probe specificity and thus should be considered during probe design include (1) nucleotide similarity of probe with nontarget sequences; (2) long stretches of a probe that are complementary to a nontarget sequence, which can lead to substantial nonspecific hybridization (Kane et al., 2000; Hughes et al., 2001); (3) the position of mismatches—more specific binding occurs when the mismatches are those distributed across the probe instead of concentrated in a single location (Letowski et al., 2004); and (4) the amount of free energy of probe-target duplexes (Li and Stormo, 2001; Held et al., 2003; Taroncher-Oldenburg et al., 2003). Specificity is also affected by the hybridization conditions (temperature, formamide concentration, salt concentration, etc.).

Recent research has shown that specific probes for FGAs could be produced using more relaxed design criteria when multiple probe-target characteristics were simultaneously considered during the design process (Liebich et al., unpublished). This indicated that specific hybridization at 50°C with 50% formamide could be achieved using 50-mer probes with a free energy release of $\leq$–35 kcal/mol and $\leq$90% similarity and $\leq$20 bp continuous stretches to nontarget sequences. Relaxing

the design criteria, even slightly, should increase the percentage of target genes for which probes can be designed.

Several software programs are currently available for the design of FGA oligo probes including: ArrayOligoSelector (Bozdech et al., 2003); OligoArray (Rouillard et al., 2002); OligoArray 2.0 (Rouillard et al., 2003); Oligopicker (Wang and Seed, 2003); OligoWiz (Nielsen et al., 2003); PRIMEGENS (Xu et al., 2002); PROBEmer (Emrich et al., 2003); ProbeSelect (Li and Stormo, 2001); and ROSO (Reymond et al., 2004). Although these programs work well for designing probes from whole-genome sequences, recent research in J. Zhou's laboratory (Li et al., unpublished) has found that a large portion of the probes designed by some of these programs from orthologous gene sequences, such as those produced by clone libraries, were not specific to the target sequence. Therefore, a new probe design software program, called CommOligo, was designed to correct this problem (Li et al., unpublished). This program incorporates a new global alignment algorithm to identify unique probes for each gene using multiple, simultaneously evaluated criteria that can be defined by the user. One major advantage of CommOligo is that it can also design group-specific probes for sets of sequences that are too similar for the design of unique probes, thus increasing the number of sequences covered by probes. Until improved design software is available, researchers should exercise caution when using software that was originally designed for use with whole-genome data for the design of probes from environmental sequences.

In addition to the actual experimental probes, it may be beneficial to design and include control probes on the array that have varying similarity to a control sequence. The control DNA can then be spiked into the hybridization solution to ensure that the correct hybridization stringency is achieved.

Community Genome Arrays

Unlike the other types of arrays, entire genomes of isolated organisms are used as probes for CGAs. These arrays are conceptually equivalent to membrane-based reverse sample genome probing (RSGP) (Greene and Voordouw, 2003), but they use a nonporous hybridization surface and fluorescence-based detection, which allows for high-throughput analyses but reduces sensitivity (Wu et al., 2004). CGAs can achieve strain-level differentiation of isolates and thus can be used to ascertain the genomic similarity of isolated bacteria or microbial communities in relation to the organisms represented on the array. The primary disadvantage of CGAs is that only cultured organisms (probes) are included on the arrays.

Metagenomic Arrays

Instead of genomic DNA from cultured organisms, MGAs use DNAs directly cloned from an environment of interest as the probes. This approach has only been used on a limited scale (Sebat et al., 2003), but it has great potential for many applications because the vast amount of unknown sequences in many samples

is one of the primary limitations for microarray analysis. This approach could also be used to produce a site-specific FGA for measuring microbial activity if sufficient mRNA could be obtained and reverse-transcribed to cDNA for the array probes.

Applications

The high-throughput capacity of microarray technology has numerous applications in diagnostic microbiology, specifically in relation to rapid pathogen detection, identification, and characterization. Many of these methods also have utility for clinical research. The following are specific examples of recent and emerging microarray applications, but the technology can be used to investigate virtually any microorganism or microbial process.

Microbial Detection and Identification

One of the potentially most powerful clinical uses of microarrays is for the rapid, simultaneous assay and detection of thousands of microorganisms. Arrays have been designed and used to detect many pathogenic microorganisms including bacteria, viruses, fungi, and protozoa (Straub et al., 2002; Wilson et al., 2002b; Diaz and Fell, 2005; Korimbocus et al., 2005). Although PCR-amplicons or longer oligos (50–70-mers) have been successfully used for arrays of limited scope, it may be necessary to use shorter oligo probes (~20-mers) for more comprehensive arrays due to reasons previously discussed. One of the most comprehensive arrays published to date for microbial detection was a POA containing 31,179 hierarchical probes perfectly matching their targets (and a corresponding number of mismatched probes) representing 1945 prokaryotic and 431 eukaryotic sequences (Wilson et al., 2002a). The array could successfully identify 15 of the 17 tested pure bacterial cultures. The diagnostic ability of the POA was then tested using microorganisms collected from a 1.4-million-liter air sample. Although the results generally agreed with those from an rDNA clone library, the array could only resolve differences to the third level of phylogenetic rank, as defined by RDP, and could not identify individual species. This illustrates the challenge for developing comprehensive, yet highly specific arrays for use with complex samples. The approach above used universal primers to amplify a single region of the target DNA for hybridization. An alternative approach, which may improve detection specificity, is to use species-specific primers to amplify multiple diagnostic regions (e.g., pathogenicity and virulence genes) for each organism of interest and then hybridize the pooled products to an array containing tiled probes covering the entirety of each of the diagnostic regions (Wilson et al., 2002b). In addition to non–culture-based detection, microarrays can also be used to specifically genotype isolated organisms (or enriched sequences) to the strain level or even differentiate point mutations depending on the array format (Vinje and Koopmans, 2000; Straub et al., 2002; Willse et al., 2004; Wu et al., 2004; Lin et al., 2005).

Community Dynamics and Activity

Microarrays now provide the researcher with the unprecedented ability to detect and monitor most, if not all, of the populations even in complex communities such as the human intestine. Microorganisms inhabiting the intestines play instrumental roles in the maintenance of health and development of disease, yet only recently have they begun to be investigated on a large scale (Eckburg et al., 2005). Researchers have used microarrays (primarily POAs) to monitor dominant bacteria and those with certain phenotypes (e.g., production of carcinogens) in intestinal and fecal samples (Wang et al., 2002, 2004a, 2004b), but to our knowledge, the communities in these environments have yet to be analyzed with comprehensive arrays representing thousands of organisms. Similarly, microarrays have also been used to measure gene expression in the intestine, but these studies have largely used human gene–based arrays to determine the response to beneficial or pathogenic bacteria (Caro et al., 2005; Galindo et al., 2005). Microarrays, specifically FGAs, could likewise be used to determine the activity of specific microbial populations (or even communities) in the intestines of different hosts or in response to different diets or stimuli (Stintzi et al., 2005).

Antibiotic Resistance

FGAs can be used to detect virtually any gene of interest including those encoding pathogenicity and virulence factors such as antibiotic resistance (Korczak et al., 2005). In contrast with traditional antibiotic resistance assays, which require isolation and growth of the organisms of interest, microarrays have the potential to rapidly screen a sample for the presence of multiple antibiotic resistance genes without the need for culturing. However, direct detection without amplification may be more applicable to antibiotic efflux- or modification-based resistance mechanisms than to mutation-based resistance given the difficulty in differentiating single (or a few) nucleotide differences with the longer probes, which provide better sensitivity. Very short oligo ($\sim$20-mers) are more appropriate for detecting resistance due to mutations but will likely require amplification of these specific genes. Once the antibiotic resistant organisms are isolated, FGAs can be used to genotype the antibiotic resistance genes, potentially revealing information on the gene's development and/or acquisition (Troesch et al., 1999; Call et al., 2003; Grimm et al., 2004; Perreten et al., 2005). This has application not only to clinical diagnostics but also to any research or regulatory program concerned with the spread of antibiotic resistance genes and the development of multidrug-resistant bacteria.

Challenges for Microarray Analysis

Clinical and environmental samples present several challenges for microarray analysis that are not encountered during the analysis of pure cultures. Although several recent studies have used microarrays to interrogate these types of samples, many

analytical challenges remain with respect to sensitivity, specificity, quantitation, and data analysis.

Specificity

One of the major challenges for microarray analysis is the design of probes specific to a given target. This is largely due to the conserved nature of many genes and the large amount of unknown sequence data present in many samples. Although longer probes may increase sensitivity, they also increase the potential for cross-hybridization with nontarget sequences. By using oligo probes, it is possible to avoid conserved regions of genes or areas containing stable secondary structure during the probe design process. The shorter oligo probes (~20-mers) can differentiate a single mismatch in a probe-target hybridization, making them ideal for use with highly conserved genes such as 16S rRNA in POAs (Wilson et al., 2002a; Urakawa et al., 2003; Zhou et al., 2004). A common format for these arrays includes sets of probes that perfectly match a target sequence and corresponding sets of probes containing one mismatched nucleotide, usually at a central position. Greater signal intensity for the perfect probes versus the mismatched probes indicates detection of the target sequence. Even though the mismatched probes typically have greatly decreased ability to bind the target of interest (Zhou et al., 2004), spurious results are sometimes obtained. This is likely due to the presence of similar yet unknown sequences and can make it difficult to achieve complete discrimination. One way to address this problem is to design and use multiple perfectly matched and mismatched probe combinations for each organism or gene of interest. The results from the probe pairs are then compared statistically, and those with abnormal results (higher signal intensity for the mismatched probe) are discarded during data analysis. It may also be possible to improve the differentiation of perfectly matched and mismatched probes by determining the thermal dissociation curve for each probe–target hybridization on a three-dimensional array platform (Liu et al., 2001; El Fantroussi et al., 2003; Urakawa et al., 2003), but this may be difficult for high-density planar arrays given the current technology.

Most functional genes are more variable than rRNA genes thus enabling the use of longer oligo probes (~40- to 70-mers), which have greater sensitivity, while still achieving species-level specificity. These longer oligo-based probes have been reported to discriminate sequences less than 80–90% similar to the probes (Taroncher-Oldenburg et al., 2003; Rhee et al., 2004). Specificity can also be increased or decreased, to an extent, by adjusting the stringency of the hybridization conditions (temperature, formamide concentration, salt concentration, etc.) (Wu et al., 2004). However, caution should be exercised when using an array under more or less stringent conditions than that for which it was designed, as this could cause overestimated or underestimated results and ultimately inaccurate conclusions.

Sensitivity

The different array types have not been directly compared with regard to sensitivity, but limits of 0.2 ng of target genomic DNA for a CGA (Wu et al., 2004), 1 ng for a

PCR-based FGA (Wu et al., 2001), and 5–8 ng for 50-mer oligo FGAs (Rhee et al., 2004; Tiquia et al., 2004) in the absence of background DNA have been reported. However, when background DNA is added to simulate environmental samples, these sensitivities are decreased around 10-fold (Rhee et al., 2004; Tiquia et al., 2004; Wu et al., 2004). The relative sensitivity of the array is correlated with probe length with shorter oligo probes typically being ~10- to 100-fold less sensitive than longer PCR-based or CGA probes (Wu et al., 2001, 2004; Denef et al., 2003; Rhee et al., 2004; He et al., 2005). Depending on the specific research objective, it may be desirable to use probes that are as long as reasonably possible without compromising specificity.

The most common approach used to increase sensitivity and detect less dominant populations is to PCR-amplify these specific organisms or groups. However, this potentially introduces other well-documented biases and limitations, making it preferable to avoid amplification if possible (Reysenbach et al., 1992; Farrelly et al., 1995; Crosby and Criddle, 2003). Magnetic beads or other capture techniques may also be useful for selecting specific populations (Tsai et al., 2003).

Although some populations or genes can be amplified with specific PCR primers as mentioned above, this option is not available for all genes and may not be the optimal approach if hundreds to thousands of genes are being simultaneously considered. In these situations, a nonspecific amplification approach is needed to amplify the community DNA or RNA. A whole community genome amplification (WCGA) procedure based on rolling circle amplification has recently been developed for use with microarray analysis (Wu et al., 2005). The method representatively detected individual genes or genomes starting from 1 to 100 ng DNA of individual or mixed genomes of equal or unequal abundance, and 1 to 500 ng of environmental DNAs. It could detect initial target DNA concentrations as low as 10 fg, but the representativeness of amplification was affected by the lower template concentrations. Hybridization of amplification products to several types of arrays indicated significantly linear relationships between initial DNA concentration and signal intensity across a range of DNA concentrations from pure cultures ($r^2 = 0.65$ to 0.99) and environmental samples ($r^2 = 0.96$ to 0.98). Other researchers are developing methods for amplification of prokaryotic mRNA including one approach that adds a poly(A) tail to the mRNA for subsequent amplification (Botero et al., 2005).

Researchers have used different nucleic acid labeling methods to increase sensitivity (Denef et al., 2003; Steward et al., 2004; Zhou and Zhou, 2004). For example, one study (Denef et al., 2003) used tyramide signal amplification labeling to increase the signal intensity of a 70-mer FGA ~10-fold, compared with the commonly used Cy dye-labeling techniques, which ultimately lowered the detection limit to ~1% of cells in the total community. Planar glass slides are commonly used for microarray analysis because they enable higher printing densities than the three-dimensional arrays. However, hybridization on these nonporous surfaces are several orders of magnitude less sensitive than membrane-based hybridizations due to the limited amount of probe material that can be attached to the nonorous surfaces (Cho and Tiedje, 2002). The development and use of new slide chemistries,

including ultrathin three-dimensional platforms, which enable increased binding capacities with high-density arrays, may also help to increase sensitivity (Guschin et al., 1997; Urakawa et al., 2003; Zhou et al., 2004).

Quantitation and Data Analysis

Due to the potential variability in steps including DNA extraction, labeling, hybridization, and analysis, there has been some debate whether microarray analysis is quantitative. Recent research has indicated that some array formats, including FGAs and CGAs, can be quantitative over a range of concentrations (Wu et al., 2001; Rhee et al., 2004; Wu et al., 2004). However, it is not currently known if arrays based on perfect match–mismatch probes sets (e.g., POAs) are quantitative.

Due to the same variations mentioned above, it can be difficult to compare data between, and even within, microarray experiments. Techniques such as the two-color dye-swap method works well for measuring relative levels of gene expression in pure cultures, but these may not be directly applicable to the types of analyses needed for many clinical and environmental samples. Different methods are needed to standardize data between slides and experiments. Alternative approaches have been developed where known amounts of labeled DNA fragments or oligos are spiked into the hybridization solution as a control (Cho and Tiedje, 2002; Dudley et al., 2002; Bodrossy et al., 2003). The array results are then normalized based on the hybridization signal intensity of this control DNA and corresponding control probes on the array.

However, it could be difficult to quantitatively correlate differences in hybridization signals with changes in specific microorganisms or genes due to the large amount of unknown nucleic acid sequences in many clinical and environmental samples—even if the microarray probes and experiments have been carefully designed and performed. Although it is typically assumed that the abundance of the target organism is directly proportional to the observed microarray hybridization signal intensity, nonspecific hybridization to uncharacterized microorganisms in the samples could occur and complicate interpretation. It may be beneficial to analyze key genes in selected samples with other methods, such as real-time PCR, to validate the conclusions drawn from microarray data (Rhee et al., 2004).

Conclusion

Microarrays have the unprecedented potential to simultaneously detect and monitor thousands of genes or organisms. Although microarrays were primarily developed for measuring gene expression in pure cultures, considerable effort has been expended over the past few years to adapt the technology for other applications. Several recent experiments have applied microarray technology to issues relevant to clinical microbiology including the rapid detection and identification of microorganisms and the genotyping of antibiotic resistance genes. The integration of microarray technology into clinical diagnostics is still in the early stages.

Although methodological and technological improvements are needed to broaden the applicability of the technology, future advances will undoubtedly expand the use of microarray technology in the clinical laboratory.

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18
Diagnostic Microbiology Using Real-Time PCR Based on FRET Technology

XUAN QIN

Introduction

Molecular amplification of specific nucleic acid–based targets associated with microbial organisms has advanced our existing tools in infectious diseases diagnosis (Fredricks, 1999; Louie, 2000; Peruski, 2003; Yang, 2004). Laboratory diagnosis of infectious diseases in the past has relied on cultivation of the microorganisms *in vitro*. Hence the viability of the organisms and the laboratory conditions used to mimic the *in vivo* environment ultimately dictates the successfulness of *in vitro* amplification of the intact organism(s) outside of the infected host. Nucleic acid–based technologies allow detection by amplification of specific microbial genetic material irrespective of viability or integrity of the organism (Nissen, 2002; Gulliken, 2004; Mackay, 2004).

Fluorescence resonance energy transfer (FRET)-based nucleic acid amplification technology has emerged from the marriage between polymerase chain reaction (PCR) and real-time monitoring of fluorescent chemistry. Historically, fluorescence-based detection is well established in the biosciences and has successfully replaced radioactive isotope labeling. Fluorescent dyes have an "environmental advantage": they have a longer shelf life, are inexpensive to discard, and safer to handle. Fluorescence detection of nucleic acid molecules itself is not more sensitive than immunological or radioactive isotope detection. In fact, in most nano-detection systems, radioactive labeling displays 10- to a 1000-fold higher sensitivity.

There are two major factors that make fluorescent real-time PCR advantageous. One is the potential of multiple parallel measurements by using different-colored dyes and the other is the potential of time-resolved continuous data acquisition. The increase of fluorescent signal is detectable in a closed system during amplification cycles. Therefore, the absolute sensitivity is no longer a decisive factor for these detection methods, particularly because most applications are based on nucleic acid amplification.

Real-time PCR monitors the fluorescence emitted during the reaction as an indicator of amplicon accumulated by every PCR cycle as opposed to the end-point analysis (Higuchi, 1992, 1993). Various FRET systems and instrumentation that

allow real-time monitoring of fluorescence within PCR vessels have shown great promise in infectious diseases diagnosis. The significance of real-time PCR as a diagnostic tool can be summarized in two important aspects. First, determination of amplification products of the targets by probes and melting analysis is highly accurate compared with size analysis by post-PCR gel electrophoresis that is prone to amplicon carry-over contamination. Second, quantitative analysis of a wide range of concentration levels of the initial target material is made possible by progressive monitoring of the dynamic accumulation of FRET signals over time, provided that the appropriate standards are available. Several real-time PCR platforms have been employed and marketed to meet various demands of assays designed for specific analysis.

Principles of FRET Technique

The real-time PCR system is based on the detection and quantitative measurement of fluorescent reporter molecules either intercalated between DNA double helix or covalently attached to specific probes (Lee, 1993; Livak, 1995). Fluorescent signal increases in direct proportion to the amount of PCR product in a reaction. The time or PCR cycle where the fluorescence signal significantly increases above background is in proportion to the initial amount of starting material in the sample well. By monitoring the amount of fluorescence emitted at the end of each cycle, it is possible to capture the PCR reaction during its exponential phase. The increase of fluorescence in the logarithmic phase can be extrapolated from a common trapezoidal curve (Fig. 18.1) where the first significant increase in the amount of PCR product correlates to the initial amount of template materials. The higher the starting copy number of the nucleic acid target, the fewer cycles needed to register a significant increase in fluorescence (C_t: cycle time at

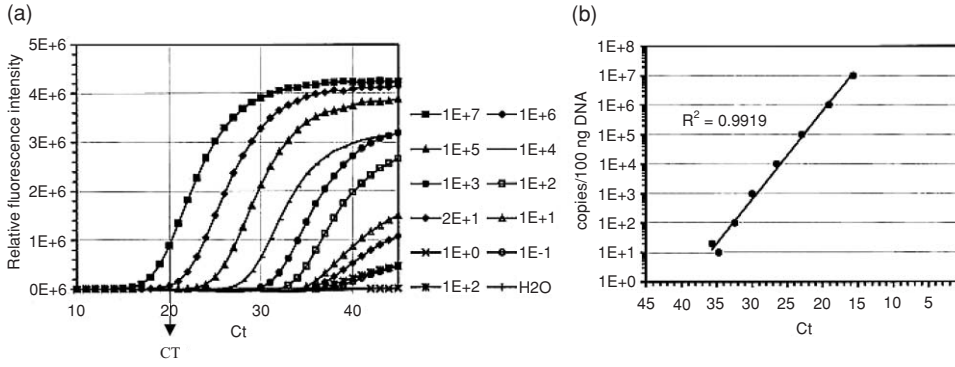

FIGURE 18.1. Quantitative PCR. (a) CT, cycle at which the observed fluorescence is 10-fold above backround (10× amplification). (b) Generating a standard curve using known amounts of target DNA and measuring the CT for each reaction.

TABLE 18.1. Comparison of four fluorescence resonance energy transfer (FRET) real-time PCR applications.

FRET real-time PCR	Application	Advantages	Disadvantages	Additional considerations	Examples
Intercalating dyes (SYBR Green)	Quantitative and qualitative measurement.	• Sensitive • Easy to design/optimize • Inexpensive • Accommodate polymorphism internal to amplicon	Signal measurement may not be specific without melt-peak analysis	Melt-peak analysis.	Bacterial and viral detection (qualitative or quantitative) when more than one target is analyzed with melt-peak analysis. Bacterial resistance gene detection such as *mecA, vanA*, and *vanB*.
TaqMan probes	Quantitative and qualitative measurement.	• Very specific for the target sequences • Good for viral load analysis	Do not tolerate polymorphism in the probe region.	Choose non-polymorphic targets and design probes with knowledge.	Viral load analysis and assays that need copy number precision.
Dual hybridization probes	Quantitative and qualitative measurement in conjunction with mutational analysis.	• Very specific for the target sequences • Useful for mutational analysis • Multiplexing	The size of the amplicon needs to be long enough to accommodate the two probes.	Choose target sequences with known mismatches for mutational analysis.	Viral load as well as point mutation analysis in CMV and HIV diagnosis.
Molecular beacons	Quantitative and qualitative measurement with an emphasis on mutation detection.	Mutational analysis made easy by real-time quantitative measurement and multiplexing.	The mutational base(s) has to be known or known to reside in the region.	Choose target sequences with knowledge of the hot spot mutations covered by the loop region of the probe.	The rifampin resistance mutations in *rpoB* of *M. tuberculosis* and drug-resistant hepatitis B viral variants.

which the observed fluorescence is 10-fold above background). Different detection chemistries are developed concerning various diagnostic preferences, which may include DNA intercalating dyes, hydrolysis probes, hybridization probes, and molecular beacons (Table 18.1).

Intercalating Dyes

A simple and cheaper detection method in real-time PCR requires a dye that emits fluorescent light when intercalated into double-stranded DNA (dsDNA). The light unit of the fluorescence signal is proportional to the amount of all dsDNA present in the reaction, including specific, nonspecific amplification products and primer–dimer complex. Therefore, this method is not a sequence-specific fluorescent measurement. However, the dye employed does not bind to single-stranded DNA (ssDNA). SYBR Green is a fluorogenic minor groove binding dye that exhibits little fluorescence when in solution but emits a strong fluorescent signal upon binding to double-stranded DNA (Morrison, 1998). Because these dyes do not make a distinction between the various dsDNA molecules in a PCR reaction, the production of nonspecific amplicons must be prevented. Therefore, primer design and optimization of the reaction conditions require extensive pilot testing. Melt-curve analysis after completion of a PCR reaction can provide additional specificity with standard controls (Ririe, 1997).

Hydrolysis Probes (TaqMan Probes)

The hydrolysis or TaqMan probe chemistry depends on the 5′–3′ exonuclease activity of the engineered *Thermus aquaticus* DNA-polymerase (Fig.18.2a). A DNA probe, labeled with a reporter dye and a quencher dye at opposite ends of the sequence, is designed to hybridize internal to the amplicon (Hiyoshi, 1994; Chen, 1997). When irradiated in the absence of a specific amplicon, the excited fluorescent dye transfers energy to the nearby quenching dye molecule (this is called FRET) rather than fluorescing. Thus, the close proximity of the reporter and quencher prevents emission of any fluorescence while the probe is intact. In the presence of specific amplicon, as the polymerase replicates a template on which a TaqMan probe is bound, its 5′ exonuclease activity cleaves the probe (Holland, 1991). Departuring from the activity of quencher (no FRET), the reporter dye starts to emit fluorescence that increases in each cycle growing at the rate of probe cleavage. Accumulation of PCR products is detected by monitoring the increase in fluorescence of the reporter dye (note that primers are not labeled). The probe is usually longer than the primers (20–30 bases long with a T_m value of 10°C higher) that contain a fluorescent dye preferred on the 5′ base and a quenching dye typically on the 3′ base. FAM (6-carboxyfluorescein) and TAMRA (6-carboxy-tetramethyl-rhodamin) are most frequently used as reporter and as quencher, respectively. The process of hybridization and cleavage does not interfere with the exponential accumulation of the amplification product (the probe and primer sites do not overlap). One specific requirement for fluorogenic probes is

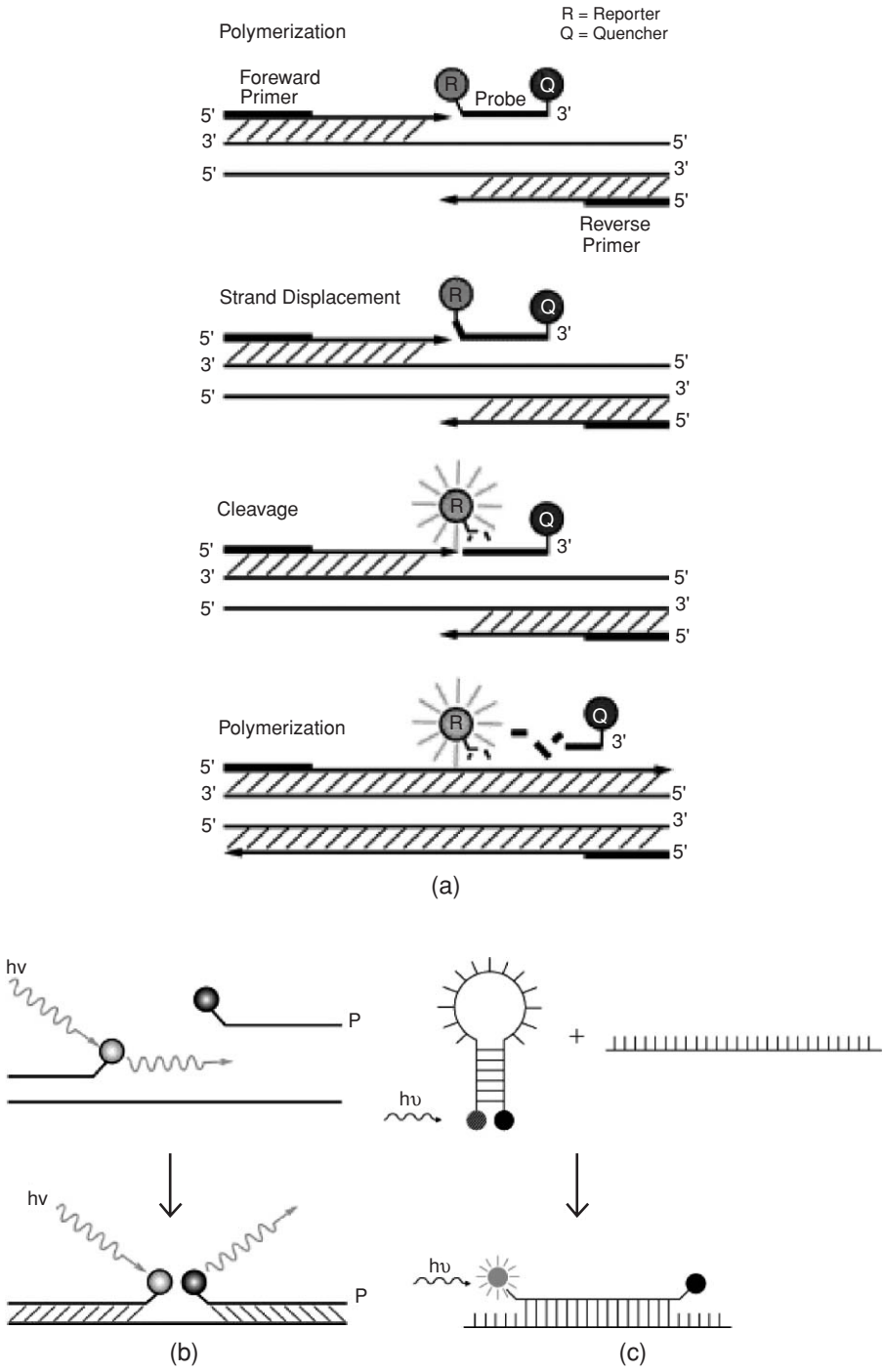

FIGURE 18.2. (a) TaqMan PCR probes. (b) FRET Probes. (c) Molecular beacons.

that there would be no G at the 5′ end. A "G" adjacent to the reporter dye quenches reporter fluorescence even after cleavage.

TaqMan probes are relatively sensitive to single base variations (mismatch). This could be extremely important when amplifying biological samples, where such a genetic variability could be present that a successful amplification may fail to result in a positive signal. Unfortunately, this sensitivity may render TaqMan probes inappropriate for genotyping, because a "nonsignal" will have to be attributed to an unknown genotype.

Dual Hybridization Probes

This detection method relies on FRET of two adjacent oligonucleotide probes (Fig. 18.2b). When both probes are specifically bound to the target amplicon, the energy emitted by the donor dyes excites the acceptor dye of the second probe, which then emits fluorescent light at a longer wavelength. One probe is labeled with a donor fluorochrome (fluorescein) at the 3′ end, and the other probe is labeled with an acceptor dye (Cy5, LC Red 640) at the 5′ end. Both probes can hybridize to the target sequences, and the two probes are usually no more than 3 bases apart (4 to 25 Å molecular distance). The first dye (fluorescein) is excited by the LED (light emitting diode) filtered light source and emits green fluorescent light at a slightly longer wavelength. When the two dyes are in close proximity as the probes simultaneously hybridize to their target, the emitted energy excites the acceptor (i.e., LC Red 640) attached to the second hybridization probe that subsequently emits red fluorescent light at a longer wavelength. The occurrence of FRET is characterized by a decrease in observed donor emission and a simultaneously increased acceptor emission. The ratio between donor fluorescence and acceptor fluorescence increases during the PCR and is proportional to the amount of target DNA generated (Wittwer, 1997; Nitsche, 1999).

An advantage hybridization probes thus have over the hydrolysis probes is their relative tolerance to single base variations; therefore their suitability for genotyping in combination with melt-peak analysis. A disadvantage is the need for a larger sequence area necessary to accommodate two adjacent probes.

Molecular Beacons

Molecular beacons are stem-loop (hairpin) shaped hybridization probes with a fluorescent dye and a quencher dye on the opposite extremities brought to their proximity by the complementary stem (Fig. 18.2c). The commonly used fluorescent dyes are FAM, TAMRA, TET, and ROX paired with a quenching dye, typically DABCYL. While in the absence of amplicon target, the FRET between the fluorescent dye and the quencher prevents light excitation and emission. In the presence of amplicon target, the complementary loop fragment of the probe is able to hybridize to the template sequence and stretches out the two ends, thus diminishing the quenching effect and resulting in detectable fluorescence (FRET does not occur).

Because the hybrid hairpin configuration is very thermostable, molecular beacons have a high specificity to hybridize to a target, which are used to distinguish single nucleotide differences. Therefore, molecular beacons are suitable for mutation analysis and single nucleotide polymorphism detection when specific mutations are known (McKilli, 2000; Szuhai, 2001; Abravaya, 2003; Bustamante, 2004; Petersen, 2004; Vet, 2005).

All real-time PCR chemistries allow detection of multiple DNA species (multiplexing) by designing each probe/beacon with a spectrally unique fluor/quench pair. All of the above can be used in conjunction to melting curve analysis or when SYBR Green is used only. By multiplexing, the target(s) and endogenous control can be amplified in a single tube. (Bernard, 1998; Lee, 1999; Vet, 1999; Elnifro, 2000; Read, 2001; Grace, 2003; Rickert, 2004)

Real-Time PCR in Infectious Disease Diagnosis

Molecular diagnostic tools and detection methods such as nucleic acid amplification are being used increasingly in the clinical microbiology laboratory to enhance the diagnosis of microbial pathogens (Lanciotti, 2001; Mackay, 2004). Nucleic acid–based technology is also used to assess drug resistance and epidemiological surveillance (Piatek, 1998; Makinen, 2001; Huletsky, 2004; Sloan, 2004). The principle of the real-time PCR is primarily used to detect and amplify a unique gene or a signature sequence of the microorganism. Quantitative measurements of viral load can also be made simple. Sensitive detection and accurate identification can speed up reporting of microbial pathogens without reliance on their phenotypic characteristics or viability after antibiotic treatment.

The application of real-time PCR in infectious diseases enables the diagnosis of microbial pathogens both with accuracy and expediency. The clinical significance of using molecular diagnosis of infectious agents can be characterized by the following aspects. (1) Pathogens that show fastidious slow growth or inability to grow *in vitro*: *Mycobacterium*, *Legionella, Bartonella, Leptospira, Borrelia, Bordetella, Mycoplasma*, and *Tropheryma whippelii* may require days or weeks of incubation under specific conditions; (2) obligatory intracellular organisms (*Chlamydia, Rickettsia, Coxiella, Ehrlichia,* DNA and RNA viruses); (3) prior antibiotic use; (4) biochemically inert for phenotypic characterization; (5) additional waiting time for drug-resistance determination, (6) diagnostic speed from bench to bedside.

Qualitative real-time amplification has outpaced conventional culture methods in detection of a long list of specific pathogens that are difficult to cultivate: *Bartonella henselae, Bordetella pertussis, Borrelia burgdorferi, Coxiella burnetii, Ehrlichia* spp., *Legionella* spp., *Mycoplasma pneumoniae, Chlamydia trachomatis, Rickettsia, Toxoplasma gondii, Microsporidium, Cryptosporidium, Tropheryma whippelii, Mycobacterium tuberculosis* and its drug-resistant determinants (Franzen, 1999; Pretorius, 2000; Hammerschlag, 2001; Bell, 2002; Fournier, 2002; Gerard, 2002; Kovacova, 2002; Exner, 2003; Templeton, 2003; Wang, 2003; Fenollar, 2004; Koenig, 2004; Simon, 2004; Wada, 2004; Khanna,

2005). Furthermore, the rapid turn-around time supported by real-time PCR may directly benefit the patient care and reduce mortality in areas of invasive infections caused by common pathogens. The examples are infective meningitis of bacterial or viral etiology, such as *Streptococcus pneumoniae, Streptococcus agalactiae, Neisseria meningitidis, Haemophilus influenzae, Listeria monocytogenes*, enteroviruses, herpes simplex viruses (HSV), and so forth (Corless, 2001; van Haeften, 2003; Archimbaud, 2004; Bryant, 2004; Guarner, 2004; Mengelle, 2004; Mohamed, 2004; Picard, 2004; Uzuka, 2004; Aberle, 2005).

Quantitative measurement of viral load using real-time PCR is another significant methodology improvement, and its diagnostic implication is infinite. First of all, HIV viral copy numbers in blood and body fluids are important disease and treatment markers directly tied into actions of clinical management. RNA reverse transcription and PCR (RT-PCR) can be established in a single-tube reaction, and copy numbers can be extrapolated from a standard curve in a single run (Kostrikis, 2002; Erikkson, 2003; Lee, 2004; Watzinger, 2004). Similarly, other viral etiology such as cytomegalovirus (CMV; Jebbink, 2003), HSV-1, HSV-2, varicella-zoster virus (VZV), Epstein–Barr virus (EBV; Legoff, 2004), parvovirus B19 (Hokynar, 2004; Plentz, 2004; Liefeldt, 2005), human polyomaviruses of BK and JC, and human herpesviruses 6, 7, and 8 can be measured both qualitatively and quantitatively according to clinical needs (Whiley, 2001; Beck, 2004; Watzinger, 2004). RT-PCR can be performed to detect and quantify hepatitis A virus (HAV Costa-Mattioli, 2002), hepatitis B (HBV; Payungporn, 2004; Sum, 2004; Yeh, 2004; Pas, 2005; Zhao, 2005), and hepatitis C (HCV; Candotti, 2004; Castelain, 2004; Cook, 2004; Koidl, 2004; Walkins-Riedel, 2004) in whole-blood samples. Real-time RT-PCR panels are increasingly becoming commonplace for respiratory viral diagnosis of influenza A and B viruses, parainfluenza viruses, human adenoviruses, human metapneumovirus, and respiratory syncytial virus, respectively (Kahn, 2003; Boivin, 2004; Cattoli, 2004; Daum, 2004; Frisbie, 2004; Moore, 2004; O'shea, 2004; Stone, 2004; Templeton, 2004; Ward, 2004).

Despite apparent high sensitivity and specificity, molecular amplification techniques are not error-free. Contamination as a result of amplicon carry-over is a top concern of its practice in clinical diagnostics. Physical and chemical control of amplified products has to be designed and implemented before the tests are validated. Strict separation of pre- and post-PCR (negative-pressure room for post-PCR analysis) environments through laboratory design and personnel training has to be the first step. Amplification chemistry employing uracil and uracil-*N*-glycosylase is an effective end-product degradation control (Pennings, 2001; Pierce, 2004). Second, microbial DNA extraction is a rate-limiting step deciding the ultimate test sensitivity. The wide spectrum of cell wall makeup pertaining to specific microorganisms makes it impossible to limit the method of extraction to any single standard approach. Specific emphasis has to be made to optimally recover DNA materials from certain species of bacteria or parasites. Sonication and/or freeze–thaw methods can be used in conjunction with enzyme digestion for DNA extraction from mycobacteria and cyst-forming protozoa parasites (Harris, 1999; Kostrzynska, 1999; Lanigan, 2004). Third, sampling error is intrinsic to PCR-based approach

due to its small specimen input nature. A false-negative reaction can be a result of low copy-number of the target material or simply missing the target material from the infected foci. Fourth, microbial genome database is rapidly growing but incomplete. Diagnosis based on single target amplification, followed by sizing, melting peak analysis, or even sequencing may not be sufficient to pin down a specific microbial agent. Sequence polymorphism as well as unknown etiology has repeatedly surprised the interested microbial miner in the field of infectious diseases. Finally, molecular sequence-based diagnosis does not provide viable organisms for additional phenotypic or genotypic investigation. Traditional culture methods remain of indispensable value for biological genetic research of emerging virulence or drug-resistance traits and epidemiological surveillance.

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19
Amplification Product Inactivation

SUSAN SEFERS AND YI-WEI TANG

Introduction

The extreme sensitivity of PCR has proved to be one of the strong points of this laboratory technique, making it possible to detect even small numbers of infectious agents present in a clinical sample. It is unfortunate that this strength can also be a weakness. Billions of copies of DNA are produced by PCR, yet only one double-stranded DNA molecule is necessary to carry out the reaction. This sets the stage for possible contamination of new reactions with previously produced PCR product.

False-positive PCR results due to contamination are very serious, and these kinds of errors can lead to fatal consequences (Persing, 1991; Patel et al., 2000). Sources of contamination in PCR can be either from unamplified, "natural" nucleic acids or amplified nucleic acids. Unamplified contamination most commonly occurs when a specimen has a very high titer of target nucleic acid. A pipette or technologist's glove contacts the specimen and this is carried over to another specimen. There have been cases documented where medical equipment has become contaminated with a patient sample. A bronchoscope was being used for collection of bronchial aspirates to be tested for *Mycobacterium tuberculosis* PCR. This bronchoscope was inadequately washed between uses, and subsequent samples collected with this instrument had become contaminated with target nucleic acid (Kaul et al., 1996). When bronchoscopes were adequately washed, the contamination ceased (Shim et al., 2002).

Most contamination in a molecular laboratory occurs when amplified nucleic acid is carried over from a previous PCR to a new reaction (Kwok and Higuchi, 1989). It is recommended to physically separate preamplification work areas from postamplification work areas. There should be dedicated refrigerators, centrifuges, lab coats, gloves, pipettes, pens, and so forth, for each work area so that amplicons (PCR amplification products) will not be carried over from postamplification areas to preamplification work areas (Kitchin et al., 1990).

Besides physical separation, some measures can be taken in the preamplification area to prevent contamination of new reactions with amplicons (Persing, 1991; Persing and Cimino, 1993). Ten percent bleach (sodium hypochlorite) can be used to clean work surfaces and destroy DNA and RNA (Prince and Andrus,

1992). Bleach causes oxidative damage to nucleic acids, making them unsuitable as templates for future reactions. A rinse with 70% ethanol after a bleach cleaning can reduce the corrosive effects of bleach on laboratory equipment (Persing and Cimino, 1993).

Another method to control (but not necessarily inactivate) amplicons has become available with the advent of "real-time" PCR. These systems can detect amplification products as they are being produced with the use of a flourescent probe. This will allow the detection of amplification products without the need to open a tube postamplification to run an agarose gel or run an ELISA. These test methods have a "closed system"—PCR tubes are never opened postamplification therefore will not generate large copies of amplicons to possibly contaminate furture amplification reactions.

The methods described thus far help with controlling contamination, but it is still necessary to implement a more comprehensive method for inactivating amplicons after they have been produced through PCR. Using at least one or more of the following methods along with physical separation parameters and bleach cleaning will be the most effective way to eliminate contamination in the molecular laboratory (Sefers et al., 2005).

Commonly Used Methods

Ultraviolet Light

Ultraviolet (UV) light has been used preamplification to cut down on amplicon carryover (Sarkar and Sommer, 1990). UV light irradiation produces pyrimidine dimer adducts between adjacent pyrimidine bases. These adducts prohibit *Taq* polymerase from processing along the amplicons. This renders nucleic acids unsuitable as templates for future reactions. Figure 19.1 shows a pictorial of the UV irradiation process.

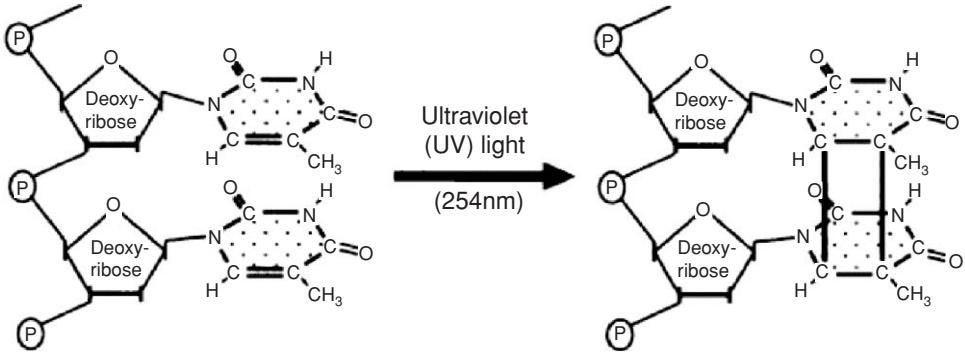

FIGURE 19.1. Action of UV light on nucleic acids. UV light irradiation produces pyrimidine dimer adducts between adjacent pyrimidine bases. This renders nucleic acids unsuitable as templates for future reactions.

Most dead-air boxes today come equipped with a UV light to facilitate the UV irradiation process. The boxes produce an area of "dead air" that limits amplicon drift into new PCR reactions. The UV light can be turned on in between uses to treat surfaces and reagents that could possibly contain contaminating amplicons. Also, the boxes come equipped with a glass shield to limit lab technologist exposure to UV light.

A UV light treatment at 254 nm for 10 min seems to be suitable to eliminate contaminating amplicons. Obviously, the action of UV light may be effected by the percentage of pyrimidines in the amplicons produced and the distance from the UV light source. Contaminating DNA was found to be eliminated at a distance of 5 cm (Ou et al., 1991). As the distance was increased, UV light was less effective at controlling contamination. It has been found that there is better success if the amplicon is greater than 500 bp (Belak and Ballagi-Pordany, 1993).

Uracil-N-Glycosylase

A preamplification system that works well is to use uracil-*N*-glycosylase (UNG). This enzyme removes uracil bases from DNA by cleaving the uracil glycosidic bond between the base and the sugar phosphate backbone. These amplicons are susceptible to strand scission when alkaline hydrolysis occurs where the uracil residues have been cleaved. This yields the DNA unsuitable for amplification by DNA polymerases. UNG has no action on RNA and is only functional on single or double stranded DNA. Figure 19.2 shows the biochemistry of the UNG inactivation method.

This method of product inactivation requires some modification of master mix and thermal-cycling conditions. Master mix must be altered to contain UNG, and there needs to be at least some, if not all, substitution of dTTP with dUTP. UNG is only active up to a temperature of 55°C, and the enzyme is inactivated at 95°C for 10 min. Therefore, the thermal-cycling program needs to be modified so that there is an initial period for UNG activity then a warm up to 95°C for 10 min. Also, it is best if thermal-cycling conditions remain above 55°C in subsequent cycles (Longo et al., 1990). After cycling, it is advantageous to hold reactions at 72°C (above 55°C) or 4°C to limit any residual UNG activity (Rys and Persing, 1993). Figure 19.3 shows the entire UNG protocol.

The success of UNG activity depends on the amount of uracil incorporated in the product, so products that are A+T rich will give better results with this method. The UNG protocol was not found to work well with small amplicons (<100 bp) (Espy et al., 1993). Also, if it is desired to add UNG to an existing protocol, it may take some time to adjust master mix and thermal-cycling programs to come to an optimal mean between good product yield and good UNG activity. The UNG method has been shown to inhibit up to 3×10^9 copies of contaminating DNA. This protocol has no effect on gel mobility, ethidium bromide staining, and hybridization reactions used in PCR product detection (Rys and Persing, 1993).

UNG has been used in PCR protocols for many different targets and has shown to be effective in stopping amplicon contamination. In most experiments, 1 μL of 1 U/μL UNG is included in a 100 μL reaction volume (Espy et al., 1993;

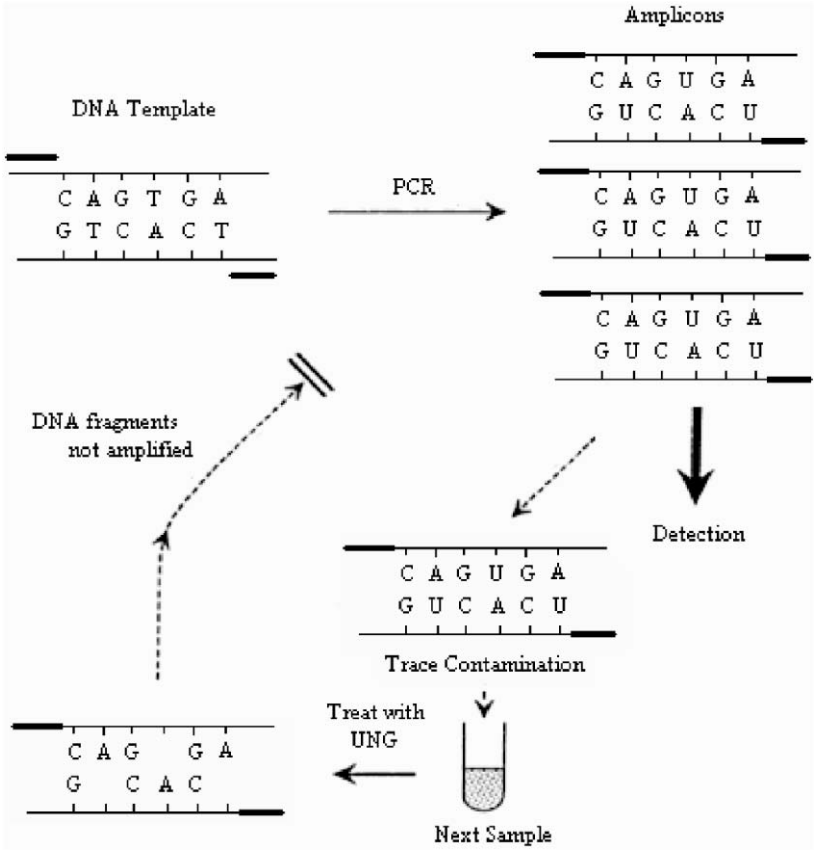

FIGURE 19.2. Biochemistry of UNG. During PCR, *Taq* polymerase includes dUTP into the amplicon. Detection is performed. In this example, trace contamination of the next sample occurs. UNG has been incorporated into the master mix, which "cuts" the contaminating DNA strand at the uracil residues. This makes these contaminating strands unable to be amplified.

Rys and Persing, 1993). A heat-labile UNG is also available that has proved to be more effective in some experiments (Taggart et al., 2002; Pierce and Wangh, 2004). Sodium hydroxide used to denature DNA also has a dual purpose in helping to halt UNG activity more completely than just heat alone (Martin et al., 2000). Concentrations of UNG higher than 0.1 units per reaction have been shown to inhibit amplification (Mohamed et al., 2004). PCR protocols using UNG have been developed for a variety of targets including enterovirus (Kao et al., 1995; Taggart et al., 2002; Mohamed et al., 2004), tuberculosis (Sarmiento et al., 2003), *Toxoplasma gondii* (Martin et al., 2000), influenza (Poddar et al., 1997), *Cryptosporidium* (Gobet et al., 1997), *Borrelia* (Loewy et al., 1994) , HSV, CMV, EBV (Espy et al., 1993), and many others. Figure 19.4 shows an example the ability of UNG to inhibit CMV DNA amplification products (Espy et al., 1993).

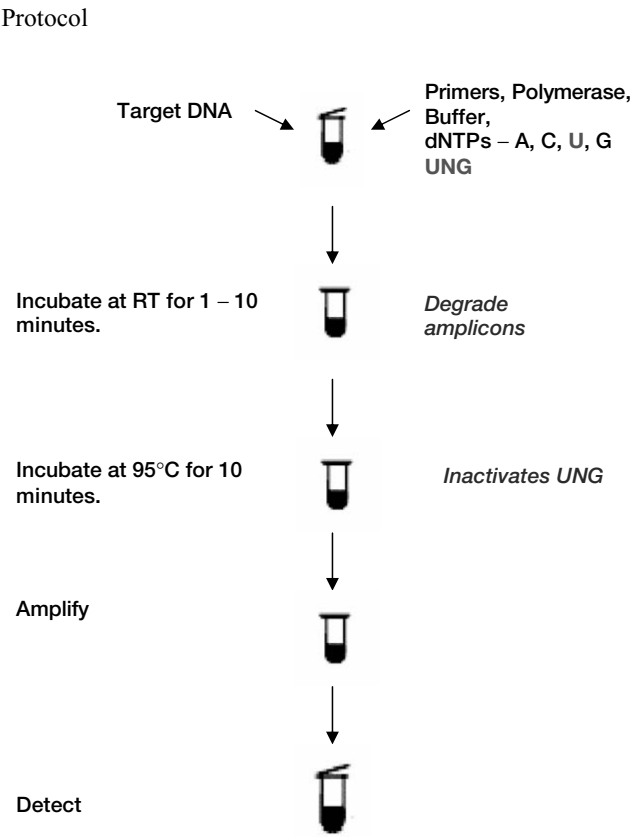

FIGURE 19.3. Steps used in UNG amplification product inactivation protocol. The master mix has been formulated to contain UNG and dUTP (as opposed to dTTP). The master mix is allowed to incubate at room temperature for 1 to 10 min to allow for UNG activity. Then, the mixture is warmed to 95°C for 10 min to inactivate UNG. The amplification and detection are then allowed to proceed as normal.

Photolinkers: Psoralen and Isopsoralen

A postamplification method for product inactivation is the use of photochemical cross-linkers known as psoralens. Two commonly used psoralens are isopsoralen and methoxypsoralen. A photolinker is added to the reaction preamplification and is thermally stable throughout thermal cycling. It is then activated by exposure to UV light postamplification. The activated photolinker forms adducts between pyrimidine residues. This blocks *Taq* polymerase so that it is unable to use these products as templates for future reactions (Cimino et al., 1991). The biochemistry of psoralens are shown in Figs. 19.5 and 19.6.

Isopsoralen is most often preferred over psoralen due to the fact that psoralen can cross-link DNA whereas isopsoralen cannot. DNA inactivated with isopsoralen

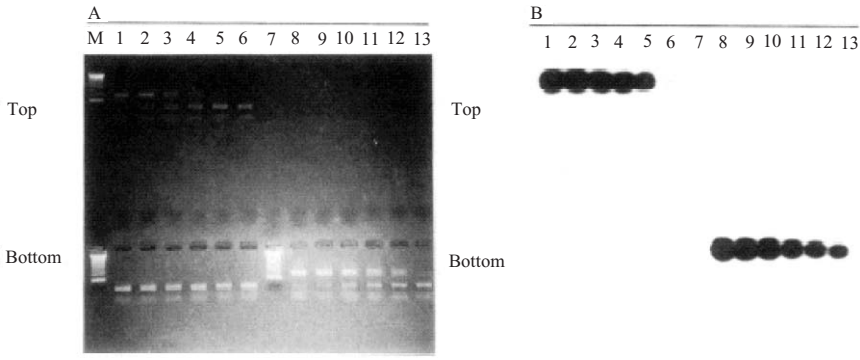

FIGURE 19.4. CMV DNA inactivation by UNG. Gel electrophoresis (A) and Southern blotting (B) of CMV DNA amplification products. CMV DNA was amplified in the presence of dUTP, which replaced dTTP. Native CMV amplified without UNG (lanes 1 to 6, top) and with UNG (lanes 8 to 13, bottom) pretreatment. A dilution series of CMV DNA amplicons containing dUTP reamplified after UNG pretreatment is also shown (lanes 1 to 6, bottom) (Espy et al., 1993).

is able to function with hybridization reactions easily whereas psoralen inactivated amplicons cannot. Concentrations of isopsoralen used range from 100 to 200 μg/mL typically. Postamplification treatment usually consists of UV irradiation at 300–400 nm for 15 min at either ambient or 4°C temperatures (Isaacs et al., 1991; Rys and Persing, 1993). The use of a HRI-300 photothermal reaction chamber (Simms Instruments, Palo Alto, CA, USA) at 5°C has been found the best and most effective means of activation of isopsoralen (Fahle et al., 1999) as opposed to a regular UV box. The entire isopsoralen procedure is show in Fig. 19.7.

Isopsoralen works best if the amplicon has a high A+T content. Isopsoralen was also found to be ineffective in amplicons that are less then 100 bp. Isopsoralen will increase the molecular weight of the amplicon, which will impact its migration on gels (Espy et al., 1993). At higher concentrations, isopsoralen will decrease the intensity of ethidium bromide staining on gels (Rys and Persing, 1993). It has been shown that increasing concentrations of isopsoralen will result in better amplicon inactivation, but at higher concentrations isopsoralen will inhibit amplification. The addition of 10% glycerol or 3–5% DMSO to the reaction has been shown to combat this negative effect (Isaacs et al., 1991). One procedure showed good results were obtained when isopsoralen was added postamplification with the help of a wax bead that kept the amplicon from becoming aerosolized (De la Vuida et al., 1996). Isopsoralen is effective on inhibiting up to 3×10^9 copies of contaminating DNA (Rys and Persing, 1993). Isopsoralen has effectively been used for amplicon inactivation in PCR assays used to detect many types of templates including cytomegalovirus (Fahle et al., 1999), HIV-1 (Isaacs et al., 1991), *Borrelia burgdorferi* (De la Vuida et al., 1996), *T. whippelii* (Whipple's disease) (Ramzan et al., 1997), CMV and EBV (Espy et al., 1993). Figure 19.8 illustrates the ability of isopsoralen to inhibit amplified HSV and CMV DNA.

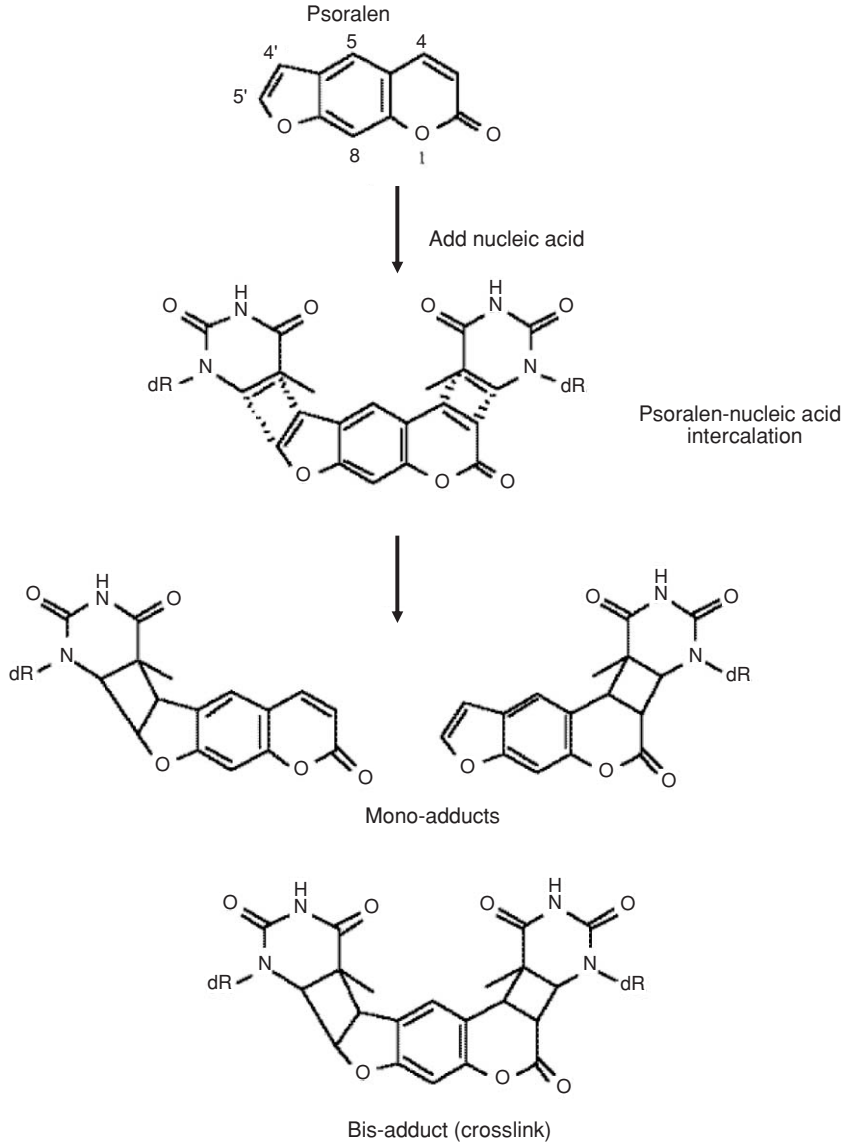

FIGURE 19.5. Structure and binding of psoralens with DNA. Psoralens activated by UV light can create mono- or bis-adducts between nucleic acids.

Others

Hydroxylamine hydrochloride is another method that can be used for amplification product inactivation postamplification. Hydroxylamine binds and chemically modifies nucleic acid. It reacts with cytosine and inhibits it from pairing with

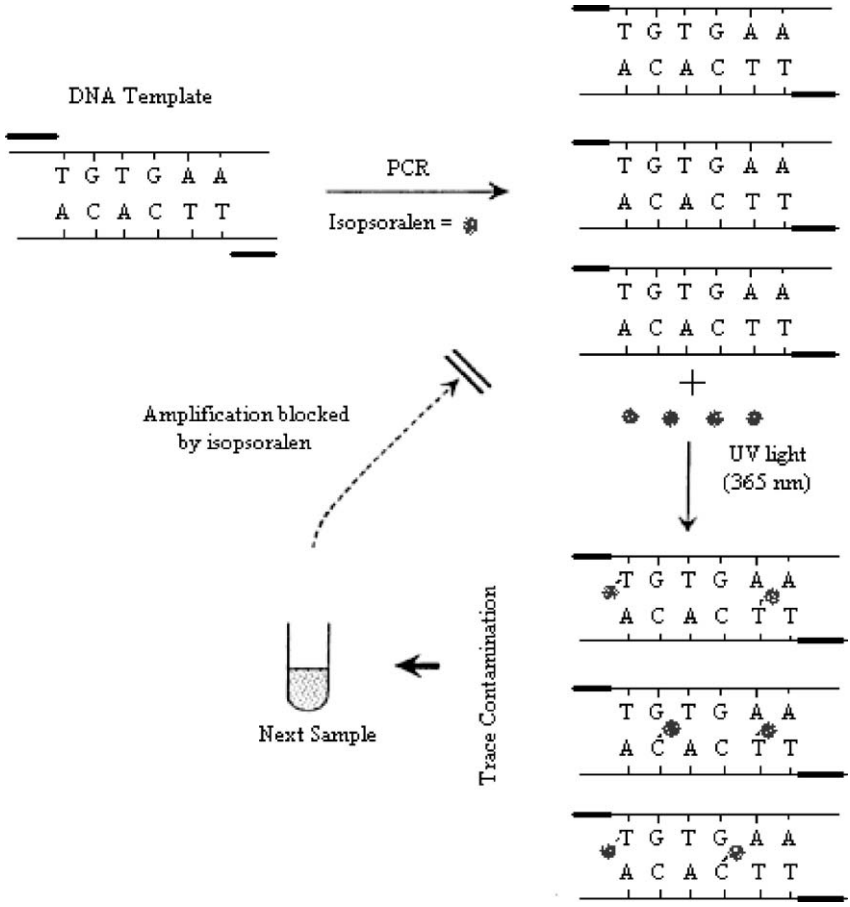

FIGURE 19.6. Biochemistry of Psoralens. Isopsoralen has been included in the master mix along with the DNA template when amplification takes place. Postamplification, the amplicons are treated with UV light to activate isopsoralen. Adducts are created at pyrimidine residues. Trace contamination of amplicon occurs in the next sample. These treated amplicons are unable to be amplified by *Taq* polymerase due to the isopsoralen adducts.

guanine. The resulting compound can bind with adenine and causes its replacement with thymine if DNA synthesis occurs after treatment (Aslanzadeh, 1993). This method has been found to work well with smaller amplicons (close to 100 bp) but is somewhat dependent on G+C concentration. Also, the treatment increases the molecular weight of the amplicon, which will affect its migration on gels (Rys and Persing, 1993). A major drawback of this method is that the reaction tube needs to be opened in order to add hydroxylamine. This can introduce aerosols that would not have been introduced if some other method was used for product inactivation. This method has been successfully used with protocols detecting DNA from HSV and *B. burgdorferi* (Aslanzadeh, 1993).

Isopsoralen protocol

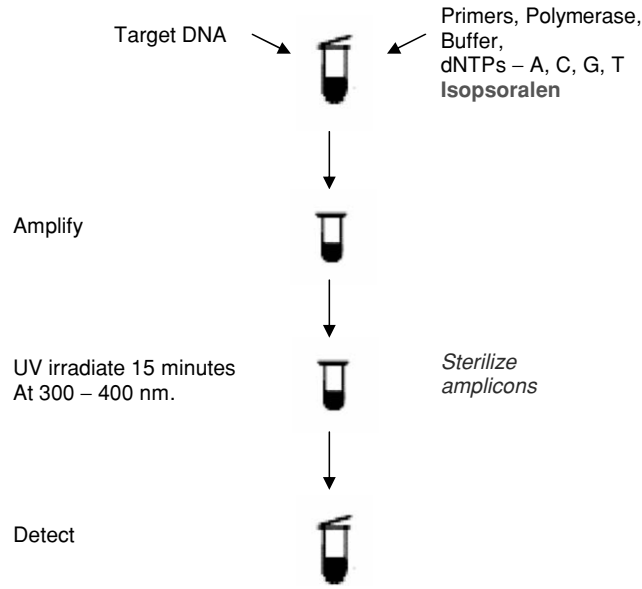

FIGURE 19.7. Steps used when using isopsoralen (IP) as a method for amplification product inactivation. Isopsoralen is incorporated in the master mix. The amplification is allowed to proceed as normal. After amplification, the unopened tube containing amplicons is exposed to UV light for 15 min. The detection step can then be performed with "sterilized" amplicons.

Primer hydrolysis (alkaline hydrolysis) is a method of product inactivation that occurs postamplification also. For this method, primers are produced that have ribose residues at or near the 3′ end. These modified primers are susceptible to cleavage when placed in an alkaline environment. If NaOH is added postamplification, the DNA is cleaved at the primer sites and leaves the subsequent DNA unsuitable for replication. HCl can be added after 30 min to neutralize the reaction mixture.

The disadvantage of this method is the addition of two reagents added to tubes postamplification. This can easily produce aerosols that could allow cross-contamination between samples. With primer hydrolysis, there was no effect seen in gel migration and it seems to be effective on even small products. The degree of inactivation does seem to vary with inhibition of anywhere between 10^4 to 10^9 copies. This type of inactivation has been used in combination with PCR for *B. burgdorferi* (Rys et al., 1993).

Another pre-PCR inactivation method is treatment with restriction endonucleases (RE). In this technique, a specific RE was added to the master mix (without the DNA polymerase) prior to PCR. The RE was allowed to incubate with the reaction mixture for a specified amount of time. Then the DNA polymerase was added to

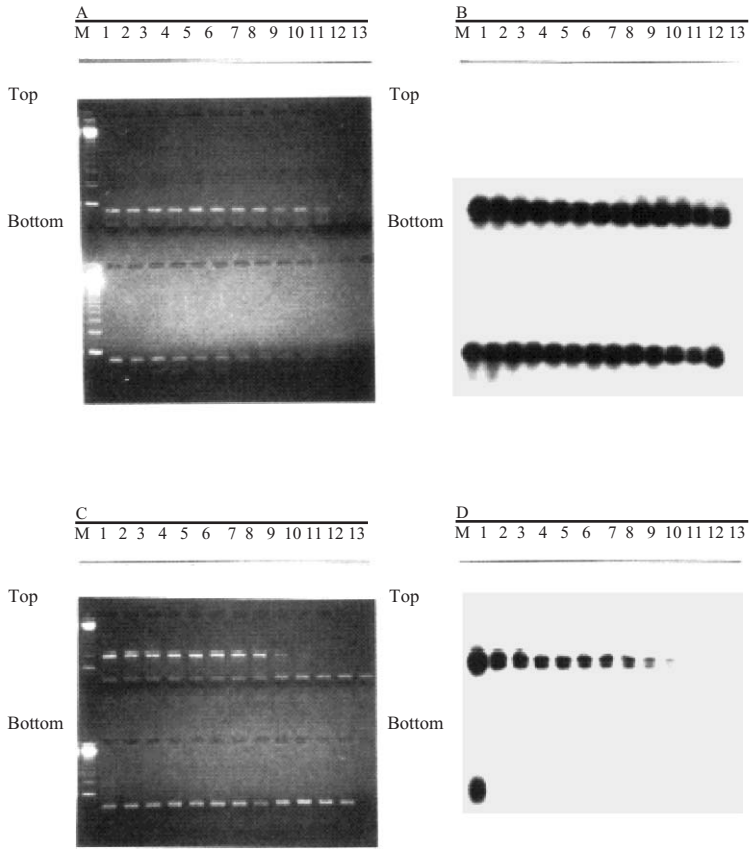

FIGURE 19.8. Inhibition of CMV DNA amplicons with isopsoralen. Gel electrophoresis (A and C) and Southern blotting (B and D) of HSV (A and B) and CMV (C and D) amplicons without (top) and with (bottom) isopsoralen (25 μg/mL) treatment. Lane designations correlate with dilution (e.g., lane 1, 10^{-1} dilution; lane 12, 10^{-12} dilution). M, molecular size markers (Espy et al., 1993).

the mixture and the PCR process initiated. This method has been shown to work successfully at destroying contaminating amplicons. There are several drawbacks of this method, one of which is that the RE incubation may be lengthy (several hours). Another is that it may take more than one RE to totally restrict all possible contaminating nucleic acid. Careful planning is necessary to make sure your RE is appropriate for the amplicon that is being inactivated (DeFilippes, 1991).

Besides inactivating the amplicons themselves, it is also possible to "treat" PCR reagents with procedures that will inactivate contaminating DNA that may have found its way into these reagents. There have been several reports of Taq DNA polymerase becoming contaminated with DNA (Bottger, 1990). UV irradiation is a quick, easy method to deal with this problem. Another method that can be

used is treatment with 8-methoxypsoralen (8-MOP) in conjunction with UV light exposure. Also, enzyme treatment with Dnases and restriction endonucleases have been used. These methods are extremely useful if employed while doing universal 16S rRNA PCR (Hughes et al., 1994; Corless et al., 2000).

Comparison of Methods

When setting up a system for amplification product inactivation, it is important to invesigate which method will work best with the procedures in your laboratory. Of the methods discussed in this chapter, several factors must be explored and prioritized such as cost, ease of use, and effectiveness with your particular PCR assay. Besides physical separation and bleach/ethanol cleaning procedures, it is best if at least two of the inactivation procedures are used to control contamination in your laboratory.

In our laboratory at Vanderbilt University Medical Center, we have been using UV light irradiation and UNG product inactivation (Sefers et al. 2005). UV irradiation works well because it is relatively inexpensive and does not require a special protocol. Our PCR assays have also been optimized for use with UNG. The disadvantage of this method is the cost. UNG can cost anywhere from 40 to 80 cents per PCR reaction. Once the assay is set up to include UNG, it is very easy to use. A laboratory should see very, very few (possibly none) contamination events when using UNG.

Also, several of our assays have been adapted to PCR real-time formats. PCR products are detected while the thermal cycling process occurs. Therefore, amplified PCR product can be discarded without opening a tube for detection purposes.

Psoralens are not as expensive as UNG, but there is some expense involved. The cost of psoralen in the master mix can be as low as 14 cents per reaction, but it is necessary to find a source of UV light to activate the psoralens post-PCR. This price can vary depending on manufacturer but will drive up the cost of this method. Psoralens are very easy to use with only slight changes in master mix formulation and no changes needed in thermal-cycling profiles. If gel electrophoresis is used to detect PCR product, psoralens will affect the migration due to the increase in molecular mass. If hybridization is used for detection of PCR product, this will remain unchanged (Isaacs et al., 1991; Rys and Persing, 1993).

Primer hydrolysis and hydroxylamine treatment have a major weakness in that it is necessary to open a PCR tube postamplification to add reagents. This could cause more headaches than if the tube was not opened at all. Also, hydroxylamine is known as a mutagenic agent and should be used carefully by laboratory personel. Restriction endonuclease treatment can be effective, yet the added incubation times needed to effectively use these enzymes may pose a problem, especially to a clinical laboratory where timeliness is important.

Table 19.1 has a listing of the major inactivation methods discussed in this chapter and advantages and disadvantages of each method. By using these methods, contamination will be kept at a minimum and will allow the laboratory to operate efficiently.

TABLE 19.1. Comparison of inactivation protocols used in nucleic acid tests.

Method	Application	Conditions	Advantages	Disadvantages	References
UV light irradiation	Preamplification	Lower G + C content, >500 bp amplicon	Inexpensive. Simple procedure. No changes to protocol.	Efficacy varies	Sakar and Sommers, 1990; Ou et al., 1991; Belak and Ballagi-Pordany, 1993
UNG	Preamplification	Lower G + C content, >100 bp amplicon	Simple procedure	Changes necessary in amplification cycling and master mix. More expensive than other methods.	Espy et al., 1993; Rys and Persing, 1993
Photochemical cross-linkers	Postamplification	Lower G + C content, >100 bp amplicon	Simple procedure	Additional instruments required. Can change molecular mass of products, which affects electrophoresis.	Cimino et al., 1993; Espy et al., 1993; Rys and Persing, 1993
Primer hydrolysis	Postamplification	No specific requirements	No effect on amplicon analysis	Necessary for reagent addition postamplification. Efficacy varies.	Rys and Persing, 1993
Hydroxylamine treatment	Postamplification	Higher G + C content, >100 bp amplicon	Effective on some amplicons	Necessary for reagent addition postamplification. Some amplicon changes may affect analysis. Hydroxylamine is a mutagenic agent.	Aslanzadeh, 1993
Restriction endonuclease	Preamplification	No specific requirements	Effective on some amplicons	May lengthen processing time.	DeFilippes, 1991

UNG, uracil-*N*-glycosylase.

Conclusion

This chapter summarizes a very important piece of molecular testing—amplification product inactivation protocols. Using a good system to control contamination that consists of at least one of the methods discussed plus the common molecular lab practice of physically separated work areas and cleaning procedures will keep amplicon contamination in control. With successful implementation of these methods, molecular labs should be able to operate at the greatest efficiency with no contamination events.

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Part II
Applications

20
Bacterial Identification Based on 16S Ribosomal RNA Gene Sequence Analysis

XIANG Y. HAN

Introduction

Clinical microbiology laboratory is responsible for the isolation or detection of microorganisms to establish the diagnosis of infection. Rapid and accurate identification of these organisms and subsequent antibacterial drug susceptibility tests also guide antibiotic therapy. Although these goals can be fulfilled most of the time, some bacteria may be difficult to be identified due to fastidious growth, morphological variations, unusual biochemical reactions, lack of previous recognition, or a combination of these. Subculture failure, though it rarely happens, virtually makes routine identification impossible. Fortunately, technological advances have largely overcome these limitations for bacterial identification. One of the advances realized in the past decade or so has been the analysis of the nucleotide sequences of the 16S ribosomal RNA gene (16S rDNA), which has emerged as the single best method to identify bacteria (Kolbert and Persing, 1999; Drancourt et al., 2000). This chapter reviews its theoretical basis, methodology, clinical application, and limitations. A thorough and in-depth review with many practical points has just been published elsewhere (Clarridge, 2004).

Theoretical Basis

Ribosomes are protein synthesis machines for living organisms and are required for survival. Many antibiotics target the bacterial ribosome to achieve a bacteriocidal effect. A bacterial ribosome is composed of multiple ribosomal proteins and three ribosomal RNAs (rRNA) (i.e., 23S rRNA, 16S rRNA, and 5S rRNA). The rRNAs are encoded by their respective genes, usually organized as an operon, termed *rrn*, in the genome. With the genomes of >100 various bacteria having been sequenced, it is realized that a bacterial genome may have multiple *rrn* operons depending on the size of the genome and the species. Table 20.1 lists several examples of the number of *rrn* operons and genome size. Generally, every mega–base pair (Mbp) contains 1–3 *rrn* (mean 1.93 and median 1.92).

TABLE 20.1. The number of ribosomal operons (*rrn*) and genome size (mega–base pairs, or Mbp) for several representative bacteria.

Bacterium	*rrn*	Mbp	*rrn*/Mbp	Reference
Mycoplasma pneumoniae	1	0.82	1.21	Himmelreich et al., 1996
Helicobacter pylori	2	1.67	1.20	Tomb et al., 1997
Mycobacterium tuberculosis	3	4.41	0.68	Cole et al., 1998
Streptococcus pneumoniae	4	2.16	1.85	Tettelin et al., 2001
Corynebacterium diphtheriae	5	2.49	2.01	Cerdeno-Tarraga et al., 2003
Haemophilus influenzae	6	1.83	3.28	Fleischmann et al., 1995
Escherichia coli	7	4.64	1.51	Frederick et al., 1997
Vibrio cholerae	8	4.03	1.99	Heidelberg et al., 2000
Clostridium perfringens	10	3.03	3.30	Shimizu et al., 2002
Bacillus cereus	13	5.43	2.39	Ivanova et al., 2003
Total	59	30.51	1.93	

The first bacterial 16S rDNA was sequenced by Ehresmann et al. in 1972 for *Escherichia coli*. This prototypic 16S rDNA (GenBank accession no. J01859) contains 1542 nucleotides. As more 16S rDNAs were sequenced and studied, it was realized that (1) the nucleotide sequences among various bacteria are highly conserved; (2) the conservation and divergence reflect bacterial evolution; and (3) each bacterial species has its unique 16S rDNA sequences (Fox et al., 1980). Therefore, 16S rDNA sequencing became a tool for studies of bacterial phylogeny. However, such 16S rDNA sequencing was laborious and sophisticated and could be performed only in a limited number of research laboratories. This changed with the invention of polymerase chain reaction (PCR) technology in the mid-1980s. As PCR became popular for its amplification power, speed, simplicity, and economy, its application for bacterial 16S rDNA has flourished.

Depending on the needs, the entire 16S rDNA or a portion of it may be amplified by PCR. Conserved regions of 16S rDNA allow design of highly conserved primers for nearly universal amplification of most bacterial species (Greisen et al.,1994; Han et al., 2002). The nucleotide sequences of the amplicon are determined, which, when compared with a database, yield homology matches and consequent identification of a particular bacterium. It is the variable regions of 16S rDNA that give discriminatory power. The longer the sequences are determined, the more accurate the identification is. Generally, at least 200 bp are required to yield meaningful results. A comprehensive and accurate database is essential for homology matches and identification of bacteria. There are multiple public and private databases available, such as GenBank, Ribosomal Database Project (RDP), Ribosomal Differentiation of Medical Microorganisms (RIDOM), and others. As of October 2004, the number of 16S rDNA sequences in the GenBank approached 170,000. For a total of 7000 or so validated bacterial species, the vast majority can be found in the GenBank database.

Methodology

Four steps are required to reach bacterial identification through 16S rDNA sequencing: DNA extraction, PCR amplification, nucleotide sequencing, and database homology search and reporting.

DNA Extraction

Pure culture of a bacterium is generally required for its identification. One or two isolated colonies or a visible pellet from pure liquid culture contain up to 10^{10} cells, and the extracted DNA will be sufficient for many subsequent PCRs. Various DNA extraction methods can be used, such as traditional phenol chloroform method, commercial DNA extraction kits, and so forth. Pure culture and relatively large quantity of target DNA makes contamination by background DNA from reagents and other sources almost negligible. Because the extracted DNA will be amplified by PCR, its purity requirement is not strict either. Therefore, for routine clinical application, the simpler and quicker the method is, the better. Here is a simple method used in our laboratory (Han et al., 2002). Two colonies or the pellet of 1 mL positive liquid medium (upon confirmation of purity by staining) are resuspended in 200 μL extraction solution (Prepman Ultra;) (Applied Biosystems, Foster City, CA, USA). The suspension is boiled for 10 min and centrifuged for 5 min at 8000 $\times$ g, and the genomic DNA is extracted in the supernatant. One percent of the supernatant (2 μL) is used for subsequent PCR.

PCR

The amplification by PCR is no different from other PCR methods. Thermal cycles and conditions vary slightly depending on polymerase and primers. PCR product can be examined and the amount estimated visually on an agarose gel electrophoresis. This step, however, being intermediate, is not essential. In our experience with universal primers and mycobacteria-specific primers (Han et al., 2002), PCR are robust and yield sufficient amplicons for sequencing to obviate the electrophoresis step.

Nucleotide Sequencing

The PCR amplicon can be sequenced directly after removal of unpolymerized primers and deoxynucleoside triphosphates that is achieved by enzymatic digestion with exonuclease and shrimp alkaline phosphatase. No further purification or concentration of the amplicon is generally necessary. Automated sequencing can be completed in no more than a few hours.

Homology Search and Reporting

The nucleotide sequences are searched for homology against a local or public database. Typically, multiple homology matches will show up, especially from a public databases, such as GenBank. These matches need to be interpreted cautiously; specifically, consensus of the matches and/or the match with type strain should be sought. Preferably, reference sequences from type strains are of good quality without unresolved nucleotides or artificial gaps, have full length (>1400 bp), and come from a reputable laboratory if they are not published. Generally, a match with >99% homology renders species-level identification if culture features are also compatible, and 97% to <99% matches corresponds to genus identification. Although <93% matches usually suggest a new genus and hence entail additional studies for confirmation, matches between 93% to <97% fall into either new genus or new species. Generally, more mismatches means farther phylogenetic distance.

The matched result is then compared with culture characteristics of the organism to reach final identification. Such features as growth rate, colony morphology, pigmentation, and Gram-stain reaction and cellular morphology are usually readily available once the organism is cultured. Uncultured organisms or subculture failures need to be handled on individual basis.

Application

Compared with traditional phenotypic tests, the 16S rDNA sequencing method offers a number of advantages. First, it shortens turn-around time. Theoretically, the process takes about 7 h: 20 min for DNA extraction, 2 h or less for PCR, 4 h or less for sequencing, and 30 min for match and reporting. Practically, it takes 1–3 days. Thus, this method is especially useful to identify mycobacteria and other fastidious organisms. Second, the results are definitive for those organisms that, due to various reasons, are difficult to be identified by routine methods. Frequently, at least genus-level identification can be offered (Drancourt et al., 2000). Third, new or unusual taxa can be discovered. Fourth, for those uncultivated bacteria or subculture failures, this method remains the only way to find out the identity of the organism (Relman, 1999). Fifth, this culture-independent method remains the only approach to study the diversity of noncultivable bacteria in various nonclinical settings (Hugenholtz et al., 1998). Therefore, the 16S sequencing method has at least the following applications in clinical microbiology.

Establishing New Taxa

Currently, a polyphasic approach is needed to establish new bacterial taxa. This includes analysis of 16S rDNA sequences, genome-wide DNA reassociation among closely related species within a genus, cellular fatty acid profiling, morphology and Gram-stain features, and biochemical reactions and other phenotypic

tests. The 16S rDNA sequencing analysis forms the backbone of the polyphasic approach (Ludwig and Klenk, 2001). For the description of a new taxon, full-length sequence of the gene is usually required, as opposed to partial sequence for regular clinical application. The sequence provides an almost unified scale to measure the phylogenetic closeness or distance among a wide range of bacteria at and above species level. The author performed a quick scan of the articles published in recent issues of *International Journal of Systematic and Evolutionary Microbiology* and found that virtually every new bacterial taxa contained 16S rDNA sequences. With a comprehensive database of sequences, like GenBank, being available to bacteriologists throughout the world, naming wrong or overlapping taxa, occasionally seen before the sequencing era, is far less likely to happen at present or in the future.

Identification of Mycobacteria

Mycobacteria are fastidious, and traditional biochemical tests are cumbersome and time consuming. Although the nucleic acid hybridization tests by Accu-probes (Gen-probe, San Diego, CA, USA) have made identification easier and much quicker for four *Mycobacterium* species or complexes (i.e., *M. tuberculosis* complex, *M. kansasii*, *M. gordonae*, and *M. avium-intracellulare* complex), many other species still depend largely on biochemical tests or cellular fatty acid analysis. Several studies from diverse settings have shown that the 16S rDNA sequencing method is especially useful to identify mycobacteria (Rogall et al., 1990; Holberg-Petersen et al., 1999; Patel et al., 2000; Tortoli et al., 2001; Turenne et al., 2001; Han et al., 2002). The 16S rDNA of almost all 100 or so mycobacterial species have been sequenced and analyzed (mostly by Rogall et al., 1990, and Turenne et al., 2001). This wealth of knowledge and the availability of these sequences through public databases (GenBank) provide a solid basis for potentially wide clinical application. The advantages are obvious: shorter turn-around time for laboratory efficiency and timely patient management. As such, all mycobacterial isolates in our laboratory have been routinely sequenced since October 2002.

Most nontuberculous mycobacteria (NTM) are of environmental origin and opportunistic pathogens. Thus, isolation of a NTM species does not necessarily indicate infection, and correlation with clinical and radiologic findings is required. As experience with accurate identification of various NTM accumulates, more knowledge about these organisms and better clinical management may be seen as well in the near future.

Identification of Other Fastidious or Uncultivated Bacteria

Timely identification of other fastidious organisms can be challenging as well, and the sequencing method is equally powerful and useful. Similarly, culture-negative infections are not uncommonly seen in clinical practice. In these situations, DNA from the infected tissue is extracted and amplified by conserved bacterial primers, and the sequences of the PCR amplicon may yield identification of known taxa

TABLE 20.2. Examples of using 16S rDNA sequencing to identify noncultivated or fastidious bacteria.

Disease	Bacterium	Reference
Bacillary angiomatosis	*Bartonella henselae*	Relman et al., 1990
Bacteremia	*Bartonella henselae*	Regnery et al., 1992
Whipple's disease	*Tropheryma whipplei*	Relman et al., 1992
Babesiosis	*Babesia*	Persing et al., 1995
Culture-negative endocarditis	Multiple bacteria	Many in MEDLINE
Abscess and others	*Actinomyces*	Clarridge and Zhang, 2000
Various infections	Coryneform gram-positive rods	Tang et al., 2000
Bacteremia	*Roseomonas* spp.	Han et al., 2003
Peritonitis	*Francisella tularensis*	Han et al., 2004a
Endocarditis	*Cardiobacterium valvarum* sp. nov	Han et al., 2004b
Bacteremia	*Moraxella osloensis*	Han & Tarrand, 2004

or novel taxa. There are many such examples for this application, and a few are shown in Table 20.2.

Limitations and Pitfalls

Like other microbiologic tests, the 16S sequencing method has limitations and pitfalls, and knowing them may prevent making mistakes in identification and taxonomy.

1. **Identical 16S rDNA sequences**. Some species within a genus or even different genera may have identical 16S rDNA sequences, which make solely sequencing-based differentiation difficult. For instance, *E. coli* and *Shigella flexneri* have identical 16S rDNA and overall high genome-wide similarity. Yet, simple biochemical reactions, such as indole test and the selective medium *Salmonella–Shigella* agar, may differentiate them. Within the genus *Mycobacterium*, *M. kansasii* and *M. gastri* have identical 16S rDNA, so do the component species within the *M. tuberculosis* complex. The closely related *Bacillus* species, *B. anthracis*, *B. cereus*, and *B. thuringiensis*, also have identical 16S rDNA sequences. At subspecies level, identical 16S rDNA sequences are more common. If a portion of the gene, instead of the full length, is used, more vigilance is needed to tell apart the closely related sequences. Therefore, the presence of identical 16S rDNA sequences stresses the importance of correlation with culture and phenotypic features in making the final identification.
2. **Lack of reference sequences in database**. Before the sequencing method became widely available, bacterial taxa were established by other classic and conventional methods. Occasionally, the type strains of some of those established taxa have not been sequenced. Thus, when a new isolate does not show good matches with known sequences in the GenBank, a false impression of a new taxa may ensue. Such was true in our experience with *Roseomonas* spp. (Han et al., 2003), a relatively obscure but medically significant genus established in

1993 by Rihs et al. Sequencing analysis of our collection of 36 strains did not yield good matches (<93%) with known species; however, it was the cellular fatty acid analysis that pointed to the correct genus identification of these organisms. This finding led us to sequence the original type strains. Therefore, multiple tests by differing methods are helpful to prevent some mistakes.

3. **Contamination**. The ultra-sensitive PCR technique can be a double-edge sword in some situations. The ubiquitous nature of bacteria and the bacterial origin of PCR enzyme Taq polymerase make it difficult to completely eliminate the background bacterial DNA. Using highly conserved bacterial 16S rDNA primers will exaggerate this problem. In our experience with the universal primers (Han et al., 2002), the blank control frequently gives a faint band of the targeted amplicon. The band, being too faint or heterogeneous or both, does not yield any reliable sequences when direct sequencing is applied. This can change, however, if the amplicon is cloned into a plasmid-vector, and a single amplicon from a heterogeneous mix is propagated and sequenced later, resulting in identification of a contaminant that may be mistaken as the culprit. Thus, it is advisable that, when this cloning approach is used, multiple clones are to be sequenced to show homogeneity of the amplicons or the dominant ones. For amplification of extracted DNA from tissue that tends to have low number of disease-causing bacteria, such precaution needs to be kept in mind. Direct electron or light microscopy of infected tissue may verify the presence of microorganism with or without special stains. For instance, Auramine–rhodamine stain and Ziehl–Neelsen stain are useful to discern tissue mycobacteria, particularly in the background of granulomatous inflammation. Frequently, however, tissue reaction may be nonspecific, and more common etiologies must be excluded first. For example, a rheumatoid nodule may mimic a granuloma for its necrotic center, palisating architecture, and occasional multinucleated giant cells in the periphery. If, from such a granuloma-like lesion, an obscure NTM is detected merely by PCR-cloning and sequencing without a positive microscopic finding, the organism could well be a contaminant that is picked up by the ultra-sensitive PCR-cloning method.
4. **Koch's postulates**. When the etiologic bacteria (or viruses or other microbes) are not cultivated, it is difficult to satisfy Koch's postulates to draw the cause–disease conclusion, and a modified version of the principles may be needed (Fredericks and Relman, 1996). Salient points are summarized as follows. First, the microbial nucleic acids should be detected in most, if not all, such infectious processes, and in the diseased sites rather than normal tissue. Direct visualization of the organism by microscopy or demonstration of its components, such as nucleic acids or proteins by hybridization or immunohistochemistry or other techniques, will significantly strengthen the cause–disease relationship. Second, the detection of microbial nucleic acid and its intensity or copy number should correlate with the occurrence of disease and its resolution and relapse. And third, the sequence-based evidence for microbial causation should be compatible with other data, such as pathological features of disease, clinical characteristics, and its response to treatment. The putative causation should be reproducible.

Conclusion

In summary, the 16S rDNA sequencing method has evolved into a powerful and useful method for the practice of clinical microbiology. With its accuracy, robustness, decreasing cost, and simplified procedure, it is finding its way to routine use in microbiology laboratories in addition to large reference or research-oriented centers. Undoubtedly, more impact on patient care will be seen.

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21
Molecular Techniques for Blood and Blood Product Screening

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Introduction

"Blood banking has become a manufacturing industry, an industry that must conform to high standards and quality control requirements comparable to those of pharmaceutical companies or other regulated industries," said David A. Kessler, M.D., former FDA commissioner (Revelle, 1995). Screening donated blood for infectious diseases that can be transmitted through blood transfusion is very important in ensuring safety. The United States has the safest blood supply in the world (Revelle, 1995), and the Food and Drug Administration (FDA) is striving to keep it safe by decreasing the risk of infectious disease transmission. The regulatory agency is continuously updating its requirements and standards for collecting and processing blood. An important step in ensuring safety is the screening of donated blood for infectious diseases. In the United States, tests for infectious diseases are routinely conducted on each unit of donated blood, and these tests are designed to comply with regulatory requirements (Table 21.1). The field of clinical microbiology and virology is now moving into the focus of molecular technology. Currently, nucleic acid testing techniques have been developed to screen blood and plasma products for evidence of very recent viral infections that could be missed by conventional serologic tests. It is time for all blood safety staffs to use molecular detection techniques. This approach can significantly aid in blood safety to reduce the risk of transmission of serious disease by transfusion. This chapter will review the current antigen/antibody–based technology, molecular biological technology, and published regulatory policy data for blood safety.

Limitations for Current Technologies Used in Blood Safety

Direct detection of viral antigens and virus-specific antibodies have been the common tools for diagnosis of virus infections in the past 10 years. There are some limitations. For direct detection of virus antigens, shortly after virus infection, only a few viruses release antigens in amounts sufficiently detectable in the body by antibody assay. For indirect virus detection by virus specific antibodies [e.g., an

TABLE 21.1. Licensed / Approved Clinical HIV, HTLV and Hepatitis Tests (Source: Center for Biologics Evaluation and Research, US Food and Drug Administration)

Tradename(s)	Format	Sample	Use	Manufacturer	Approval Date
Antibody to Hepatitis B Surface Antigen (HBsAg Assay)					
Auszyme Monoclonal	EIA	Serum/Plasma	Donor Screen & Conf Kit	Abbott Laboratories Abbott Park, IL US License 0043	4/1/1985
Genetic Systems HBsAg EIA 3.0	EIA	Serum/Plasma/ Cadaveric Serum	Donor Screen & Conf Kit	Bio-Rad Laboratories Redmond, WA 98052 US License 1109	1/23/2003
ORTHO Antibody to HBsAg ELISA Test System 3	EIA	Serum/Plasma	Donor Screen / Diagnosis & Conf Kit	Ortho-Clinical Diagnostics, Inc Raritan, NJ 08869 US License 1236	4/23/2003
Antibody to Human Immunodeficiency Virus (HIV-1 Antigen Assay)					
Coulter HIV-1 p24 Ag Assay; HIV-1 p24 Antigen ELISA Test System	EIA	Serum/Plasma	Donor Screen/Prognosis & Neut Kit	Coulter Corporation Miami, FL US License 1185	3/14/1996
Coulter HIV-1 p24 Ag Assay	EIA	Viral Culture Supernatant	Prognosis (Quantitative) & Neut Kit	Coulter Corp	3/14/1996
Anti-HIV-1 Oral Specimen Collection Device					
Epitope OraSure HIV-1 Oral Specimen Collection Device	Oral Specimen Collection Device	Oral Fluid	For Use in Designated Non-Donor Screen and Non-Donor Supplemental Assays	Epitope, Inc Beaverton, OR	12/23/1994
Anti-HIV-1 Testing Service					
Home Access HIV-1 Test System	Dried Blood Spot Collection Device	Dried Blood Spot	Non-Donor Screen	Home Access Health Corp Hoffman Estates, IL	7/22/1996

Hepatitis B Surface Antigen (Anti-HBs Assay)					
Ausab	RIA	Serum/Plasma	Anti-HBs	Abbott Laboratories Abbott Park, IL US License 0043	2/5/1975
Ausab EIA	EIA	Serum/Plasma	Anti-HBs	Abbott Laboratories	11/18/1982
Hepatitis B Virus Core Antigen (Anti-HBc Assay)					
Corzyme	EIA	Serum/Plasma	Donor Screen	Abbott Laboratories Abbott Park, IL US License 0043	3/19/1991
Ortho HBc ELISA Test System	EIA	Serum/Plasma	Donor Screen	Ortho-Clinical Diagnostics, Inc Raritan, NJ US License 1236	4/18/1991
Hepatitis C Virus Encoded Antigen (Anti-HCV Assay)					
Abbott HCV EIA 2.0	EIA	Serum/Plasma	Donor Screen	Abbott Laboratories Abbott Park, IL US License 0043	5/6/1992
Ortho HCV Version 3.0 ELISA Test System	EIA	Serum/Plasma	Donor Screen	Ortho-Clinical Diagnostics, Inc Raritan, NJ US License 1236	5/20/1996
Chiron RIBA HCV 3.0 Strip Immunoblot Assay	SIA	Serum/Plasma	Donor Supplemental	Chiron Corp Emeryville, CA US License 1106	2/11/1999
HIV-1/HCV Nucleic Acid Testing					
Roche Amplicor HIV-1 Monitor Test	PCR	Plasma	Prognosis / Patient Management HIV-1 Viral Load Assay	Roche Molecular Systems, Inc Branchburg Township, NJ	3/2/1999
NucliSens HIV-1 QT	NASBA	Plasma	Prognosis / Patient Management HIV-1 Viral Load Assay	bioMerieux, Inc Durham, NC 27712	11/19/2001
COBAS Ampliscreen HIV-1 Test	PCR	Plasma	Donor Screen	Roche Molecular Systems, Inc Pleasanton, CA 94566	12/20/2002
Procleix	HIV-1/ HCV Nucleic Acid Test (TMA)	Plasma	Donor Screen	Gen-Probe San Diego, CA 92121	2/8/2002

(*continued*)

TABLE 21.1. (*Continued*)

Tradename(s)	Format	Sample	Use	Manufacturer	Approval Date
Trugene HIV-1 Genotyping Kit and Open Gene DNA Sequencing System	HIV-1 Genotyping	Plasma	Patient Monitoring	Visible Genetics, Inc Toronto, CA	4/24/2002
UltraQual HIV-1 RT-PCR Assay	PCR	Plasma	Donor Screen	National Genetics Institute Los Angeles, CA 92121	9/18/2001
UltraQual HCV RT-PCR Assay	PCR	Plasma	Donor Screen	National Genetics Institute Los Angeles, CA 92121	9/18/2001
ViroSeq HIV-1 Genotyping System with the 3700 Genetic Analyzer	HIV-1 Genotyping	Plasma	Detecting HIV genomic mutations that confer resistance to specific types of antiretroviral drugs, such as an aid in monitoring and treating HIV infection	Celera Diagnostics Alameda, CA 94502	6/11/2003
Versant HIV-1 RNA 3.0 (bDNA)	Signal amplification nucleic acid probe	Plasma	Patient Monitoring	Bayer Corp Berkeley, CA 94702	9/11/2002
COBAS AmpliScreen HCV Test	PCR	Plasma	Donor Screen	Roche Molecular Systems, Inc Pleasanton, CA 94566	12/3/2002
		Human Immunodeficiency Virus Type 1 (Anti-HIV-1 Assay)			
HIVAB HIV-1 EIA	EIA	Serum/Plasma	Donor Screen	Abbott Laboratories Abbott Park, IL US License 0043	3/1/1985
Genetic Systems rLAV EIA	EIA	Serum/Plasma	Donor Screen	Bio-Rad Laboratories Blood Virus Division Redmond, WA US License 1109	6/29/1998
Murex SUDS HIV-1 Test	Rapid EIA	Serum/Plasma	Donor Screen	Murex Diagnostics, Inc Norcross, GA US License 1152	5/22/1992

Vironostika HIV-1 Microelisa System	EIA	Serum/Plasma	Donor Screen	bioMerieux, Inc Durham, NC 27712 US License 1624	12/18/1987
Vironostika HIV-1 Plus O Microelisa System	EIA	Plasma/Serum/Dried Blood Spots	Diagnostic Non-Donor Screen	bioMerieux, Inc Durham, NC 27712 US License 1624	6/6/2003
Cambridge Biotech HIV-1 Western Blot Kit	WB	Serum/Plasma	Donor Supplemental	Calypte Biomedical Corp Berkeley, CA US License 1207	1/3/1991
Genetic Systems HIV-1 Western Blot	WB	Serum/Plasma	Donor Supplemental	Bio-Rad Laboratories Blood Virus Division	11/13/1998
Fluorognost HIV-1 IFA	IFA	Serum/Plasma	Donor Supplemental	Waldheim Pharmazeutika GmbH Vienna, Austria US License 1150	2/5/1992
HIVAB HIV-1 EIA	EIA	Dried Blood Spot	Non-Donor Screen	Abbott Laboratories	4/22/1992
HIV-1 Urine EIA	EIA	Urine Screen	Non-Donor Screen	Calypte Biomedical Corp	8/6/1996
Genetic Systems rLAV EIA	EIA	Dried Blood Spot	Non-Donor Screen	Bio-Rad Laboratories Blood Virus Division	6/29/1998
Vironostika HIV-1 Microelisa System	EIA	Dried Blood Spot	Non-Donor Screen	bioMerieux, Inc Durham, NC 27712	4/11/1990
Oral Fluid Vironostika HIV-1 Microelisa System	EIA	Oral Fluid	Non-Donor Screen	bioMerieux, Inc Durham, NC 27712	12/23/1994
Cambridge Biotech HIV-1 Western Blot Kit	WB	Urine	Non-Donor Supplemental	Calypte Biomedical Corp	5/28/1998
Genetic Systems HIV-1 Western Blot	WB	Dried Blood Spot	Non-Donor Supplemental	Bio-Rad Laboratories Blood Virus Division	11/13/1998
OraSure HIV-1 Western Blot Kit	WB	Oral Fluid	Non-Donor Supplemental	Epitope, Inc	6/3/1996
Fluorognost HIV-1 IFA	IFA	Dried Blood Spot	Non-Donor Supplemental	Waldheim Pharmazeutika GmbH	5/14/1996
OraQuick Rapid HIV-1 Antibody Test	Rapid EIA	Fingerstick	Non-Donor Screen	OraSure Technologies, Inc Bethlehem, PA 18015	11/7/2002

(*continued*)

TABLE 21.1. (*Continued*)

Tradename(s)	Format	Sample	Use	Manufacturer	Approval Date
Reveal Rapid HIV-1 Antibody Test	Rapid Immunoassay	Serum/Plasma	Non-Donor Screen	MedMira Laboratories, Inc Halifax, Nova Scotia Canada B3S 1B3	4/16/2003
Uni-Gold Recombigen HIV	Rapid Immunoassay	Serum/Plasma/Whole Blood	Non-Donor Screen	Trinity Biotech, plc Bray Co., Wicklow Ireland	12/23/2003
Human Immunodeficiency Virus Types 1 & 2 (Anti-HIV-1/2 Assay)					
Abbott HIVAB HIV-1/HIV-2 (rDNA) EIA	EIA	Serum/Plasma	Donor Screen	Abbott Laboratories Abbott Park, IL US License 0043	2/14/1992
Genetic Systems HIV-1/HIV-2 Peptide EIA	EIA	Serum/Plasma/ Cadaveric Serum	Donor Screen	Bio-Rad Laboratories Blood Virus Division Redmond, WA US License 1109	12/9/2000
Genetic Systems HIV-1/HIV-2 Plus O EIA	EIA	Serum/Plasma	Donor Screen	Bio-Rad Laboratories Inc Hercules, CA US License 1109	8/5/2003
Human Immunodeficiency Virus Type 2 (Anti-HIV-2 Assay)					
Genetic Systems HIV-2 EIA	EIA	Serum/Plasma	Donor Screen	Bio-Rad Laboratories Blood Virus Division Redmond, WA US License 1109	4/25/1990
Human T-Lymphotropic Virus Types I & II (Anti-HTLV-I/II Assay)					
Abbott HTLV-I/HTLV-II EIA	EIA	Serum/Plasma	Donor Screen	Abbott Laboratories Abbott Park, IL US License 0043	8/15/1997
Vironostika HTLV-I/II Microelisa System	EIA	Serum/Plasma	Donor Screen	bioMerieux, Inc Durham, NC 27712 US License 1624	1/17/1998

immunofluorescence assay or enzyme immunoassay (EIA), etc.], there is a problem in that shortly after infection by a pathogenic virus, there is a window period in which antibody generation is insufficient for detection (Chamberland, 2001). To reduce this window period, direct nucleic acid tests are needed.

Application of Advanced Molecular Techniques in Blood Safety Applications

Through the application of molecular biology, biological and biochemical analyses have been revolutionized, and nucleic acid, gene-based techniques have been developed to screen blood and plasma donations for evidence of very recent and earlier viral infections that could be missed by conventional serologic tests. The nucleic acid tests can also provide evidence for genetic variation in viruses. Molecular methods include the use of nucleic acid probes as well as amplification based and DNA sequence–based techniques. More and more molecular diagnostic methods are now available commercially. In comparison to classical methods, molecular biological methods are superior in terms of rapidness, specificity, and sensitivity. The current nucleic acid detection methods in the field may be grouped into two major classes: amplifying techniques such as PCR and nonamplifying techniques such as Southern blot hybridization. Amplifying techniques are more sensitive than nonamplifying techniques. There are two different amplifying methods (Hayden, 2005), target amplification methods and signal amplification methods. Target amplifying techniques include PCR, nucleic acid sequence–based amplification (NASBA) (Guichon et al., 2004, Starkey et al., 2004), self-sustaining sequence amplification (3SR), transcription-based amplification (TAS), transcription-mediated amplification (TMA), strand displacement amplification (SDA), and ligase chain reaction (LCR). Signal amplification methods include branched DNA signal amplification (bDNA) (Peter and Sevall, 2004), cleavage-based signal amplification (cycling probe technologies and invader assay), Qß replicase, hybrid capture, cycling probe technologies (CPT), and rolling-circle amplification (RCA) (Tang et al., 1997). To further insure the safety of blood products, it is of importance to further develop nucleic acid testing.

Major Different Generations of Nucleic Acid Detection Techniques

Southern Blot Hybridization (1970s)

Southern blotting (Mornet, 2004) was named after Edward M. Southern who developed this procedure at Edinburgh University in the 1970s. This technique is used to detect specific sequences within mixtures of DNA, which is size-fractionated by gel electrophoresis and then transferred by capillary action to a suitable membrane. After blocking of nonspecific binding sites, the nitrocellulose replica of the original

gel electrophoresis experiment is then allowed to hybridize with an oligonucleotide probe representing the specific DNA sequence of interest. Should specific DNA be present on the blot, it will combine with the labeled probe and be detectable. By co-electrophoresing DNA fragments of known molecular weight, the size(s) of the hybridizing band(s) can then be determined. Southern blotting hybridization technology is one of the major tools that have already helped clinical staffs world-wide interpret genomic information. Other competing methodologies include *in situ* hybridization and solution hybridization. Important clinical examples of the use of this technology are DNA fingerprinting and the ability to detect DNA gene rearrangements.

Polymerase Chain Reaction (1980s)

In 1983, Dr. Kary Mullis at Cetus Corporation conceived of polymerase chain reaction (Hersing, 1993). There is not any technique that has had a greater impact on the practice of molecular biology than PCR. With this technique, we can detect infectious disease agents at an extremely low level. It is based on the ability of sense and anti-sense DNA primers to hybridize to a DNA of interest. After extension from the primers on the DNA template by DNA polymerase, the reaction is heat-denatured and allowed to anneal with the primers once again. Another round of extension leads to a multiplicative increase in DNA products. Therefore, a minute amount of DNA can be efficiently amplified in an exponential fashion to result in easily manipulable amounts of DNA. By including critical controls, the technique can be made quantitative. The current level of the sensitivity and detection limit is as low as 10–50 copies per mL in HIV testing (Ginocchio et al., 2003). Important clinical examples of the use of PCR are detection of HIV and HCV (Abbott, 2003; Roche, 2003; Katsoulidou, 2004). PCR techniques have evolved into different branches. Some of them are now widely in use for virus detection in clinical diagnostics. These are real-time PCR by Taqman (Roche), Light Cycler (Roche), and Smart Cycler (Cepheid), and *in situ* PCR, nested-PCR, broad-range PCR, multiplex PCR, RT-PCR, arbitrarily primer PCR, long PCR, and quantitative PCR. Real-time sequence technology will be coming soon for more detailed detection. In the past, identification of viral serotypes was restricted to investigative methods using antibody detection and restriction fragment length polymorphism (RFLP). With real-time sequences technology, we will be able to detect a virus early as well as obtain the viral sequence.

Microarrays (1990s)

Microarrays were developed at Stanford University by Schena and co-workers in the early 1990s (Schena, 2002). For medical applications, a microarray analysis offers a very accurate screening technology. It allows hundreds or thousands of nucleic acid hybridization reaction to be performed on a solid substrate. It will be a fast and accurate diagnostic tool in the field of clinical microbiology and virology.

Applied to infection safety for blood and blood products, it will be able to screen for the presence of viral pathogens by matching genetic sequences. Compared with existing technologies, it allows for a wider variety of specific tests to be carried out simultaneously to determine the quality of the blood and will provide consumers with extra safety. With the use of molecular biology protocols, the microarray will permit the detection of lower concentrations of microorganisms in the blood and the accurate identification of many types of pathogenic contaminants. In the future, progress can be expected in the application of microarray technology for screening of donated blood for infectious agents. It can provide vast information about the identity of bloodborne pathogens as well as their gene expression profiles (yu et al., 2004).

Screening of Donor Blood for Infectious Agents

To ensure a safe blood supply for everyone who may need a transfusion, an important step in ensuring safety is the screening of donated blood for infectious agents. After donation, each unit of donated blood undergoes a series of tests for bloodborne agents such as human immunodeficiency virus (HIV)-1, HIV-2, hepatitis B virus (HBV), hepatitis C virus (HCV), human T-cell lymphotropic virus (HTLV)-1 and HTLV-II, West Nile virus (WNV), and the agent of syphilis.

Confirmatory Testing of Donor Blood for Infectious Agents

All of the above tests are referred to as screening tests and are designed to detect as many infectious agents as possible. Because these tests are so sensitive, some donors may have a false-positive result, even when the donor was never exposed to the particular infection. In order to sort out true infections from false-positive test results, screening tests that are reactive may be followed up with more specific tests called confirmatory tests. Thus, confirmatory tests help determine whether a donor is truly infected. If any one of these tests fails, affected blood products are considered unsuitable for transfusion (U.S. FDA, 2004).

Application of Nucleic Acid Testing for Infectious Agents

Nucleic acid testing (NAT) employs testing technology that directly detects the genomes of viruses. Because NAT detects a virus's genetic material instead of waiting for the body's response— the formation of antibodies, as with many current tests—it offers the opportunity to reduce the window period during which an infecting agent is undetectable by traditional tests (Stramer et al., 2004), thus further improving blood safety. Nucleic acid testing will become the gold standard because of greater sensitivity compared to antibody tests.

Since 1999, NAT has been approved by the FDA and used to detect HIV-1 and HCV, and this technology is under investigation for detecting other infectious disease agents. We know that viral RNA appears very early in the infection, in 1 to 2 weeks, but the antibody doesn't appear until 10–12 weeks (e.g., HIV and HCV) (CDC, 1997). In order to virtually prevent infection by all the transfusion associated viruses, we need to detect the viruses in their window period, and a NAT or gene-based testing method is needed. NAT also provides an opportunity for the viral (e.g. HIV or HCV) infected donor to seek early treatment. On the other hand, NAT is not only a sensitive method but also a rapid method that is suitable for a blood bank laboratory because the turn-around time for maintaining blood donations is extremely critical.

Hepatitis B Virus

The hepatitis B virus (HBV) is a highly infectious and often nonsymptomatic virus that is transmitted primarily through blood and blood-derived fluids and is a leading cause of liver infection worldwide. The World Health Organization (WHO) estimates that 2 billion people worldwide have been infected with HBV and 350,000,000 people are chronically infected. Chronic infection results in a high risk for liver cancer and cirrhosis of the liver, which cause about 1,000,000 deaths each year. Each year up to 200,000 people become newly infected in the United States alone. Since screening for HBV began in 1969, the rate of infection through blood transfusions has greatly decreased. However, as of 2000, HBV is still transmitted through blood transfusions in 1 out of 137,000 units of blood. One reason for this is that currently available blood screening technologies detect core antibodies or surface antigens, which appear up to 8 weeks after infection. Serologic tests for hepatitis B virus include hepatitis B surface antigen (HBsAg) and hepatitis B core antibody (HBcAb).

Hepatitis B Surface Antigen

HBV, which mainly infects the liver, has an inner core and an outer envelope (the surface). The HBsAg test detects the outer envelope, identifying an individual infected with the hepatitis B virus. This virus can cause inflammation of the liver, and in the earliest stage of the disease, infected people may feel ill or even have yellow discoloration of the skin or eyes, a condition known as jaundice. Fortunately, most patients recover completely and test negative for HBsAg within a few months after the illness. A small percentage of people become chronic carriers of the virus, and in these cases, the test may remain positive for years. Chronically infected people can develop severe liver disease as time passes and need to be followed carefully by an experienced doctor. To reduce the occurrence of post-transfusion hepatitis, it is essential to screen all blood donations for hepatitis B surface antigen by the most sensitive and specific assays. Blood donations that are found to be reactive in the HBsAg test are automatically confirmed by the HBsAg confirmatory assay. If the specimen is neutralizable in the confirmatory test, the specimen is

considered positive for HBsAg. Hepatitis B surface antigen testing of donated blood was begun in 1975 (Table 21.1).

Currently, all blood donors are screened for HBsAg, but occasional transmission of HBV still occurs due to the inclusion of window period donations. (i.e., blood from recently infected donors who are antibody negative but still viremic). Detection of early HBV infection of blood donors is still a major problem of blood transfusion. Using the current third-generation licensed HBsAg tests (mostly radioimmunoassay and enzyme immunoassays) cannot detect HBV in the window period for HBV infection. This is a strong motivation for introducing molecular detection techniques to the field. There are some commercially available test methods for detecting HBV DNA in the market now, such as Chiron's Quantiplex HBV DNA (Krajden et al., 1998), Digene's Hybrid Capture, Abbott's HBV DNA assay, and Roche's Amplicor HBV Monitor. Using these commercial hybridization or PCR-based assays, HBV DNA can be detected 1 to 3 weeks before the appearance of HBsAg (Hollinger and Jake, 2001). All HBsAg positive patients (blood donors) have detectable HBV DNA in their serum. Some chronically infected patients who have lost their HBsAg remain HBV DNA positive but are disqualified as potential blood donors. Molecular detection of HBV DNA is more sensitive than current methods employed for HBsAg screening.

Antibodies to the Hepatitis B Core Antigen (Anti-HBc)

Determination of anti-HBc (total) is also used to monitor the progress of the hepatitis B viral infection. Determination of anti-HBc (IgM) is employed to distinguish an acute hepatitis B infection from a chronic infection. The anti-HBc test developed in 1987 detects an antibody to the hepatitis B virus that is produced during and after infection. If an individual has a positive anti-HBc test, but the HBsAg test is negative, it may mean that the person once had hepatitis B but has recovered from the infection. Of the individuals with a positive test for anti-HBc, many have not been exposed to the hepatitis B virus; thus, there is a frequent problem of false positives. Although the individual may be permanently deferred from donating blood, it is unlikely that the person's health will be negatively affected. (Note: This antibody is not produced following vaccination against hepatitis B.)

Hepatitis C Virus

The hepatitis C virus (HCV) is a member of the Flaviviridae family of viruses, which are associated with both human and animal diseases. Hepatitis caused by HCV is the most common chronic bloodborne infection in the United States. Over 4 million Americans are believed to be infected. HCV can also be transmitted through blood transfusion. HCV causes inflammation of the liver, and up to 80% of those exposed to the virus develop a chronic infection, which can lead to liver inflammation, cirrhosis, cancer, and death. Eventually, up to 20% of people with HCV may develop cirrhosis of the liver or other severe liver diseases. As in other forms of hepatitis, individuals may be infected with the virus but may not realize

they are carriers because they do not have any symptoms. Because of the risk of serious illness, people with HCV need to be followed closely by a physician with experience evaluating this infection. Since the full length HCV cDNA was first cloned in 1989, significant progress has been made in characterizing its molecular biology (Lamballerie, 1996). But, the natural history of HCV infection is still largely unclear and current treatment options for patients are limited. There is no vaccine for HCV, and the only available treatment, a combination of alpha-interferon and ribavirin, is efficacious in only a minority of patients (Wang and Heinz, 2001). The life cycle of the HCV is poorly understood due to the lack of an efficient cell culture system (Cohen, 1999). There is an urgent need to develop a highly sensitive detection method for studying possible extrahepatic sites for the replication of hepatitis C virus. We recently established a cell culture system for the replication of HCV by using human T and B leukemia cell lines. (Hu et al., 2003) This model should represent a valuable tool for the detailed study of the initial steps of the HCV replication cycle and for the evaluation of antiviral molecules. Currently, appropriate therapeutic and vaccine strategies for HCV have not been developed. Early detection and prevention of HCV infection are most important for blood safety.

It is a formidable task to design primers and probes for sensitive nucleic acid level diagnostic assays throughout the open reading frame of the HCV genome because of a high mutation rate in this genomic region. However, the untranslated region of about 341 nucleotides contains highly conserved domains, which allows for stable primer design and sensitive diagnostic tests, both qualitative and quantitative, which have equivalent sensitivity against the known six various genotypes of HCV.

Antibodies to the Hepatitis C Virus (Anti-HCV)

In 1990, the first specific test for HCV, the major cause of "non-A, non-B" hepatitis was introduced. Now, a third-generation ELISA kit is available to detect antibodies to HCV, and screening blood for HCV antibodies is recommended. These assays are based on detection of serum antibody to various HCV antigens because these antibodies are nearly universally present in patients who are chronically infected with HCV (Major et al., 2001). The HCV screening tests are known to have significant limitations, and positive samples should be further tested by HCV confirmatory tests.

HCV Confirmatory Tests

Guidelines provided by the CDC recommend that HCV antibody screening test positive samples should be confirmed with serologic or nucleic acid supplemental testing. HCV confirmatory tests include the recombinant immunoblot assay in which several recombinant peptide antigens are applied on a strip that is then probed with the patient's serum. In this way, the response to individual antigens can be recognized, and some false-positive ELISA results can be eliminated

(e.g., RIBA, Chiron HCV 3.0 and PCR assay) (e.g., Roche COBAS AMPLICOR HCV Test, version 2.0). Laboratories can choose to perform this testing on all positive specimens or based on screening test positive (signal to cutoff) ratios. The positive predictive values (s/co) can vary depending on the prevalence of infection in the population being screened.

HCV antibodies are not generally detectable for at least 6 weeks and may not appear for several months. Acute HCV infections are relatively rare among blood donors, but the antibody tests often fail to detect these patients in the window period between the time of infection and the time of appearance of antibody detectable by the above assays. High-sensitivity detection of HCV during the window period is a long-term technical challenge in the field. Tests for HCV RNA genome detection based on the PCR or other highly sensitive RNA detection systems have been used for the diagnosis of acute hepatitis (Major et al., 2001). Sensitive detection of HCV RNA based on RT-PCR or other nucleic acid amplification techniques can be readily accomplished with kits that are now available commercially. For example, in 1999 the FDA approved Roche's Amplicor HIV-1 Monitor Ultra Sensitive quantitative assay. It can measure HIV levels at as few as 50 copies/mL and another commercial kit, the LCx HIV RNA Quantitative Assay from Abbott Laboratories, also has a detection limit at 50 copies/mL. Some research papers even showed a sensitivity limit at 1 copy (Palmer et al., 2003). In fact, a qualitative assay should be much more sensitive than a quantitative assay for HIV/HCV screening. A sensitive qualitative HCV molecular detection assay will possibly interdict and virtually prevent all transfusion-associated HIV/HCV. The current sensitivity standard for clinical diagnostics is 100 copies per mL, but since there has been an improvement in technology, this would be the time to change sensitivity standard to 50 copies per mL.

Human Retroviruses

Antibodies to the Human Immunodeficiency Virus, Types 1 and 2 (Anti-HIV-1, -2)

HIV-1 and/or HIV-2 virus cause acquired immunodeficiency syndrome, or AIDS. The test is designed to detect antibodies directed against antigens of the HIV-1 or HIV-2 viruses. HIV-1 is much more common in the United States, whereas HIV-2 is prevalent in Western Africa. Donors are tested for both viruses because both are transmitted by infected blood, and a few cases of HIV-2 have been identified in U.S. residents. In 1985, the first blood-screening EIA test to detect HIV was licensed and quickly implemented by blood banks to protect the blood supply. And, in 1992, testing of donor blood for both HIV-1 and HIV-2 antibodies (anti-HIV-1 and anti-HIV-2) was implemented. In 1996, HIV p24 antigen testing of donated blood was mandated although the test does not completely close the HIV window. Now, the p24 antigen testing is going to be compared with a PCR-based test for their ability to detect HIV in the window period.

Antibodies to Human T-Lymphotropic Virus, Types I and II (Anti-HTLV-I, -II)

HTLV retroviruses are endemic in Japan and the Caribbean but relatively uncommon in the United States. They cause adult T-cell leukemia/lymphoma and a neurological disorder similar to multiple sclerosis. The infection can persist for a lifetime but rarely causes major illnesses in most people who are infected. In rare instances, the virus may, after many years of infection, cause nervous system disease or an unusual type of leukemia. HTLV-II infections are usually associated with intravenous drug usage, especially among people who share needles or syringes. Disease associations with HTLV-II have been hard to confirm, but the virus may cause subtle abnormalities of immunity that lead to frequent infections, or rare cases of neurological disease.

In 1989, human T-lymphotropic virus antibody testing of donated blood was begun. Blood is now routinely screened for antibodies to HTLV-I, II. These tests screen for antibodies directed against epitopes of the HTLV-I and HTLV-II viruses. Several commercial assays based on the enzyme-linked immunosorbent assay (ELISA) or particle agglutination formats are used for screening of HTLV antibodies, followed by confirmatory assays using Western blotting. In some infected individuals, the serologic response to HTLV infection is very low. These problems have been solved by the application of PCR amplification of specific sequences in the virus genome. PCR can be used to detect HTLV-I, II provirus and is now the method of choice for detection of HTLV DNA directly from blood and many other tissues. Commercial PCR kits for HTLV are available (Green and Chen, 2001).

West Nile Virus

West Nile virus (WNV) is a single-stranded RNA virus of the Flaviviridae family and is the most recent emerging infectious disease threat to public health and, potentially, to the safety of our blood supply. In 2002, WNV was identified as transfusion transmissible. It is transmitted by mosquitoes to birds and other animals through a mosquito bite. The virus can infect people, horses, many types of birds, and some other animals. WNV was shown in 2002 to be transmissible by blood (Biggerstaff and Peterson, 2003), with an estimated mean risk of 2/10,000 to 5/10,000 in outbreak regions in the United States. The most common symptoms of transfusion-transmitted cases of WNV were fever and headache. Detection of WNV includes either a measurement of WNV antibodies or of WNV nucleic acid (detecting genetic material from the virus itself). There are two types of WNV antibody testing: IgM and IgG. In most individuals, IgM antibodies will be present within 8 days after the initial exposure to WNV, followed by IgG production several weeks later. But, the antibodies tested to detect WNV are not expedient for donor blood screening. Nucleic acid testing involves amplifying and measuring the West Nile virus's genetic material to detect the presence of the virus in blood or tissue. WNV NAT will be negative in the blood once clinical illness has occurred. In this situation, both NAT and IgM antibody testing may be needed. Nucleic

acid tests to screen blood for WNV are commercially available and in current use. But, the viral yield for WNV infection is much lower than other viruses. Consequently, a more sensitive WNV NAT system for donor blood screening will be required, which could further reduce the risks of transfusion transmitted WNV.

Syphilis

Serum samples from all blood units should be subjected to either the VDRL (Venereal Disease Research Laboratory) test or a treponemal test, such as the *Treponema pallidum* hemagglutination (TPHA) test before transfusion. Any unit found positive should be discarded as per standard safety procedures. This test is done to detect evidence of infection with the spirochete that causes syphilis. Blood centers began testing for this shortly after World War II, when syphilis rates in the general population were much higher. The risk of transmitting syphilis through a blood transfusion is exceedingly small (no cases have been recognized in this country for many years) because the infection is very rare in blood donors, and because the spirochete is fragile and unlikely to survive blood storage conditions. Sensitivity and specificity of serologic tests vary depending on the type of test performed and the stage of the disease. If the donor has spirochetemia, their serologic tests are usually negative, and if the donors are antibody positive, their blood is not infectious. Syphilis serological tests for donors have less clinical significance. A nucleic acid test for accurately detecting syphilis is needed. It can be used to determine whether a blood donor is currently or has recently been infected with the spirochete.

Other Concerns

Hepatitis Viruses

In recent years, numerous infectious agents found worldwide have been identified as potential threats to the blood supply and among these are several newly discovered hepatitis viruses that present unique challenges in assessing possible risks. Even if the hepatitis virus test is negative for all known A–E hepatitis agents, there are some unidentified hepatitis viruses, called non A–E hepatitis viruses that can still be transmitted by blood transfusion. In the future, advances in NAT may allow rapid discovery of the unknown hepatitis viruses.

TT Virus

TT virus (TTV) (Cossart, 2000), named for the patient from whom it was first isolated with non A–E and G post-transfusion hepatitis in Japan in 1997, is a newly discovered transfusion transmitted, single-stranded and circular DNA virus (Okamoto et al., 1999). TTV is non-enveloped and its entire sequence of ~3.9 kb has been determined. It is also often interpreted as a transfusion-transmitted virus (Cossart, 2000). At least 16 genotypes have been identified, and TTV is now found

all over the world. TTV infection was sought by detection of TTV DNA in serum by polymerase chain reaction using primers generated from a conserved region of the TTV genome (e.g., the UTR region) (Das et al., 2004). Donor blood and blood product can be screened for TTV DNA by using PCR or real-time PCR. The significance of positive findings is still unclear, because high-level TTV carriers in healthy populations are currently found (Nishizawa et al., 1997; Blut, 2000). Whether TTV actually causes hepatitis remains to be determined.

Cytomegalovirus

Cytomegalovirus (CMV) is a virus belonging to the herpes group that is rarely transmitted by blood transfusion. Donor blood is not routinely tested for CMV, and the prevalence of CMV antibody ranges from 50% to 80% of the population. But, blood contaminated with CMV can cause problems in neonates or immunocompromised patients. It also remains a major pathogen for solid-organ transplant recipients causing febrile syndromes, hepatitis, pneumonitis, retinitis, and colitis. Potential problems in selected patient populations can be prevented by transfusing CMV negative blood or frozen, deglycerolized red blood cells. Serologic tests for antibody to CMV are useful for determining whether a patient had CMV infection in the past, a determination of great clinical importance for organ and blood donors, and in the pretransplant evaluation of prospective transplant recipients (Pass, 2001). Commercial NAT kits are available for CMV (Hayden, 2004), these include the Amplicor PCR CMV Monitor test and Hybrid capture system CMV DNA test.

Malaria

Sensitive screening tests for malaria are neither commercially available nor officially approved yet. The most effective way of screening donors is to take a proper history of malaria or of fever that could be due to malaria. Donor selection criteria should be designed to exclude potentially infectious individuals from donating red blood cells for transfusion. Because there are no practical laboratory tests available to test donor blood, donors traveling to high-risk malaria areas are excluded from donating blood for 6 months. However, there is a need to develop suitable screening tests, especially for use in an endemic area. A number of clinical research approaches have been developed for the extraction, amplification, and detection of malaria parasite DNA from blood products (Pass, 2001).

Severe Acute Respiratory Syndrome

Coronavirus is an RNA virus known to be associated with respiratory disease. Severe acute respiratory syndrome (SARS) virus is a newly recognized coronavirus whose genome sequence does not belong to any of the known coronavirus groups and which quickly spread all over the world from Asia in 2003. There has been no evidence that this infection is transmitted from blood donors

to transfusion recipients, but the virus associated with SARS is present in the blood of people who are sick, and it is possible that the virus could be present in blood immediately before a person gets sick, so that an individual with infection but no symptoms possibly could transmit SARS through a blood donation. To help determine whether or not an individual might be infected with SARS, a blood collection facility will ask a potential donor orally or in writing about any travel to a SARS-affected country or a history of SARS or possible exposure to SARS. Enzyme-linked immunoassays for detection of specific IgG and IgM antibodies and RT-PCR for detection of SARS coronavirus–specific RNA in SARS patients has been developed. Rapid, sensitive, and specific identification of SARS and other novel coronaviruses by molecular methods will be very important in the future.

Discovery of Unrecognized and Uncharacterized Viral Agents

Based on past history, it is not just a hypothetical risk that many people have been infected with unrecognized viruses, for example, many patients with symptoms of non A–E, G, and TTV post-transfusion hepatitis. It is still possible that unexplained cases of post-transfusion hepatitis may be caused by a new, undiscovered pathogen. In recent years, numerous new infectious agents found worldwide have been identified through time-consuming procedures. By the time a new virus, such as HCV, HIV, and SARS, is found, many people are infected and there could be a large number of fatalities. There is an urgent need to develop methods for rapid identification and characterization of previously unknown pathogenic viruses. The most recent technologies for detecting and identifying previously unrecognized pathogens are expression library screening, representational difference analysis, and broad-range polymerase chain reaction. But they are all time-consuming approaches. The new unrecognized and uncharacterized viral agents can be rapidly identified by some of the new molecular approaches, for example, subtraction hybridization (Hu and Hirshfield, 2005) and DNA microarray.

Conclusion

Ensuring the safety and efficacy of blood and blood products is a critical regulatory challenge. The high safety level of the blood supply is the result of continued improvements in blood donor screening and testing. It will be achieved by introducing more updated nucleic acid tests to the field of blood safety. Nucleic acid testing is a method of testing blood that is more sensitive and specific than conventional tests that require the presence of antibodies to trigger a positive test result. Also, NAT works by detecting the low levels of viral genetic material present when an infection occurs but before the body develops an immune response to a virus. This improved sensitivity should enable us to significantly decrease the infection window period, allowing for earlier detection of the infection and diminishing the

chances for transmission of the agent via transfusion. We are not only to protect the blood supply from known pathogens but also the emergence of new and unrecognized and uncharacterized infectious agents. The NAT methods are more sensitive and specific compared with non-NAT. In the future, NAT technology, such as PCR, may allow routine screening of donors for all the known and unknown pathogens of concern to blood safety.

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22
Review of Molecular Techniques for Sexually Transmitted Diseases Diagnosis

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Introduction

Sexually transmitted diseases (STDs) constitute the most common infectious diseases around the world and bear significant consequences for both the individual and public health of the community. More than 20 STDs have now been identified, and they affect more than 13 million men and women in the United States each year (CDC, 2002). Data from the Centers for Disease Control and Prevention (CDC) show that more than 7 million cases of *Chlamydia trachomatis* infection and more than 350,000 cases of *Neisseria gonorrhoeae* infection were reported in 2000 (CDC, 2001). In the past decade, the rapid development of molecular techniques has gradually shifted the paradigm of laboratory diagnosis from traditional biological to molecular amplification and detection of major causative agents of sexually transmitted infections.

A milestone in biotechnology that heralded the beginning of molecular diagnostics was the development of the polymerase chain reaction (PCR) by Mullis and colleagues (Saiki et al., 1988). Since then, numerous molecular detection techniques have been designed to detect specific nucleic acids without relying on the ability to culture or directly observe intact organisms. As a result, stringency in transport of clinical samples, in terms of preserving organism viability, has become less strict. With automation, a faster turn-around time of molecular tests became an advantage that significantly enhances this paradigm shift. Silent pathogens such as human papillomavirus (HPV) that cannot be cultivated *in vitro* can now be detected and typed by using molecular detection techniques that can also determine oncogenic potential and prognostic outcome of different infections. These powerful molecular techniques have a significant impact on strategies and public health programs designed for the control and prevention of STDs worldwide.

An estimated 50% of STDs occur asymptomatically, and this forms a major reservoir of infectious source that persists in the community. More sensitive detection techniques are often required for detecting asymptomatic individuals with low microbial load (Yoshida et al., 2002). Currently available molecular techniques using nucleic acid amplification can now offer high sensitivity in screening for these infections and disrupt the transmission chains within the community. This would,

in turn, lead to decrease in case burden and ultimately eliminate the reservoir of infection.

Because of difficulties in selection of representative samples for evaluation of commercially available test kits, interpretation of test performance (specificity, sensitivity) must be done with clear definition of the prevalence of disease in the test population. Also, because of obvious social stigma that can be associated with various STDs, accuracy (positive predictive values) of tests should be of highest priority in selection of those that are appropriate for the patient. Presence of nonviable gene fragments can, in principle, generate positive signals in all the molecular tests. This means that vigorous evaluation and correlation studies must be done before the significance of their presence can be adequately interpreted.

Although rapid tests do significantly reduce turn-around time and this will expectedly generate pressures from both clinician and patient, the choice of molecular tests should not depend on this consideration alone. In less well developed countries, cost implications of some molecular tests can become an insurmountable one that must be balanced with available resources. However, with rapid evolution and refinement of different test platforms, it is to be expected that unit prices of many molecular tests will decline significantly in the very near future.

This paper intends to review the currently developed and available molecular diagnostics of common STDs including (1) *Neisseria gonorrhoeae*; (2) *Chlamydia trachomatis*; (3) *Treponema pallidum*; (4) *Haemophilus ducreyi*; (5) *Mycoplasma* and *Ureaplasma*; (6) *Trichomonas vaginalis*; (7) herpes simplex virus; and (8) human papilloma virus.

Neisseria gonorrhoeae

Neisseria gonorrhoeae is the etiological agent of gonorrhea and remains a major sexually transmitted infection worldwide. In the United Kingdom, the number of gonorrhea cases has been rising each year since 1995 (more than 60 million cases) (Ward et al., 2000) with a 56% increase overall, including a 25% rise in 1999 (Ward et al., 2000).

Due to the extensive antigenic variability of many gonococcal surface components, no effective vaccine for *N. gonorrhoeae* is available (Unemo et al., 2003). Geographic and international surveillance of the epidemiological characteristics as well as the antibiotic susceptibilities of the pathogen become essential in the prevention and control of this infection. From both local and global perspectives, *N. gonorrhoeae* strains continue to evolve phenotypically and genotypically over time. It is therefore essential to develop discriminative and precise characterization laboratory techniques that can help control the transmission of infections, especially those caused by antibiotic-resistant strains.

Traditional Diagnostic Methods

There are several traditional diagnostic methods for *N. gonorrhoeae* such as direct stained smear examination, antigen detection, and culture. Sensitivity of direct

male urethral smears ranges 90–95% while that of female endocervical smears is 50–70% (Janda et al., 2003). Owing to colonization by other Gram-negative coccobacillary organisms, endocervical smears as well as rectal specimens require extra care in interpretation. Enzyme immunoassay (EIA) (Gonozyme, Abbott Laboratories, Abbott Park, IL, USA) is a presumptive diagnostic method for gonococcal antigen detection. The sensitivity and specificity is comparable to direct smear examination, although it is less sensitive for endocervical specimens (Knapp, 1988). Isolation and identification of *N. gonorrhoeae* from cultures is still considered the gold standard for the diagnosis of gonococcal infections. Both nonselective (chocolate agar) and selective media such as modified Thayer–Martin (MTM), Martin–Lewis (ML), or New York City (NYC) are often used for primary isolation.

Molecular Detection Methods

The commercial detection kits currently available for *N. gonorrhoeae* include those using probe hybridization (Pace 2, GenProbe, San Diego, CA, USA), PCR (COBAS AMPLICOR; Roche Molecular Systems, Branchburg, NJ, USA), ligase chain reaction (LCR; Abbott Laboratories, Abbott Park, IL, USA), and strand displacement amplification (SDA; Becton Dickinson, Sparks, MD, USA). Besides, two PCR assays have been published that target the gene encoding outer membrane protein III (*ompIII*) (Liebling et al., 1994) and the *cppB* gene (Ho et al., 1992) of *N. gonorrhoeae*.

Probe Hybridization

No amplification of nucleic acid takes place in the probe hybridization method. This method is based on annealing of complementary nucleic acid strands on a stable double-strand. There are two nucleic acid probe assays: the GenProbe PACE 2 and PACE 2C assays (GenProbe) and the Hybrid Capture II assay (Digene Corp., Gaithersburg, MD, USA) (Modarress et al., 1999). Both assays have been approved by the Food and Drug Administration (FDA) in the United States for detecting *N. gonorrhoeae*. In the GenProbe assay, target sequence of ribosomal RNA of *N. gonorrhoeae* is hybridized by an acridinium ester–labeled complementary DNA probe (Kluytmans et al., 1991). After adsorption of DNA-RNA hybrids to magnetic particles and removal of the unbound probe, the acridinium ester-DNA-RNA hybrid is measured in a luminometer. PACE 2C is a single-tube assay that can screen for presence of both *Chlamydia trachomatis* and/or *N. gonorrhoeae* (Iwen et al., 1995). If positive result is initially obtained, individual organism can be identified by performing separate tests. The initial positive result can also be verified by probe competition assay with unlabeled probe. In Hybrid Capture II assay, specific RNA hybridization probes are used to detect both genomic DNA and cryptic plasmid DNA sequences of *C. trachomatis* and *N. gonorrhoeae* (Girdner et al., 1999; Schachter et al., 1999). The RNA-DNA hybrids are captured by hybrid-specific antibodies in microtiter plates and detected in luminometer by adding chemiluminescent substrate with alkaline phosphatase–labeled antibodies. However, there

is no supplemental test to verify the initial positive results. Sensitivity and specificity of probe hybridization ranged from 96.3% to 100% and 98.8% to 99.6%, respectively (Girdner et al., 1999; Schachter et al., 1999).

PCR

Molecular techniques using direct nucleic acid amplification from clinical samples are so powerful that theoretically a single copy of target DNA or RNA in the sample can be detected. These techniques obviate the requirement for presence of viable organisms in specimens, much like other nonculture approaches, in the diagnosis of infections as well as for molecular epidemiological typing studies. The COBAS AMPLICOR (Roche Molecular Systems) is one of three commercial molecular detection tests that are FDA approved for *N. gonorrhoeae*. The COBAS AMPLICOR NG test targets a 201-bp sequence in the cytosine methyltransferase gene of *N. gonorrhoeae*. Sensitivity and specificity of PCR ranged from 94.2% to 98.1% and 98.4% to 100%, respectively (Martin et al., 2000; van Doornum et al., 2001). However, it has been reported that the COBAS AMPLICOR NG test for *N. gonorrhoeae* cross-reacts with certain strains of nonpathogenic *Neisseria* species, such as *N. subflava* and *N. cinerea* (Farrell, 1994). Approximately 26% of COBAS AMPLICOR NG–positive results were false positives in one test population and corresponded to approximately 3% of the total population (Farrell, 1994). However, the same laboratory observed less than 1% false-positive results among urogenital specimens from a second study population (Farrell, 1994).

Two PCR assays targeting the gene encoding outer membrane protein III (*omp*III) (Liebling et al., 1994) and the cppB gene (Ho et al., 1992) of *N. gonorrhoeae* have been published. Specificity of the *omp*III assay was 96.4% and sensitivity 78.6% (Liebling et al., 1994). No false-positive or false-negative results have been described in *omp*III PCR assay (Palmer et al., 2003), whereas *cppB* PCR can give false-positive results from several different *Neisseria* spp. (Palmer et al., 2003). Less frequent genetic exchange events possibly occur at *omp*III, making it a potentially good target for use in molecular diagnostic tests (Palmer et al., 2003).

Ligase Chain Reaction

The *N. gonorrhoeae* ligase chain reaction (LCR) assay was developed by Abbott Laboratories as LCx *Neisseria gonorrhoeae* assay (Abbott Laboratories). This uses a ligase chain reaction amplification in the LCx probe system for detection of a specific 48-bp nucleotide sequence in the *opa*-encoding gene (*opa*-1) of *N. gonorrhoeae* (Stern et al., 1996). The assay uses four probes complementary to *opa* genes that may be present in up to 11 copies per bacterial cell. Sensitivity and specificity of the LCx assay ranged from 81% to 97.3% and 99.4% to 99.8%, respectively (Ching et al., 1995; Kehl et al., 1998). A study demonstrated that significant reproducibility problems can occur in the LCx probe system that would not be detected by the manufacturer's quality control procedures (Gronowski et al., 2000). Procedures for detecting and preventing contamination and possible solutions to

reproducibility problems have been suggested (Gronowski et al., 2000). In early 2003, the Abbott LCx for *N. gonorrhoeae* (and also for *Chlamydia trachomatis*) was globally withdrawn due to continuing batch to batch problems with the performance of the test.

Strand Displacement Amplification

The BDProbeTec amplified DNA assay (Becton Dickinson) uses strand displacement amplification (SDA) and fluorescent resonance energy transfer probes that target DNA sequences homologous to genomic DNA of *N. gonorrhoeae* (Koenig et al., 2004). The system uses sealed microwells to minimize the release of amplicons to the environment. Sensitivity and specificity of the BDProbeTec assay ranged from 84.9% to 98.5% and 92.5% to 98.6%, respectively (Little et al., 1999; Van Der Pol et al., 2001). The closed system design and assay flow are advantages when compared with other systems. Assay is performed on samples collected without transport medium, and transport at room temperature provides a significant advantage over other nucleic acid amplification tests.

Chlamydia trachomatis

Chlamydia trachomatis is the major causative agent of non-gonococcal urethritis, as well as epididymitis in men, and is the most common sexually transmitted bacterial pathogen worldwide. Genital *C. trachomatis* infection is a key global issue facing female reproductive health. It can cause cervicitis, pelvic inflammatory disease, infertility, and ectopic pregnancy. Asymptomatic infections are common in both men and women. Currently, routine screening for chlamydia are recommended by the Centers for Disease Control and Prevention on sexually active teenagers and adults of $\leq$24 years (CDC, 2002). Because of the requirements for possible population-based screening, an accurate and sensitive laboratory test using noninvasive procedures for the diagnosis of chlamydia is often required.

Traditional Diagnostic Methods

Inoculation of cell cultures with a genital specimen has been the conventional method for the laboratory diagnosis of *C. trachomatis*. This method was popularized during 1970s and 1980s. Relative to newer methods, the conventional approach entails more expense and is more labor intensive and time consuming (2 to 3 days). It also requires considerable manual expertise to perform the meticulous handling of the specimen during transport to maintain viability of inoculated organisms as well as cell cultures. Enzyme immunoassays (EIAs) and direct fluorescence assays (DFAs) are antigen detection methods that are still commonly used in many clinical diagnostic laboratories (Newhall et al., 1999; Van Dyck et al., 2001). Both methods bear less stringent demands on transport requirements, and test results can often be obtained on the same day. As specimens are transported

stably under ambient conditions, EIA is suitable for public health laboratories covering large geographic areas. It is also inexpensive because specimens can be processed in batch using automated equipment (Newhall et al., 1999). Because the test is based on the detection of chlamydial genus-specific lipopolysaccharide (LPS) using monoclonal or polyclonal antibodies linked to a solid-phase support, chlamydial LPS antibodies may also cross-react with the LPS of other Gram-negative bacteria to give false-positive results. Either DFA or blocking assays are needed to confirm positive EIA results and thus improve specificity. On the other hand, EIA lacks sensitivity as screening assay, especially for asymptomatic men, possibly due to its lower detection limit of 10,000 elementary bodies (Lin et al., 1992).

Molecular Detection Methods

Several amplified nucleic acid assays were developed in the early 1990s for detection of *C. trachomatis* gene fragments. These included polymerase chain reaction (PCR), ligase chain reaction (LCR), Q-β replicase-amplified hybridization (QBRAH), transcription-mediated amplification (TMA), and strand displacement amplification (SDA). Extensive evaluations of PCR and LCR have been done and showed that these amplified assays are at least 20–30% more sensitive than culture, antigen detection, or non–nucleic acid amplification detection methods (Darwin et al., 2002; Koumans et al., 2003).

Probe Hybridization

Two nucleic acid probe assays, the PACE 2C assay (GenProbe) and the Hybrid Capture II assay (Digene Corp.), are currently commercially available for detection of *C. trachomatis*. Both assays can also detect *N. gonorrhoeae* using the same specimen and have been mentioned in the previous section.

PCR

Several in-house PCR assays have been developed for detection of *C. trachomatis* in clinical specimens. Target regions of the primers employed include the cryptic plasmid (Wong et al., 1995), major outer membrane protein (MOMP) (Ngeow et al., 1997), cysteine-rich protein (Watson et al., 1991), or 16S RNA genes (Madico et al., 2000). COBAS AMPLICOR (Roche Molecular Systems) is a commercially available assay for detection of *C. trachomatis* in clinical specimens. This is a semiautomated plasmid-based assay using primers to amplify a 207-bp fragment captured with oligonucleotide probes immobilized onto magnetic beads. Uracil-*N*-glycosylase and dUTP are incorporated in the assay to counter the danger of amplicon contamination. Optionally, an internal control reagent is available for verification of negative results by detecting inhibitors of the amplification reaction and preexisting in the clinical specimens. Sensitivity and specificity of COBAS AMPLICOR for detection of *C. trachomatis* have been extensively reported and

ranged respectively from 94.5% to 100% and 98.5% to 100% (Vincelette et al., 1999; Lister et al., 2004).

Ligase Chain Reaction

Use of the ligase chain reaction assay to detect *C. trachomatis* was commercialized by Abbott Diagnostics and requires a dedicated thermocylcer and detection module. Like the PCR, LCR targets the cryptic plasmid of *C. trachomatis* using four oligonucleotide primers, a thermophilic ligase for continuous primers ligation, and *Taq* polymerase for PCR. The primers incorporate two labels, one for capture and the other for detection. The ligated product is captured on microparticles and detected by an alkaline phosphatase–labeled antibody and a fluorescence-producing substrate. The sensitivity and specificity of LCR assay for *C. trachomatis* ranged from 86.4% to 100% and 99.6% to 100%, respectively (Stary et al., 1998; Moncada et al., 2003). In early 2003, the Abbott LCx for *C. trachomatis* and *N. gonorrhoeae* was globally withdrawn due to continuing batch to batch problems with the performance of the test.

Q-β Replicase-Amplified Hybridization

Developed on the principles of sandwich hybridization, reversible target capture, and Q-β replicase amplification, the Q-β replicase-amplified hybridization (QBRAH) assay (Gene-Trak, Framingham, MA, USA) (An et al., 1995) is a 4-h test that detects *C. trachomatis* rRNA or rDNA. Two types of probes are used in this assay including a test-specific capture probe immobilized to magnetic beads and a replicatable RNA detector molecule containing a sequence complementary and adjacent to the capture probe site on the target. After reversible target capture, the signal is detected by replication of the detector molecule with Q-β replicase in the presence of propidium iodide. The lower detection limit of QBRAH is said to be five elementary bodies (EB) (An et al., 1995).

Transcription-Mediated Amplification

AMP-CT and APTIMA Combo 2 are transcription-mediated amplification (TMA) assays developed by GenProbe. The assay targets the 16S rRNA of *C. trachomatis* with hybridization of a DNA primer at its 5′ end to a phage RNA polymerase promoter sequence. After primer extension by reverse transcription, RNA complement is removed by RNase H. A second primer binds to the end of the DNA and is extended backwards to form a double-stranded DNA template that is transcribed by a phage T7 polymerase to give multiple transcripts. The RNA product is then detected in a luminometer by a hybridization protection assay (HPA) using an acridinium-labeled DNA probe and an enzyme-labeled anti–DNA-RNA duplex antibody. TMA has recently been shown to be just as sensitive and specific as PCR and LCR, with sensitivities ranging from 88.5% to 100% and specificities from 98.7% to 100% in urine samples as well as in both female and male urethral swab samples (Crotchfelt et al., 1998; Verkooyen et al., 2003).

Strand Displacement Amplification

As mentioned in the previous *N. gonorrhoeae* section, a semiautomated thermophilic strand displacement amplification (SDA) assay (Becton Dickinson) has been used to simultaneously amplify and detect *C. trachomatis* and *N. gonorrhoeae* in the BDProbeTec system (Little et al., 1999). The multicopy cryptic plasmid in *C. trachomatis* was chosen as the target region for detection. Both sensitivity and specificity of the assay, ranging from 95.2% to 100% and 92.6% to 100%, respectively, are comparable with the PCR assay (McCartney et al., 2001; Van Der Pol et al., 2001). However, it has been reported that the sensitivity of the pooled SDA tests can reduced from 100% to 86.5% when compared with individual SDA tests (Bang et al., 2003).

Treponema pallidum

Syphilis is a chronic and systemic sexually transmitted infection caused by the spirochaete *Treponema pallidum* subsp. *pallidum*. It has diverse clinical manifestations presenting in distinct stages: primary chancre or ulcer, rash of secondary syphilis, the relative asymptomatic latent stage, and then the potentially destructive tertiary stage. Despite repeated attempts over the years, *T. pallidum* cannot be cultured *in vitro* nor easily stained using simple techniques. Other laboratory methods have been developed to identify infection in different stages of syphilis. Routine laboratory tests of syphilis are based largely on either the visual detection of *T. pallidum* organism in a characteristic lesion or the presence of reactive antibodies in patient's serum (Larsen et al., 1989). Rabbit infectivity test (RIT) is the oldest and most sensitive method to identify infection with *T. pallidum* (Larsen et al., 1995). Although RIT is expensive and requires 3 to 6 months to complete and is therefore not practical for routine laboratory use, it has been the gold standard for measuring the sensitivities of other methods (Centurion-Lara et al., 1997; Wicher et al., 1998). It must be clear at the outset that not all laboratory methods can be used to diagnose all the distinct clinical stages of syphilis. Antibodies are often not present early in serum and are only detectable 1 to 4 weeks after a lesion has been formed. Lesions are also not always present in all stages (Larsen et al., 1995).

Traditional Diagnostic Methods

Routine diagnostic laboratory tests of syphilis can be broadly classified into four categories: (i) direct microscopic examination where a lesion is present; (ii) nontreponemal serologic test for screening; (iii) treponemal serologic test for confirmation; and (iv) direct antigen detection tests. In direct microscopic examination, *T. pallidum* is detected in primary or secondary syphilitic lesions by dark-field microscopy and identified by its characteristic morphology and motility (Larsen et al., 1989). Direct fluorescent antibody testing for *T. pallidum* (DFA-TP) method has

also been commonly used (Romanowski et al., 1987). Both dark-field microscopy and DFA-TP, however, do not distinguish *T. pallidum* from the other pathogenic species of *Treponema* (Larsen et al., 1995). On the other hand, nontreponemal serologic tests are based on detection of antibodies to a cardiolipid–cholesterol–lecithin antigen. They include the Venereal Diseases Research Laboratory (VDRL) and the Rapid Plasma Reagin (RPR) card test. Both are modified from the original Wasserman reaction (Larsen et al., 1989; Young, 1992). Limitations of these tests include the lack of sensitivity in early and latent stages of syphilis and the well-known possibility of biological false-positive reactions. Treponemal serologic tests are based on detection of treponemal specific antibodies to cellular component of *T. pallidum*. They include the serum fluorescent treponemal antibody absorption test (FTA-ABS), the microhemagglutination test (MHA-TP), and the *Treponema pallidum* particle agglutination (TPPA) test (Rodolph 1976). These tests have higher sensitivities and specificities than nontreponemal serologic tests and are used as confirmatory tests for syphilis. Although false-positive results are rare in FTA-ABS compared with nontreponemal serologic tests, they have been well documented to occur in association with autoimmune diseases, viral infections, and pregnancy (Sparling, 1971). Enzyme immunoassay (EIA) is a semiautomated method used to detect treponemal antibodies (Young et al., 1998). A format that can directly detect *T. pallidum* antigens in early syphilitic lesions has also been developed (Young et al., 1998). Antigens are extracted from swabs containing lesion exudates within 72 h and the antigen–antibody complexes are measured in an automated spectrophotometer device. Advantages of the EIA test are more objectivity in interpretation; less labor intensive; and potentially automatable.

Molecular Detection Methods

Polymerase chain reaction (PCR) is the newest laboratory technique for direct detection of *T. pallidum*. Only a few laboratories use PCR for routine case detection. The CDC has developed a PCR test that is available for specimens from patients with suspected neurosyphilis or with genital ulcer diseases (CDC, 2002). PCR has also been found to be useful for detection of congenital syphilis, and the sensitivity of the test is comparable to RIT for amniotic fluid specimens (Hollier, 2001). In addition, a syphilitic aortitis case (which often has atypical clinical presentation, and available serological tests are nonspecific) has recently been described that was diagnosed by the use of PCR (O'Regan et al., 2002). Dot blot hybridization is another technique also being used for detection of *T. pallidum* (Jethwa et al., 1995).

PCR

Several PCR-based tests of *T. pallidum* have been developed on the basis of membrane lipoproteins (Orle et al., 1996), *TmpA* and 4*D* genes (Hay et al., 1990), 16S RNA (Centurion-Lara et al., 1997), DNA polymerase I (*polA*) gene (Liu et al., 2001). Levels of detection of these assays ranged between 10^{-3} equivalents of

organisms by reverse-transcription PCR (RT-PCR) (Centurion-Lara et al., 1997) to 10–50 organisms by amplifying the gene fragment that encodes the 47-kDa membrane lipoprotein (Orle et al., 1996). Although the 47-kDa major membrane lipoprotein immunogen has been hypothesized to be involved in cell wall synthesis and expected to be conserved, the exact function in *T. pallidum* is still unknown (Weigel et al., 1994). The homology of sequences, therefore, cannot yet be compared for primer optimization. The RT-PCR test itself, however, is highly sensitive in detecting *T. pallidum*. Special care is required to prevent contamination from unrelated organisms in specimens during the time-consuming RNA isolation steps. A potential development that can be used in clinical application of PCR is a multiplex PCR for simultaneous detection of *T. pallidum*, *Haemophilus ducreyi,* and herpes simplex virus (Orle et al., 1996). In *polA* gene test, the detection limit of *polA* is about 10–25 organisms when analyzed on gel, and single organism can theoretically be detected when automated DNA analyzer is used for detecting the fluorescence-labeled amplicons. With further development and validation, this test has the potential to be applicable for routine diagnosis of syphilis.

Dot Blot Hybridization

Dot blot hybridization is a DNA-DNA hybridization method whereby PCR products are analyzed by dot blotting onto nylon membranes with a 496 PCR-generated, digoxigenin (DIG)-dUTP probe internal to *tpp47* gene of membrane lipoprotein (Jethwa et al., 1995). The PCR–dot blot appears to show good concordance with DFA. The detection limit of this test is about 10^2 organisms, but the reproducibility of the test method needs to be further improved (Jethwa et al., 1995).

Haemophilus ducreyi

Chancroid is a genital ulcer caused by *Haemophilus ducreyi* and is prevalent mainly in developing countries. Detection and treatment of genital ulcer disease have become increasingly important because of strong associations with increased risk of transmission of human immunodeficiency virus (HIV) infection (Pepin et al., 1992; CDC, 2002). As the disease can very often be definitively diagnosed only by isolation of the bacterium from the genital ulcer, this disease is probably underdiagnosed. Several studies have shown that the accuracy of clinical diagnosis for chancroid ranged from 33% to 80% (Dangor et al., 1990; Chapel et al., 1977). A definitive bacteriological diagnosis of the disease requires identification of the bacteria on specially prepared culture media that is not widely available from commercial sources. Even in experienced and well-equipped laboratories, the optimal sensitivity of bacteriological culture can only be up to 50% to 90% of clinically diagnosed chancroid cases (Dylewski et al., 1986; CDC, 2002). Because of lack of a gold standard on the diagnosis of chancroid, the sensitivity of culture can only be estimated.

Traditional Diagnostic Methods

Clinical diagnosis with *in vitro* culture of *H. ducreyi* currently remains the main tool for the diagnosis of chancroid. Laboratory culture technique has been used as the "reference standard" for evaluating new methods of diagnosis for many years. On the other hand, direct microscopic examination of clinical material by Gram stain can be misleading due to the polymicrobial flora that are often present in most genital ulcers. Direct microscopy is, therefore, not recommended to be used as a routine diagnostic tool for chancroid (Albritton, 1989). *H. ducreyi* grows relatively slowly with small-sized colonies, rendering it easily overlooked on culture plates. This is especially so with respect to specimens taken from sites with mixed bacterial flora. Selective agents and/or special incubation procedure have been advocated to solve this problem, such as incubation at 33°C with 5–10% CO_2 on Mueller–Hinton agar supplement with 5% chocolatized horse blood, 1% IsoVitaleX, and 3 mg of vancomycin per liter (MH-HB) (Dangor et al., 1992). Antigen detection assays have also been made available for diagnostic use. These assays use a monoclonal antibody raised against outer membrane protein (OMP) or lipo-oligosaccharide (LOS) in *H. ducreyi* for immunofluorescence detection of *H. ducreyi* (Hansen et al., 1995; Patterson et al., 2002). These methods have been shown to be more sensitive than culture in detecting *H. ducreyi* in genital ulcer smears. Serological tests for *H. ducreyi* include enzyme immunoassays (EIAs), dot immunobinding, agglutination, complement fixation, and precipitation (Schalla et al., 1986; Museyi et al., 1988; Alfa et al., 1992; Totten et al., 2000). Sensitivity of serological approach to diagnose chancroid is rather low for the detection of circulating antibodies to *H. ducreyi* in individual symptomatic patients, although they may still be useful tools for epidemiological studies in at-risk communities.

Molecular Detection Methods

Several DNA amplification based techniques that detect *H. ducreyi* directly in patient samples have been developed and significantly improved the sensitivity of laboratory diagnostic tests for chancroid. Specific DNA primers have been designed to target and identify the bacteria in these techniques, which include probe hybridization and PCR amplification assays.

Probe Hybridization

Two probe hybridization assays for detection of *H. ducreyi* have been developed (Parsons et al., 1989; Rossau et al., 1991). In one assay, three ^{32}P-labeled DNA probes designed on basis of encoding *H. ducreyi*–specific proteins have been demonstrated to react strongly with *H. ducreyi* DNA in both bacterial suspensions as well as in infected rabbit lesion material blotted onto nitrocellulose membranes (Parsons et al., 1989). The sensitivity of this probe hybridization assay is around 10^3–10^4 CFU of *H. ducreyi* in both pure and mixed cultures. The other assay approach was based on the development of specific rRNA-derived oligonucleotide

probes for *H. ducreyi* (Rossau et al., 1991). Hybridization probes were chemically synthesized on eight oligonucleotide sequences complementary to different regions in the 16S and 23S rRNA molecules. This DNA-RNA hybridization assay has been demonstrated to show high specificity on culture grown isolates, but the sensitivity of the technique has not been provided. There has been no complete evaluation on the usefulness of these DNA or RNA probe hybridization techniques in the diagnosis of chancroid by *H. ducreyi* detection using clinical specimens.

PCR

Several PCR techniques have been developed to improve on the sensitivity of laboratory diagnosis of chancroid (Johnson et al., 1994; Parsons et al., 1995; Orle et al., 1996). Target regions of the primers of these assays include 16S rRNA gene (Orle et al., 1996), the *rrs* (16S)–*rrl* (23S) ribosomal intergenic spacer region (Gu et al., 1998), an anonymous fragment of cloned DNA (Johnson et al., 1994), and the *groEL* gene encoding the GroEL heat shock protein (Parsons et al., 1995). As mentioned previously for *T. pallidum*, a multiplex PCR (M-PCR) assay with colorimetric detection has been developed for the simultaneous amplification of DNA targets from *H. ducreyi*, *T. pallidum*, and herpes simplex virus (HSV) type 1 and 2 (Orle et al., 1996). Sensitivity and specificity of M-PCR detection of *H. ducreyi* was 98.4% and 99.6%, respectively, as compared with 74.2% and 100% for culture. Expectedly, the sensitivity of culture is relatively low in comparison with PCR. Provided adequate clinical correlation studies can be carried out, the PCR assay has the potential to become an accurate and easily available reference method for the detection of *H. ducreyi*.

Mass Spectrometric Methods

Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI/TOF-MS) technique has been used for rapid identification and speciation of *Haemophilus* bacteria (Haag et al., 1998). This MALDI/TOF-MS technique can identify *H. ducreyi* and at the same time determine strain differences between different *H. ducreyi* isolates. The acquisition time for this technique is only about 10 min for identification of *Haemophilus* spp., which is far shorter than all other traditional biological methods. It is the first mass spectral fingerprinting method described in the literature for *Haemophilus* bacteria and can become a most powerful tool in diagnostic bacteriology.

Mycoplasma and *Ureaplasma*

Based on differing treatment strategies, urethritis has commonly been categorized etiologically as gonococcal or non-gonococcal urethritis (NGU). Both infections are usually acquired via sexual contact, and the latter infection is mainly attributable to the pathogenic role of *Chlamydia trachomatis*, which accounts for 30–50% of

men with NGU (Taylor-Robinson, 1996). Besides NGU, *Mycoplasma hominis, M. genitalium*, *Ureaplasma parvum*, and *U. urealyticum* are important etiological agents of postpartum fever, infertility, and pelvic inflammatory disease (Waites et al., 2003). A number of different approaches for detection of mycoplasmas and ureaplasmas have been developed, and each method has its advantages and disadvantages with respect to cost, time, reliability, specificity, and sensitivity. These methods commonly include culture (Jensen et al., 1996), antigen detection (Bebear et al., 1997), DNA probes (Razin, 1994; Yoshida et al., 2003) and PCR (Jensen et al., 1996; Mahony et al., 1997; Yoshida et al., 2002).

Traditional Diagnostic Methods

Culture method is the traditional technique that has been widely used for detection of mycoplasmas (Jensen et al., 1996). These techniques can be highly specific for detection of some mycoplasmal infections, but they are relatively insensitive because of the many difficulties in culturing various *Mycoplasma* and *Ureaplasma* agents (Waites et al., 2003). Conventional serological detection of mycoplasmal infections is often quite difficult mainly due to the lack of humoral immune responses in infected patients. More recently, an enzyme-linked immunosorbent assay (ELISA) has been developed based on *M. hominis* lipid-associated membrane proteins (LAMP) and can rapidly detect specific antibodies in sera of patients infected with *M. hominis* (Lo et al., 2003). Several antigen detection methods have also been developed for detection of *Mycoplasma* antigens in patient specimens (Bebear et al., 1997). As *Mycoplasma* spp. are capable of inhabiting an intracellular site, these antigen detection methods may not be completely reliable.

Molecular Detection Methods

Introduction of molecular detection techniques provides a new horizon in the identification and detection of *Mycoplasma* and *Ureaplasma* species. These provide improved specificity and sensitivity compared with traditional culture methods. The molecular detection techniques on *Mycoplasma* and *Ureaplasma* include probe hybridization and PCR methods.

Probe Hybridization

Sequences based on the variable species-specific regions of 16S rRNA genes have been widely used for synthesis of specific probes for hybridization. Several rRNA probe hybridization techniques have been developed for detecting mycoplasmas that are of human origin, although many rRNA probes have been designed for the purpose of contamination detection in tissue cell cultures (Razin, 1994; Kessler et al., 1997). Specific probes that are designed from genomic libraries of *Mycoplasma, M. pneumoniae* and *M. genitalium*, have also been reported (Hyman et al., 1987). Dot blot hybridization has been variously designed in these probe methods with ^{32}P-labeled, digoxigenin, or biotin-labeled probe for detection of *Mycoplasma*. A

major limitation has been that the detection limit of these assays is approximately 1 ng of specific mycoplasma DNA or 10^4–10^5 CFU, which is often not sufficiently sensitive for use in the clinical laboratory (Marmion et al., 1993; Razin, 1994). Recently, a rapid detection method for *M. genitalium, M. hominis, U. parvum,* and*U. urealyticum* in genitourinary samples by PCR-microtiter plate hybridization has been developed (Yoshida et al., 2003). In this assay, four species-specific capture probes were used to detect the targets by PCR amplification of a part of the 16S rRNA gene followed by 96-well microtiter plate hybridization. Sensitivity of this assay was up to detection of approximately 10 copies of the 16S rRNA gene of each of the four species. Further evaluation may therefore prove this to be a useful tool for diagnosis of genitourinary infections with *Mycoplasma* or *Ureaplasma.*

PCR

Compared with what was available a decade ago, many PCR systems have been developed for detection of clinically relevant *Mycoplasma* and *Ureaplasma* (Blanchard et al., 1993; Bebear et al., 1997; Yoshida et al., 2002). Specific primers have been designed for different target regions including 16S rRNA genes and other repetitive sequences, such as MgPa adhesion gene of *M. genitalium* (Jensen et al., 1991; Baseman et al., 2004). In particular, *Ureaplasma* species were detectable by specific PCR amplification on urease genes (Blanchard et al., 1993). As PCR assay is highly sensitive compared with culture, it is a potentially useful tool for detection of slow-growing and fastidious organisms, such as *M. genitalium*, where only very small amount of bacteria DNA is required. *M. genitalium* is also the smallest known self-replicating cell with a genome size of 580 kb only. This imposes severe biosynthetic limitations. The highly fastidious nature of *M. genitalium* is also reflected in the absence of direct isolations from urethral specimens (Tully et al., 1981). With the many difficulties in cultivating *M. genitalium*, PCR assay has become an important tool to establish links between *M. genitalium* and human diseases. Two different TaqMan-based real-time PCR assays for quantifying *M. genitalium* were recently developed and have been validated in detection of a fragment of MgPa adhesion gene or 16S rRNA (Jensen et al., 2004; Yoshida et al., 2002). These TaqMan assays can provide valuable information for understanding the pathogenic mechanisms of *M. genitalium* in causing urogenital tract disease.

Apparently, PCR assays seem to be less valuable in other more rapidly growing and easily cultivable *Mycoplasma*, such as *M. hominis*, and *Ureaplasma* species. *U. urealyticum* can often be isolated with ease, although it may be difficult to culture from certain specimens such as amniotic fluids and endotracheal aspirates of newborns (Blanchard et al., 1993). The use of PCR technology in these situations, in contrast to culture, can also enhance the detection of other mycoplasmas. A multiplex PCR assay has been developed to detect *C. trachomatis, N. gonorrhoeae, U. urealyticum*, and *M. genitalium* in first-void urine specimens (Mahony et al., 1997). The development of multiplex PCR tests may prove to be useful

in improving our understanding of the epidemiology of these important sexually transmitted diseases in areas where these are endemic. A denaturing gradient gel electrophoresis (DGGE) fingerprinting of 16S rDNA of 32 mycoplasma species has also been developed for rapid identification of *Mycoplasma* species, and this can be useful when specific PCR is not available (McAuliffe et al., 2003).

Trichomonas vaginalis

Trichomonas vaginalis is a parasitic protozoan that causes human trichomoniasis and is estimated to be the most prevalent nonviral sexually transmitted disease in the world (Petrin et al., 1998). Infections in women cause vaginitis, urethritis, and cervicitis (Riley et al., 1992), and complications include premature labor, low birth-weight offspring, and postabortion or posthysterectomy infection (Shaio et al., 1997). There are approximately 180 million cases of trichomoniasis each year worldwide; of which an estimated 5 million women and 1 million men in the United States are infected each year (Leber et al., 2003). The actual figures are, however, expected to be higher than these estimates because (i) the infection can be asymptomatic, particularly in men; (ii) trichomoniasis is not a reportable disease in the United States and other countries; and (iii) the sensitivities of different diagnostic tests varied between different laboratories, which often have little quality control on these methods.

Traditional Detection Methods

Diagnosis of *T. vaginalis* infection has traditionally been based on the examination of wet preparations of vaginal and urethral discharges, prostatic secretions, and urinary sediments (van der Schee et al., 1999; Schwebke and Lawing, 2002). Depending on experience and technical skill, the sensitivity of microscopic examination of the wet mount preparation is only between 50% and 70% (Fouts and Kraus, 1980). In comparison, culture method has greater sensitivity ($>80\%$) than the wet mount method and is, therefore, considered the gold standard method (Gelbart et al., 1990). Diagnostic improvements on culture have been suggested by using more sensitive culture media such as modified Diamond medium and Kupferberg Trichosel medium (Gelbart et al., 1989, 1990). There are, however, variations in optimal cultural conditions, and daily examination is required for up to 7 days. Moreover, some isolates may not be cultivable because of strains with differing nutritional requirements, low numbers of parasites in original specimen, or presence of predominant damaged or nonviable organisms (Draper et al., 1993). Direct detection of *T. vaginalis* antigens in clinical specimens by using monoclonal antibodies is a rapid method in the laboratory diagnosis of trichomoniasis (Krieger et al., 1985). Results from an evaluation of a monoclonal antibody–based enzyme-linked immunosorbent assay for *T. vaginalis* have found it to be as sensitive and specific as those of wet-mount microscopy and culture (Lisi et al., 1988).

Molecular Detection Methods

For the molecular detection of *T. vaginalis*, recent methods include the use of probe hybridization and PCR assays. These assays have been devised to detect various regions or genes of the genome including 2.3-kb *T. vaginalis* fragment (Rubino et al., 1991), the feredoxin gene (Riley et al., 1992), beta-tubulin gene (Madico et al., 1998), highly repeated DNA sequences (Kengne et al., 1994), and 18S ribosomal gene (Mayta et al., 2000).

Probe Hybridization

A commercially available kit, Affirm VP system (MicroProbe, Bothweel, WA, USA), is currently available and uses synthetic DNA probes to directly detect *Gardnerella vaginalis* and *T. vaginalis* from a single vaginal swab (Briselden and Hillier, 1994). This Affirm VP deoxyribonucleic acid probe test was found to be better than wet-mount preparation and has a sensitivity of 90% and a specificity of 99.8% for the identification of *T. vaginalis* organisms in women with a high prevalence of trichomoniasis (DeMeo et al., 1996). However, false-negative results have been encountered compared with culture technique (Briselden and Hillier, 1994). A dot blot hybridization technique has also been developed by using a 2.3-kb *T. vaginalis* DNA fragment as a probe to detect *T. vaginalis* DNA from vaginal exudates (Rubino et al., 1991). However, the drawbacks of this technique are the instability of the probe and the necessity to handle and dispose of radioactive materials. To overcome these limitations, fluorescence-labeled DNA probe can be used for identification of *T. vaginalis* by DNA *in situ* hybridization technique. For asymptomatic carriers, the usefulness of these techniques still requires more definitive evaluation.

PCR

Several PCR systems have been developed to detect *T. vaginalis* from clinical samples. Specific TVA5–TVA6 primers targeting the unique sequences of the genome of *T. vaginalis* have been designed. A 102-bp genomic fragment was amplified and termed as A6p sequence, which appears highly selective for a broad range of *T. vaginalis* isolates (Riley et al., 1992). Beta-tubulin gene of *T. vaginalis* is a well-conserved region that has been used to develop a PCR assay (Madico et al., 1998). The sensitivity and specificity of the beta-tubulin gene PCR assay were 97% and 98%, respectively, while the sensitivities of culture and wet preparation were 70% and 36%. Another target region of *T. vaginalis* that has been used for PCR amplification was 2000-bp repeated fragment of *T. vaginalis* (Kengne et al., 1994). Two sets of primers (TVK3–TVK4 and TVK3–TVK7) were used and have been shown to be highly specific for *T. vaginalis* without reacting with human DNA or other infectious agents tested. Another PCR assay that used primers targeting a specific region of the 18S rRNA gene of *T. vaginalis* has also been developed (Mayta et al., 2000). The PCR amplification product was subsequently confirmed by enzyme digestion with *Hae*III. Overall sensitivity and specificity of the 18S

rRNA PCR assay of vaginal swab samples were 100% and 98%, respectively, which compared favorably to *T. vaginalis* culture.

Recently, two real-time PCR assays (TaqMan-based and FRET-based) for *T. vaginalis* DNA detection were developed using double-labeled fluorescent probes (Jordan et al., 2001; Hardick et al., 2003). High levels of agreement between these real-time PCR assays and culture have been obtained for detecting *T. vaginalis*. The real-time process has effectively eliminated the need for post-PCR processing for PCR product detection. This has resulted in significant decrease in turn-around time and increased throughput. The closed system detection format also would allow prevention of laboratory cross-contamination. Thus, the real-time PCR assay has advantages over the conventional PCR assays. Recently, a urine-based PCR-EIA assay has also been developed and validated for the detection of trichomoniasis in men and women (Kaydos et al., 2002). Sensitivity and specificity of this assay ranged from 86.4% to 92.7% and 88.6% to 95.2%, respectively.

Herpes Simplex Virus

Herpes simplex virus (HSV) infects mucocutaneous sites and is one of the major causes of genital ulcer disease. It causes both symptomatic and asymptomatic infections and subsequent latent infection of nerve cells. HSV causes a wide spectrum of clinical manifestations in the central nervous system (CNS) of infants (encephalitis with or without disseminated visceral infection) and adults. In the United States, at least 10% to 20% of all viral encephalitis has been estimated to be caused by HSV (Hofgartner et al., 1999). Presently, effective antiviral therapy is possible if these are administered early (Whitley et al., 1998). Thus, rapid laboratory diagnosis is important for minimizing the death rate and the morbid sequelae of HSV infection.

Traditional Detection Methods

Traditional diagnosis of HSV in routine clinical laboratories depended on viral culture in susceptible cell lines and considered to be the gold standard for virological diagnosis. Inoculated cells are examined regularly for cytopathic effect (CPE), which takes about 2 to 7 days depending on the inoculation titer present at time of infection (Jerome et al., 2003). Confirmation of HSV culture results as well as discrimination between HSV type 1 (HSV-1) and type 2 (HSV-2) is normally performed by immunofluorescent staining with monoclonal antibodies. HSV antigens can be detected by direct immunofluorescence assay (DFA) (Reina et al., 1997). Cells from lesions are concentrated and spotted onto a slide followed by incubation with a fluorescein-conjugated monoclonal HSV type-specific antibody. Intracellular fluorescence is then observed under fluorescent microscope. Sensitivity of DFA ranged from 10% to 87% when compared with culture and is higher in specimens from vesicular lesions and poorer in specimens from healing lesions (Lafferty et al., 1987). Enzyme immunoassay is a technique similar to DFA except

a second antibody is used that is conjugated with an enzyme, which then reacts with a colorimetric substrate used for detection (Morrow et al., 2003). However, the sensitivity of EIA was only 47% to 89% compared with culture (Brinker and Herrmann, 1995).

Molecular Detection Methods

Molecular detection techniques have often been used in HSV detection due to their higher sensitivities and shorter turn-around time compared with the culture, DFA, and EIA methods. Probe hybridization and PCR-based techniques have been well developed for detection of HSV. PCR techniques are now widely recognized as the reference standard laboratory assay method for the sensitive and specific diagnosis of CNS infections caused by HSV (Mitchell et al., 1997; Read and Kurtz, 1999; Tang et al., 1999; Quereda et al., 2000).

Probe Hybridization

A number of probe hybridization methods have been used in direct detection of HSV DNA from specimens (Gentilomi et al., 1997; Morioka et al., 1999), such as *in situ* hybridization of HSV biotinylated probe in Papanicolaou-stained cervicovaginal smears (Iwa and Noguchi, 2003). Type-specific nonradioactive probes were normally used in these assays (Gentilomi et al., 1997; Morioka et al., 1999). Hybrid Capture II (HC II) signal amplification probe test is a second-generation nucleic acid–based test for rapid detection of HSV DNA and has been developed by Digene Corporation (Silver Spring, MD, USA) as a commercially available detection kit for HSV (Cullen et al., 1997). Basically, this technique captures the RNA-DNA hybrids by hybrid-specific antibodies that have been encoated on microtiter plates. Subsequent detection is by a luminometer after adding chemiluminescent substrate with alkaline phosphatase–labeled antibodies. Sensitivity and specificity of the HC II test were reported to be 93.2% and 100%, respectively when compared with the census results of culture and PCR (Cullen et al., 1997).

PCR

PCR has become the mainstay laboratory diagnostic method for HSV encephalitis over the past decade and is considered the most sensitive method for direct detection of HSV (Tang et al., 1999). According to the different primers and detection methods that have been set up, PCR can detect both HSV-1 and HSV-2 (Ryncarz et al., 1999) or distinguish between HSV-1 and HSV-2 (Kimura et al., 1990; Ryncarz et al., 1999). Target regions of PCR primers on HSV genes include those coding TK (U_L23) (Kimura et al., 1990; Mitchell et al., 1997), DNA polymerase (U_L30) (Mitchell et al., 1997), DNA-binding protein (U_L42) (Puchhammer-Stockl et al., 1990; Cassinotti et al., 1996), and glycoproteins B, C, D, and G (U_L27, U_L44, U_s6, and U_s4), respectively (Cassinotti et al., 1996; Cinque et al., 1998; Tang et al., 1999). Several multiplex PCR assays have been developed that enable simultaneous

screening of three to five viruses or bacteria in one assay. These multiplex PCR assays include PCR amplification of *T. pallidum*, *H. ducreyi*, HSV-1, and HSV-2 (Orle et al., 1996); PCR amplification of HSV-1, HSV-2, varicella-zoster virus (VZV), and enteroviruses (Read and Kurtz, 1999); PCR amplification of HSV-1, HSV-2, VZV, human cytomegalovirus (CMV), and Epstein–Barr virus (EBV) (Ryncarz et al., 1999; Markoulatos et al., 2001); and multiplex herpesvirus PCR assay on CMV, EBV, VZV, HSV, and human herpesvirus 6 (HHV-6) (Quereda et al., 2000). In addition, real-time quantification PCR assays (SYBR Green I and FRET probe-based) for HSV have also been established (Espy et al., 2000; Aldea et al., 2002). These assays provide sensitive and rapid laboratory diagnostic methods for HSV that can minimize the effects of contamination of sample by eliminating post-PCR processing steps. Recently, parallel detection of five human herpesviruses (CMV, EBV, HSV-1, HSV-2, and VZV) in a single run LightCycler assay has been developed (Stocher et al., 2003; Weidmann et al., 2003). These real-time PCR assays provide both rapid and accurate identification and quantification of the human herpesviruses and bear important improvements in initiation of antiviral therapy in patient monitoring as well as new drug discovery research.

Human Papillomavirus

Human papillomaviruses (HPVs) are the most studied members of the family Papillomaviridae. These are DNA viruses that have an unenveloped icosahedral structure as well as the basic organization and replication strategy of the circular, double-stranded, 8-kb DNA genome. Infection with certain HPV types has been shown to be the single most important risk factor for the development of cervical cancer (zur Hausen, 2000). More than 80 types of HPV have been identified to date. Specific types that have been associated with anogenital tract disease can be classified on the basis of phylogenetic relationship (Van Ranst et al., 1992). Extensive studies have shown association frequencies with benign or malignant cervical lesions as high-risk types (HPV-16, -18, -31, -33, -35, -39, -45, -51, -52, -54, -56, -58, -59, and -66) (Van Ranst et al., 1992; van der Bruk et al., 1993) and low-risk types (HPV-6, -11, -34, -40, -42, -43, and -44) (Jacobs et al., 1997). The most commonly reported high-risk types are HPV-16, -18, -31, -33, and -45 (Nobbenhuis et al., 1999), while the most common low risk types are HPV-6 and -11 (Jacobs et al., 1997).

Traditional Detection Methods

As HPV cannot be grown in cell cultures, clinical, cytological, and histological methods have traditionally been used to provide indirect evidence of HPV infection by demonstration of cellular dysplasia, which is one of the results of HPV infection. Colposcopic examination by visual inspection to detect abnormalities in the exterior parts of the genital tract is convenient and easily done at the bedside. However, this method is very insensitive, and hidden lesions in the

cervix often remain undetected (Feng and Kiviat, 2003). Microscopic examination of exfoliated cell samples (Pap smears) or tissue biopsy specimens have long been used in cytological and histological methods for revealing HPV infections. Changes in HPV-infected cells can be rather subtle and there are nonspecific manifestations, such as koilocytosis or dyskeratosis of the squamous cells. These changes, however, are not always clearly presented and detectable by microscopy (Hippelainen et al., 1994). Poor intra- and interlaboratory reproducibility of the cytologic diagnosis of HPV infection has also been reported (Kiviat et al., 1992). On the other hand, serological assay based on serum antibodies mainly against the L1, L2, E6, and E7 HPV proteins, such as Western blot assay and enzyme-linked immunosorbent assays (ELSA), has been devised for detection of HPV infection. This approach has limited capability for HPV detection due mainly to difficulties associated with the large number of HPV types, cross-reactions between HPV, the diversity of clinical lesions, as well as target sites for infection (Coursaget, 2003). Advent of various virus-like particles (VLP) has enabled the use of these as valuable reagents for development of more sensitive and specific assays (Studentsov et al., 2003). As there is virtually no oncogenic viral genome present and that high titers of neutralizing antibodies appeared to be generated, these VLPs have also been used for development of prophylactic vaccine (Roden et al., 1996).

Molecular Detection Methods

Molecular detection methods have become increasingly important because of their higher sensitivity and the potential to detect presence of virus before onset of clinical manifestations. Different probe hybridization and PCR-based methods have now been well developed for detection of HPV DNA from clinical specimens.

Probe Hybridization

It is now possible to detect HPV DNA sequences by hybridization with either DNA or RNA probes. Different probe hybridization techniques have been developed and include *in situ* hybridization (Park et al., 1991; Wolber and Clement, 1991), RFLP-Southern blot, and dot blot and hybridization in solution (Hybrid Capture assay). *In situ* hybridization was used to detect HPV DNA on the microscopic slide, such as cervical smears or slices of biopsy material. One advantage of this method is that exact localization in infected cells can be obtained to determine whether HPV has been integrated into host cell chromosome or just episomal (Park et al., 1991; Wolber and Clement, 1991). Sensitivity of this *in situ* hybridization technique has been reported to be around 25 copies, which means it can be possible to detect gene-specific mRNA transcripts (Stoler, 1993). RFLP-Southern blot technique has been achieved to directly detect HPV through hybridization of restriction-digested DNA on a membrane by labeled HPV probe (Brown et al., 1993). This technique is

fairly sensitive (from 0.1 to 0.001 HPV genome copy per cell) and can also provide information on whether the HPV is integrated or episomal (Brown et al., 1993; Abramson et al., 2004). Disadvantages of the Southern blot technique are that it is too labor-intensive and requires fairly large amounts of sample materials. In the dot-blot analysis technique, which is similar to Southern blot, HPV DNA is applied directly to a membrane as a dot followed by hybridization with type-specific HPV probes (Duggan et al., 1993).

Hybrid Capture II assay (Digene) is a hybridization in solution method and is the only kit currently approved by the U.S. FDA for the detection of HPV DNA in cervical samples (Castle et al., 2003). In this assay, RNA-DNA hybrids are captured by hybrid-specific antibodies coated on microtiter plates. Positive signals are then detected in a luminometer after adding chemiluminescent substrate with alkaline phosphatase–labeled antibodies.

PCR

The sensitivity of PCR-based amplification tests are higher than other non–amplification–based laboratory techniques and have been commonly used in prevalence studies of HPV infection (Giuliano et al., 2001). Several hybridization techniques, mentioned in the previous section, have been modified by including PCR amplification steps to improve their sensitivities, such as PCR–dot blot and PCR-RFLP (Gravitt and Manos, 1992; Kay et al., 2002).

Consensus primers have been used to amplify a broad spectrum of HPV types on the conserved L1 capsid gene. HPV L1 ORF was targeted by MY09–MY11 primers to yield a 450-bp fragment, and GP5–GP6 primers, which target a fragment within the MY09–MY11 region, are commonly used in nested PCRs with MY09–MY11 primers (Nelson et al., 2000). In addition, type-specific primers have also been designed, within HPV E6 and E7, that are often retained and expressed in cervical cancer tissues (Baay et al., 1996; Karlsen et al., 1996). CPI and CPII are consensus primers that have also been used to detect HPV E1 regions (Tieben et al., 1993). Recently, a general primer set, SPF_{10}, was used to amplify a 65-bp fragment of the HPV L1 region (Quint et al., 2001). As the small-sized amplicons are usually more efficiently amplified, this assay was considered to be especially suitable for those formalin-fixed, paraffin-embedded tissue samples that have been difficult or poorly amplified before.

Because only 5–10% of HPV-infected patients eventually develop cervical cancer, it has been suggested that the level of HPV present, and perhaps its changes over time, could be associated with increased risk of neoplasia (Swan et al., 1999). A rapid method of quantitative real-time PCR was recently developed to quantify the copy numbers of E2 and E6 genes for analyzing the physical status of HPV (Nagao et al., 2002). High-level expression of HPV E6 and E7 oncoproteins appeared to be required for progression of cancer cell replication (Dyson et al., 1989; Scheffner et al., 1993). Moreover, integration of HPV DNA with destruction of the E2 gene has been related to and used for evaluation of expression of

E6 and E7 proteins. The regression activities on the HPV promoters of E2 gene product seem to be able to direct the expression of these genes (Romanczuk et al., 1990).

Conclusion

Molecular biology has now been demonstrated to be a powerful tool for research and laboratory diagnosis of sexually transmitted diseases and has become increasingly important for disease control and prevention (Peeling et al., 1999). From an epidemiological perspective, accurate delineation of sexual networks and disease transmission patterns within populations can be constructed and understood by molecular typing methods. With the experiences gained in the different molecular techniques and approaches in several STDs, including *N. gonorrhoeae, C. trachomatis, T. pallidum, H. ducreyi, Mycoplasma* and *Ureaplasma, T. vaginalis,* herpes simplex virus, and human papillomavirus, the advantages of molecular detection of STDs are readily apparent compared with traditional methodologies, such as direct examination, culture, and serology.

The major advantages of molecular approaches to diagnosis of STDs have usually included increased sensitivity, improved specificity, and reduced turn-around time for test results. However, the astute laboratorian should be well versed with the pitfalls that are inherent in most molecular techniques. Cross-contamination of amplified products carried over in samples, presence of amplification inhibitors such as heme, and genomic variations in target organisms can easily lead to inaccurate results. Development of real-time PCR systems may possibly eliminate some of the cross-contamination problems by using a closed-tube format without need for post-PCR processing. Uracil-*N*-glycosylase (UNG) is a DNA repair enzyme that has the ability to remove uracil residues and has been used in real-time PCR systems as well as other PCR-based assays to remove dUTPs incorporated PCR products that arose from carrying-over contamination. However, it should be clear that UNG would not be effective in controlling contamination in RNA amplification assays because it does not remove uracil from RNA molecules.

Automation will be one of the main future directions in the development of molecular diagnostic assays, as many of the currently available molecular tests are still labor-intensive. For most automated systems, the biggest challenge will remain the initial sample preparation and processing procedures, although the recent development of molecular detection workstations may be able to provide solutions for this long-standing issue. In recent years, the extensive development of microarray technology has provided new impetus and a new horizon on the molecular diagnostic front. It will not be surprising that in the not too distant future, a small chip will be used for the automated screening of several infectious pathogens. Formal and complete evaluation of these new tests is needed before one can contemplate their use in practicing molecular diagnostic laboratories.

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23
Advances in the Diagnosis of *Mycobacterium tuberculosis* and Detection of Drug Resistance

ABDULLAH KILIC AND WONDER DRAKE

Introduction

The first technique for diagnosing tuberculosis (TB) was reported in 1882 when Dr. Robert Koch, along with Dr. Paul Erlich, developed the acid-fast stain as a means to identify *Mycobacterium tuberculosis* (MTB). TB remains a disease associated with crowded living conditions, depressed immunity, and poverty. MTB infects one third of the world's population, and approximately 8 million new tuberculosis cases are reported each year, with a resultant 2 million deaths (Dye *et al*., 1999).

Mycobacterial Cell Wall

MTB is an aerobic, non–spore forming, non-motile, pleomorphic bacilli that is typically 1 to 5 μm long and 0.2 to 5 μm wide (Starke, 2004). Its generation time is 15 to 20 h; so, visible growth takes 3 to 6 weeks on solid media (Haas, 2000). The mycobacterial cell wall is composed of superficial lipids (e.g., cord factor), mycolic acids, arabinogalactan, peptidoglycan, and lipoarabinomannan. The cell wall confers shape, size, protection against osmotic pressure, and probably protects the plasma membrane from deleterious molecules in the cellular environment (Fig. 23.1) (Inderlied, 2004). The cell wall components of mycobacteria determine their most prominent feature: resistance to acid alcohol. Despite staining with decolorizing agents containing 95% ethyl alcohol–3% hydrochloric acid, carbolfuchsin cannot be readily removed. Mycobacteria are not classified as either Gram-positive or Gram-negative. They are commonly referred to as acid-fast bacilli (AFB) (Geo. F. Brooks, 2001). This important feature of MTB allows differential staining in contaminated specimens like sputum. Polysaccharides in the MTB and Gram-positive bacteria cell walls are similar; however, *Mycobacterium* cell wall contains lipid but Gram-positive bacteria have proteins in their cell wall (Portaels, 1995; Inderlied, 2004).

Mycosides including mycolic acids (long-chain fatty acids C78–C90), waxes, and phosphatides are in the exterior surface of the MTB cell envelope. In the

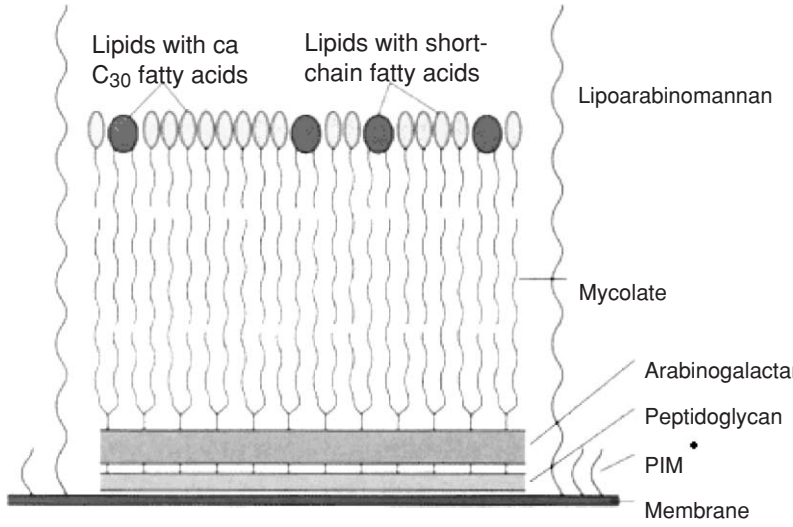

FIGURE 23.1. Mycobacteria cell wall. *Adapted from* Cohen & Powderly: Infectious Diseases (Inderlied, 2004):
$^{+}$Phosphatidylinositol mannosides

cell wall, the mycolic acids are largely bound to peptidoglycan by phosphodiester bridges and to arabinogalactan by esterified glycolipid linkages. The agglutination serotype of strain and colony morphology is related to the mycosides (McMurray, 2000; Inderlied, 2004). Another important cell component of mycobacteria is cord factor (trehalose 6,6′-dimycolate) that is thought to correlate with virulence. Cord factor may cause chronic granulomas and inhibits migration of leukocytes (Geo. F. Brooks, 2001).

Epidemiology of Tuberculosis Infections

After acquired immunodeficiency syndrome (AIDS), tuberculosis is the most dangerous infectious disease resulting in death (Frieden et al., 2003). Tuberculosis cases occur predominantly in individuals 15 to 59 years of age (Starke, 2004). According to the World Health Organization (WHO), in 2002 about 9 million new cases and 2 million MTB deaths occurred in the world (http://www.who.int/mediacentre/news/releases/2004/pr17/en/). Newly acquired tuberculosis infections occur at a frequency of every second in the world. In addition, one third of the world population is currently infected with MTB. Due to malnutrition, crowded living conditions, and poor access to health care, the highest incidence rate of TB cases is among developing countries. Most cases are in Southeast Asia and India. Co-infection with human immunodeficiency virus (HIV) has led to an even greater increase in both developed and developing countries (Huggett et al., 2003).

Sub-Saharan Africa has the highest incidence rate of tuberculosis (350 cases per 100,000 population). In England and Wales due to immigration and HIV infection, the incidence of new TB cases increased from 8.4/100,000 in 1993 to 9.2/100,000 in 1998. The greatest increase was in London at 11% (Kumar et al., 1997). Five to ten percent of immunocompetent persons infected with MTB will develop active disease sometime during their lifetime. Each year, approximately 10% of people who are co-infected with MTB and HIV develop active tuberculosis (Frieden et al., 2003).

Other risk factors for the development of TB include being foreign-born, traveling to a high-prevalence country, intravenous drug use, imprisonment, homelessness, health care workers who care for high-risk patients, and children exposed to adults in high-risk groups (except health care workers) (Starke, 2004).

Epidemiology of Multidrug-Resistant TB

Drug resistance in TB is a global problem. Multidrug-resistant TB (MDR-TB) is defined as bacilli resistant to at least two first-line agents, isoniazid (INH) and rifampin (RIF). Drug resistance has increased especially in regions where TB control programs are poorly enforced (http://www.who.int/mediacentre/news/releases/2004/pr17/en/). According to WHO, every year 300,000 new cases of MDR-TB occur in the world. In 1994, the WHO, the International Union Against TB and Lung Diseases (IUATLD), and other partners conducted an anti-TB drug-resistance program and began to standardize the sampling techniques and laboratory methodologies used worldwide to measure and control drug-resistant TB. Surveillance by WHO/IUATLD reveals that Eastern Europe, Latin America, Africa, and Asia have more than 4% incidence of MDR-TB among new cases (WHO/74, 1997). According to the WHO report, in Eastern Europe and Central Asia, TB patients have 10 times more inclination to have MDR-TB than patients in other parts of the world. In Estonia, Kazakhstan, Latvia, Lithuania, Uzbekistan, and the Russia Federation the incidence rate of drug resistance is 14% (http://www.who.int/mediacentre/news/releases/2004/pr17/en/).

MDR-TB development not only hinders treatment but also increases the costs of treatment by 100-fold and lengthens the treatment time (WHO/74, 1997). The basic treatment of mycobacterial infection is chemotherapy. In TB treatment, because single drug therapy can cause an increase in drug resistance strains, a combined treatment should be applied. With a combined treatment, cure can be $>95\%$ (Geo. F. Brooks, 2001). INH, RIF, pyrazinamide (PZA), ethambutol (EMB), and streptomycin (SM) are the first-line agents. These drugs are used for drug-susceptible TB infections. The most important risk factor for MDR-TB is failure to complete treatment for tuberculosis. Six months short-course therapy, starting with treatment with INH, RIF, ETH, and PZA for 2 months, followed by 4 months treatment with INH and RIF, is still being used. The American Thoracic Society, the Centers for Diseases Control and Prevention (CDC), the Infectious Diseases Society of

America, WHO, and IUATLD advise slight modifications to this treatment protocol (Parsons et al., 2004).

In case of MDR-TB, unfortunately, second-line drugs are the choice for treatment of infection (Geo. F. Brooks, 2001). There are eight second-line drugs: kanamycin, capreomycin, ethionamide, cycloserine, ofloxacin, clofazamine, levofloxacin, para-aminosalicylic acid, and ciprofloxacin (Starke, 2004). A TB cavity usually contains 10^7 to 10^9 bacilli. Between 1 in 10^6 and 1 in 10^8 replications of tubercle bacilli may result in spontaneous mutation that confers resistance to antituberculous therapy. When INH and RIF are used together, spontaneous mutations resulting in resistance would be extremely rare (1 in 10^{14}). If the single drug or multidrug therapy is used episodically, resistant tubercle bacilli multiply under selective pressure and emerge rapidly (Geo. F. Brooks, 2001; Sharma and Mohan, 2004).

The genetic mutations responsible for resistance to tuberculosis therapy are myriad. The genes indan enoyl acp reductase (*inhA*), catalase-peroxidase (*katG*), alkyl hydroperoxide (*ahpC*), and oxidative stress regulator (*oxyR*) are responsible for INH resistance (Sharma and Mohan, 2004). RIF resistance is caused by mutations in RNA polymerase subunit 12 (*rpsl*) genes. Mutations in *pncA* gene lead to resistance against PZA. SM resistance is associated with mutations in ribosomal protein subunit 12 (*rpsL*), 16s ribosomal RNA (*rrs*), and aminoglycoside phosphotransferase gene (*strA*). Resistance to EMB and fluoroquinolones occurs secondary to mutations in arabinosyl transferase (*emb A*, *B*, and *C*) gene and DNA gyrase (*gyr A* and *B*) gene, respectively (Sharma and Mohan, 2004).

Optimal Specimen Collection of MTB

Early diagnosis of TB is important for infection control, as well as to introduce the treatment in a timely manner and determine the resistance profile. During the past two decades, dedicated efforts have resulted in enhancing TB diagnosis techniques. In this review, we discuss the application of currently available methods and their impact on the diagnosis of TB and detection of drug resistance. Samples such as sputum, bronchial or gastric washings, pleural fluid, urine, or cerebrospinal fluid (CSF) are collected from patients suspected of having TB (McMurray, 2000). Collection of good-quality specimens containing the highest number of mycobacteria is important for diagnosis. A specimen must be sent to the laboratory within 30 min or at most within 24 h after collection (Tenover et al., 1993).

Sputum

The properly collected sputum is the best specimen for diagnosis of pulmonary TB. Expectorated sputum specimen should be collected early in the morning on three occasions and sent to laboratory in a wide-mouthed, sterile, plastic container (wax free) with a tight fitting cap at 0–4°C. The U.S. CDC recommends that TB be identified and first-line drug susceptibility testing be completed within 30 days of

specimen collection. However, by traditional culture methods, TB identification and drug susceptibility testing can take 4–6 weeks.

Gastric Lavage

Other specimens that may yield a diagnosis include the CSF and gastric lavage (Gray, 2004). Gastric lavage for swallowed sputum is generally collected from young children who do not have suitable sputum. Because *Mycobacterium* cannot survive for a long period in acidic gastric washing, gastric lavage should be sent to a laboratory promptly in 10% sodium carbonate. Gastric lavage specimen should be collected before breakfast on three separate occasions (Inderlied, 2004).

Cerebral Spinal Fluid

Cerebral spinal fluid (CSF) requires high-volume aspirates to successfully stain and culture MTB. Typically, microbiology labs require at least 5 ML for a TB culture.

Urine

Midstream urine specimens should be collected in a sterile plastic container (wax free) with a leak-proof cap for three early mornings. Blood specimens should be collected in a sodium polyanethol sulfonate (SPS) tube and must be treated with lytic agent such as deoxycholate and should be concentrated by centrifugation before inoculating the media. Fluids should be processed promptly and inoculated into a liquid growth medium as well as a solid medium. Biopsy specimens should be immediately sent to the lab (Inderlied, 2004).

It can be expected that specimens collected from nonsterile sites will be contaminated by normal microflora. Therefore, in order to reduce contamination by normal flora, decontamination processes are necessary. The most commonly used mucolytic agents for sputum specimens are freshly-prepared *N*-acetyl-L-cysteine (NALC) and dithiothreitol (DTT or sputolysin). NALC and sodium hydroxide are generally used for digesting and decontaminating because they kill most bacteria and fungi. Following decontamination, specimens are neutralized with buffer and concentrated by centrifugation. Because swab specimens do not generally contain sufficient materials for culture, 1 g of tissue or 10 ML of fluid are preferred (Inderlied, 2004).

Conventional Methods for Identifying MTB

Microscopic Techniques

Acid-fast staining is a fast, cheap, and convenient method for direct detection of mycobacteria from clinical specimens (Inderlied, 2004). Although microscopy provides preliminary information, it is not an adequate method for differentiating

MTB from other *Mycobacterium* species. Also, microscopic techniques are not suitable for examination of specimens, such as urine, which are contaminated with nonpathogenic bacteria (Gray, 2004).

In microscopy, Ziehl–Neelsen (ZN), Kinyoun's stain, and fluorochrome stain, methods are used. It is necessary that a reference laboratory report results of acid-fast stain within 24 h of receiving the specimens. In this method, due to mycolic acid-rich cell wall, carbol fuchsin dye is retained after washing with acid alcohol. This method is advised by WHO and IUATLD (Frieden et al., 2003). An alternative method is a fluorochrome stain made of auramine–rhodamine, which stains mycolic acids in the AFB cell wall. To visualize one AFB, approximately 5×10^3 bacilli per ML sputum should be present. In smear examination, it is necessary to assess 300 fields before reporting a specimen as AFB negative (Tenover et al., 1993). With respect to culture, microscopy specificity is 99%, and sensitivity is 25–75%. In some patients who receive antituberculosis therapy, it is possible to have positive smears and negative cultures, which reflect nonviable bacilli (McMurray, 2000).

Traditional Culture Techniques

There are nonselective and selective mediums for culture of mycobacteria. Selective media contains antibiotics that inhibit growth of normal flora (Adjers-Koskela and Katila, 2003). In order to culture mycobacteria from clinical specimens, there are various kinds of solid and liquid media such as Lowenstein–Jensen, Kirchner, and the various Middlebrook formulations (7H9, 7H10, and 7H11) (Watterson and Drobniewski, 2000). Specimens contaminated with normal bacterial flora such as sputum are inoculated in a selective medium containing antimicrobial agents; sterile body fluids are inoculated with solid and a broth media (McMurray, 2000). The growth of solid culture media is 6 weeks or longer, whereas that of liquid culture media is usually 7–21 days. Therefore, specimens should be cultured on solid and liquid media (Frieden et al., 2003) at 35–37°C, with 5–10% CO_2. All cultures should be examined weekly for 8 weeks. The major advantages of solid cultures are that they make it possible to examine the morphology of colonies and visualize the pigmentation. These advantages are useful to differentiate the MTB from other non-tuberculous mycobacteria (NTM) (McMurray, 2000).

Biochemical Tests and Morphological Features

There are various kinds of biochemical tests and morphological features for identification of mycobacteria. Based on pigment production, mycobacteria are classified into three groups: photochroomogens, scotochromogens, and nonchromogens. Photochroomogens produce pigmented colonies in the light. Scotochromogens produce pigmented colonies when grown in the dark. Nonchromogens are nonpigmented in both light and dark, but only have light tan or buff-colored colonies (Geo. F. Brooks, 2001; Ve'ronique Vincent, 2003). Pigmented mycobacteria are classified as nontuberculous mycobacteria (NTM) because *M. tuberculosis* does not produce pigments (Ve'ronique Vincent, 2003).

Growth rate, colony morphology, and biochemical tests are other important differentiating factors among mycobacteria. Mycobacteria that grow less than 14 days are called rapid growers, and those that grow after 14 days are called slow growers. Examination of the morphology of colonies is important especially in mixed cultures. After 15 days growth, MTB produces thin, nonpigmented, rough colonies on 7H11 agar. Biochemical tests also aid in the identification of MTB from NTM. TB has the ability to reduce nitrate to nitrite. There are also other tests such as production of catalase or urease, arylsulfatase test, iron uptake, tween hydrolysis, tellurite reduction, and positive niacin test, which aid in the diagnosis of tuberculosis (Ve'ronique Vincent, 2003).

Modern Methods for Identifying MTB

BACTEC 460TB

Automated or semiautomated liquid culture systems that detect growth of mycobacteria species earlier than direct visualization have been developed. BACTEC 460TB system was commercially developed in the early 1980s by Becton Dickinson Company Sparks, MD, USA (Watterson and Drobniewski, 2000; Inderlied, 2004). This method is based on radiometric analysis of liquid growth medium containing palmitic acid, labeled with radioactive carbon (^{14}C-palmitic acid) as the substrate (Tenover et al., 1993). As the mycobacteria metabolize these fatty acids, radioactive carbon dioxide ($^{14}CO_2$) is released and measured by the system (Sharma and Mohan, 2004). This system has evolved to become the "gold standard" (Scarparo et al., 2002). The advantage of this method is reduced detection time of both smear-positive and -negative samples by nearly 50%; also, the bacterial growth can be detected in 5–10 days (Brunello et al., 1999; Katoch, 2004). Although this system considerably reduces the mycobacteria detection time, it is labor intensive and has other limitations, including cumbersome manual loading and unloading, potential risk of cross contamination, lack of computerized data management, use of radioactive material, and accumulation of low-level radioactive waste (Cruciani et al., 2004).

BACTEC 9000 MB

BACTEC 9000 MB is a fully automated, nonradiometric method developed by Becton Dickinson Co. This fluorescence-based system uses an oxygen-specific sensor to detect the mycobacterial growth (Flanagan, et al., 1999; Caws and Drobniewski, 2001). In every vial, there is a silicon rubber disk impregnated with a ruthenium metal complex as oxygen-specific sensor (van Griethuysen et al., 1996). Although this system is more rapid and less labor-intensive than solid media, it requires using a solid media, MYCO/F medium, a modified Middlebrook 7H9 broth to suppress the growth of contaminating microorganisms and can be supplemented

with antibiotics. The lower risk of cross-contamination and data management has greatly facilitated its use (Pfyffer et al., 1997a).

BACTEC MGIT 960

The Mycobacterial Growth Indicator Tube (MGIT; Becton Dickinson) system is a completely automated, nonradiometric, continuously monitoring, noninvasive instrument that requires neither needles nor other sharp instrument culture system (Hanna et al., 1999). Similar to Bactec 9000 MB, it uses the same oxygen-quenching fluorescence technology (Flanagan et al., 1999). It has greater capacity (960 bottles vs. 240 bottles) than other automated culture systems such as Bactec 460TB. This method detects MTB in 2 days (Epstein et al., 1998). Every 60 min, culture vials are monitored by instrument and based on specific growth algorithms are tagged as positive (Scarparo et al., 2002). This system is accepted as an alternative to radiometric culture system (Pfyffer et al., 1997b). Also, it is preferred by laboratory personnel as it is a safe and easy method (Cornfield et al., 1997). However, although it has significant safety advantages and is less labor intensive, the detection time for MTB, especially in smear-negative specimens, is longer than Bactec 460 TB (Chew et al., 1998). Morever, some studies have shown that this system has a higher contamination rate than radiometric Bactec 460 TB system (Hanna et al., 1999; Tortoli et al., 1999).

MB/BacT

MB/BacT is a continuously monitored nonradiometric system, developed by Organon Teknika Company, Marcy L'Etoile, France, which contains a computerized database management system. It uses a gas-permeable sensor embedded in a colorimetric indicator at the bottom of culture vials. Carbon dioxide released by growing mycobacteria is detected by sensor (Piersimoni et al., 2001). Every sensor in the bottom of the culture vial changes from dark-green to yellow depending on the CO_2 concentration. Every incubating drawer in the instrument is monitored by a reflometric detection unit (Brunello and Fontana, 2000). Calculated values are transmitted into a computer in every 10 min, and mycobacterial growth is indicated based on a predetermined algorithm (Rohner et al., 1997). In previous studies, it was shown that this radiometric system is an automated, sensitive, rapid method and requires less labor for detection of TB in clinical specimens. It serves as a good alternative to radiometric systems and solid media (Benjamin et al., 1998; Alcaide et al., 2000; Piersimoni et al., 2001; Mirovic and Lepsanovic, 2002).

Septi-Chek AFB

Septi-Chek AFB (Roche, Basel, Switzerland) contains biphasic culture medium and a self-contained CO_2(20%) environment. This system uses 20.0 ML of

modified Middlebrook 7H9 broth, a paddle with agar media enclosed in a plastic tube, and enrichment broth. One side of the paddle is covered with nonselective Middlebrook 7H11 agar. The reverse side is separated into two sections: one section contains chocolate agar for detection of contaminants, and the other side contains modified L-J medium. Vials are inoculated with 0.5 mL of the processed sediment and incubated at 35–37 °C for 10 weeks. Vials are inspected four times per week during the first 2 weeks for growth, then inspected weekly for an additional 6–8 weeks. It does not require specialized equipment or use of radioactive material (D'Amato et al., 1991; Sewell et al., 1993; Sharp et al., 1997). Also, this system is quite useful and has potential advantages for detection and isolation of mycobacteria (Isenberg et al., 1991). However, it requires more incubator space and is more labor intensive than the manual method (Sharp et al., 1996).

Trek Diagnostic ESP Culture System II

The ESP Culture System II developed by AccuMed (Chicago, IL, USA) is a fully automated, continuously monitoring method based on the detection of pressure changes, which are related to either gas production or gas consumption as a result of mycobacterial growth (Tortoli et al., 1998). A special detection algorithm has been developed for very slowly growing mycobacteria (Tholcken et al., 1997; Woods et al., 1997). Bottles contain a modified Middlebrook 7H9 medium, which is enriched prior to use with OADC (oleic acid, albumin, dextrose, and catalase) growth supplement (Myco GS) and with Myco PVNA (polymyxin B, vancomycin, nalidixic acid, and amphotericin B). The bottles also contain a compressed sponge submerged in the broth, which provides a growth support platform, and they should be incubated at 35°C for 42 days or until a positive signal is achieved. If this system is used with solid media, it has the same advantages as BACTEC in terms of being fully automated and does not use radioisotopes (Tortoli et al., 1998). However, when compared with BACTEC MGIT, times to detection of MTB are significantly longer in this system (Williams-Bouyer et al., 2000).

High-Performance Liquid Chromatography

High-performance liquid chromatography (HPLC) is based on variation of mycobacterium species cell wall mycolic acid profiles. This method can detect mycobacteria in smear-positive clinical specimens and positive liquid cultures (Inderlied, 2004). Thibert et al. demonstrated that HPLC proved to be a more reliable, easy to perform, cost effective, and rapid analysis technique than other identification methods after 18 months of routine use (Thibert and Lapierre, 1993). However, its routine use has not been implemented in large hospitals, reference and public health laboratories due to the equipment cost and the expertise required to perform the assays (Inderlied, 2004).

Molecular Methods for Identifying MTB

Nucleic Acid Amplification Tests

The extremely slow growing nature of *Mycobacterium tuberculosis* in culture method delays the diagnosis of TB disease. Nucleic acid amplification tests (NAATs) are developed for rapid diagnosis of TB disease. In this method, mycobacterial DNA and RNA are directly detected in clinical specimens (Soini and Musser, 2001). There are four commercial NAATs for direct detection of TB in clinical samples. However, the U.S. Food and Drug administration (FDA) has approved only two of them for direct detection of TB in acid-fast bacilli (AFB) smear-positive respiratory specimens: Enhanced Amplified Mycobacterium Tuberculosis Direct Test (MTD) (Gen-Probe, San Diego, CA, USA), and COBAS Amplicor MTB test (Amplicor, Roche Diagnostic Systems, Indianapolis, IN, USA) (Shamputa et al., 2004). No assay has been licensed for use in nonrespiratory specimens (Pai et al., 2004).

Enhanced Amplified Mycobacterium tuberculosis Direct Test

On September 30, 1999, the FDA approved the Enhanced Amplified Mycobacterium Tuberculosis Direct (MTD) test for detection of TB in AFB smear-positive and smear-negative respiratory samples. This test is based on detection of RNA by isothermal transcription-mediated amplification system developed by Kwoh (Shamputa et al., 2004). In this test, the mycobacterial rRNA is released from target cells by sonication, followed by promoter-primer binding to the target rRNA. Reverse transcriptase is used to copy rRNA to a cDNA-RNA hybrid. After degrading the initial RNA strand, a second primer binds to the cDNA and it is extended, resulting in the formation of double-stranded cDNA, which is then transcribed by DNA-dependent RNA polymerase to produce more rRNA molecules. The new transcripts then serve as targets for amplification and reverse transcription. The RNA amplicons are detected with an acridinium ester–labeled DNA probe in a hybridization assay solution. The esterified acridinium on the hybridized probe is hydrolyzed by addition of alkaline hydrogen peroxide, resulting in the production of visible light, which is measured in a luminometer. The test is performed at 42°C in a single tube, to reduce contamination (Soini and Musser, 2001; Shamputa, et al., 2004). The system works well with both AFB smear-positive and smear-negative specimens. The overall sensitivity for respiratory specimens compared with culture is 85.7–97.8%. Sensitivity of smear positive specimens (91.7–100.0%) is higher than smear-negative specimens (40.0–92.9%) (Shamputa et al., 2004). Test specificity of test was 100.0% (CDC, 1996). The major disadvantage of this test is lack of internal control for accessing amplification inhibitors (Soini and Musser, 2001).

Polymerase Chain Reaction–Based Amplicor and COBAS Amplicor MTB Test

The Amplicor test is based on the PCR amplification of a 584-bp segment of the gene encoding the 16S rRNA. After amplification, amplicons are denaturated and added to a microtiter plate containing a bound, MTB complex-specific oligonucleotide probe. Detection is achieved with the help of avidin–horseradish peroxidase conjugate and 3,3′, 5,5′-tetramethyl benzidine in dimethylformamide. The results are measured with spectrophotometer. The test has an internal amplification control for the detection of PCR inhibition. Uracil-*N*-glycosylase (UNG) carry-over prevention system is used in order to detect contamination by DNA remaining from previous runs. The results are available in 6–7 h. An automated version of this test, Cobas Amplicor, is available in Europe (Soini and Musser, 2001; Shamputa et al., 2004). The overall sensitivity and specificity of this test after resolving discordant results for respiratory specimens is 79.4–91.9% and 85.7–100.0%, and for nonrespiratory specimens is 27.3–98.6% and 85.7–100.0%, respectively (Shamputa et al., 2004). Because the sensitivity of this test is lower (40.0–73.1%) for smear-negative specimens, FDA has approved this test only for smear-positive respiratory specimens (Shamputa et al., 2004).

Noncommercial Diagnostic Assays

Classical Polymerase Chain Reaction

PCR technique has been used to detect MTB in clinical specimens since 1989, a year after it was first described. This method has been developed for detection of mycobacteria because of the slow growth rate of most pathogenic mycobacteria (Watterson and Drobniewski, 2000). PCR not only allows rapid diagnosis but also it theoretically can detect one DNA copy (Rodrigues et al., 2004). PCR offers better accuracy than AFB smears and has greater speed than culture (Su, 2002). Different PCR methods used for detection of MTB have been developed. In these PCR methods, targets can be either DNA or rRNA (Katoch, 2004). IS*6110*, MPB64, 16S rRNA genes have been used as a target in PCR assays. Overall sensitivity and specificity are 84.2–100% and 83–100%, respectively, using IS*6110* as target (Shamputa et al., 2004).

Real-time PCR

Real-time PCR has been developed for rapid and specific detection of MTB in clinical specimens (Katoch, 2004). In past few years, real-time PCR assays have been implemented in clinical microbiological laboratories (Bruijnesteijn Van Coppenraet et al., 2004). Real-time PCR is based on hybridization of amplified nucleic acids with fluorescent-labeled probes spanning DNA regions of interest

and monitored by inclusion of optical devices or CCD cameras in thermocyclers (Shamputa et al., 2004). This technique has been used for identification of a variety of the bacteria and viruses (Miller et al., 2002). Different format of probes are used for detection of MTB. The most frequently used ones are TaqMan probe, molecular beacons, and fluorescence resonance energy transfer (FRET) probes (Garcia de Viedma et al., 2002). Real-time PCR sensitivity is 71.6–98.1% and the specificity is 100.0% (Shamputa et al., 2004).

Nucleic Acid Probe Methods

The AccuProbe (GenProbe, San Diego, CA, USA) is based on the use of species-specific DNA probes that hybridize with rRNA. Briefly, the rRNA is released from mycobacteria by sonication and heat. The labeled DNA probe is combined with the rRNA-containing lysate to form a DNA-rRNA hybrid. The labeled product is detected in a luminometer (Ve'ronique Vincent, 2003). The results can be available in 2 h for culture-positive specimens (Soini and Musser, 2001). The test is only used for culture identification. It cannot be used for direct detection of mycobacteria in clinical specimens (Inderlied, 2004). This method is rapid and very easy to perform, and special instrumentation is not required. Overall sensitivity and specificity are both 100% when MTB colonies are tested (Ve'ronique Vincent, 2003). Another commercial probe test is SNAP (Syngene, San Diego, CA, USA). This assay is used for MTB and MAC culture identification, using covalently labeled to alkaline phosphatase oligonucleotide probes directed against ribosomal RNA. Sensitivity and specificity of this test are 100% and 99%, respectively. This test is also highly versatile and is useful in many clinical and public health laboratories (Lim et al., 1991).

DNA Microarrays

High-density oligonucleotide arrays (DNA microarrays) provide an opportunity for rapid examination of large amounts of DNA sequence in a single hybridization step. This method is based on hybridization of fluorochrome-labeled PCR amplicons to DNA produced from bacterial colonies to DNA arrays. Oligonucleotide probes based on the 16S rRNA, DNA gyrase subunit B (*gyrB*) or the *rpoB* genes are used. The bound amplicons emit a fluorescent signal that is detected by a scanner (Shamputa et al., 2004). This method has been used for identification of simultaneous species and detection of RIF resistance mutants of mycobacteria. This test contains 82 unique 16S rRNA sequence probes and allows the differentiation of 54 mycobacterial species and 51 sequences that contain unique *rpoB* gene mutations. It is easy to perform and permits identification of a large number of mycobacteria in one reaction. This method is used in research and clinical laboratories for identification of mycobacteria. The results of the test are reliable, reproducible, and are obtained in only 4 h for culture-positive specimens (Soini and Musser, 2001).

Immunodiagnostic Methods

A number of immunodiagnostic tests have been described. None of these tests has found widespread clinical use. Serological methods test the 38-kDa antigen, lipoarabinomannan, antigen 60, the antigen 85 complex, glycolipids including phenolic glycolipid, Tb1, 2,3-diacyltrehalose, and lipooligosaccharide as antigens (Ve'ronique Vincent, 2003). Because of low sensitivity and specificity of the antigen tests, it cannot be recommended at this time (Gray, 2004).

Typing Methods

IS 6110-*Based Restriction Fragment Length Polymorphism*

The restriction fragment length polymorphism (RFLP) technique using the IS*6110* repetitive as a probe was published in 1990 and was widely used for typing strains of mycobacteria. This method is considered the gold standard for typing strains of mycobacteria. IS*6110* is a 1355-bp insertion sequence in the MTB genome; zero to 25 copies of IS*6110* are found in the most strains of MTB. It is not known to be in other organisms (Narayanan, 2004). Genomic DNA is extracted and then digested with specific restriction enzyme. Then, the restricted fragments are separated with electrophoresis (Inderlied, 2004). This method has several disadvantages. First, it requires large amount of high-quality DNA, extracted from a large number of bacteria grown from clinical material. Second, this method does not have good discrimination of isolates that contain less than 6 copies of IS*6110* (Narayanan, 2004).

Spoligotyping

Spoligotyping is a PCR-based typing method. A small direct repeat (DR) with 36 bp in the genomic DR region of MTB DNA is used for PCR amplifications with appropriate primers (Inderlied, 2004). The resulting PCR products are hybridized to 43 different oligonucleotides fixed to a membrane. The presence and absence of spacers in a given biotinylated strain are determined by hybridization with a set of 43 oligonucleotides derived from spacer sequences in *M. tuberculosis* H37Rv. Although this method is weaker than IS*6110* typing for differentiation of MTB strains, it has higher sensitivity for strains with low copy numbers of IS*6110* (Narayanan, 2004).

Variable Number of Tandem Repeats

One variable number of tandem repeats (VNTR) method is based on 12 loci of a type of VNTR sequences called mycobacterial interspersed repetitive units (MIRUs) (Narayanan, 2004). MIRUs are located in the intergenic region separating two genes, SenX3–RegX3 which encode a mycobacterial two-component

system; they are present as tandem repeats. The number of the repeats are different among MTB strains. The PCR amplicons are detected in agarose gel or by a DNA sequencer, allowing numbers of the repeats to be detected. Compared with culture, this method was found to be 100% sensitive and 100% specific (Shamputa et al., 2004).

Susceptibility Tests for MTB

Mycobacterial susceptibility testing is important in determining the appropriate treatment of mycobacterial infections (Woods, 2000). CDC advises that susceptibility results should be available an average 28–30 days after receipt of a specimen from a microbiology laboratory (Brunello and Fontana, 2000). Different types of susceptibility tests are performed in clinical laboratories. It is recommended that the National Committee for Clinical Laboratory Standards (NCCLS) be used as a standardization method. NCCLS document M24-T has a tentative standard method for using only MTB. The second edition of this method (NCCLS document M24-T2) has been developed (Woods, 2000). Among the methods used for susceptibility testing of MTB, the agar proportion method (MOP) is universally accepted as the gold standard (Kontos et al., 2004). The result of this method takes generally 3–4 weeks; new methods such as radiometric or nonradiometric methods are being developed (Pfyffer, 2003).

Conventional Methods for Susceptibility Testing of MTB

Three methods are used for susceptibility testing of MTB; MOP, absolute concentration method, and resistance ratio method. MOP is most frequently used in the United States and Western Europe (Inderlied, 2004). MOP for mycobacterial susceptibility testing was developed in the 1960s by G. Canetti (Pfyffer, 2003). This method was modified with a standard method published by NCCLS in 2003 (Parsons et al., 2004). The preferred medium for this test is Middlebrook 7H10 agar plates because it has a simple composition, is easy to prepare, and allows the early detection and quantitation of colonies. The Lowenstein–Jensen medium is recommended by WHO and IUATLD as an alternative medium (Pfyffer, 2003). The inoculum can be prepared as either a direct or an indirect test. A smear-positive specimen is used as the source of inoculum in the direct test. In the indirect test, the pure culture is used as the inoculum source. Several dilutions of a standardized suspension are inoculated onto suitable agar plates. The number of colony-forming units (CFU) on the drug-containing plates are compared with the number of colony-forming units on a drug-free plate. Strains of tubercle bacilli that exceed greater than 1% growth on drug-containing media, compared with growth on drug-free media, are considered resistant to that agent (Inderlied, 2004).

In the absolute concentration method, the minimum inhibition concentration (MIC) of the agent is detected. The inoculating control media and drug-containing media are used for serial twofold dilutions of each agent. The lowest concentration

of antibiotic that inhibits growth of the agent indicates resistance (Sharma and Mohan, 2004).

In the resistance ratio method, MIC of the isolate is shown as a multiple of the MIC of a standard susceptible strain in order to avoid intra- and inter-laboratory variations. Inoculum size should be strictly controlled in both tests. These tests are not suitable for direct sensitivity testing of concentrated clinical specimens (Sharma and Mohan, 2004).

BACTEC 460TB

In 1975, it was reported that MTB metabolic end-products could be detected by using a ^{14}C-labeled substrate (Parsons et al., 2004). This system has been widely performed in clinical laboratories in the past two decades and is considered the gold standard in many ways (Tortoli et al., 2002). Radioactive BACTEC 460TB system (Becton Dickinson) has been developed based on detection of $^{14}CO_2$ produced by consumption of a ^{14}C-labeled substrate. This system is used to test the metabolism of the organism in the presence of all first-line drugs (SM, INH, RIF, EMB, PZA) (Tortoli et al., 2000). This system is reliable and rapid for detection of susceptibility of MTB to these drugs (Scarparo et al., 2004). Results of susceptibility test can be available in 1 week compared with 3–4 weeks when solid media is used (Parsons et al., 2004). BACTEC 460TB is the proposed reference method for PZA susceptibility testing by NCCLS (Scarparo et al., 2004), but this system uses radioactive substrates (Johansen et al., 2004).

MB/BacT Alert 3D

MB/BacT Alert 3D (Biomerieux) is a fully automated, nonradiometric system that allows susceptibility testing of MTB to EMB, INH, PZA, RIF, and SM (Inderlied, 2004). The system is based on the detection of CO_2 released into the medium by actively growing mycobacteria. The CO_2 generated decreases the pH, and color change occurs in a sensor in the bottom of the vial. Color changes are detected by a reflect metric detection unit in the instrument (Angeby et al., 2003). This system is likely to increase biosafety profiles, and it does not contain radioactive waste (Tortoli et al., 2000).

BACTEC MGIT 960

BACTEC MGIT 960 (Becton Dickinson) system is a nonradiometric, fully automated continuous-monitoring system that allows susceptibility testing for INH, RIF, SM, and EMB. Recently, the automated BACTEC MGIT 960 system was developed to make the susceptibility testing of MTB to PZA possible (Kontos et al., 2003). This system consists of using a modified Middlebrook 7H9 broth in a test tube in conjunction with a fluorescence-quenching oxygen sensor. The fluorescent compound is sensitive to the presence of oxygen in the test tube. Fluorescence light emitted from growing mycobacteria is detected using a 365-nm UV

transilluminator (Ardito et al., 2001). This system has been reported as a rapid, reliable, and automated method for susceptibility testing of MTB to first-line drugs in many studies (Bemer et al., 2002; Kontos et al., 2003; Marttila et al., 2003; Johansen et al., 2004; Kontos et al., 2004).

ESP Culture System II

ESP Culture System II (Trek Diagnostic, Westlake, OH, USA) is a fully automated continuously monitoring system for testing the susceptibility of MTB to INH, RIF, EMB, and SM (Pfyffer, 2003). This system is based on detection of produced or consumed gas due to microbial growth within the headspace above the broth culture medium in a sealed bottle (Bergmann and Woods, 1998). It was reported as a reliable, rapid, and automated method for performing susceptibility testing of MTB (Ruiz et al., 2000).

Molecular Methods for Susceptibility Testing of MTB

PCR-DNA Sequencing

PCR-DNA sequencing is the main method and gold standard for detection of the genetic mechanisms of drug resistance in MTB (Shamputa et al., 2004). This method includes PCR amplification of the DNA region of interest in clinical isolates or samples. Each isolate needs several sequencing reactions to be performed. Unfortunately, this method is not used for routine identification of drug resistance in mycobacteria because the mutations responsible for antibiotic resistance involve many different genes. However, *rpoB* resistance is contained in a very short segment of the gene, and this method is useful to accurately identify mutations that are associated with RIF resistance (Soini and Musser, 2001). It is labor intensive and identifies previously recognized mutations. This system does not require expensive equipment but needs a high level of expertise. Although automatic sequencers are increasingly present in health care institutions, they are not available in many microbiology laboratories (Garcia de Viedma et al., 2002).

INNO LiPA Rif.TB

RIF is an important drug in the treatment of tuberculosis and a marker for MDR-TB. The *rpoB* gene is target for amplification and detection point mutations, small deletions or insertions in *rpoB* gene, which lead to RIF resistance. LiPA has been developed for detection of RIF resistance. It is based on the reverse hybridization method and consists of PCR amplification of a segment of the *rpoB* gene, denaturation, and hybridization of the biotinylated PCR amplicons to capture probes bound to a membrane strip. The formed hybrids are colorimetrically detected (Rossau et al., 1997). LiPA test strip includes four probes for specific *rpoB* mutations and five probes for wild-type *rpoB* sequences. It also contains conjugated control and MTB control probes. MTB or the *rpoB* mutation is detected in the interpretation of the banding pattern on the strip. In this test, MTB culture or clinical specimens

are used directly. Results are available in less than 48 h (Soini and Musser, 2001). This method is simple, rapid, and does not require expertise in molecular biology (Garcia de Viedma, 2003). However, this method is expensive and impractical for routine use (Lemus et al., 2004) and also cannot detect rare mutations (Soini and Musser, 2001).

Rifampicin Oligonucleotide Typing (Rifoligotyping)

Rifoligotyping is analyzed by hybridization by the reverse line blotting technique. The 437-bp fragment of the *rpoB* gene is amplified with primer, one of which is labeled with biotin. The amplicons are hybridized to a set of wild-type and mutant oligonucleotides covalently bound to a membrane by reverse line blotting and are detected by enzyme chemiluminescence. The results of this method are available in one day and performance is easy. In each run, a total of 43 samples can be studied. Also, if more than one mutation is known, the number of the probes should increase (Shamputa et al., 2004).

PCR-Based Single-Strand Conformational Polymorphism (SSCP)

SSCP technique is one of the methods based on PCR-electrophoresis. The principal of SSCP is conformational distortion resulting from a nucleotide substitution in a single-strand DNA fragment. A difference in sequence, even a change in a single base, leads to an electrophoretic mobility different to that of the wild-type single-strand fragment (Gong et al., 2002). DNA fragment is denatured to single strand and amplified by PCR. It is run on a polyacrylamide gel together with the denatured wild-type reference sample. When a mutation occurs, mobility shifts would occur in the clinical sample (Garcia de Viedma, 2003). This system has frequently been used for detection of mutation in RIF and INH and may be the most cost-effective method to detect point mutations within the 69-nucleotide region of RIF-resistant MTB (Kim et al., 2001). This system is cheap, easy, rapid, and suitable for analysis of a large number of samples (Garcia de Viedma, 2003).

PCR-Heteroduplex Formation (HDF) Assay

This assay was developed by Williams and co-workers for detecting RIF resistance. The assay is based on the amplification and detection of mutation in the *rpoB* gene (Mayta et al., 2003). The amplicon of a 305-bp fragment of the *rpoB* gene is denatured and then mixed with an equivalent amount of a denatured amplicon from a reference wild-type strain. Then the mixture of amplicons is heated to denature the DNA, after which, the DNA is allowed to reconform on ice, and then the aliquot of the amplified DNA is analyzed by electrophoresis. If in clinical strains mutations have occurred, electrophoretic mobility between the susceptible strain and the clinical strain is different (Garcia de Viedma, 2003; Shamputa et al., 2004). This assay is rapid and sensitive for detection of RIF genotype of MTB directly from sputum specimens (Mayta et al., 2003).

Real-Time PCR

In many studies, real-time PCR was used for detection of drug resistance in MTB (Torres et al., 2000; Garcia de Viedma et al., 2002; van Doorn et al., 2003; Sajduda et al., 2004). Van Doorn et al. designed a real-time PCR assay that can be performed directly on clinical samples to detect INH resistance. The sensitivities and specificities of the probes were 82% and 100% for the mutant probe and 70% and 94% for the wild-type probe. They reported that this assay allows rapid identification of a mutant *katG* allele and could be easily implemented in clinical microbiology laboratories (van Doorn et al., 2003). Torres et al. developed a single-tube method for detecting mutations associated with resistance to RIF and INH. They demonstrated that this assay is the fastest available method for detection of RIF and INH resistance-associated mutations in MTB (Torres et al., 2000). In another study, Sajduda et al. reported that real-time PCR method is fast and reliable for detection of RIF and INH resistance-associated mutations in MTB (Sajduda et al., 2004). Viedma et al. designed a new genotypic approach that can be simultaneously used for detecting resistance to RIF and INH in a single reaction tube. They reported that their design could be a model for new, rapid genotypic methods and is able to simultaneously detect a wide variety of antibiotic resistance mutations (Garcia de Viedma et al., 2002).

DNA Microarrays

DNA microarray technology is used for rapid detection of mutation associated with TB drugs (Gingeras et al., 1998; Troesch et al., 1999). A sequence database was described based on the use of a DNA probe array for detecting MTB RIF resistance (*rpoB* alleles). Seventy mycobacterial isolates from 27 different species and 15 RIF-resistant MTB strains were tested. A total of 26 of 27 species were correctly identified, including all of the *rpoB* mutants (Troesch et al., 1999). Yue et al. demonstrated that this method is an efficient, specialized technique to implement and can be used as a rapid method for detecting RIF resistance to complement standard culture-based method (Yue et al., 2004).

Conclusion

Although in most developed countries, TB prevalence is at a low level, MDR-TB is increasing due to immigration of the foreign-born. Drug-resistant TB prevalence has alarmingly increased in some of these developed countries (Raviglione et al., 2001). So, the mycobacteriology laboratories have an important role in the diagnosis and control of TB (Su, 2002). The turn-around time for microscopy is $\sim$24 h in most laboratories. Every laboratory should have experienced, well-trained technicians established in the mycobacteriology labs (Espinal et al., 2001). Molecular techniques are being used in mycobacteriology laboratories in developed nations for detection of MTB, and drug susceptibility testing is being performed directly from clinical specimens. These tests allow rapid identification of MTB and detection of drug resistance. However, these techniques are complicated

and costly. Also, they require experienced personnel, separate rooms to prevent cross-contamination, and expensive tools including thermocyclers, electrophoresis equipment, sequencing and other automated systems (Shamputa et al., 2004).

Current opinions support molecular methods in combination with smear results, culture data, and clinical suspicion to diagnose tuberculosis (Shamputa et al., 2004). Molecular techniques are currently unable to replace the traditional smears and culture (Frieden et al., 2003).

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24
Rapid Screening and Identification of Methicillin-Resistant *Staphylococcus aureus*

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Clinical Relevance

Staphylococcus aureus is a major pathogen responsible for both nosocomial and community-acquired infections. Although the first *S. aureus* isolates displaying resistance to methicillin (MRSA) were reported in the early 1960s (Barber, 1961), endemic strains of MRSA carrying multiple resistance determinants did not become a worldwide nosocomial problem until the early 1980s (Hryniewicz, 1999). The presence of MRSA in an institution is paralleled by an increased rate of bacteremia or other severe MRSA infections (Harbarth et al., 2000). MRSA-related bacteremia carries a threefold attributable cost and a threefold excess length of hospital stay when compared with methicillin-susceptible *S. aureus* (MSSA) bacteremia (Abramson and Sexton, 1999).

Recently, the apparition of endemic community-acquired MRSA (CA-MRSA) has been reported (Naimi et al., 2003; Saiman et al., 2003). In contrast to hospital-acquired MRSA (HA-MRSA), CA-MRSA are frequently isolated from healthy people and strains are generally susceptible to several older, but clinically important antibiotics (Saimen et al., 2003; Vandenesch et al., 2003; Harbarth et al., 2005). Population dynamic analysis of CA-MRSA revealed that this part of the MRSA population is in constant expansion (Carleton et al., 2004) and was only recently detected as an emerging but worldwide infectious concern (Said-Salim et al., 2003; Vandenesch et al., 2003).

The spread of MRSA in health care centers is difficult to control and requires elaborate infection control guidelines (Pittet et al., 1996, 2000; Herwaldt, 2000). The difficulty to eradicate nosocomial MRSA infections may be explained by (i) the presence of an unknown hidden reservoir of MRSA carriers (ii) the emergence of novel highly epidemic *S. aureus* clonotypes such as EMRSA-15, EMRSA-16, or EMRSA-17 (Cox et al., 1995; Richardson and Reith, 1993; Aucken et al., 2002), with unspecified selective advantages, and/or (iii) failure in enforcement of infection control prescriptions. Some authors reported that the screening of high-risk patients for MRSA colonization was a cost-effective measure for limiting the spread of the organism in hospitals (Papia et al., 1999). As said before, early detection of MRSA carriers is also crucial for therapeutic decisions with last-line

antibiotics against MRSA (e.g., glycopeptides and oxazolidinones) (Sakoulas et al., 2002).

An extensive screening of MRSA carriers at hospital admission, despite its important cost, appears to have a major impact in reducing MRSA nosocomial infection rates (Wernitz et al., 2005), as recently shown by Wernitz and colleagues. Indeed, MRSA carriage or colonization is a major risk factor for becoming infected. The preferred colonization sites are the nose, the throat, and the skin surface (Kluytmans et al., 1997). The spread of MRSA occurs generally after contact with carriers (Grundmann et al., 2005) or "MRSA reservoirs" (parts of which are probably unknown). The spread of MRSA in health care centers is difficult to control and requires elaborate infection control guidelines (Cohen et al., 1991; Nettleman et al., 1991; Pittet et al., 1996; Cosseron-Zerbib et al., 1998; Chaix et al., 1999; Papia et al., 1999; Harbarth et al., 2000) including (i) large-scale screening of suspected carriers, (ii) automated computerized alerts, (iii) specific recommendations for at-risk patients, such as contact isolation (Pittet et al., 1996, 1997; Harbarth and Pittet, 1998), and (iv) significant improvement of hand hygiene compliance (Pittet et al., 2000). These data, together with successful containment effort programs (Cohen et al., 1991; Nettleman et al., 1991; Pittet et al., 1996; Cosseron-Zerbib et al., 1998; Chaix et al., 1999; Papia et al., 1999; Harbarth et al., 2000) prompt for screening high-risk patients even in a highly endemic setting (Rubinovitch and Pittet, 2001). Several international guidelines now recommend the screening of potential MRSA-positive patients at hospital admission (BSAC, 1998; KKI, 1999; Muto et al., 2003). However, despite intensive efforts in the application of such guidelines, MRSA spread remains difficult to control.

Molecular Epidemiology

Molecular techniques dedicated to bacterial detection and identification have been recently reviewed (Nolte et al., 2003; Diekema et al., 2004). In the case of MRSA, the *mecA* gene encoding for the low-affinity penicillin-binding protein PBP2′ is the genetic basis of methicillin resistance in MRSA isolates. This gene, originating from a mobile genetic element designated SCC*mec* [staphylococcal cassette chromosome *mec* (Katayama et al., 2003)], flanked by terminal inverted and direct repeats (Ito et al., 2001), is invariably inserted into the *orfX* gene of *S. aureus* chromosome (Fig. 24.1). This element contains two site-specific cassette chromosome recombinases, *ccrA* and *ccrB*, responsible for the precise excision and integration of SCC*mec* within the bacterial chromosome (Katayama et al., 2003).

To date, five differently organized SCC*mec* elements have been characterized (Ito et al., 2003). Three types of SCC*mec* elements are typically found in HA-MRSA strains (i) type I, a 34-kb element that was prevalent in MRSA isolates in the 1960s, (ii) type II, a 53-kb element that was identified in 1982 and is ubiquitous in Japan, Korea, and the United states, and (iii) type III, the largest (67-Kb) element, identified in 1985, currently prevalent in Germany, Austria, India, and other South Asian and Pacific areas (Hiramatsu et al., 2001; Ito et al., 2003). In contrast to

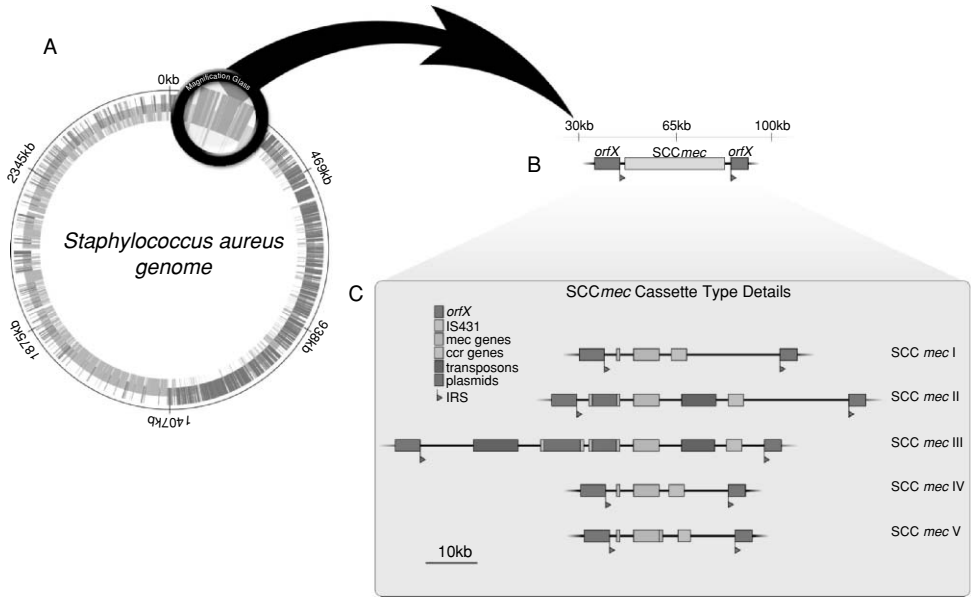

FIGURE 24.1. General molecular organization of the *Staphylococcus aureus* genomic region containing the determinant of methicillin-resistance. The circular chromosomes of several *Staphylococcus aureus* strains are fully sequenced (A) allowing prediction of all ORFs and their respective coding sequences (red for ORFs encoded on the plus strand, blue on the minus strand). The SCC*mec* element encoding the methicillin-resistance determinant *mecA* is located in the *orfX* region at the very beginning of the chromosome (B). The five characterized variants of the SCC*mec* element show variable lengths; they are all flanked by inverted repeat regions (red arrows), contain recombinase(s) (yellow blocks) and the methicillin-resistance determinant (cyan blocks) (C). Variability in sequence contents concerns presence of mobile elements harboring other antibiotic resistance determinants (green blocks). [*See* Color Plate I]

HA-MRSA, CA-MRSA isolates generally carry the SCC*mec* type IV element, whose size is much smaller than those of SCC*mec* types I, II, and III (Ma et al., 2002; Ito et al., 2003). At least four subtypes of the type IV SCC*mec* element, whose size varies from 20 to 24 kb, have been reported so far (IVa to IVc) (Ito et al., 2003) and IVd (accession number AB097677). The molecular structure of the recently described type V cassette (Ito et al., 2004) encoding a new *ccrC* recombinase presents some analogy with the type IV allele: both are more frequently associated with CA-MRSA, do not contain additional antibiotic-resistance determinants, and are the smallest SCC*mec* cassettes with a size <30 kb (Fig. 24.1).

Molecular composition of the four SCC*mec* elements reveals key components useful for SCC*mec* typing (Ito et al., 2001; Francois et al., 2004). Variations in this gene set have allowed identifying five classes of *mecA* gene complexes (Hiramatsu et al., 2001; Ito et al., 2003, 2004), as discussed before. A second essential region is

the *ccr* gene complex. Types I and III harbor *ccrA1-B1* and *ccrA3-B3* recombinases, respectively; whereas types II and IV contain *ccrA2-B2* recombinases, showing some difference in the amino acid sequences (Ito et al., 2001). Distinct from these four types, SCC*mec* V strains harbor a new *ccrC* recombinase type (Ito et al., 2004) (Fig. 24.1).

Recent efforts in the field of high-throughput sequencing yielded to the release of numerous bacterial genome sequences. To date, seven fully sequenced strains of *Staphylococcus aureus* are publicly available (Kuroda et al., 2001; Baba et al., 2002; Gill, 2004; Holden et al; 2004) providing opportunities for searching conserved or variable targets within the genome of MRSA, thus allowing improved molecular identification and characterization (Charbonnier et al., 2005; Couzinet et al., 2005b). For example, the markers described above (e.g., *mecA* and SCC*mec* organization) are important epidemiological indicators of strain origin (Dufour et al., 2002; Naimi et al., 2003).

Culture-Based Methods Dedicated to MRSA Identification

Liquid cultures have been instrumental to the nascent field of microbiology and are still used routinely in laboratories to recover minute amounts of bacteria or are employed as backup media. For example, Mueller–Hinton supplemented by oxacillin is still currently used to detect or confirm presence of MRSA from swabs sampled for surveillance programs. This medium appears in the guidelines for the prevention and control of antibiotic-resistant organism from the NCCLS recommendations (NCCLS,2002). However, solid media are now commonly used for organism isolation and identification, allowing MRSA identification in approximately 24 h. Agar plates provide numerous advantages, such as the possibility for microbiologists to detect the presence of relevant colony morphologies, isolate them by sub-plating, and assess their purity on isolation plates. Pure isolates are essential for further phenotypic testing, including speciation (when required), antimicrobial susceptibility testing, and typing. Agar plates developed for MRSA isolation or detection represent a particularly active field for microbiology companies. To date, numerous selective media containing β-lactam antibiotics and chromogenic substances are commercially available. The general principles are simple and consist in providing selective medium supplemented in (i) Gram-negative growth inhibitor (required for samples containing mixed flora), (ii) antibiotic (allowing the selection of methicillin-resistant organisms only), and (iii) a chromogenic substrate allowing the specific detection of growing *Staphylococcus aureus* colonies. ORSAB plates (Oxoid Ltd; Basingstoke, UK), a solid variant of the liquid mannitol–salt medium containing oxacillin and aniline blue, allows detection of mannitol-fermenting organisms as blue colonies, due to medium acidification. This medium appears adapted to high-risk population (Simor et al., 2001), but presents limitation for surveillance applications (Becker et al., 2002) as some

coagulase-negative staphylococci (mainly *S. haemolyticus*, a frequent skin colonizer) also appear blue (Becker et al., 2002). Thus, the use of this plate requires additional tests for robust identification. To date, ChromAgar/MRSA (ChromaAgar, Paris, France) stands among the most popular agar plates dedicated to the detection of MRSA. This medium, where MRSA colonies appear mauve while other bacteria display different colors (Fig. 24.2), has been extensively tested and shows appreciable sensitivity and specificity (Kluytmans et al., 2002; Diederen et al., 2005). Other chromogenic agar media showing interesting performance are MRSA ID (bioMérieux, Marcy l'Etoile, France), containing cefoxitin and a chromogenic substrate of α-glucosidase. Recent evaluation of this medium has shown improved performance compared with ChromAgar/MRSA (Perry et al., 2004). Comparable results were reported with MRSA Select (Bio-Rad, Reinach, Switzerland) (www.rapidmicrobiology.com/PG/MRSA.php). All these media represent appreciable improvement of the current situation in the field of MRSA screening and identification, albeit these culture-based methods require at least 20–24 h to yield identification results. During this period of time, infection control measures cannot be optimally applied. And, in case of empirical treatment, options include usually glycopeptide prescription leading to important costs and suboptimal use of last-barrier drugs.

Molecular Methods for MRSA Identification

Rapid detection of MRSA by standard clinical microbiological procedures appears then tedious and time consuming, as it first requires identification of isolated *S. aureus* colonies from mixed flora samples before assessing their antibiotic susceptibility profile. Direct or indirect particle agglutination assays using antibody-coated beads offer a rapid alternative to oxacillin susceptibility testing. For example, MRSA-Screen (Denka Seiken, Tokyo, Japan) provides sensitive and specific immuno-detection of MRSA in a pure culture by using anti-PBP2′ antibody-coated latex beads, and reveals similar to standard oxacillin disk diffusion or oxacillin salt agar screening (Cavassini et al., 1999; Hussain et al., 2000). However, the specific immuno-detection of MRSA based on PBP2′ cannot be performed in the presence of other methicillin-resistant staphylococcal species, organisms that are frequently recovered as commensal contaminant of mixed flora samples (Cavassini et al., 1999). Indeed, the high level of sequence homology of the *mecA* gene present in *S. aureus*, *S. epidermidis*, and potentially other coagulase-negative staphylococci (CNS) species (Ryffel et al., 1990; Wielders et al., 2001) precludes discrimination of methicillin-resistant strains of *S. aureus* from CNS. Furthermore, the sensitivity of the latex agglutination based on detection of PBP2′ antigenic motif requires the induction of its expression to reliably obtain detectable levels of the protein (Rohrer et al., 2001). Because the *mecA* gene, encoding the low-affinity penicillin binding protein (PBP2′) (Ryffel et al., 1992) represents the "gold standard" for detecting methicillin resistance (Murakami et al., 1991), several assays based on the direct detection of the *mecA* gene have been described, using chemiluminescent

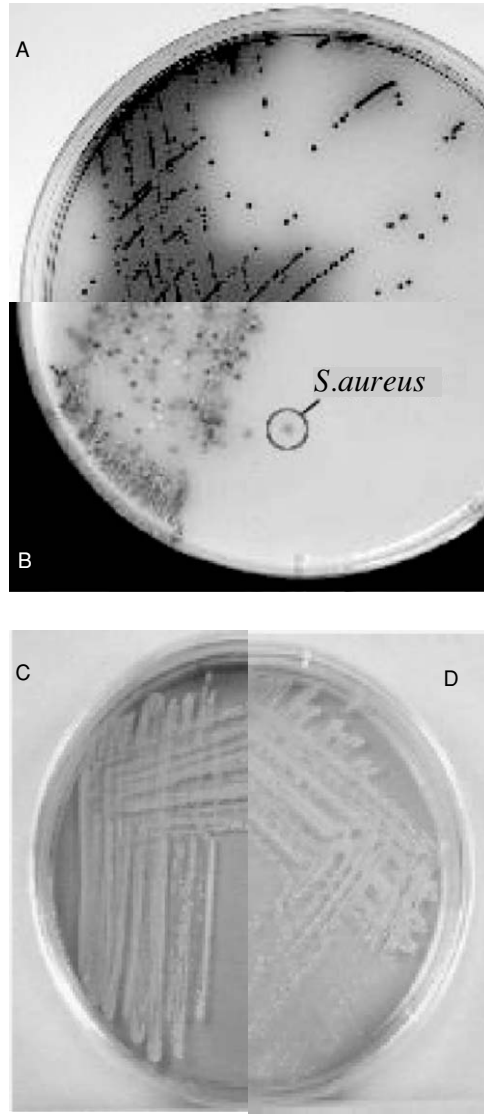

FIGURE 24.2. Illustrative examples of chromogenic media. (A). ORSAB plates (Oxoid) contain oxacillin and a tracer of mannitol fermentation yielding blue *Staphylococcus aureus* colonies. (B). Selective ChromAgar plate (ChromAgar), yielding pink *Staphylococcus aureus* colonies (pictures are visible on the respective Web sites of the companies). Standard mannitol–salt agar plate showing colony aspects resulting from negative (C) or positive (D) MRSA cultures. [*See* Color Plate II]

probes (Youmans et al., 1993), cycling probe technology (Hamels et al., 2001) or a two-step immuno-PCR assay based on the amplification and immuno-detection of *mecA* and *femB* amplicons, and performed after overnight culture enrichment of clinical samples (Towner et al., 1998). These techniques appear promising in terms of sensitivity but also of turn-around time as they generally require a few hours before obtaining results. However, a common drawback is the requirement for induction of protein expression and the fact that they are not designed to identify MRSA among other MR-CNS; rather, they appear much more adapted to isolated pathogens or sterile samples containing a single bacterial species.

Improved knowledge about genome sequences has allowed the design of numerous PCR-based methods. Rohrer and colleagues reported the use of a duplex PCR recognizing the genes *mecA* and *femA* [a gene specific for and recovered in all *S. aureus* strains (Berger-Bachi et al., 1989; Hurlimann-Dalel et al., 1992)] on a collection of isolated MRSA (Rohrer et al., 2001). Other similar multiplexed PCR assays, using different targets (coagulase and *femA* genes) (Vannuffel et al., 1998; Kearns et al., 1999) or *S. aureus* toxins (Schmitz et al., 1998), yielded promising results with good specificity. More recently, other promising results were also obtained by using triplex PCR assays based on the detection of *S. aureus* rRNA, *mecA*, and *nuc* (Louie et al., 2002; Maes et al., 2002) or clfA genes (Mason et al., 2001). Very recently, a smart approach was reported by Cuny and colleagues using a simplex PCR amplification using primers complementary to a conserved region of *orfX* and a *S. aureus*–specific region selected by the authors (Cuny et al., 2005). Overall, PCR-based methods perform extremely well when applied to isolated strains or samples containing a pure culture, not exactly what is required for direct MRSA screening.

Multiplexed real-time qPCR techniques using fluorescently labeled detection of PCR products (Lee et al., 1999) combine accurate identification with limits of detection close to a single gene copy/sample. They have provided a significant technological advantage for the rapid and large-scale identification of various microorganisms (Corless et al., 2001; Mackay et al., 2002; Brancart et al., 2005; Gieseler et al., 2005; He and Jiang, 2005; Hsu et al., 2005; Lee et al., 2005; Svenstrup et al., 2005). These techniques outperform conventional detection methods by providing rapid and sensitive detection and avoiding the use of acrylamide gel. Using the *nuc* gene as target, Fang and Hedin reported a fast screening and identification assay applicable to isolated bacteria (Fang and Hedin, 2003). More recently, using five specific primer pairs in the SCC*mec* and *orfX* regions, Huletsky and colleagues reported a very fast assay allowing direct identification of MRSA in 2 h, even in the presence of other staphylococcal species (Huletsky et al., 2004). Note, however that the emergence of new SCC*mec* elements warrants further modifications of the design and revalidation of the whole assay as new molecular variants continuously emerge (Berglund et al., 2005). Donnio and colleagues nicely described that the partial excision of the SCC*mec* cassette occurs not infrequently in MSSA strains and might compromise the efficiency of this assay (Donnio et al., 2005). In this field, our group reported recently the use of a novel immuno-qPCR procedure allowing rapid detection of MRSA from mixed flora samples (Francois et al.,

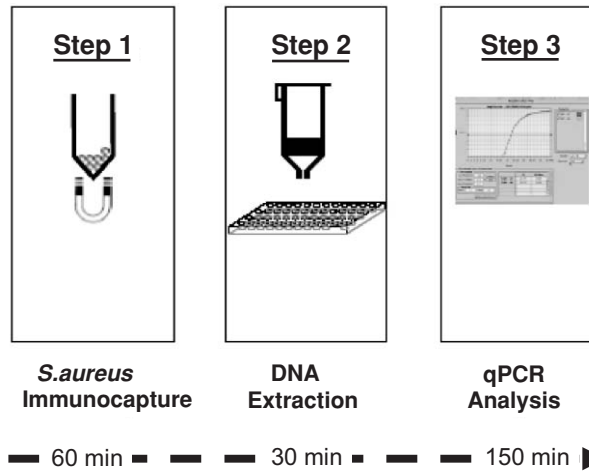

FIGURE 24.3. Flowchart of an immuno-qPCR assay for rapid MRSA identification. After sampling and conditioning, mixed-flora containing samples are adsorbed using *S. aureus*–specific antibodies coupled to metallic particles (step 1). After washes, antibody-coated bacteria are disrupted by bead beater and then purified on columns (step 2). The lysis medium is subjected to qPCR analysis (step 3). Note that turn-around time required for the complete procedure requires only a few hours, whereas culture-based methods imply a minimum of 24 h.

2003b). The procedure consists in a direct one-step enrichment of MRSA present in either nasal or inguinal swabs, followed by DNA extraction of immunocaptured bacteria and their identification by a triplex qPCR (Figure 24.3). The specificity of MRSA identification is based on the quantitative correlation of the *mecA* gene and that of the *S. aureus*–specific *femA* signal, a probe that does not cross-react with other bacterial species, including *S. epidermidis*. This assay allows detection and identification of MRSA in less than 6 h after sample collection, thus allowing same-day identification. We have successfully applied this method to the rapid screening of patients admitted to the ICU and have shown that infection control measures could be implemented earlier than with optimized culture-based methods (22.2 vs. 93.1 h, respectively). Finally, this molecular screening strategy resulted in decreased MRSA infections in the medical intensive care unit (MICU) after coupled implementation of strict control measures (Masuet-Aumatell et al., 2004).

Impact of Recent Hybridization Technologies on the Identification of MRSA

Hybridization technologies benefit from the efforts in high-throughput sequencing and allow the deposition onto solid surfaces of thousands of capture

oligonucleotides able to hybridize with complementary target sequences. PCR-amplified products are detectable with low-density oligoarrays, enabling the parallel detection of several targets during the same experiment. The basic principle of DNA probe technology is hybridization, relying on denaturation of double-stranded DNA and detection of hybridized labeled-DNA. Recent reports relying on ribosomal or other specific markers demonstrated the identification of *Staphylococcus* at the species level (Hamels et al., 2001; Couzinet et al., 2005a) and even provided an overview of the virulence factors harbored by clinical isolates during the same experiment (Saunders et al., 2004; Couzinet et al., 2005b).

Despite parallelism and robustness, these analyses based on PCR products hybridization require several time-consuming steps (e.g., enzymatic amplification, product isolation, and labeling), thus impairing real-time screening demand. Sensitivity issue is another major challenge that has to be addressed before transferring direct hybridization technologies into routine laboratories. Efforts have been recently realized in this field, using direct labeling of target nucleic acids and/or optimized optic for the detection of hybridized products (Francois et al., 2003a).

Conclusion

Nosocomial infections due to MRSA are frequent and represent an economical burden, requiring prompt isolation measures and use of last-barrier drugs. Rapid detection and identification of MRSA is an absolute prerequisite. Tight collaboration with clinicians involved in prevention or infection control can contribute to the reduction of transmission and control of costs. Until recently, microbiological methods dedicated to MRSA identification relied on the use of selective growth media, which are time-consuming and preclude same-day diagnosis. Nucleic acid–based assays have brought the promise to revolutionize diagnosis of infectious diseases during the 1090s. For more than one decade, nucleic acid–based assays have demonstrated their usefulness for the detection of hardly cultivable, non-cultivable, and even killed microorganisms, as well as for the identification of specific pathogens against the background of a mixed microflora. The current view is still that molecular methods are used to supplement, but not to replace cultures. MRSA molecular detection nicely illustrates this paradigm: it provides early warning, but cultures are still required for further antimicrobial susceptibility testing or epidemiological typing. Molecular assays based on target nucleic acid amplification, and especially real-time PCR, have proved rapid, affordable, and successful in terms of sensitivity and specificity. Detection of amplification reactions is still improving, and future development coupled to the parallelism of hybridization techniques might provide more broadly usable tools. Such microarrays might be adapted to identify bacteria as well as to provide their genetically encoded antibiotic resistance and virulence determinants, ultimately improving knowledge in microbial ecology and enabling optimal use of infection control and therapeutic resources.

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25
Bead-Based Flow Cytometric Assays: A Multiplex Assay Platform with Applications in Diagnostic Microbiology

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Introduction

Researchers have focused on developing specific assays for conclusively identifying and measuring the levels of bacteria, fungi, protozoa, viruses (microbes), and their associated products (biomarkers) that cause disease in humans and animals (Murray et al., 2003). Traditional methods using microscopy and chemical or immunological stains, test cultures with selective media or target cells, or serological assays have been used effectively to identify infectious agents in biological specimens or environmental samples. However, due to increasing veterinary, medical, and public health concerns, faster and more accurate diagnostic tools have been sought. Multiplex array-based assays allow for a range of biomarkers to be rapidly and simultaneously measured within specimens (Robertson and Nicholson, 2005). Recently, multiplex bead-based flow cytometric immunoassays have been developed and applied that show great promise for improving the study, diagnosis, and therapeutic management of infectious diseases (Alvarez-Barrientos et al., 2000; Jani et al., 2002).

The advent of genetic and molecular engineering and monoclonal antibody generation has driven the development of many assays that employ immunoassay-based technology for specifically measuring various biomarkers. Highly purified recombinant or synthetic molecules have been used as antigens to generate specific antibody preparations and to provide target antigens or calibrated standards for these assays. Radioimmunoassays (RIAs) and enzyme-linked immunosorbent assays (ELISAs) are widely used for specific and sensitive analyte measurements that have diagnostic value (Andreotti et al., 2003). Although this methodology is well suited for single-analyte analysis in a relatively large sample volume, methods that simultaneously quantitate multiple analytes from a relatively small sample size are needed to improve the speed, utility, and confidence of diagnostic assays. The combination of traditional immunoassay-based technologies with multiparameter flow cytometry has promoted the development of bead-based, high-throughput multiplex immunoassays. In this method, polymer microspheres (beads) that are

several micrometers in diameter replace the traditional immunoassay plates and serve as the solid phase for the flow cytometric assay (Horan and Wheeless, 1977). Many different types of bead-based flow cytometric immunoassays have been described including those that have indirect or sandwich immunoassay formats (McHugh et al., 1994; Vignali, 2000).

The indirect immunoassay format is especially useful for measuring specific antibody levels in biological fluids (e.g., for determining rising or falling serum antibody titers during the course of an infectious disease). In this format, capture beads are coated or covalently conjugated with target antigen molecules (Fig. 25.1A). For diagnostic microbiology assays, these may be either crude microbial extracts or highly purified molecular preparations. The beads are mixed with test biological fluids (tissue culture supernatants, serum, wound exudates, bronchial lavage fluids) that may contain specific antibodies. Solutions or samples containing quantified levels of antibodies that are specific for the analyte of interest can serve as the assay's calibration standards. Adding fluorescent anti-immunoglobulin detection antibodies (DAb) develops the assay. These fluorescent DAb may be specific for all immunoglobulin isotypes. In these assays, total antibody levels are measured. Alternatively, the DAb may be specific for immunoglobulin isotypes and thus allow measurement of antibodies with IgM, IgA, IgG, or IgE isotypes. Isotype-specific measurements of antibody levels may be very important for monitoring the course of an infectious disease and for characterizing the nature of immune responses to vaccinations (Wild, 2001). The levels and isotype profile for antibodies may indicate whether the humoral immune response is weakly or strongly protective or nonprotective for a particular infectious disease.

In the sandwich immunoassay format, specific capture antibodies can be noncovalently adsorbed or covalently bound to plastic beads (Fig. 25.1B). The capture beads are then mixed with biological fluids that contain target analytes of interest (e.g., soluble microbial antigens including structural molecules, enzymes, or toxins). During or after incubation of the capture beads with the samples, a fluorescent DAb (or biotinylated DAb developed with a fluorescent avidin conjugate) is added that serves as the reporter fluorophore. The analyte levels in bead-based flow cytometric immunoassays are proportional to the mean fluorescence intensity (MFI) levels generated by the bead/analyte-bound fluorescent DAb. With the inclusion of a standard antigen of known concentration, such as natural, synthesized, or recombinant molecules, standard dilution curves can be generated from which the corresponding levels of target analyte are interpolated.

Bead-based single analyte flow immunoassays were first described for measuring the levels of α-fetoprotein (Frengen et al., 1993) and beta2-microglobulin (Bishop and Davis, 1997). Multiplex bead-based flow cytometric immunoassays were made possible by using beads of different sizes. McHugh(1994) and Fulwyler et al. (1988) used beads of different sizes as carriers for target antigens or antibodies. The beads were differentiated by their sizes that were related to their forward light-scattering characteristics determined by flow cytometric analysis. Appropriate electronic gating strategies were used during data acquisition and

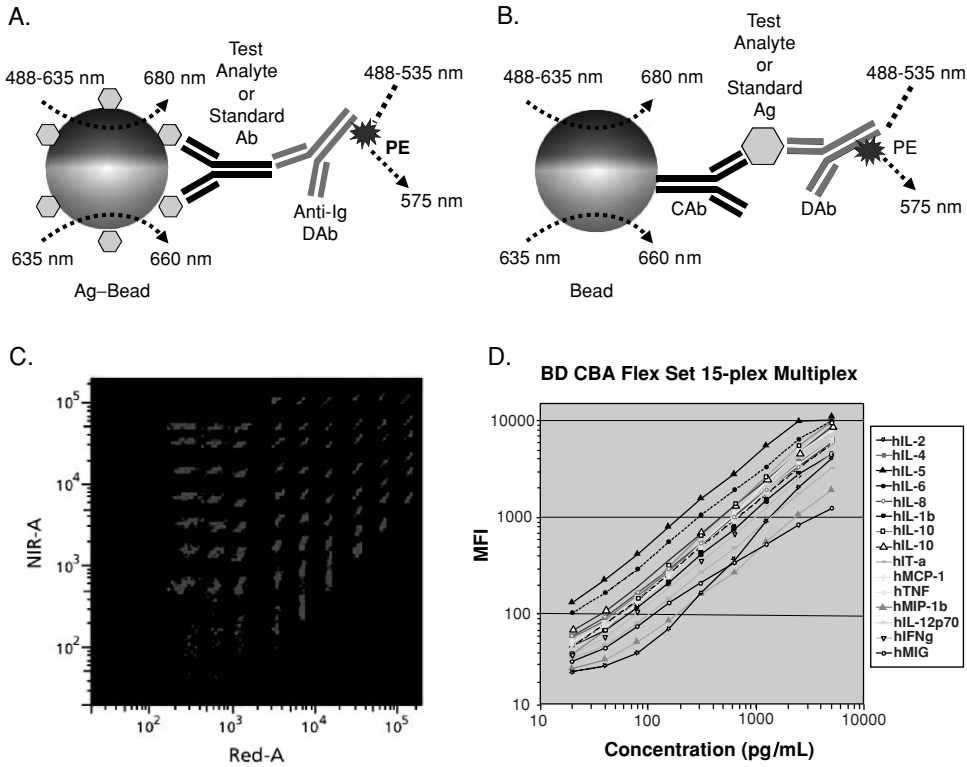

FIGURE 25.1. Multiplex flow cytometric immunoassays. Multiplex flow cytometric immunoassay systems using a BD Cytometric Bead Array (CBA) Flex Set format are shown. Beads dyed with various levels of two different fluorescent dyes are stylistically represented in the top panels. Panel A: For indirect flow cytometric immunoassay format, beads are coated or conjugated with target antigens. When mixed with specimens or calibrated solutions, antibodies specific for the target antigen bind to the capture beads and in turn are recognized by fluorescent anti-immunoglobulin reagents. Upon flow cytometric analysis, two incident light beams of different wavelengths excite the two fluorescent bead dyes. The beads emit fluorescent signals that are distinguished in terms of their wavelength (depending on the type of dye) and fluorescence intensity (depending on the incorporated dye level). Panel B: Alternatively, in flow cytometric sandwich immunoassays, the dyed beads are conjugated with a specific capture antibody (CAb). When mixed with specimens or standard solutions, the capture beads specifically bind and localize antigens to their surface. Fluorescent detection antibodies (DAb) are added that bind to another antigen site. Panel C: A large number of different bead positions can be resolved in two-color dot plots, for example, the logarithmically amplified FL3 and FL4 channels of a BD FACSCalibur flow cytometer or the NIR and Red channels of a BD FACSArray bioanalyzer (72 different bead sets). Beads with different two-color fluorescent positions can be combined in assays to create a multiplex assay. Incident light causes the fluorescent DAb that is directly conjugated with a fluorochrome such as phycoerythrin (PE) to emit distinct fluorescence signals. These reporter signal intensities are commensurate with the amount of analyte-bound DAb connected with distinct fluorescent capture bead sets. The flow cytometric data can then be reanalyzed to generate standard curves and to quantify the levels of specific analytes in test specimens. Panel D: Standard curves generated from a 15-plex Cytokine CBA Flex Set analysis are shown.

reanalysis to determine the mean fluorescent intensities emitted by the fluorescent DAb associated with each distinct capture bead group.

Multiplex flow cytometric immunoassays can also be made using beads of the same size with unique fluorescence properties. In these assays, distinct capture bead sets are produced by labeling them with different levels of one type of fluorescent dye. The different capture bead sets can then be distinguished by flow cytometric analysis based on their distinct MFI. Fluorescent DAb provide the second type of fluorescence that constitutes the reporter immunoassay signals (Camilla et al., 1998). This approach is useful for creating low-complexity bead-sets (Chen et al., 1999). However, larger distinguishable bead sets can be prepared by labeling them with distinct proportions of two fluorescent dyes (Fulwyler et al., 1988), for example, red and far-red dyes as detected by a flow cytometer (Fig. 25.1C). In this case, DAb coupled to a third fluorescent dye [e.g., phycoerythrin (PE)] generate the immunoassay signals. With this approach, it is possible to develop a number of different capture bead/DAb pairs. These pairs are initially developed and tested separately for their sensitivity and range of analyte measurement. As part of the validation process, the pairs are mixed together with other bead pairs and tested for their capacities to specifically measure analytes in mixtures such as serum samples or cocktails of diluted standards. Capture bead/DAb pairs must be capable of specifically measuring the same analyte levels when tested separately or in mixtures. With the selection of good antibody pairs that do not cross-react with other analytes or antibodies in the system, flow cytometric immunoassays can be used to measure the levels of multiple analytes (e.g., biological markers associated with one or more microorganisms).

The bead-captured analyte levels measured by multiplex flow cytometric immunoassays are proportional to the mean fluorescence intensity levels generated by the bead/analyte-bound fluorescent DAb. With the inclusion of calibrated analyte solutions (e.g., solutions that contain target antigens or antibodies of known concentration), standard dilution curves can be generated from which the corresponding levels of target analytes are interpolated. Data reanalysis using immunoassay software provides a selection of a variety of standard curve-fitting choices. These allow for the best fit of immunoassay signals as a function of standard and sample concentrations. These may include either linear-log, log-log, or four- or five-parameter transformations (Fig. 25.1D).

Multiplex flow cytometric immunoassays are ideal for measuring biomarkers associated with multiple infectious agents (Jani et al., 2002). This may be particularly important in complex diseases such as the generalized acquired immunodeficiency caused by the human immunodeficiency virus (HIV). Immunodeficient individuals are susceptible to infections caused by multiple opportunistic pathogens. Alternatively, multiple biomarkers associated with a particular infectious agent can be analyzed for a conclusive diagnosis or prognosis. In the latter case, biomarkers for incubation, acute, convalescent, and recovery phases from a disease caused by an infectious agent can be carefully monitored.

Multiplex flow cytometric immunoassays simplify analyte profile determinations. Only a single sample is needed to detect and quantify a number of analytes,

an advantage when the sample volume is limited (e.g., pediatric or cerebrospinal fluid samples). The multiple independent measurements within each bead population assure good precision. The high sensitivity and wide detection range afforded by logarithmically amplified fluorescent signals or high-resolution digital data systems is another advantage (e.g., fewer sample dilutions are required). Moreover, the greater surface area and exposure to soluble analytes improves the bead-based assay kinetics when compared with traditional microwell plate–based immunoassays.

In the following sections, some examples of multiplex bead-based flow cytometric immunoassays as applied to diagnostic microbiology are presented. These include methods for measuring the levels of specific antibodies generated by hosts to specific microbial pathogens as well as detection of the microbial pathogens and their effector molecules. In addition, the potential use of these multiplex assays for measuring biological response modifiers such as cytokines and chemokines that are produced as a consequence of infection, leading to disease states in some instances, are presented.

Flow Cytometry–Based Assays for Detection of Microbe-Specific Antibodies and Microbial Antigens

Measurement of Antimicrobial Antibody Responses

Flow cytometric immunoassays are well suited to monitor antibody responses against antigens from infectious agents. Determinations of stable, rising, or dropping antibody titers and the immunoglobulin isotype of the antimicrobial antibodies provide valuable information concerning the nature or status of an infection or the efficacy of an antimicrobial therapy or vaccine. A growing list of publications for this technology is being reported (Jani et al., 2002).

Best and colleagues (1992) successfully developed an indirect bead assay for the serodiagnosis of *Helicobacter pylori*. For this assay, the investigators coated plastic microspheres with a crude multicomponent antigen mixture prepared from *H. pylori* to react with antibodies that were present in patient sera. Fluorescein-conjugated anti-human IgG DAb were used to measure the levels of IgG anti–*H. pylori* antibodies that were present in serum samples from infected (positive by culture or histological examination) or noninfected control patients. Pooled negative sera served to determine the assay's limit of detection and relative serum antibody titers with weakly and strongly positive control sera included in each assay to control for intra- and inter-assay variability. The bead-based assay was 100% sensitive and 89% specific (positive and negative predictive values of 90% and 100%) and gave no equivocal results. Parallel testing with a commercial ELISA was 96% sensitive and 89% specific (positive and negative predictive values of 90% and 96%) and gave five equivocal results.

Lal et al. (2004) developed a simple and rapid cytometric bead assay that simultaneously quantitated serum IgG antibodies that were directed against the

Neisseria meningitidis serogroups, A, C, Y, or W-135. Different bead sets were conjugated with meningococcal capsular polysaccharides A, C, Y, or W-135. A calibrated anti-meningococcal antiserum served as the reference. The tetraplex assay had good intra- and inter-assay variations and showed a strong correlation with ELISAs specific for the same antigens. The authors suggested that assays of this type would be useful to study the efficacy of multivalent vaccines designed to protect against meningococcal infections. Biagini et al. (2003) similarly reported the development of a multiplex bead-based assay that was capable of measuring the antibody response directed against 23 pneumococcal capsular polysaccharides. These pneumococcal polysaccharide serotypes were present in the pneumococcal polysaccharide vaccine (PPV23) licensed by the U.S. Food and Drug Administration.

An indirect multiplexed flow cytometric immunoassay has also been reported that can quantitate specific IgG antibody levels (PE-conjugated anti-human IgG) directed against *Haemophilus influenzae* type b polysaccharide and toxoids from *Clostridium tetani* and *Corynebacterium diphtheriae* (Pickering et al., 2002). These investigators used a pooled serum standard that was calibrated against standard antisera from the World Health Organization (units/mL) and the U.S. Food and Drug Administration (μg/mL) for antibody quantitation. The antibody levels measured in pre- and post-vaccination antisera by flow cytometric immunoassay agreed closely with those determined by individual ELISAs. The usefulness of this type of assay for the evaluation of vaccine efficacy was demonstrated.

McHugh et al. (1997) produced a cytometric bead assay for the detection and quantitation of serum antibodies directed against the hepatitis C virus (HCV). Microspheres of different size were coated with proteins from either the viral core or the nonstructural region 3 (NS3). These beads were incubated with serum or plasma samples and developed with PE-conjugated anti-human IgG DAb. The assay mixtures were then analyzed by multiparameter flow cytometric analysis. The forward light-scattering characteristics of the beads were used to identify the smaller (HCV core) and larger (NS3) beads and their associated immunofluorescent (PE) signals. The assay developed by McHugh and colleagues was more sensitive than a commercial ELISA and could resolve indeterminate clinical samples. This type of assay has great potential for screening donor blood samples.

Measurement of Microbial Agents

Multiplex flow cytometric immunoassays have also been developed to identify microbes that are present in specimens. Park et al. (2000) created a competitive inhibition multiplex flow cytometric immunoassay to simultaneously identify 15 *Streptococcus pneumoniae* serotypes. Latex bead sets, of different size and incorporated levels of red fluorescent dye, were coated with different pneumococcal capsular polysaccharides. In brief, the beads were mixed with individual pneumococcal lysates followed by the addition of pooled rabbit antisera against

all serotypes plus fluorescein-conjugated anti-rabbit immunoglobulin DAb. Upon flow cytometric analysis, the fluorescent DAb signals were only inhibited when the homologous serotype of pneumococcus was present. The method was capable of correctly identifying 100% of the pneumococcal isolates tested.

Molecular biological techniques are also compatible with bead-based flow cytometric assays. Different PCR amplification strategies have been used to demonstrate flow methods to measure HIV viral load (Mehrpouyan et al., 1997; Van Cleve et al., 1998). Defoort (2000) developed a multiplex flow cytometric bead-based hybridization assay for the detection of human immunodeficiency virus type 1 (HIV-1) and hepatitis C (HCV) RNA and hepatitis B virus (HBV) DNA using specific oligonucleotide probe-coated, green fluorescent microspheres. The assay was devoid of cross-hybridization between oligonucleotide probes and biotinylated PCR products that were generated by multiplex reverse transcription–PCR. Bound PCR products were detected with PE-cyanin 5-conjugated streptavidin. Methods of this type can be extremely useful for the detection and quantitation of several different pathogens or serotypes in each plasma sample. Flow cytometric assays based on nucleic acid technologies are useful for identifying pathogens not amenable to culture or for when an immunodominant microbial antigen that can serve for serological diagnosis of an ongoing infection has not been characterized. Requirements for highly trained technicians, expensive equipment and materials, and concerns that nucleic acid technologies do not discriminate between viable and dead microbes are potential issues that affect the use of this method.

Simultaneous Measurement of Microbes and Toxins

Novel methods for simultaneously measuring the levels of specific microbes and their products in complex specimens are also possible. As an example, a cytometric bacteria and protein assay (CBPA) was developed (Bolton et al., 2002). For the sandwich flow cytometric immunoassay component of the CBPA, antibodies were generated against *Staphylococcus aureus* enterotoxin B (SEB) and ricin. The antibodies were used to prepare two distinct sets of single-color fluorescent capture beads and PE-conjugated DAb. Purified preparations of SEB and *Ricin communis* agglutinin II served as calibrators. In this case, the identification of bacteria used the capability of the flow cytometer to detect bacteria directly by light scatter or dye-mediated nucleic acid fluorescence. The specific identification was achieved with fluorochrome-labeled species-specific antibodies. The use of membrane permeable and impermeable nucleic acid dyes allowed for the discrimination of live and dead cells. For the simultaneous analysis of killed *Bacillus anthracis* and living *Bacillus subtilis* bacteria, rabbit anti–*B. anthracis* antibodies were conjugated with PE. For the discrimination of total and dead bacteria, the nucleic acid dyes, Syto 59 and propidium iodide were used, respectively.

To perform the CBPA, known amounts of the toxins, bacteria, and capture beads were mixed together in assay diluent along with the PE-conjugated anti-SEB,

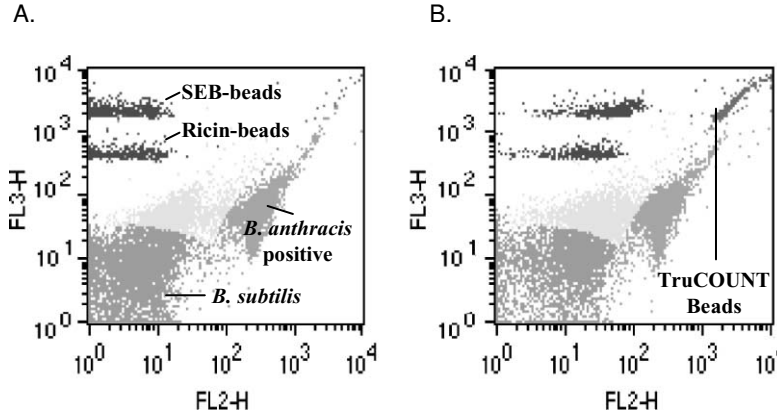

FIGURE 25.2. Analysis of bacteria and toxins by CBPA. Panel A shows the dot plot analysis of two different intensity fluorescent bead populations coupled with antibodies specific for SEB and ricin and two bacteria *B. anthracis* and *B. subtilis*. Dead bacteria (i.e., PI/FL3-positive) that are not labeled with the PE-anti–*B. anthracis* detector antibody appear as light grey dots. Panel B shows the increased FL2 fluorescence of the SEB and ricin beads upon addition of the SEB and ricin toxins and anti-SEB and anti-ricin PE labeled detector antibodies. TruCOUNT beads have also been added to the assay in Panel B.

anti-ricin, and anti-*B. anthracis* antibodies. The mixture was serially diluted and added to TruCount tubes (BD Biosciences, San Jose, CA) for 25 min (room temperature). These tubes contained a known number of fluorescent microspheres to enable the flow cytometric enumeration of bacteria (Alsharif and Godfrey, 2001). PI was added for 30 min followed by the addition of Syto59 (Millard et al., 1997). The mixtures were then analyzed by multicolor flow cytometry with a FACSort (BD Biosciences) cytometer equipped with blue (488 nm) and red (635) lasers to generate the multiparameter fluorescence and forward- and side-light scatter signals.

The dot plots shown in Figure 25.2 (panels A and B) reveal the CBPA's capacity to simultaneously discriminate the bead-based measurement of SEB and ricin toxins level and viable *B. subtilis* and nonviable *B. anthracis*. Figure 25.2 (panel A) shows the dot plot analysis (FL3 vs. FL2) of the single tube assay containing two bacteria, *B. anthracis* and *B. subtilis* stained with Syto59 and PI dyes and two different intensity fluorescent bead populations coupled to capture antibodies specific for SEB and ricin toxins. Panel B shows the result of the assay after adding anti-SEB and anti-ricin PE labeled detector antibodies, which causes an increase in the FL2 fluorescence and shifts the beads to the right and TruCOUNT beads, added to aid in bacterial quantitation. The standard curves for the serially diluted toxins (panel A) and *B. subtilis* counts (panel B) are shown in Fig. 25.3. Bacterial concentrations were calculated from the ratio of counts of bacteria and TruCount microspheres.

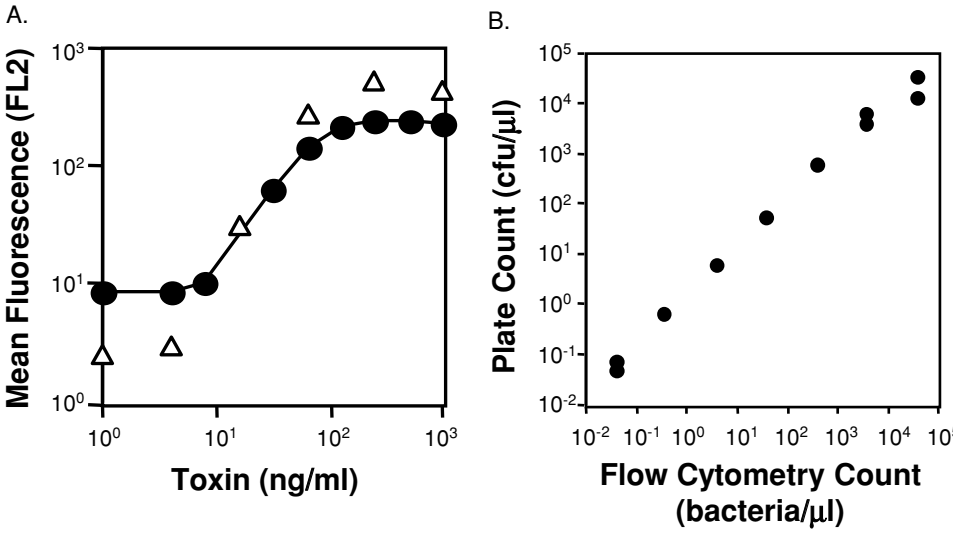

FIGURE 25.3. Standard curves for toxins and viable bacterial counts of *B. subtilis* measured by CBPA. Panel A: Standard curves for toxins: Ricin (-Δ-Δ-) and SEB (-•-•-) proteins were added to the assay at the concentrations indicated and the mean fluorescence intensity (FL2) of the PE-labeled DAb signals were determined for each different protein concentration in a FACSort. All other components of the assay were present during the titrations. Panel B: Correlation between the concentration of *B. subtilis* determined by flow cytometry and by plating on agar. Serial dilution was made on a *B. subtilis* culture grown in trypticase soy broth. The diluted suspensions were analyzed either by flow cytometry using the nucleic acid dyes plus TruCOUNT beads or were plated on trypticase soy agar to enumerate the bacteria.

Assessment of Biomarkers Using Multiplexed Bead-Based Assays in Infectious Disease States

Cytokine Measurement in Infection and Sepsis

Numerous investigators (Oliver et al., 1998; Chen et al., 1999), have described multiplex methods to measure panels of secreted cytokines in serum. For example, Carson and Vignali (1999) demonstrated a multiplex bead assay for the simultaneous quantitation of 15 cytokines. The multiplex measurement of cytokine profiles by flow cytometric bead arrays panels may be used to monitor the host response to various infectious disease processes.

Infection and sepsis are a frequent cause of neonatal morbidity and mortality (Tarnok et al., 2003). The early diagnosis of these disease processes is difficult because the clinical signs are highly variable, subtle, and similar to other conditions. Advances in diagnostic detection of pediatric infection and sepsis have been slow, but recent advances in multiplex cytometric bead array analysis of multiple cytokines show promise for improved early diagnoses. Elevated levels of serum

IL-6, IL-8, and IL-10 are among the proposed early indicators of infection and sepsis (Morgan et al., 2004).

Hodge et al. (2005) measured the serum levels of inflammatory cytokines as biomarkers for following the course of pediatric sepsis cases. These investigators used the BD CBA Human Th1/Th2 Cytokine (measures IL-2, IL-4, IL-5, IL-10, TNF-α, and INF-γ levels) and Human Inflammatory Cytokine (IL-1ß, IL-6, IL-8, IL-10, IL-12, and TNF-α) kits for the simultaneous measurement of multiple cytokines in small-volume (0.1 mL) samples. These assays were chosen for their diagnostic potential because of the associated rise in plasma-derived cytokine levels with pediatric sepsis. Cytokines from a group of 18 infected term neonates (culture positive) and a noninfected control group (culture negative) were analyzed. When the cytokine levels of the infected group were compared with the normal ranges of plasma cytokine levels expressed by the control group, all but two patients failed to show significant elevation of the cytokines tested. All other patients showed elevated levels of between one and nine cytokines tested. Although the different cytokine profiles did not correlate with the identity of a specific infectious agent, the authors concluded that these types of assays could be developed to rapidly identify neonates with sepsis.

Evaluation of the Immune Pathogenesis of Human SARS Using Multiplexed Bead-Based Flow Cytometric Assays

Multiplexed bead assays have become very valuable biological tools due to their ability to measure multiple proteins simultaneously in laboratory samples with speed, accuracy, and specificity. Given that the technology is adaptable to the measurement of cytokines, chemokines, or other immune mediators in small volumes of sera or plasma, a role for the multiplex bead assay in profiling the immune pathogenesis or host immune responses involved in human disease has recently emerged.

Severe acute respiratory syndrome (SARS) is a highly contagious respiratory disease caused by the newly identified SARS coronavirus (SARS CoV) that emerged in late 2002 from Guangdong Province, China (CDC Update, 2003; Peiris et al., 2004; Skowronski et al. 2005). SARS has infected more then 8400 persons worldwide to date, killing more than 10% of patients. Those infected with SARS suffer fever, cough, muscle aches, and shortness of breath and often progress to severe lung inflammation. Most patients recover from this illness within 2 weeks, however a large percentage of SARS patients develop severe complications. Although the SARS outbreak of 2003 was largely contained through public health measures, it is clear that the future threat of similar emergent viruses or other microbial agents causing severe respiratory disease will not easily be eliminated.

SARS CoV infection appears to be hallmarked by a poorly defined “cytokine storm” in the lungs and circulatory system of SARS patients (Huang et al.,

2005). Most of the current SARS immunological literature is based on one or a few cytokines, making it unclear how immune responses and progressive lung injury culminates in poor outcomes. In contrast to what one might expect from the immune system when faced with a common microbial invader, it may be that infection and lung injury progresses in SARS patients as a result of uncontrollable innate and adaptive immune responses as reflected by rampant lung inflammation and uncoordinated cytokine production. Because the varying severity of SARS CoV infection appears to be rooted in a patient's own host immune response, SARS represents a unique opportunity to look at pathogenesis of a critical infectious disease and host defense in a new light using multiplexed bead assays, such as BD Biosciences Cytometric Bead Array (CBA) kits.

The public health directive associated with the Toronto SARS outbreak in spring 2003 required all individuals who had contact with SARS-infected patients and who developed any SARS symptoms to present to a hospital for assessment, treatment, and close surveillance. This directive, likely to be a common feature of future outbreaks of emergent or unknown infectious diseases, allowed the collection of specimens within 3 days of onset of symptoms from acutely ill SARS patients as well as a longitudinal study of patients delineated by the severity of SARS disease course (i.e., from mild to moderate symptoms to severe forms of the disease requiring intubation and ICU admittance). CBA results from a representative non-ICU patient shown in Fig. 25.4 shows the high levels of proinflammatory cytokines and chemokines in the plasma of a SARS patient in the early phase (<3 days since onset of symptoms) of disease compared with the convalescent sample and average healthy control levels. As shown, early SARS infection is characterized by increased expression of numerous cytokines and chemokines representing a vigorous acute-phase response (IL-1β, IL-6, and TNF-α) as well as strong innate (IL-1β, IL-6, CXCL8, IL-12, and TNF-α) and IFN-γ– driven adaptive immune responses. Levels of expression of these cytokines and chemokines appeared to peak within the first 2 weeks of SARS disease and had generally resolved to healthy control levels at convalescence, probably assisted by aggressive steroid treatment begun at day 7 in this patient.

With elevated levels of IFN-γ measurable at the onset of symptoms in the majority of SARS patients, this would suggest that the early skewing of the SARS immunity in favor of a T helper (Th)1-like response during a vigorous early innate immune response may be a critical piece of the puzzle. Because IFN-γ and other interferon-response genes are crucial in starting and directing different classes of innate and adaptive immune responses, this would indicate that they also could determine the overall effectiveness, or conversely overall mismanagement, of immune responses against the SARS CoV. Interestingly, other cytokines involved in adaptive immunity and T cell subset regulation, such as IL-2, IL-5, and IL-10, were not expressed at significant levels, although levels of IL-4 did increase slightly during the second week of illness. In other words, to describe SARS as mediated by a generalized "cytokine storm" is probably oversimplified. Instead, the

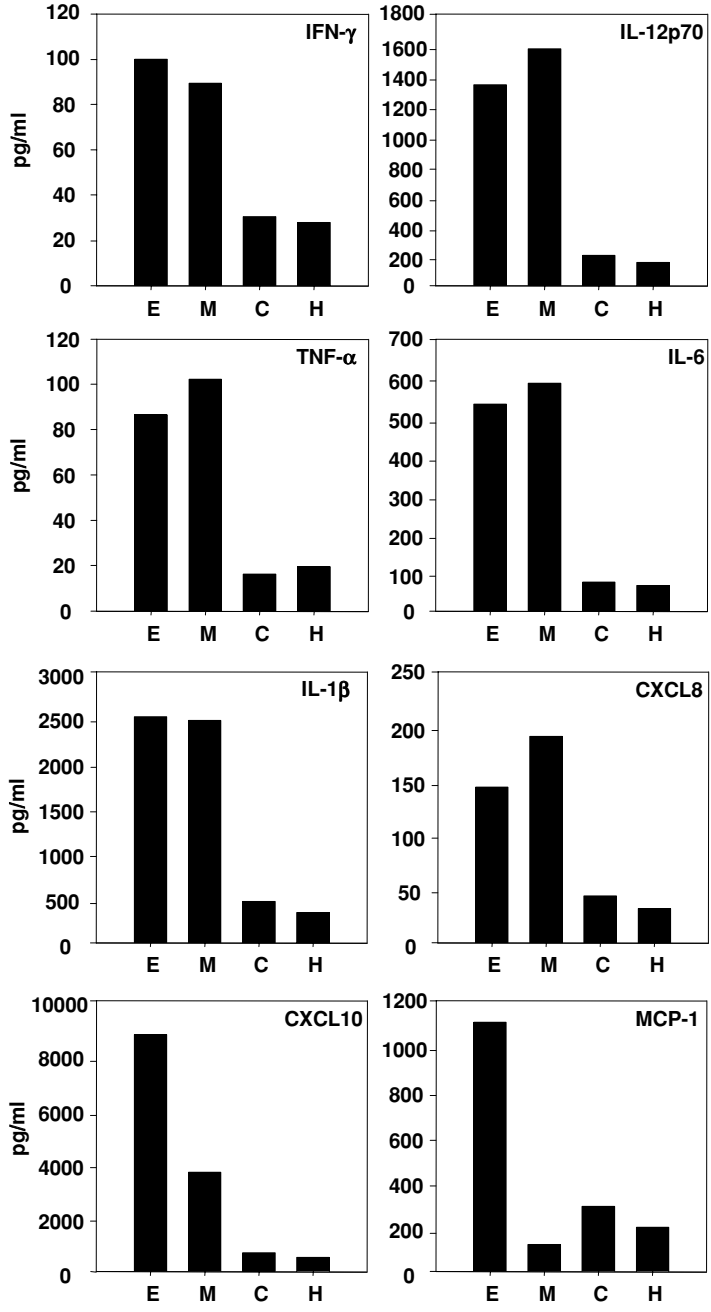

FIGURE 25.4. Plasma concentrations of cytokines and chemokines in a representative SARS CoV–infected patient with a mild to moderate disease course. Cytokine and chemokine levels were measured in 50 μL of plasma from one SARS patient within 48 h of onset of symptoms (Early, E), at midpoint (M) of SARS infection (12–14 days), and at convalescence at 30 days (C) by BD Biosciences CBA according to the manufacturer's instructions. A healthy control average ($n = 10$) is also displayed (H).

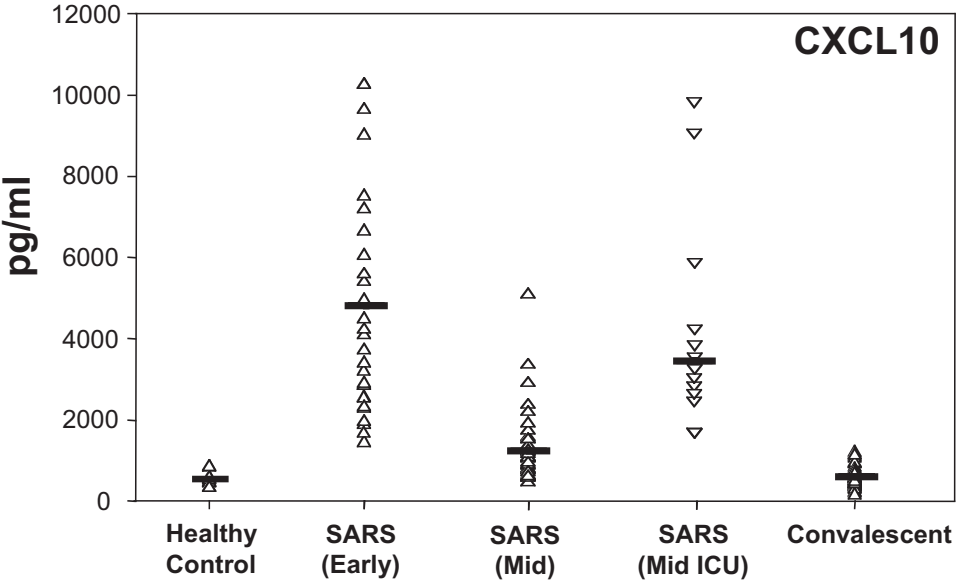

FIGURE 25.5. High plasma concentrations of CXCL10 in SARS patients. CXCL10 levels measured in 50 μL of plasma from early SARS patients ($n = 34$) within 48 h of onset of symptoms, 25 SARS patients with mild to moderate symptoms at midpoint of SARS infection (12–14 days), 15 confirmed SARS patients requiring intubation and admittance to the ICU at midpoint of SARS infection (12–14 days), and healthy controls ($n = 14$) by BD Biosciences CBA according to the manufacturer's instructions. Higher levels of CXCL10 were observed in SARS patients relative to controls ($P < 0.0001$). CXCL10 was also significantly lower at 12–14 days in mild to moderate SARS patients compared with those in the ICU ($P < 0.05$).

immunology of SARS appears to be driven over time by a select subset of cytokines and chemokines causing unabated innate and adaptive immune responses in response to the virus.

One chemokine produced at unusually high levels very early in SARS infection is CXCL10 [interferon (IFN)-γ inducible protein 10/IP-10]. CXCL10 showed elevated levels in 100% of SARS cases at early onset (Fig. 25.5). In SARS patients with mild to moderate symptoms, CXCL10 levels decrease to near convalescent levels by the mid stage (12–14 days), whereas levels in SARS patients with severe symptoms requiring intubation and admittance to the ICU remain significantly elevated beyond 30 days regardless of aggressive steroid treatment. It is not yet known whether infected tissues are the main source of CXCL10, although the persistence of elevated levels of CXCL10 in patients with severe illness and unresolved SARS CoV infection indicates that this may be the case. Nonetheless, CXCL10 appears to play a unique role in the progress of SARS illness. Chemokines are known for their roles in cell recruitment, but they can also activate immune cells and shape Th1/Th2-type immune responses (Luster, 1998; Rossi and Zlotnik, 2000).

Although interferons and interferon-response genes (i.e., CXCL10) may play a general role in the modulation of respiratory diseases involving lung injury, CXCL10 appears to play a special dual role in SARS. Interferons and CXCL10 responses are necessary to clear SARS CoV if properly timed and modulated but in a minority of patients, CXCL10 participates in the pathogenesis of SARS and poor outcome through uncontrolled inflammation and continued recruitment of activated T cell and mononuclear infiltrates. Indeed, CXCL10 controls the outcome of coronavirus infections in mouse models of other diseases (Liu et al., 2001a, 2001b).

The host immune response to SARS may be the first to be mapped in detail in terms of an emerging infectious disease. Using multiplex bead-based flow cytometric immunoassay technology to study other host responses to infectious diseases may identify novel host response genes that are diagnostic and/or prognostic during the acute phases of the disease. By modeling host gene expression in different subsets of patients from the onset of symptoms through convalescence, multiple genetic pathways may be found that show changes in expression patterns and prognostic capabilities over varying courses of a disease. The role that interferons and interferon response genes play in regulating long-lasting uncontrolled inflammatory events, such as those involved in SARS, is worth careful examination.

In summary, multiplex bead-based flow cytometric immunoassays are emerging as a powerful tool for profiling the nature of microbial agents in biological samples and of host responses in infectious diseases. With the development of new fluorescent dyes, specific high-affinity antibodies, and high-throughput multiparameter methods and flow cytometers, these bead-based immunoassays can have a significant impact on the establishment of prophylactic and therapeutic measures to counter acute and chronic infectious diseases caused by microbial pathogens. With the production of more affordable and user-friendly equipment and materials, people in the developed and developing countries could benefit dramatically from this advanced technique in diagnostic microbiology.

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26
Molecular Strain Typing Using Repetitive Sequence–Based PCR

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Introduction

Microbial strain typing is increasingly important in routine clinical microbiology laboratories as a method to track hospital-acquired infections. Although many methods can be used, this chapter focuses on one particular method used for typing of bacteria and fungi: repetitive sequence–based PCR (rep-PCR). A number of valuable reviews are available for more in-depth discussion of other technologies (Olive and Bean, 1999; Soll, 2000; Zaidi et al., 2003). The technology behind rep-PCR will be introduced with a review of traditional, manual rep-PCR. The latest advances will be highlighted with a discussion of the DiversiLab System, Bacterial Borcodes, Inc., Athens, GA, which is an automated rep-PCR technology. Additionally, we describe a comparison of the technologies, current applications in clinical microbiology laboratories, and future potential of rep-PCR as a routine clinical test.

Principles of Rep-PCR and the DiversiLab System

Microbial Typing

With the rising spread of antibiotic-resistant organisms, clinical laboratories must focus more and more on the epidemiology of hospital-acquired infections. Strain typing is an extremely useful tool in tracking the spread of nosocomial infections (Watterson and Drobniewski, 2000; Taha, 2002; Wu and Della-Latta, 2002). In addition to tracking the source of hospital-associated infections, strain typing is useful for studying community-acquired infections, discriminating between recurrent infections caused by new exposure or by colonization, and investigations to determine the presence of single source; multiple-site infections or other environmental source. Finally, strain typing can be a useful tool to identify and pinpoint laboratory contamination. Because of its many uses, strain typing has become a common procedure in many clinical laboratories. This has required clinical laboratories to shift from using solely phenotypic methods to using genotypic methods

that incorporate molecular biology. Common genotyping methods include plasmid analysis, restriction endonuclease analysis, PCR assays, multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), DNA sequencing, ribotyping, PCR ribotyping, restriction fragment length polymorphism (RFLP) studies, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and repetitive sequence–based PCR (rep-PCR) (Versalovic and Lupski, 1996, 2002; van Belkum et al., 2001; van Belkum, 2003; Zaidi et al., 2003).

Rep-PCR

Rep-PCR exploits repetitive sequences present in numerous prokaryotic and eukaryotic microorganisms by incorporating primers targeted to bind the repetitive elements located at multiple sites in the genome (Stern et al., 1984; Versalovic et al., 1991; Koeuth et al., 1995). As the product is amplified, the generation of different-sized fragments depends on the orientation of the primer binding and whether the distance can be spanned by *Taq* polymerase. Size fractionation of the multiple amplicons can be accomplished using various methods, for example agarose gel (Versalovic et al., 1993; Versalovic and Lupski, 2002; Spigaglia and Mastrantonio, 2003; Castro et al., 2004) or capillary electrophoresis (Versalovic and Lupski, 1995). The fragment separation reveals a band indicating the quantity and size of each fragment, and the combined bands give a unique fingerprint pattern of the repetitive elements in the organism. First reported in 1991 (Versalovic et al., 1991), manual rep-PCR fingerprint patterns became an established approach for subspecies classification and strain delineation of bacteria (Versalovic et al., 1993; Versalovic and Lupski, 2002).

Two of the most commonly used repetitive elements in manual rep-PCR publications are REP and Enterobacterial repetitive intergenic consensus (ERIC). Repetitive extragenic palindrome (rep) was the first repetitive element described in bacteria (Higgins et al., 1982; Gilson et al., 1984; Stern et al., 1984). It was initially identified through DNA sequence comparisons of intercistronic regions of different operons in *Salmonella typhimurium* and *Escherichia coli* (Higgins et al., 1982; Gilson et al., 1984; Stern et al., 1984). ERIC, which is also an interspersed repetitive DNA sequence element, was identified in *Escherichia coli*, *Salmonella typhimurium*, and other enterobacteriaceae (Hulton et al., 1991). The chromosomal locations of ERIC can differ in different species and have been demonstrated to be conserved throughout the eubacterial kingdom.

The first commercialized rep-PCR kit (manual), the rep-PRO DNA Fingerprinting Kit, provided primers targeted to various repetitive elements that required each user to optimize the assay for their organism of interest. Additionally, agarose gel electrophoresis was the recommended fragment detection system. Interpretation was either performed visually or using gel documentation analysis of scanned gel images. Manual rep-PCR was useful for typing, but it was challenged by low interlaboratory reproducibility, suboptimal turn-around times, and agarose gel–based DNA separation and detection (Johnson and Clabots, 2000).

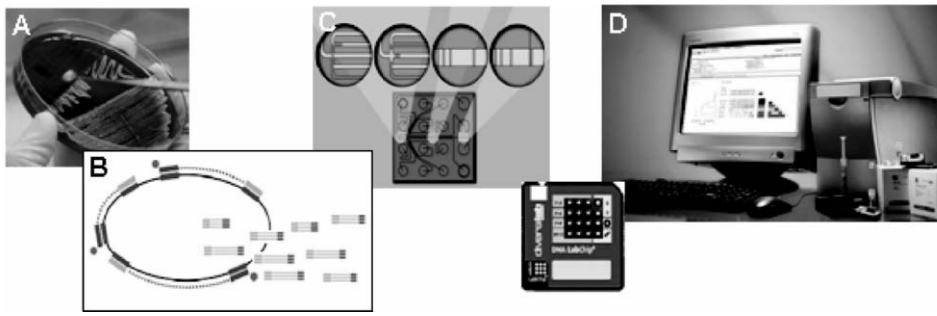

FIGURE 26.1. The DiversiLab System workflow is as follows: (A) colonies are gathered from an isolated culture and genomic DNA is extracted, (B) rep-PCR primers bind to genomic DNA at multiple binding sites, and amplification generates amplicons of various sizes, (C) amplicons are separated using the microfluidics LabChip device and detected using the 2100 bioanalyzer, (D) data are automatically collected and analyzed using the DiversiLab software.

DiversiLab System

The second-generation commercial assay, the automated DiversiLab System, improved rep-PCR technology by incorporating standardized reagent kits, microfluidics-based electrophoresis, and Web-based software for data interpretation. These combined increased reproducibility by minimizing interprofile variability, reducing fractionation time from 6 h to 1 h, and decreasing technical time and labor costs by employing a standardized workflow (Fig. 26.1).

Briefly, the first step of the DiversiLab workflow (Healy et al., 2005) is the extraction of genomic DNA from a pure microbial culture (Fig. 26.1A). Pure cultures are required because the primer binding sites are relatively universal (Versalovic et al., 1991). Approximately 50 ng of the extracted DNA is amplified using the DiversiLab DNA Fingerprinting Kit (either genus-specific or general, such as the Mold kit), *Taq* polymerase, and 10X PCR buffer. The thermal cycling parameters for each kit may vary slightly, but are generally as follows; initial denaturation of 94°C for 2 min, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 45°C to 70°C for 90 s; and a final extension at 70°C for 3 min. During amplification, the specific rep-PCR primers found in each kit bind multiple repetitive DNA sequences that generate amplicons of different sizes (Fig. 26.1B). Instead of using more traditional gel electrophoresis, amplicons are size fractionated using a disposable microfluidics chip (Fig. 26.1C). Separation occurs based on charge separation through a viscous copolymer matrix. An intercalating dye that binds to each amplicon is detected as the DNA fragments pass by a laser causing fluorescence of the dye and detection by the bioanalyzer (Fig. 26.1D).

DiversiLab Analysis

The DiversiLab System is integrated with the Web-based software that is used for rep-PCR fingerprint analysis. Data from the microfluidics chip are viewed

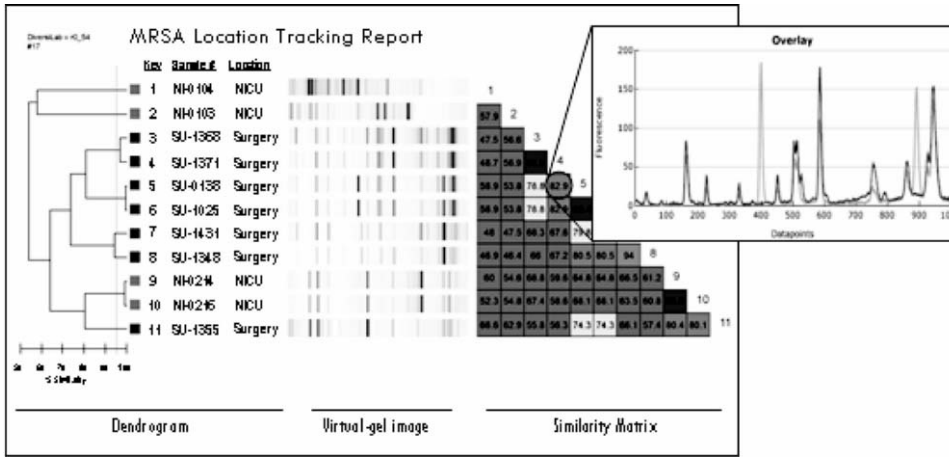

FIGURE 26.2. An example DiversiLab Report. Each report provides an interactive dendrogram and similarity matrix. For each sample, up to four demographic fields can be displayed, and any one of the fields can be color-coded to aid data interpretation. An overlay of any two samples is generated by clicking the corresponding box in the similarity matrix.

in real-time and are automatically uploaded to a personalized, secured customer Web site. The data can then be accessed either from the DiversiLab System or any Internet-connected computer. Once imported into the software, each sample pattern is normalized based on marker position and viewed for quality control. Acceptable rep-PCR results can then be used to infer relationships among microorganisms based on the similarities of the fingerprint patterns. The similarities between all possible pairs of samples in a data set are calculated by two methods: the Pearson correlation coefficient or a modified Kullback–Leibler coefficient. Both methods compare fluorescent intensity at each data point between a pair of fingerprint patterns.

The DiversiLab software facilitates sample comparisons by including a number of interpretation tools including a dendrogram, an interactive similarity matrix, a scatterplot, electropherograms, overlays, virtual gel images, and selectable demographic fields (Fig. 26.2). The dendrogram is a tree-like diagram that uses branching and branch length to indicate average percentages of similarities between samples and sample clusters that show like fingerprints. Each dendrogram can include up to four demographic fields (such as species, strain, location, and date), one of which can be color-coded to reveal sample clustering. The similarity matrix also provides percentages of similarity, but the matrix format allows the direct comparison of each sample pair. The similarity matrix also allows an overlay of any two sample graphs to easily be generated for direct examination of the fingerprint pattern differences. When comparing new data to a user-defined or DiversiLab reference library, a top-match module allows comparisons to be seen as a table instead of in a more complicated dendrogram. Improved assay reproducibility and Web-based software make it possible to share user-defined libraries between collaborators or multisite facilities, if so desired.

Automation of the DiversiLab System allows sample processing, analysis, and report generation to be completed for 13 samples in approximately 4 h with an additional hour for every 13 extra samples (Healy et al., 2005). Strain-level discrimination using the DiversiLab System has been seen with mycobacteria (Cangelosi et al., 2004; Gira et al., 2004), *Staphylococcus aureus* (Shutt et al., 2005), *Lactobacillus* (Land et al., 2005), *Aspergillus* (Healy et al., 2004), zygomycetes (D. Kontoyiannis et al., 2005), dermatophytes (Pounder et al., 2005), and *Candida* (Chau et al., 2004; Li et al., 2004). The DiversiLab System is also applicable to atypical organisms, including archeabacteria, mycobacteria (ATCC, Technical Bulletin, 2005), and anaerobes (Spigaglia and Mastrantonio, 2003). This wide utility makes DiversiLab an excellent tool for the clinical microbiology laboratory.

Technology Comparison

Current molecular typing methodologies, including manual rep-PCR, have limitations (Versalovic and Lupski, 1996; van Belkum et al., 2001; van Belkum, 2003; Zaidi et al., 2003). According to a review by Soll published in 2000 (Soll, 2000), at that time a comprehensive strain-typing method was not available because no typing method was practical for assessing contamination in real-time (Woods et al., 1996; Shopsin and Kreiswirth, 2001), for providing complete tracking data (Dahl et al., 1999; Suppola et al., 1999), or had data archiving capability, all of which were required to build libraries and share data among laboratories. The DiversiLab System was specifically designed to integrate these components into a commercially available package (Healy et al. 2005). Table 26.1 provides a comprehensive comparison of many commonly used strain-typing methods.

Discrimination to the subspecies or strain level is essential to a clinical strain-typing system because many organisms, such as methicillin-resistant *Staphylococcus aureus* (MRSA), are extremely heterogeneous. Although some typing systems may be valid for more diverse organisms, many fail to discriminate at the strain level for these clinically important organisms. For example, ribotyping often has difficulty distinguishing among different subtypes (Kostman et al., 1995; Lobato et al., 1998), and 16S rRNA sequencing often shows low levels of subspecies and strain discrimination (Sander et al., 1998; Barney et al., 2001; Callon et al., 2004). Additionally, plasmid typing and MLEE provide only an estimate of genetic relatedness (Mayer, 1988; Seifert et al., 1994a, 1994b; Trindade et al., 2003). The DiversiLab System provides genus-specific kits that have been developed for a high level of strain discrimination. However, universal kits, such as the DiversiLab Gram-negative kit, can be used when speed is more important than discrimination. Even when the genus or species identification is inconclusive, the DiversiLab System can reveal relationships among samples and thus help identify the contaminant source. PFGE is considered the gold standard because of its high discriminatory power (Tenover et al., 1995), and RAPD is also highly discriminating; however, both methods show poor interlaboratory reproducibility (Davis et al., 2003). Table 26.2 is a summary of the organism-specific applications of each technology.

TABLE 26.1. Comparison of strain-typing technologies.

Technique	Ease of performance[a]	Ease of interpretation[a]	Discrimination[a]	Time[a]	Reproducibility[a]	Cost[a]	Comercial kit
PFGE	Moderate	Moderate–High	High	High	High	Moderate–High	No
Sequencing	Low	Moderate–High	High	Moderate	High	High	No
RAPD	High	High	High	Low	Moderate	Low	No
RFLP	Moderate	High	Moderate	Moderate	High	Low	No
AFLP	Moderate	Moderate	High	Moderate	Moderate	Moderate–High	Available[b]
Ribotyping	Moderate	Moderate	Moderate	Moderate	High	High	Available[c]
MLST	Low	Moderate–High	High	Moderate	High[e]	High[e]	No
Rep-PCR	High	High	High	Low	High	Low	Available[d]

PFGE, pulsed-field gel electrophoresis; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; AFLP, amplified fragment length polymorphism; MLST, multilocus sequence typing.

[a] Table 1 (Olive and Bean, 1999 J Clin Micribiol, 37(6):1661–1669 and/or Table 2 (Van Belkum, et al., 2001 Clin Microbilol Rev, 14(3):547–560).

[b] Applied Biosystems Microbial AFLP Kits.

[c] DuPont RiboPrinter.

[d] Bacterial Barcodes Diversilab System.

[e] Trinidade, et al. 2003 Braz J Infect Dis, 7(1):32–43.

TABLE 26.2. Organism-specific applications of each technology.

Technique	Bacteria[a]	*Mycobacterium*[a]	Filamentous fungi	Yeast[a]	Protocol consistency between organisms
PFGE	Yes	Limited[h]	No	Yes	Low, RE and electrophoresis parameters change with each organism[b]
Sequencing	Yes	Yes	Yes	Yes	Moderate, species-specific gene-related assays required for strain-typing[c]
RAPD	Yes	Yes	Yes	Yes	Low, very sensitive to primer and annealing temperature changes[c]
RFLP	Yes	Yes	Yes	Yes	Moderate, gene-specific primers change with each organism[c]
AFLP	Yes	Yes	Yes	Yes	Moderate, nonspecific primer kits available[d]
Ribotyping	Yes	Yes	No	No	Moderate, nonspecific restriction enzyme kits available[e]
MLST	Yes	No	Limited[i]	Yes	Low, multiple primers change with each organism[f]
Rep-PCR	Yes	Yes	Yes	Yes	High, quality-controlled genus-specific kits available[g]

PFGE, pulsed-field gel electrophoresis; RAPD, randomly amplified polymorphic DNA; RFLP, restriction fragment length polymorphism; AFLP, amplified fragment length polymorphism; MLST, multilocus sequence typing.

[a] Data taken from Table 4, Pfaller (2001) Emerg Infect Dis, 7(2):312–318.
[b] http://www.cdc.gov/pulsenet/protocols.htm.
[c] Olive and Bean (1999) J Clin Micribiol, 37(6):1661–1669.
[d] http://www.appliedbiosystems.com.
[e] http://www.qualicon.com/riboprinter.html.
[f] Trinidade, et al. (2003) Braz J Infect Dis, 7(1):32–43.
[g] http://www.bacterialbarcodes.com.
[h] Zhang, J Clin Microbial, 2004; 42(12):5582–5587.
[i] Taylor, Curr Opin Microbiol, 2003; 6:351–356.

Reproducibility of a typing method is critical for longitudinal studies, including tracking and trending, for comparing archived fingerprint patterns, and for validation of the system. The data presented in several studies (Versalovic et al., 1992; Kang and Dunne, 2003) indicate that rep-PCR fingerprints are stable over multiple generations of growth, reproducible within a plate of isolated colonies within a strain, and distinct between strains. Additionally, automated rep-PCR has shown high interlaboratory reproducibility (Healy et al., 2005; Shutt et al., 2005).

Some sequencing techniques, including MLST, also show high reproducibility (Storms et al., 2002; Tavanti et al., 2003). Conversely, both automated ribotyping and AFLP have been shown to be reproducible only after manipulation of the fingerprint patterns to achieve high reproducibility (Bagley et al., 2001; Brisse et al., 2002).

Commercialization of a typing system can increase the reproducibility due to standardization of reagents and data collection. The DiversiLab System has been commercialized with the appropriate controls to facilitate quality-control efforts. Variation in the template DNA concentration, instruments, laboratory facilities, or operator do not affect the reproducibility of the assay, verifying the ease of use, portability of the data, and robustness of automated rep-PCR (Cangelosi et al., 2004; Healy et al., 2004; Healy et al., 2005; Shutt et al., 2005). All manufactured kits are quality controlled and include positive and negative controls. The DiversiLab System instrumentation comes with documentation of design qualification, including a Declaration of Conformity for manufacturing specifications and a CE mark. Additionally, installation of the system includes on-site training, a certification panel for technical performance, and qualification for laboratory thermal cyclers. Most typing methods must still be performed with "homebrew" reagents and nonstandardized protocols, although ribotyping has been commercialized as the DuPont RiboPrinter and AFLP has been commercialized in ready-made primer kits from Applied Biosystems to be used with their Genetic Analyzer and GeneMapper software.

Automation of a methodology often allows the technology to be more rapid, which is important for tracking infections in real-time, and easier, which is required for routine clinical labs. PFGE, RAPD, and AFLP have extensive technical hands-on time or require highly skilled technicians (Kostman et al., 1995; Tenover et al., 1995; Lobato et al., 1998; Olive and Bean, 1999; van Belkum et al., 2001; van Belkum, 2003; Zaidi et al., 2003). Although, MLST can be used as a non–culture-based typing method, it can be labor intensive and costly (Shopsin and Kreiswirth, 2001; Diep et al., 2003; van Belkum, 2003; Zaidi et al., 2003). Because much of the workflow is automated, the DiversiLab System overcomes these issues (Pounder et al., 2005; Shutt et al., 2005). However, the process still requires some manual manipulation of the samples. The current recommended DNA extraction method is the MoBio UltraClean Microbial Isolation Kit due to its high, consistent yield from Gram-positive, Gram-negative, and fungal isolates. Twenty-four samples can be processed in an hour; this method is simple, yet laborious (Shutt et al., 2005). To overcome the hands-on portion of this procedure, automated extraction methods can be coupled with the DiversiLab System; however, validation of these methods may be required.

Often, the downside of technology commercialization is the associated cost. Automated ribotyping is generally much more expensive than other typing techniques, including rep-PCR (Inglis, 2002; Silbert, 2004). Both noncommercial and commercial AFLP are expensive due to the requirement of a sequencer (Olive and Bean, 1999), which is also required for MLST and other sequencing protocols.

Noncommercial typing systems may also be expensive if they require specialized equipment, such as the specialized gel-electrophoresis unit required for PFGE. The DiversiLab System has a lower capital equipment cost and competitive cost per test.

One issue common to all fingerprinting methods, including the DiversiLab System, is the difficulty of the data interpretation, mainly because the results are qualitative and complex. For example, chromosomal RFLP and AFLP yields complex DNA profiles that can be challenging to interpret (Vos et al., 1995; Olive and Bean, 1999). Although standardized guidelines have been applied to PFGE (Tenover et al., 1995), this method is often time-consuming and difficult because it depends on visual analysis of the fingerprints. With the DiversiLab System software, interpretation continues to be subjective. However, an interpretation guide is provided and the software provides a variety of analysis tools to assist the user. To aid the infection control team in producing specialized data reports, such as a search on a particular isolate over a specified period, the DiversiLab software offers 10 fields to incorporate sample demographics, which are linked to the isolate and can be displayed on the report (Fig. 26.2). These demographics may include time, date, location, or other particulars about the sample. By using the DiversiLab software package to assist in monitoring over time and providing useful trend reports, proactive measures can be taken to prevent contamination.

Applications for Strain Typing in the Clinical Laboratory

Source Tracking of Pathogens in Outbreak and Clonal Spread

Rapid typing can significantly reduce costs associated with treatment, containment, and decontamination. The Centers for Disease Control and Prevention (CDC) estimates that 2 million patients acquire nosocomial infections each year, and 90,000 of those patients die as a result of their infections. Infection control programs that include DNA fingerprinting allow outbreaks to be tracked and controlled in real-time. As a laboratory tool, typing is used to assist in tracking sources of contamination and the spread of hospital-associated infections (HAI) (Watterson and Drobniewski, 2000; Taha, 2002; Wu and Della-Latta, 2002).

Methicillin-resistant *Staphylococcus aureus* is currently the most common antibiotic-resistant pathogen reported associated with HAI in the United States (CDC, 2004). Even in previously unaffected countries, MRSA has become a common problem (Faria et al., 2005). Strain typing of MRSA is important for performing outbreak investigations of nosocomial infections. Tracking the strains of MRSA in a facility over time has previously demonstrated a shift in the type of organism most commonly seen (Perez-Roth et al., 2004). Strain typing can also reveal the spread of a clone not only through a hospital but to a different facility. For example, strains in Hong Kong were linked to clones with United Kingdom

and Brazilian lineages (Ip et al., 2003). The resistance profiles of MRSA clones can vary widely; strain typing provides a method of distinguishing these clones (Coombs et al., 2004).

MRSA is a clonal organism that is difficult to strain type with many common genotypic techniques. PFGE is considered the gold standard for typing MRSA strains; however, PFGE is a cumbersome technology to implement routinely in the clinical laboratory. Many studies have been published that have tried to demonstrate the level of PFGE strain discrimination with an easier or quicker technology. A sequencing method that targets a polymorphic repeat region of the protein A gene, called *spa* typing, has shown a high level of concordance to PFGE (Harmsen et al., 2003; Faria et al., 2005); however, the discrimination was slightly lower for some outbreaks (Tang et al., 2000). MLST may be used in concert with *spa* typing or PFGE as a confirmation method (Perez-Roth, 2004; Faria, 2005). PCR methods such as multiplex PCR with primers targeting MRSA-specific genes have not been as discriminating as PFGE (Stranden et al., 2003). When manual rep-PCR was compared with PFGE, one rep element showed excellent discrimination, but the interlaboratory reproducibility was poor (Deplano et al., 2000). When automated rep-PCR was compared with PFGE, however, the results were concordant (Shutt et al., 2005). The reproducibility of automated rep-PCR using the DiversiLab System has been established elsewhere (Healy et al., 2005).

Another common nosocomial pathogen is multidrug-resistant *Acinetobacter baumannii* (IMRAB). As it is for MRSA, PFGE is a common typing technique for *Acinetobacter*; however, decreased isolate clonality allows for successful typing using simpler methods than PFGE. Many reports have established manual rep-PCR as an accepted typing method of *Acinetobacter* (Liu and Wu, 1997; Bou et al., 2000). Although slightly less discriminating than PFGE, the interpretation is easier. Other methods include antibiotyping and RAPD (Mathai et al., 2001), which appear to have less discrimination than rep-PCR (Martin-Lozano et al., 2002).

Acinetobacter strain typing has been successfully used to track source infections and outbreaks in health care facilities. Rep-PCR was used to determine a definite source for multiple cases of bacteremia, and in a number of the cases, the suspected source, originally determined through standard microbiological tests, was refuted (Martin-Lozano et al., 2002). Typing by rep-PCR established that another outbreak was transmitted with a humidifier temperature probe (Snelling et al., 1996). Another study, using IRS-PCR typing, identified that a single strain of multiresistant–*A. baumannii* was responsible for the prevalence of nosocomial infection among surgical patients, clearly differentiating this outbreak from the previous endemic situation (Wu and Della-Latta, 2002).

The incidence of infection from another vancomycin-resistant enterococcus (VRE) has also grown in recent years (CDC, 2004). Due to its spread, the CDC recommends typing isolates in facilities with continued or subsequent outbreaks (CDC, 1995). Similar to MRSA, resistant strains of enterococcus are less diverse than sensitive strains (Harrington et al., 2004). Strain typing of VRE is performed using methods described in previous sections, but commonly by PFGE (Pfaller,

2001). Manual rep-PCR of VRE has been successful in establishing the transmission of this organism, including the spread of the same strain through multiple health-care facilities in the same city (Dunne and Wang, 1997).

Extended spectrum beta-lactamase (ESBL)-producing *Escherichia coli* and *Klebsiella pneumoniae* are also commonly reported pathogens in nosocomial infections (CDC, 2004). Manual rep-PCR was effective in typing both *E. coli* and *K. pneumoniae*, as well as other ESBLs, including *Serratia marcesens* and *Proteus mirabilis* (Arpin et al., 2003). Additionally, a combination of RFLP and rep-PCR was used to study the epidemiology of ESBL *E. coli* and *K. pneumoniae*, which showed dissemination differences between CTX-M-type enzyme-producing organisms (Edelstein et al., 2003).

Mycobacteria are important clinical pathogens, but they are generally more difficult to strain type with traditional methods because of their slow growth and cellular structure. DNA fingerprinting of most *Mycobacterium tuberculosis* isolate populations can be accomplished with a combination of PCR-based methods, usually spoligotyping and mycobacterial interspersed repetitive unit–variable-number tandem repeat (MIRU-VNTR) typing. PCR-based methods require relatively small cell mass to generate results, a considerable advantage given the low growth rates of pathogenic mycobacteria. MIRU-VNTR assesses repeating units at multiple loci in the *M. tuberculosis* genome based on product size after PCR amplification (Kamerbeek et al., 1997; Cowan et al., 2002; Fietto et al., 2004). The discriminatory power of MIRU-VNTR approaches that of IS*6110*-targeted restriction fragment length polymorphism (IS*6110*-RFLP), a more labor-intensive, high-resolution approach but currently considered the gold standard (Barnes and Cave, 2003; Kwara et al., 2003; Sola et al., 2003). MIRU-VNTR may be used as a first-line tool for studying the genetic diversity of *M. tuberculosis* isolates in large urban settings (Sun, 2004). As seen with other organisms, the combination of techniques (for example, MIRU-VNTR and IS*6110*-RFLP) increases knowledge of specific genotypes and refines evidence of epidemiological linkage transmission studies (Niobe-Eyangoh et al., 2004; Cave et al., 2005). The DiversiLab System was a useful tool for *M. tuberculosis* typing and MAIC discrimination (Cangelosi et al., 2004).

In addition to bacteria, various fungi have been associated with nosocomial infections, and not all strain-typing systems have the ability to type these organisms (Table 26.2). *Candida* has become one of the most common blood isolates, as well as one of the leading causes of nosocomial bloodstream infections. Multiple powerful molecular typing methods have emerged as the gold standard and are widely used for strain delineation of *C. albicans* isolates (Merz, 1990; Odds et al., 1992; Vazquez et al., 1997; Espinel-Ingroff et al., 1999). These include fingerprinting methods such as karyotyping using PFGE, RFLP, RAPD, and Southern hybridization with moderately repetitive DNA probes (Asakura et al., 1991; Arnavielhe et al., 1997). In order to be useful epidemiological tools, these methods should be able to discriminate between unrelated strains and, at the same time, demonstrate the relationships among all organisms isolated from individuals infected through the same source (Olive and Bean, 1999). Methods resulting in more complex banding

patterns presumably provide increased levels of discrimination and allow detection of substrains or strain variants that may evolve from an original strain (Boerlin et al., 1996; Pujol et al., 1997; Olive and Bean, 1999). Several authors have compared different genotyping methods for *C. albicans*. In general, there is a lack of consensus on the method(s) of choice as well as the interpretation of results. The use of a single method may not be optimal, and a combination of typing techniques is often required to provide a comprehensive assessment of the epidemiology of candidiasis (Vazquez et al., 1991; Bostock et al., 1993; Diaz-Guerra et al., 1997; Pujol et al., 1997). For example, a combination of methods with high and low discriminatory power may be useful for nosocomial source tracking because higher discrimination allows detection of strain variants, whereas lower discrimination is more helpful for determining isogeneity of organisms (Lopez-Ribot et al., 2000). By using the *Candida*-specific DiversiLab Kit for strain discrimination and the general DiversiLab Yeast Kit for lower discrimination, two purposes could be served with this one technology.

Filamentous fungi such as *Fusarium* and Zygomycetes have also been shown to contribute to hospital-acquired infections. Fusariosis, an emerging and severe opportunistic mold infection, is typically a community-acquired mycosis (Raad et al., 2002) however, the potential for nosocomial transmission has recently been raised (O'Donnell et al., 2004). Godoy et al. recently showed the clinical utility of manual rep-PCR for genotyping *Fusarium* (Godoy et al., 2004). Automated rep-PCR was recently used to demonstrate discrimination of *Fusarium* species and strains (Healy et al., 2005). Strain typing of *Fusarium* isolates using MLST and AFLP established a single clonal lineage as a large part of the clinical isolates examined from a study of three U.S. hospitals (O'Donnell et al., 2004). Additionally, Zygomycetes are increasingly reported as causing lethal infections (Walsh and Groll, 1999). Methods such as DNA sequencing and RFLP have shown subspecies discrimination of different Zygomycetes species (Chakrabarti et al., 2003; Kobayashi et al., 2004). Automated rep-PCR also successfully grouped multiple Zygomycetes species (Kontoyiannis et al., 2005). There is a growing need for strain typing to track the source of outbreaks for a wide variety of organisms.

Infections Caused by Community-Acquired Organisms, Colonization, Recurrence, or Reinfection

There has been awareness of community-acquired pathogens, but that phenomenon is now being more carefully studied as it appears that the level of community acquisition is somewhat organism dependent. The prevalence of community-acquired MRSA has increased, as confirmed by the use of microbial strain typing (Herold et al., 1998; Fey et al., 2003), and the increase of community-acquired infections makes the determination of HAI increasingly difficult. In order to understand the transmission and source of MRSA outbreaks, strain typing must be used to compare community-acquired and nosocomial pathogens at the genomic level

(Stemper et al., 2004; Hanssen et al., 2005). Interestingly, for *A. baumannii* the absence of multidrug-resistant strains in the community compared with 36.8% prevalence among hospital isolates suggesting that the reservoir for epidemic strains resides in the hospital environment itself, and the community is an unlikely reservoir for hospital strains of *A. baumannii* (Zeana et al., 2003). Further, candidemia must be considered as a potential cause of sepsis in the community (Pasqualotto et al., 2005).

The etiology of infectious diseases includes the potential infection not only from community sources but also from colonization of patients or health care workers. Because MRSA can colonize healthy individuals, screening of employee strains may indicate routes of transmission. One study using PFGE found that of seven staff members, three carried the same strain as found in patients (Macfarlane et al., 1999). Using CARE-2 probe-based DNA fingerprinting, *Candida* infection isolates from burn patients, results indicated that strains collected from different patients were different. It is noteworthy that patients with disseminated candidiasis had a similar but unique strain isolated from all body locations, suggesting infection from patient colonization (Gupta et al., 2004).

Molecular subtyping aids in further understanding the pathogenesis of infections and their potential to relapse or cause new infections. Between 15% and 20% of patients have recurrences of *Clostridium difficile*–associated diarrhea, which is the most common hospital-acquired infectious diarrhea, after discontinuation of therapy (Wilcox and Spencer, 1992). Two studies using ribotyping indicated that the recurrence of infection due to the same strain as the original outbreak is high, ranging from 79% to 90% (Svenungsson et al., 2003; Noren et al., 2004). However, arbitrarily primed PCR showed a lower incidence of same-strain reinfection at approximately 66% (Tang-Feldman et al., 2003). This discrepancy may be due to the lower discrimination seen with ribotyping; rep-PCR and PFGE are more discriminating than ribotyping when performing fingerprinting of *C. difficile* (Spigaglia and Mastrantonio, 2003; Rahmati et al., 2005).

The rate of subsequent MRSA infections may be as high as 29% in 18 months, and the recurrent infection is often severe (Huang and Platt, 2003). PFGE determined that patients with long-term MRSA colonization often have several different strains of MRSA that change over time (Maslow et al., 1995). Additionally, the severe recurrent infections of *Staphylococcus aureus*, certain Gram-negative rods, *Nocardia* species, and fungi seen with chronic granulomatous disease (CGD) often present the same bacterial species but different strain type, as determined by PFGE (Guide et al., 2003). In recurrent listeriosis, molecular subtyping by automated ribotyping and PFGE confirmed either relapses of infection or reinfection due to a common source by indication of same-strain isolates (Sauders et al., 2001).

It is generally accepted that tuberculosis results from a single infection with a single *Mycobacterium tuberculosis* strain. Specific PCR-based results suggest that multiple infections are frequent, implying high reinfection rates and the absence of efficient protective immunity conferred by the initial infection. This finding could influence our understanding of the epidemiology of disease in high-incidence regions and our understanding for vaccine development (Warren et al., 2004).

Surveillance for Potential Infections

Although the debate continues over what may be the most cost-efficient and necessary approach for prospective monitoring of health care–associated infections, it is clear that surveillance is the first step to understanding and management (Peterson and Brossette, 2002). One way the microbiology laboratory can aid in surveillance is by screening patient admission isolates. MRSA colonization of nares, either present at admission to the hospital or acquired during hospitalization, increases the risk of MRSA infection. Identifying MRSA colonization at admission could target a high-risk population that may benefit from interventions to decrease the risk for subsequent MRSA infection (Davis et al., 2004). However, there remains some controversy on how to best apply the results to infection-control practices. Surveillance cultures and genotyping of MRSA and Methicillin-Resistant *Staphylococcus aureus* (MSSA) isolates demonstrated the absence of cross-transmission among patients in the medical intensive care unit (MICU), despite ongoing introduction of these pathogens. Reporting culture results and isolating colonized patients, as suggested by some guidelines, would have falsely suggested the success of such infection-control policies (Nijssen et al., 2005). Surveillance may also identify sources of infection before patients are infected such as was seen with a health care worker whose nails harbored an undetected infection passed on to patients (McNeil et al., 2001).

Monitoring the Environment

Although the environment serves as a reservoir for a variety of microorganisms, it is rarely implicated in disease transmission except in the immunocompromised population. Inadvertent exposures to environmental opportunistic pathogens (e.g., *Aspergillus* spp. and *Legionella* spp.) or airborne pathogens (e.g., *Mycobacterium tuberculosis*) may result in infections with significant morbidity and/or mortality, U.S. Department of Health, 2003). Both air and water systems can contain or be contaminated with microbes that lead to patient infections or laboratory contamination. For these reasons, some clinical laboratories are now analyzing environmental samples.

Invasive aspergillosis (IA) has emerged as a major opportunistic mycosis (Denning, 1998; Latge, 1999; Kontoyiannis and Bodey, 2002). Because environmental factors play a key part in the acquisition of IA, infection-control measures are crucial to reduce exposure to this opportunistic pathogen and to decrease the incidence of IA in epidemic and possibly in nonepidemic situations (Buffington et al., 1994; Leenders et al., 1996; Chazalet et al., 1998; Radford et al., 1998; Bart-Delabesse et al., 1999, 2001; Bertout et al., 2000; Kontoyiannis and Bodey, 2002; Lasker, 2002; Gil-Lamaignere et al., 2003). However, many controversies still exist regarding the epidemiology of IA, such as what constitutes a hospital-acquired versus a community-acquired case of IA and what are the potential sources of nosocomial IA (Kontoyiannis and Bodey, 2002). In view of the tremendous genetic diversity among *Aspergillus*

isolates (Sampson, 1994; Debeaupuis et al., 1997; Chazalet et al., 1998; Latge, 1999; Kontoyiannis and Bodey, 2002; Lasker, 2002), various DNA fingerprinting technologies such as RFLP, Restriction Enzyme Assay (REA), RAPD, and microsatellite analysis have been developed in order to delineate strains of *Aspergillus* species in order to answer these questions (Buffington et al., 1994; Soll, 2000; Kontoyiannis and Bodey, 2002; Lasker, 2002; Gil-Lamaignere et al., 2003). Each method has advantages and disadvantages (Kontoyiannis and Bodey, 2002; Lasker, 2002); therefore, no single method has emerged as ideal for investigation of IA case clustering or routine surveillance (Lasker, 2002). For example, RFLP analysis with Southern blotting is labor intensive, microsatellite analysis requires specialized equipment, RAPD analysis is prone to artifacts and lacks reproducibility, and REA frequently has subjective interpretation of the profiles (Lasker, 2002). Like many other organisms, a combination of typing methods has been suggested in order to overcome assay limitations (Lasker, 2002).

Molecular typing studies have been performed to determine the possible relationship between environmental contamination by *A. fumigatus* and occurrence of IA. *Aspergillus fumigatus* infection in hospitalized immunocompromised patients often raises suspicion regarding the potential for hospital acquisition. There have been high expectations that molecular typing methods such as sequence-specific DNA primer analysis might determine the source of *A. fumigatus*. The results of one study confirmed the huge biodiversity of the *A. fumigatus* population and consequently the difficulty in ascertaining a hospital source of the infection, as opposed to infections due to other *Aspergillus* species less frequently encountered (Symoens et al., 2002). Patient and environmental isolates genotyped using RAPD analysis also indicated great genetic diversity among isolates from infected patients and from the environment. Interestingly, there was a concurrent infection by at least two different strains in one patient, and a genetic similarity was noted between isolates obtained from one patient and from the environment (Menotti et al., 2005). A mycological survey of air and surfaces in hospitals showed massive contamination by *A. flavus*. Strains from patients and from the hospital environment were typed by RAPD and rep-PCR resulting in the same genotype, indicating the clonal single-source of the environmental contamination (Heinemann et al., 2004). Factors that may influence potential environmental contaminants include extensive building renovations or changes in the HVAC and HEPA filtration systems (Munoz et al., 2004; Curtis et al., 2005; Price et al., 2005). There is an increasing awareness of environmental opportunistic pathogens and therefore a need for methods to study modes of prevention.

In addition to air-filtration systems, pathogens can also be isolated from hospital water sources. Specific strains of *Legionella pneumophila*, as determined by PFGE, can colonize hospital water supplies and cause nosocomial infections over long periods of time (Rangel-Frausto et al., 1999). After a nosocomial outbreak caused by *L. pneumophila* serogroup 5, the hospital water system was determined to be colonized by *L. pneumophila* serogroups 5 and 6. The colonizing *Legionella* flora was examined using AFLP and RAPD analysis, which indicated that, long-term,

the diversity of *Legionella* strains in a hospital water system remains stable over years (Perola et al., 2005). When PFGE was used to type infections of *L. pneumophila,* only 2 of 11 were determined to be hospital-acquired, yet, both strains were also present in the ward water (Thouverez et al., 2003). A study showed VNTR typing was as informative as PFGE for comparison of strains, allowing a more rapid identification of the *L. pneumophila* colonies suggesting the presence of several strains in a single sample (Pourcel et al., 2003). Additionally, manual Rep-PCR, AP-PCR, REA, PFGE, and ribotyping have been used for strain typing *L. pneumophila* in water (Ishimatsu et al., 2001; Miyamoto et al., 1997) and for determing the source in a *L. pneumophila* nosocomial outbreak (Georghiou et al., 1994). As strain-typing methods advance, they can be applied tools in several arenas of microbiology. However, subtype matching of patient and environmental isolates should be interpreted with caution, and it is important to combine a molecular typing method with sound epidemiological data to ensure that the most stringent criteria are used to determine whether a hospital reservoir is responsible for nosocomial pneumonia.

Laboratory Contamination

One problem that the clinical laboratory can address for its own purposes of quality control is laboratory contamination. One example is the genotypic analysis of atypical mycobacteria, most notably the *M. avium* complex (MAC), that has been useful for investigating routes of acquisition from environmental sources and confirming contamination of laboratory cultures (Arbeit et al., 1993; von Reyn et al., 1994; Falkinham, 1996). There are no consensus PCR-based methods for typing *Mycobacterium* species other than *M. tuberculosis*. RFLP is widely used for *M. avium* subsp. *avium* strains, most of which carry the insertion element IS*1245*-RFLP (van Soolingen et al., 1998); however, like IS*6110*-RFLP it requires large cell mass and is labor intensive. Pulsed-field gel electrophoresis (Arbeit et al., 1993; von Reyn et al., 1994) also requires a large cell mass, and few laboratories have the required equipment. Not surprisingly given the limited molecular epidemiological tools available for environmental mycobacteria, our understanding of the routes of acquisition of these opportunistic pathogens remains limited as does the ability to detect contamination.

Compared with solid media, broth-based mycobacterial culture systems have increased sensitivity but also have higher false-positive rates due to cross-contamination. False-positive cultures can be identified by careful documentation of specimen data and good communication between clinical and laboratory staff. Automated broth culture systems should be supplemented with molecular analysis such as VNTR, so patients are not placed on unnecessary tuberculosis therapy or cases are not falsely identified as treatment failures (Gascoyne-Binzi et al., 2001). When unusual organisms are identified from multiple patients, concern about laboratory contamination, nosocomial spread, or even the possibility of a novel organism associated with disease are raised (Zhang et al., 2002). Advances in

molecular typing methods may provide additional tools to help discern the answers to the above questions.

Probiotics

Advances in patient treatment also demand advances in molecular typing techniques. Probiotic agents are increasingly used in clinical practice for the treatment and prevention of a variety of infectious and inflammatory conditions. Rep-PCR and other strain-typing methods can be used to identify and discriminate probiotic organisms such as bifidobacterial isolates (Masco et al., 2003; Vitali et al., 2003) and the closely related *Saccharomyces cerevisiae* and *S. boulardii* (Fietto et al., 2004). Probiotics are generally safe and infections associated with probiotic strains of lactobacilli are extremely rare, but complications of probiotic use can occur. Bacteremia occured in a child after ingestion of a *Lactobacillus rhamnosus* GG probiotic tablet. Strain typing with PFGE showed identity between the tablet and bloodstream isolates (De Groote et al., 2005). Additionally, two patients received probiotic lactobacilli and subsequently developed bacteremia and sepsis attributable to *Lactobacillus* species. Rep-PCR DNA fingerprinting analysis showed that the *Lactobacillus* strain isolated from blood samples was indistinguishable from the probiotic strain ingested by the patients. This report (Land et al., 2005) should serve as a reminder that these agents can cause invasive disease in certain populations and that advances in medical treatment are often accompanied by new requests for the laboratory.

Potential Future Applications

Additional studies demonstrate the use of the DiversiLab System for identification of bacteria and fungi (Cangelosi et al., 2004; Healy et al., 2004; Kontoyiannis et al., 2005; Pounder et al., 2005). The DiversiLab System represents an effective platform for microbial genotyping in microbiology laboratories. Traditionally, the rep-PCR methodology has been used for subspecies and strain discrimination. With the advances in bioinformatics technology, the rep-PCR methodology could be used for niche identification where rapid methods may not be useful (Healy et al., 2004, 2005; Kontoyiannis et al., 2005). Identifications are based on positive DNA fingerprint matches to a similar fingerprint from a well-characterized collection stored in the database. Therefore, future applications include the capability for additional identification assays with compilation of new databases and expansion of the current databases.

The DiversiLab System has potential to be the standardized platform for routine bacterial and fungal genotyping in the clinical microbiology laboratory. The DiversiLab System offers efficiency, excellent discriminatory power, and reproducibility in order to allow for database building, intersite comparisons, and expedited report generation. The small footprint of the automated system conserves work space, and the Web-based interface enables remote access and data portability for assessment

of population profiles within and among manufacturing sites. Finally, rep-PCR–based molecular typing is considered to have favorable costs (particularly start-up and equipment costs), as compared with other methods (Olive and Bean, 1999; Goulding et al., 2000; van Belkum et al., 2001; Diep et al., 2003; Cangelosi et al., 2004), and additional cost savings may be realized by reductions in technician time, training, or labor.

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27
Molecular Differential Diagnoses of Infectious Diseases: Is the Future Now?

JIAN HAN

Introduction

The clinical presentations for most infectious agents are often not specific enough to allow for a definitive diagnosis. Coughing and fever, for example, are symptoms that may be caused by many different bacterial or viral infections. Thus, for better treatment and disease control, a molecular differential diagnostic (MDD) assay that can identify, differentiate, and pinpoint the offending pathogen associated with a clinical syndrome (Fig. 27.1) is needed. MDDs are essential tools for effective infectious disease surveillance, biodefense, and personalized medicine.

MDDs are *needed* for emerging infectious disease surveillance and control. When outbreaks such as SARS occur, public health officials and laboratory scientists often struggle for weeks, if not longer, to identify the offending pathogen. With molecular differential diagnostic assays available, scientists involved in an outbreak investigation can quickly rule out many pathogens associated with similar clinical symptoms and focus on new, emerging infections. An MDD test can also aid in the management of a public health crisis. It can help health care personnel in triaging patients and determining which patients should be isolated, as well as identifying environmental sources of contamination within an intensive care unit (ICU) or patient room.

MDDs are *needed* for homeland security and biodefense. With the current global political atmosphere, biodefense threats are a reality. A first-response technology could quickly identify a bioterrorism agent and control the spread of the pathogen. Without the availability of MDDs for rapid pathogen identification, the bioterrorism agent may go undetermined for days. Every hour wasted in determining the causative agent provides a greater opportunity for pathogen spread and for global panic to occur.

MDDs are *needed* for delivering true personalized medicine. Personalized medicine focuses on treating the patient, rather than the disease. It is genotype-based medicine, rather than phenotype- or symptom-based. An MDD test also makes it possible to practice *theranosis* (therapy guided by a diagnosis) by developing or reclassifying drugs that specifically target the molecular cause of the disease. If pharmacogenomics is the development of drugs based on individual

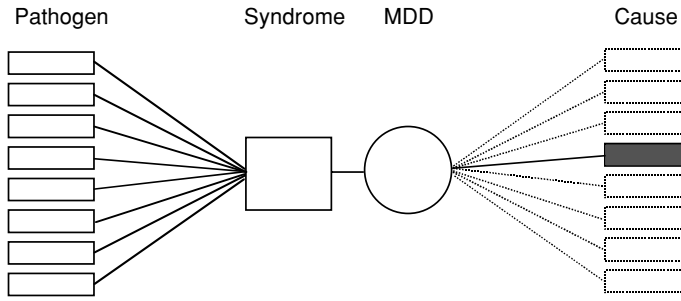


FIGURE 27.1. Multiple Pathogens can lead to the same clinical syndrome. One MDD test should be able to differentiate and identify the real cause.

genotypes, then theranosis is the administration of drugs based on individual (or infectious agent) genotypes. It is clear that MDDs are *needed*, but in order to make the assays practical, we *want* them to have the following advanced features:

- **Multiplex capabilities**. The definition of multiplexing is "receiving multiple signals from the same source." For MDDs, multiplexing refers to the ability to conduct multiple genotyping tests at the same time and within the same sample. We *want* multiplexing because it requires only small amounts of precious patient sample; it allows the clinician to run fewer tests while acquiring more relevant information; it reduces the amount of reagents, consumables, and time involved; and most importantly, it can save lives. For infectious diseases MDDs, we *want* a multiplex test that can identify all pathogens related to a clinical syndrome or that can detect all the genes and mutations responsible for the drug-resistance phenotype.
- **Specificity**. Even though multiple microorganisms are studied simultaneously, we *want* only the pathogens associated with the infection be identified with a high level of confidence.
- **Sensitivity**. We *want* a MDD to be able to identify a pathogen or drug resistance *directly* from a patient sample or enrichment culture. Using the patient sample directly reduces the time required for bacterial or viral culture preparation and enzymatic testing. Yet, bypassing this propagation step forces the assay to be sensitive enough to detect only a small amount of pathogen material present in the patient sample.
- **Reliability**. For clinical application of MDDs, a consistent performance from assay to assay and from lot to lot is required.
- **Speed**. For an MDD to be practical for infectious disease diagnosis and treatment, it must be locally available and produce results within a few hours.
- **Simplicity**. An MDD should not require a Ph.D. laboratory scientist to conduct the assay. An MDD should be user-friendly and even automatable. No special training should be required to perform the assay. The MDD system should be easily integrated into standard molecular laboratory practice.
- **Affordability**. MDDs should be efficient and cost-effective.

The technology advancements in this post-genomic era have made sequence information readily available for almost all known pathogenic microorganisms. Based on this information and armed with standard molecular tools, scientists have developed molecular assays, usually PCR-based, for almost every infectious pathogen. A simple Internet keyword search for a pathogen name and the word "PCR" will produce several pages referencing specific tests for that pathogen. From this exercise, it seems possible that the basic *needs* for molecular differential diagnosis can be met. However, to produce the MDD assay we really *want*, some unique technical challenges should be addressed.

The most difficult challenge of all is multiplexing. PCR technology has been established for nearly 20 years. However, multiplex PCR is still very difficult to accomplish. The following is a list of common challenges associated with multiplexing:

- **Incompatible loci**. Each target in a multiplex PCR demands its own optimal condition; therefore, increasing the number of multiplex targets becomes difficult and, in many instances, impossible.
- **Lack of specificity**. Multiple sets of high-concentration primers in a system often generate primer dimers or give nonspecific, background amplification. Lack of specificity also adds operational burdens by requiring post-PCR clean-up and multiple posthybridization washes.
- **Lack of sensitivity**. Crowded primers reduce amplification efficiency and waste resources by occupying enzymes and consuming substrates.
- **Uneven amplification**. Differences in amplification efficiency may lead to large discrepancies in amplicon yields. In a multiplex system, some loci may amplify very well, whereas others may amplify poorly or even fail to amplify. Uneven amplification also makes it impossible to accurately perform end-point quantitative analysis.
- **Lot-to-lot variation**. Due to the fact that large amounts of primers are consumed in each reaction and that manufacturers can generate only a limited amount of assays per lot, quality control and quality assurance can be difficult.

In the following discussions of this chapter, we will present a new multiplex PCR technology developed by Genaco (Huntsville, AL, USA) scientists, called Templex. The Templex technology answers many of the challenges that have already been described and delivers what infectious disease control professionals truly want. This chapter will also discuss technology integration strategies and application examples. Finally, the implementation and impact of MDDs (also called MD^2 by Genaco) will be discussed.

A Novel Multiplex PCR Technology: The Templex Advantage

Templex technology was developed to meet the challenges of multiplex PCR. Templex is a multiplex PCR strategy using the Genaco proprietary Tem-PCR (target enriched multiplex PCR) method. Figure 27.2 describes the Tem-PCR method.

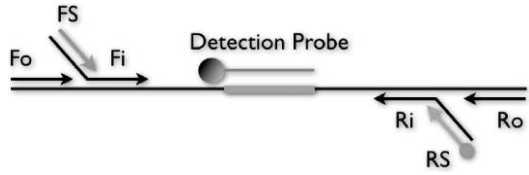


FIGURE 27.2. In Tem-PCR method (Target-enriched multiplex PCR), nested gene-specific primers are designed to enrich the targets during the initial cycles. Later, a pair of Super-Primer is used to amplify all targets.

For each target in the multiplex PCR, nested gene-specific primers are designed and included in the reaction (Fo, forward out; Fi, forward in; Ri, reverse in; and Ro reverse out). These primers are used at extremely low concentrations and are used only to enrich the targets during the first few cycles of PCR. Some of these gene-specific primers have tag sequences that can be recognized by a universal set of primers, called SuperPrimers . Only the SuperPrimers are included at a concentration necessary for exponential amplification, and only the reverse SuperPrimer is labeled. Labeled PCR products are detected with a complementary capture probe that is covalently coupled to a color-coded bead.

Templex works because it addresses two of the most difficult problems inherent in multiplex PCR: (1) incompatibility of amplification conditions among different primer sets and (2) background amplification associated with high concentrations of primers.

First, as shown in Fig. 27.3, in a standard multiplex PCR reaction, if there are six targets to be amplified, each may require a different optimal annealing temperature or buffer formula. When the number of multiplex targets increases, it forces all

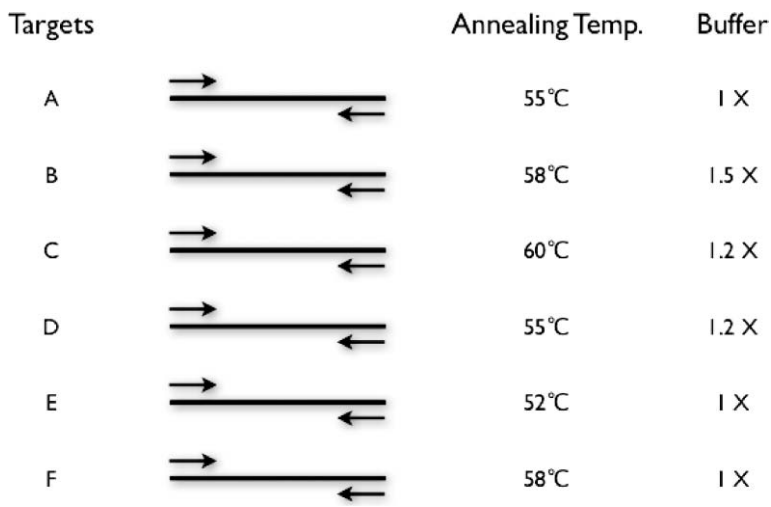
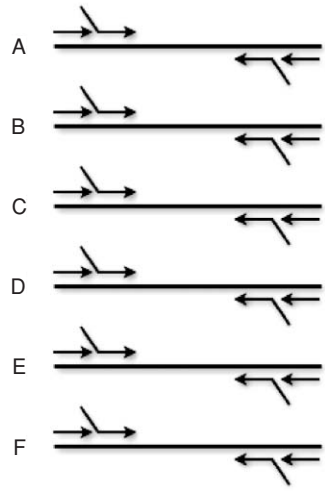


FIGURE 27.3. In regular multiplex PCR reactions, for multiple targets that need to be co-amplified, each may demand its unique annealing temperature or different buffer strength.



Optimal Conditions for different primer combinations

	Fo/Ro	Fo/Ri	Fi/Ro	Fi/Ri
A	55°C 1X	58°C 1X	55°C 1.2X	57°C 1X
B	58°C 1.5X	58°C 1X	55°C 1X	60°C 1.5X
C	60°C 1.2X	55°C 1X	55°C 1.2X	58°C 1.2X
D	55°C 1X	55°C 1.2X	55°C 1.5X	58°C 1.2X
E	52°C 1X	58°C 1X	55°C 1X	52°C 1.2X
F	55°C 1X	60°C 1X	58°C 1X	55°C 1X

FIGURE 27.4. With Templex, each target may be "enriched" with four different primer combinations (Fo/Ro, Fo/Ri, Fi/Ro, and Fi/Ri). Each combination may have its own optimal condition. But with four to choose from, it becomes easier to find universal condition that fits all targets.

primer sets to work under a single amplification profile, and multiplex PCR is nearly impossible under standard conditions. With Templex (Fig. 27.4), there are two sets of nested primers for each target in the enrichment stage. This design gives rise to four possible forward and reverse primer combinations for amplification. Each combination may have its own optimal amplification profile, but given four amplification opportunities, a common condition that satisfies all targets can be attained. For example, if multiplex PCR is a baseball game, traditional multiplex PCR is equivalent to the expectation that every player (or target to be co-amplified) on the team will hit a home run on the first and the only swing. Templex, on the other hand, provides each player with four chances to hit a home run. Though it is still difficult, the possibility of each player hitting a home run is more likely.

Second, standard multiplex PCR uses multiple sets of high-concentration, labeled primers. These primers can associate with one another to form dimers or create nonspecific, background amplification. Reduced amplification efficiency can also occur when primers occupy active sites on the polymerase. In addition, unused labeled primers produce background signal and use up reagents during the detection portion of the assay. Because of these issues, post-PCR clean-up (such as spin column purification) is often required to remove these labeled primers before they can be used as probes. Yet, high-concentration primers are only required in the

last cycles of a PCR reaction. With Templex, the amount of gene-specific primers used is only enough to "enrich" the targets and incorporate the SuperPrimer tag into the PCR products. After enrichment and tag incorporation, amplification is carried out with only one pair of primers. Because only one pair of primers is labeled, the background is low; therefore, no post-PCR clean-up is required. The PCR reaction is also very specific and sensitive. No posthybridization washes are necessary. This feature makes it feasible to fully automate the laboratory procedures and perform high-throughput clinical studies.

Templex also allows semiquantitative analysis of co-infections. With traditional multiplex PCR, each primer set, or each locus, has its own amplification efficiency. Hence, at the end of amplification cycling, the signal ratio of PCR products from different loci will not reflect the original ratio of the templates. With Templex, the only primers used for exponential amplification are the pair of SuperPrimers. Consequently, all co-amplified loci will have the same amplification efficiency. As a result, the end-point reading can reflect the original copy number ratios among the co-amplified targets.

Templex is a flexible technology. Increased compatibility among multiple targets means that existing panels can be reorganized and remixed to build new panels. In addition, new amplification targets can be added without significantly reducing the sensitivity of the panel.

Another benefit of Templex is its repeatability. Because only a small amount of gene-specific primer is used for each assay, and only one biotin-labeled primer is included in the reaction, one production run can generate a large number of assays. This makes assay-to-assay and lot-to-lot variation minute, and quality control is less complicated. Detailed applications and results are presented later in this chapter.

Vertical Integration: Choose Wisely

Molecular differential diagnosis is a comprehensive process that includes three steps (Fig. 27.5): nucleic acid isolation, amplification, and detection. There are many methods for completing each of the three steps in this process. Furthermore, a wide variety of instrument platforms are available to facilitate or automate each of these methods. To make molecular differential diagnosis a routine clinical practice, these choices must be weighed against each other to obtain the best possible combination of methods and platforms to carry out the task. This process of vertical integration can produce multiple possibilities. Thus, if molecular differential diagnosis is to be the next breakthrough in modern medicine, we must choose wisely which technology integration path to take.

The biotech industry is very much like the information technology industry (see Table 27.1) where an application is developed by using a combination of hardware (the platform) and software (the basic methodology and reagent system). A product-based company may choose different combinations of methods and platforms. A particular amplification method can be followed by one of many

TABLE 27.1. Comparison of information technology and biotechnology.

	IT	Biotech
Hardware	PC or APPLE	Luminex xMAP, Affymetrix chip, flow cytometry
Operation systems	Windows, Mac OS, Linux	PCR, Templex, branched DNA, etc.
Applications	Word, Excel, PowerPoint	HIV, bloodstream infection panels, HPV typing

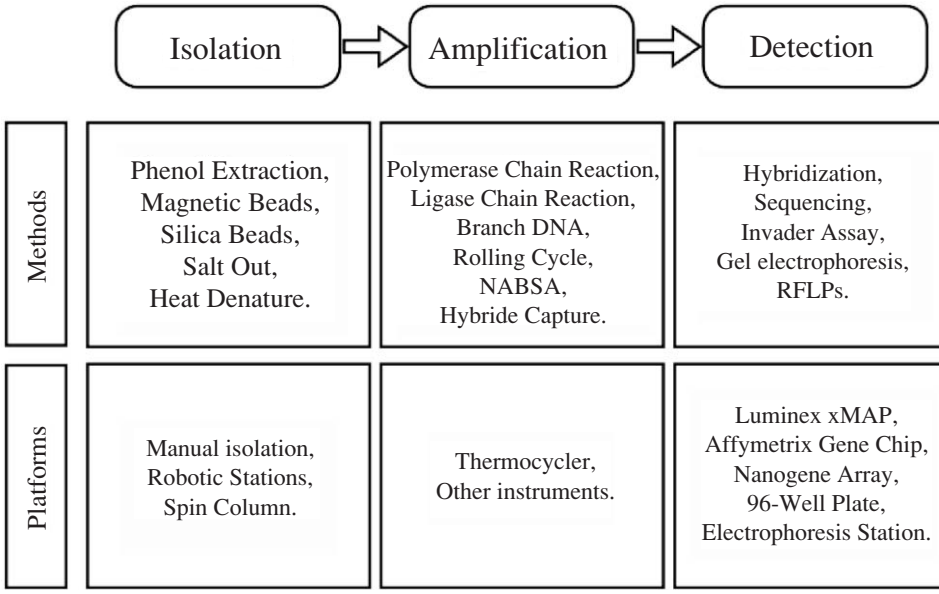


FIGURE 27.5. System integration to achieve practical solutions.

different detection means. For example, PCR amplification may be paired with multiple detection methods, such as direct hybridization, gel analysis, or sequencing, to build a molecular diagnostic system. One should also note that a particular method can be performed on multiple platforms. Successful biotech companies and clinical laboratories are those that are able to develop such applications through technology integration and innovation.

Amplification Methods and Platforms

Nucleic acid amplification is the most important step in molecular diagnosis. In many cases, especially those caused by an infectious disease, patient samples have a limited number of copies of pathogen DNA or RNA. Without amplification, it is unlikely that one can identify or differentiate the infectious pathogens present in the patient sample. In previous chapters of this book, different amplification

TABLE 27.2. Comparison of different nucleic acid amplification methods.

Method	Specificity	Sensitivity	Multiplex	Ease of use	Platform	Company	Products
PCR	++++	++++	++	+++	Thermal cycler	Roche	HIV, HCV, etc.
Real-time PCR	++++	++++	++	+++	Light cycler	Roche	HIV, HCV, etc.
Templex	++++	++++	++++	+++	Thermal cycler	Genaco	Many panels
LCR	++++	++++	++	++	Thermal cycler	Epicenter	
Branched DNA	++	++++	+	+	Incubator	Bayer	
Rolling cycle	++	++++	+	+++	Isothermal	Qiagen	
SDA	++	++++	+	+++	Isothermal	BD/Nanogene	*C. trachomatis*
Hybrid capture	+	+++	+	++	Isothermal	Digene	HPV
Invader	++	+++	+	++		Thridwave	
NASBA	++	+++	+	++	Isothermal	BioMerieux	NucliSens kits

and detection methods have been reviewed in detail (see Chapters 8–19). Table 27.2 compares some nucleic acid amplification methods along with their unique features, associated platforms, founding companies, and representative products.

Undoubtedly, the best way to duplicate DNA or RNA is to use the enzymes that nature has selected to perform the job, such as DNA or RNA polymerases (Please refer to Chapter 11 for a detailed discussion). PCR and different variations of PCR are still the gold standard of molecular diagnostic technologies. Still, other non-PCR amplification techniques (see Chapters 12 and 13) are valuable because they often create product opportunities for commercial companies by allowing them to develop intellectual property positions.

Ligase chain reaction (LCR) uses a different enzyme to amplify DNA. Instead of using a thermal stable polymerase, it uses a thermal stable ligase to link two primers together when a perfect match template sequence is present. The applications of LCR are limited because longer DNA sequences are more frequently desired for studies, and LCR only allows for the investigation of about 40 base pairs. Multiplex LCR is difficult for the same reason mentioned above.

Recently, a few isothermal DNA amplification methods have been developed. They include branched DNA, rolling cycle, NASBA (Nucleic Acid Sequence Based Amplification), and strand displacement assay (SDA) methods. One common problem facing these isothermal amplification methods is specificity. Unlike traditional PCR, the enzymes used in these reactions are not thermal stable. Temperature cannot be used to control the primer-target hybridization stringency, which is a very notable disadvantage of isothermal methods. Consequently, the amplification specificity is difficult to control. In addition, incompatibility among targets and high background make these reactions difficult to multiplex.

TABLE 27.3. Comparison of different nucleic acid detection methods and platforms.

Method	Platform	Throughput	Specificity	Sensitivity	Ease of use	Company	Price
Hybridization	Luminex	+++	++++	++++	++++	Luminex	Medium
Hybridization	Affymetrix	++++	++	+++	+++	Affymetrix	High
Hybridization	Ilumina	++++	+++	++++	+++	Illumina	High
Hybridization	Nanogene	++++	++	++	++	Nanogene	High
Invador assay	Invader	++	+++	+++	++	Thirdwave	Medium
SDA/Hybridization	Nanogene	++	+++	+++	+++	Nanogene	High
Sequencing	Sequencer	++	+++++	++	+	Applied Biosystems	High
Pyrosequencing	Sequencer	+++	++++	++++	+++	Biotage	Medium
Gel electrophoresis	Gel box	+	+	+	+	Bio-Rad	Low
RFLP	Gel box	+	++	+	+	Bio-Rad	Low

Detection Methods and Platforms

If abundant and specific DNA targets can be generated by an efficient amplification method, detection is more straightforward. The challenge then becomes providing an accurate measurement of the amplification products in a rapid, high-throughput, and low-cost format. Various detection methods and platforms have been reviewed in this book (see Chapters 15–18). Table 27.3 lists several different detection methods and their associated platforms.

The simplest detection method is hybridization. Hybridization occurs without an enzymatic reaction. One strand of DNA binds to its complementary strand, in solution, via hydrogen bonding. Specificity is controlled by temperature and salt concentration. Typically, a detectable molecule (fluorescent dye or radioactive isotope) is attached to one strand of DNA, which can be recognized by a device. Because of its ease of use, hybridization is the method of choice for many detection platforms.

A high-throughput DNA hybridization is called an array. Currently, nucleic acids are arrayed on solid supports that are either glass slides or nylon membranes. Depending on the type of array, targets can be composed of oligonucleotides, PCR products, cDNA vectors, or purified inserts. The sequences on an array may represent entire genomes, which may include both known and unknown sequences, or they may be collections of sequences such as apoptosis-related genes or cytokines. Many premade and custom arrays are available from commercial manufacturers, although many labs prepare their own arrays with the help of robotic arrayers. The methods of probe labeling, hybridization, and detection depend on the solid support to which the sequences are bound. Typically, fluorescently labeled probes are used with glass arrays, whereas radiolabeled probes are used with membranes.

Many terms exist for naming gene arrays including biochip, DNA chip, GeneChip (Affymetrix, Inc., Santa Clara, CA, USA), DNA array, microarray, and macroarray. Generally the terms biochip, DNA chip, or GeneChip refer to an array on a glass support. The terms microarray and macroarray may be used to specify spot size and also the number of spots on the support. The term gene arrays

suggests sequence identification (e.g., mutation analysis) or differential expression analysis of two or more RNA samples. This discussion will focus on the use of arrays for expression analysis.

Nylon membrane arrays are typically hybridized with ^{33}P-dNTP labeled probes and analyzed using a phosphorimager and accompanying software. A different array must be used for each sample analyzed. A typical experiment involves isolating RNA from two tissue or cell culture samples. The RNAs are reverse transcribed with radioactively labeled nucleotides using oligo dT, target-specific, or random-sequence primers to create two separate, labeled cDNA populations. The two cDNAs are hybridized onto two identical arrays. After washing, the hybridization signal from each array is detected and analyzed. The signal emitted from each gene-specific spot is compared between the two arrays. Genes expressed at different levels in the two samples generate different amounts of labeled cDNA, which results in different levels of signal for corresponding spots on the arrays.

Glass slide array analysis involves the same steps used for nylon arrays, but rather than labeling with radioisotopes during reverse transcription, probes are labeled with two distinct fluorescently labeled nucleotides. Both probes are competitively hybridized to the same array. Typically, one RNA sample is labeled with cyanine 3-dNTP (Cy3) and the other with cyanine 5-dNTP (Cy5). Each dye fluoresces in a different color. After both RNA populations are hybridized to one glass slide, the array is scanned using a fluorescence imager.

Affymetrix's GeneChips are glass slide arrays manufactured using special photolithographic methods and combinatorial chemistry, which allows the oligonucleotide spots to be synthesized directly onto the array substrate. The analysis procedure specifies that the RNA samples be converted to biotin-labeled cDNA and that each sample be hybridized to a separate GeneChip. The hybridized cDNA is then stained with a streptavidin–phycoerythrin conjugate and visualized with an array scanner.

Luminex xMAP technology is also an array (see Fig. 27.6). Unlike other arrays, microspheres in suspension provide the solid support for probe binding. Therefore, Luminex xMAP technology is also known as a "liquid chip" or "suspension array." With xMAP technology, molecular reactions take place on the surface of color-coded beads called microspheres (Dunbar, et al. 2003). For each pathogen, target-specific capture probes are covalently linked to a specific set of color-coded microspheres. Labeled PCR products are captured by the bead-bound capture probes in a hybridization suspension. A microfluidics system delivers the

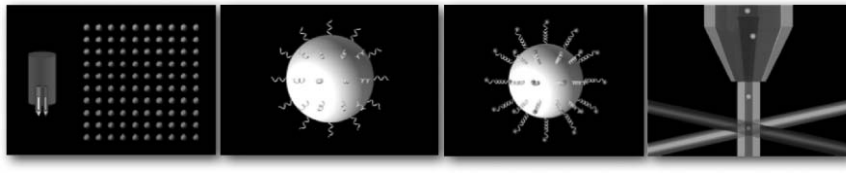


FIGURE 27.6. Basic concepts of Luminex xMAP technology platform.

suspension hybridization reaction mixture to a dual-laser detection device. A red laser identifies each bead by its color-coding, and a green laser detects the hybridization signal associated with each bead. Software is used to collect the data and report the results in a matter of seconds.

The platform is specific because only the probes that are captured by the beads are recognized by the green laser as signal. Any signal not associated with a specific set of color-coded beads is considered background. The platform is also very sensitive. Each bead has as many as 10^8 COOH groups on its surface for linking capture oligos. The green laser can detect the signal for as few as eight fluorescently labeled probes that are captured by a bead. Another important feature of the xMAP platform is its repeatability. Because everything occurs in a homogeneous solution (from bead manufacture, color-code staining, and capture probe coupling to product hybridization and data collection), highly repeatable results are obtained with this platform. The xMAP method for collecting and reporting data also contributes to repeatability. Typically, there are 5000 beads added per reaction for each color-coded bead set. Each bead set is specific for a particular disease marker, such as a mutation or a pathogen. The laser counts 100 microspheres from each bead set and reports the median fluorescent intensity (MFI). Thus, the data represents 100 microbead-associated data points, not just one data point produced by a standard array.

Sequencing-based methods and platforms provide the best specificity of all nucleic acid detection methods. However, abundant, pure templates must be generated first. The costs associated with reagents, instruments, and labor are relatively high. Sequencing is an essential technology for research laboratories, but it is usually too complicated and cost-prohibitive for routine clinical applications.

Examples of Technology Integration

It is not expected that one company, institution, or individual should develop a complete solution for all components of a molecular diagnostic process. Instead, it is more favorable for the best available methods and platforms to be combined, along with individual creativity, to realize the potential of molecular differential diagnosis. In the following section (see Table 27.4), there will be an evaluation of several companies that have created multiplex technologies and products with different strategies for technology integration, and there will be a discussion of the advantages and disadvantages of these strategies.

TABLE 27.4. Comparison of different methods/platform integration strategies.

Company	Amplification method/platform	Detection method/platform	Multiplex capability	Post-PCR clean-up	Posthybridization washes
Genaco	Templex	Luminex xMAP	Yes	No	No
TM Bioscience	PCR/TM100	Luminex xMAP	Yes	Yes	Yes
Roche	Real-time PCR	Light Cycler	Limited	No	No
Fluidigm	Real-time PCR	IFCs	Yes	No	No
Prodessa	PCR	ELISA	Yes/No	Yes	Yes
Maxim Biotech	PCR	Gel electrophoresis	Yes	No	No

Genaco

Genaco has developed the Templex technology for sensitive and specific amplification of multiple targets in one PCR reaction. For detection, Genaco uses the Luminex xMAP technology platform. Because of the high degree of specificity and sensitivity achieved with the Tem-PCR method, no post-PCR clean-up is required to remove the excess primers, and no posthybridization washes are needed to reduce the background. Templex products can be used directly for hybridization with the high-throughput xMAP platform, creating a system highly conducive to automation. This innovative technology integration creates many product opportunities. Later in this chapter, we will describe some of the panels that have already been developed.

TM Biosciences

TM Biosciences is a public Canadian company that develops DNA-based diagnostics. They use standard PCR as their amplification method with their core technology being the Tm100 Universal Array (http://www.universalarray.com/). Luminex xMAP technology is used for detection. TM Biosciences has developed a universal tag system for their bead array. Up to 1000 artificial oligonucleotides were designed and then coupled to the Luminex color-coded beads. Primers with universal tag sequences can be used for many amplification reactions such as PCR, LCR, primer extension reactions, and so forth. The amplification product can then be detected and differentiated by beads coupled with the anti-tag. Their core technology does provide some convenience in product development for scientists unfamiliar with using the Luminex bead array. Yet, their technology does not provide a significant improvement in multiplex amplification. TM Biosciences currently markets eight different Tag-It kits, including three cystic fibrosis kits, three drug metabolism kits, a hereditary disease kit, and a kit that detects mutations that are potentially associated with an increased risk of venous thromboembolism. However, lack of specificity and sensitivity at the amplification stage makes these products difficult to use. It is necessary to perform post-PCR clean-up and posthybridization washes, which complicate automation of these assays.

Roche

Roche is a leader in PCR technology that has developed the real-time PCR method. Real-time PCR is unique in that it integrates an amplification method (PCR) and a detection method (hybridization) with an all-in-one platform that combines a thermocycler with a detection instrument. The Roche real-time PCR method adds a probe directly into the PCR reaction to detect amplification products. This short probe is labeled with a fluorescent dye at one end and a quencher at the other. When in close proximity to the dye molecule, the quencher suppresses the fluorescent signal and inhibits its detection. When the probe binds to a PCR product, the

fluorescent dye is removed by DNA polymerase, which is also the enzyme used for PCR amplification. By doing so, the fluorescent signal is released from its quencher and detected by the instrument.

The advantage of the real-time PCR technology is its ability to quantitatively measure PCR products in real-time. When there is an increase in the PCR product being generated in a given cycle, there is a corresponding increase in the fluorescent signal being released. However, the real-time PCR is limited in its multiplexing capabilities. The limitation comes from both the methodology itself and the platform. From the methodology standpoint, multiplexing requires multiple sets of primers to function in the same reaction environment. These conditions demand a degree of compatibility that is difficult to achieve. Regarding the platform, multiplexing with real-time PCR requires that fluorescent probes have different colors. Moreover, the different colors must be detected by different means. As result of these limitations, developing multiplex panels with real-time PCR technology is very problematic.

Companies that have adopted the real-time PCR platform have already developed multiple kits for infectious disease diagnosis that test for HIV, HCV, and many other pathogens. A real-time PCR assay exists for almost all microorganisms. However, few high-quality multiplex products have been commercialized.

Fluidigm

Based on integrated fluidic circuits (IFCs), Fluidigm has developed MSL technology that brings real-time PCR technology to a higher level. Like their analog in the semiconductor industry, it is a network of tens of thousands of fluid-control valves and interconnected channels fabricated within a miniature device. The IFCs allow Fluidigm to run thousands of real-time PCR reactions at once in different fluidic systems with their own thermal-cycling profiles. However, the amplification template is not shared among the reaction chambers.

Prodesse

Prodesse has already developed multiplex assays for respiratory infections (Hindiyeh et al., 2001). They use the traditional multiplex PCR strategy as their amplification method and ELISA as their detection platform.

With traditional multiplex PCR, incompatible primer sets make it difficult to improve upon amplification efficiency. High background may be caused by a high concentration of labeled primers; therefore, a post-PCR clean-up step is necessary to remove the unused PCR primers in an effort to reduce hybridization background.

For signal detection, Prodesse uses a hybridization-based method. Their platform is borrowed from the immunoassay technology. Here, 96-well plates are coated with target-specific capture probes. Each well is specific for a particular

target. After clean-up, the multiplex PCR product is added into a number of wells (depending on how many targets are detected) for hybridization with the capture probes. After posthybridization washes for removal of nonspecific hybridization, streptavidin-conjugated horseradish peroxidase (HRP) is added to bind to the captured PCR products. After additional washes to remove the nonspecific enzyme binding, TMB (tetramethylbenzidine) substrate is added to HRP to generate a signal. Then, the 96-well plate is analyzed with a normal ELISA reader for signal detection.

Maxim Biotech

Maxim Biotech was once the leader in multiplex PCR. They have developed many products, including several infectious disease diagnostic products, such as HPV (for five different types), HIV, STD, and viral respiratory infections. Like Prodesse, Maxim Biotech does not have a unique multiplex PCR amplification method. Their detection platform of choice is gel electrophoresis.

They have no unique methodology for providing a solution to the problems associated with multiplex PCR. Instead, they used a trial-and-error approach to reduce the incompatibility among the primer sets, forcing the different primer sets to work under one amplification condition by adjusting primer lengths, sequences, and buffer conditions. Their detection method is the most traditional one: gel electrophoresis. Different gene products are identified by size. This method often causes laboratory contamination and produces false positives. It is also difficult to design multiple targets for compatibility in a multiplex reaction using this "trial-and-error" method. For example, in infectious diseases diagnosis the amplification primer selection is usually limited to short segments of DNA (or RNA). Outside these regions, the sequence may not be conserved enough, resulting in a reduced detection rate due to mispriming.

In general, there are many amplification and detection methods as well as platforms from which to choose. Of the available choices, PCR remains the most powerful amplification method, while hybridization is still the method of choice for easy detection. To obtain what we want (i.e., multiplexing that is specific, sensitive, simple, easily automated, and affordable), we must choose wisely. We can then integrate these systems to best serve clinical needs.

Application Examples: Systems for Syndromes

Molecular Differential Diagnostic System for Respiratory Infections

The development of multiplex PCR or RT-PCR assays for respiratory pathogens has been reported (Gröndahl et al., 1999). During the SARS outbreak of 2003, an MD^2 system was rapidly developed by Genaco and used in Beijing (Ma et al., 2004).

TABLE 27.5. List of pathogens amplified and detected by the Respiratory Infections I panel.

Pathogen	Targeted gene	Target symbol
Mycoplasma pneumoniae	MPN592	MPN
Legionella pneumophila	mip	LPN
Chlamydia pneumoniae	ompA	CPN
Neisseria meningitidis	ctrA	NMG
Streptococcus pneumoniae	lytA	SPN
Haemophilus influenzae (all strains)	ompP2	HINF1
H. influenzae (all typeable strains)	bexA	HINF2, HINF3
Adenovirus types 3, 7, 21	hexon	AVDB
Adenovirus type 4	hexon	ADVE
Adenovirus types 1, 2, 5, 6	hexon	ADVC
Acinetobacter baumannii	Rec A	ABM

Based on this panel and through collaborations with scientists at the CDC (Fields et al., 2005, personal communication), we have developed two MDD systems that, together, detect 23 pathogens that cause respiratory infections.

Tables 27.5 and 27.6 list the pathogens identified by the MDD systems for Respiratory Infections I and II. We group these pathogens into two panels: the first panel includes pathogens with DNA genomes, and the second panel includes viruses with RNA genomes. Amplification for the DNA panel needs only a PCR step; whereas the RNA panel needs a one-step or two-step RT-PCR reaction before undergoing Templex PCR. Dean et al. (2005) reported that, if a random-primed reverse transcription step is added before performing Templex, the sensitivity is at, or close to, that of singleplex real-time PCR. If however, a one-step RT-PCR enzyme system is used for Templex PCR, the sensitivity is about two logs less than that of a comparable singleplex real-time PCR reaction. For the MDD system for Respiratory Infections II (the DNA panel), Templex PCR is also less sensitive compared with the real-time PCR method (Fields et al., 2005, personal communication).

TABLE 27.6. List of pathogens amplified and detected by the Respiratory Infections II panel.

Pathogen	Targeted gene	Target symbol
SARS CoV	N, pol	SARS
Influenza A	NS	INFA
Influenza B	NS	INFB
Respiratory syncytial virus A	NS	RSVA
Respiratory syncytial virus B	NS	RSVB
Parainfluenza 1	N	PIV1
Parainfluenza 2	N	PIV2
Parainfluenza 3	N	PIV3
Parainfluenza 4	N	PIV4
Human metapneumovirus	F	hMPV
Rhinoviruses	5′ UTR	RhV
Coxsackie viruses & echoviruses	5′ UTR	CVEV

TABLE 27.7. Detection results of the Respiratory Infections I panel.

		Detectable pathogens										
No.	Samples	MPN	LPN	CPN	NMG	SPN	HINF1	HINF2	ADVB	ADVE	ADVC	ABM
1	MPN	**1294**	36	44	44	48	71	50	98	52	67	59
2	LPN	51	**4016**	52	43	42	47	33	45	47	55	79
3	CPN	42	34	**769**	45	36	73	41	52	46	36	54
4	NMG	51	30	37	**1006**	38	63	35	54	35	60	50
5	SPN 19f	39	34	40	54	**5299**	74	34	39	39	40	66
6	H.Inf a	43	45	35	26	45	**522**	40	35	39	49	56
7	H.Inf f	25	34	38	43	33	**897**	**1835**	38	43	46	43
8	ADV 3	17	21	38	26	29	42	34	**1226**	29	44	43
9	ADV 4	86	22	37	32	20	47	28	25	**641**	41	34
10	ADV 2	44	49	54	36	23	57	57	33	37	**882**	48
11	ADV 5	44	18	35	36	40	37	29	44	47	**798**	54
12	ABM	57	49	44	53	38	36	58	84	53	49	**829**
13	PCR blank	36	31	26	35	30	54	27	28	36	32	54
	Cutoff	250	250	250	250	250	250	250	250	250	250	250

* Boldface indicate values above cut-off.

Tables 27.7 and 27.8 show sample data generated by the two panels. Pathogen nucleic acid was isolated from ATCC strains or provided by the CDC. Each column indicates a pathogen target, and each row represents a sample. The numbers in the MFI table are (Medium Fluorescent Intensity) values obtained from a Luminex 100 machine. Positive results are indicated in bold. The theoretical cutoff for each target is the mean of the negative results plus five times the standard deviation. To ensure high assay specificity, we suggest using a universal cutoff of 250 MFI for all targets. Highly specific results were obtained without the denaturation of PCR products. No post-PCR clean-up or posthybridization washes were necessary. In general, the single-tube Templex PCR reaction is complete in about 3.5 h. After performing the multiplex PCR, 5 μL of PCR product is mixed with color-coded beads (covalently coupled with pathogen target-specific capture probes) and hybridized for 15 min at 52°C. Streptavidin-PE is added to introduce a fluorescent label to the PCR products and then hybridized for another 10 min. A stopping buffer is added before analysis with the Luminex instrument. The entire procedure takes less than 5 h, which includes a handling time of less than 30 min.

Fever and coughing are common symptoms of acute respiratory infections. These symptoms can be caused by either bacterial or viral infections. By using Genaco MDD systems for initial patient screenings, the appropriate antibiotics can be prescribed, thus preventing the proliferation of drug-resistant bacteria.

Molecular Differential Diagnostic System for Health Care–Associated Infections

According to the Centers for Disease Control and Prevention (CDC), 90,000 Americans die from health Care–associated infections (HAIs) and another

TABLE 27.8. Detection results of the Respiratory Infections II panel.

		Detectable pathogens												
No.	Samples	SARS1	SARS2	INFA	INFB	RSVA	RSVB	PIV1	PIV2	PIV3	PIV4	hMPV	RhV	CVEV
1	SARS	**3140**	**4180**	14	21	21	18	17	19	23	29	12	22	24
2	INFA	13	15	**2573**	11	11	13	30	33	16	23	19	22	18
3	INFB	21	12	9	**3659**	24	9	17	55	24	22	10	15	16
4	RSVA	11	35	11	16	**4042**	11	25	34	14	27	8	20	16
5	RSVB	19	11	1	8	25	**5027**	22	20	22	30	12	23	19
6	PIV-1	8	15	4	18	30	11	**4450**	57	39	11	16	12	16
7	PIV-2	19	26	20	16	44	24	16	**5137**	11	28	5	23	53
8	PIV-3	10	11	9	12	21	9	59	41	**4452**	36	0	15	15
9	PIV-4	14	23	24	26	6	41	21	16	54	**5819**	16	11	22
10	hMPV A	13	17	17	28	11	21	19	20	30	20	**2127**	4	15
11	Rhinovirus	17	88	8	30	23	19	49	38	28	23	16	**1061**	27
12	CVA	14	12	9	16	20	0	21	35	23	57	21	17	**3128**
13	CVB	12	9	6	19	16	13	20	62	26	20	8	13	**3066**
14	Echovirus	23	14	6	31	25	13	8	27	20	8	7	20	**1623**
15	RT-PCR blank	7	12	18	1	16	16	23	24	30	33	26	10	13
	Cutoff	250	250	250	250	250	250	250	250	250	250	250	250	250

*Boldface indicate values above cut-off.

TABLE 27.9. List of pathogens amplified and detected by the HAI I system.

Pathogen	Targeted gene	Target symbol
Acinetobacter baumannii	N, pol	ABM
Chlamydia pneumoniae	ompA	CPN
Enterobacter cloacae	ampC	EBC
Enterococcus faecalis	ddl	ENTFLS
Enterococcus faecium	ddl	ENTFCM
Escherichia coli	eac	ECL
Klebsiella pneumoniae	khe	KPN
Mycoplasma pneumoniae	MPN592	MPN
Proteus mirabilis	Topoiso	PROM
Pseudomonas aeruginosa	algD	PMA
Streptococcus pneumoniae	lytA	SPN
Streptococcus pyogenes	mf	SPYG
Staphylococcus aureus	nuc	STP
Staphylococcus aureus (MRSA)	mecA	STPM
Integron 5′	Integron	INT

1.9 million suffer from nosocomial infection-related illnesses each year. HAIs add an estimated 5 billion annually to the nation's health care spending bill because of the additional hospitalization and treatment required for infected patients.

In 2002, Illinois became the first state to pass a law requiring hospitals to report the rate at which their patients develop nosocomial infections. Since then, the Pennsylvania Healthcare Cost Containment Council approved a plan for infection rate reporting. More recently, Florida, Missouri, and Virginia adopted similar disclosure requirements. Currently, more than 30 states are considering similar regulations.

Tables 27.9 and 27.10 list the pathogens that can be detected using Genaco MDD systems for HAI I and II. Together, the two systems can detect 25 microorganisms

TABLE 27.10. List of pathogens amplified and detected by the HAI II system.

Pathogen	Targeted gene	Target symbol
Haemophilus influenza	ompP2	H.inf
Candida albicans	rRNA	C.alb
Candida tropicalis	rRNA	C.trop
Candida parapsilosis	rRNA	C.para
Candida krusei	rRNA	C.kru
Candida glabrata	rRNA	C.glab
Aspergillus flavus	rRNA	Asper.
Aspergillus niger	rRNA	Asper.
Rhizopus microsporus	rRNA	Rhizo
Mucor fragilis	rRNA	Mucor
Mucor hiemalis	rRNA	Mucor
Fusarium tricinctum	rRNA	Fusa

common in HAI. In addition to detecting common HAI pathogens, the MDD system for HAI I also detects two targets related to drug resistance: the *mecA* gene and Class I integron. The *mecA* gene is specific for methicillin-resistant *Staphylococcus aureus* (MRSA). The Class I integron is commonly seen in Gram-negative bacteria that have resistance to multiple antibiotics.

Tables 27.11 and 27.12 contain sample data generated with the MDD systems for HAI I and II. Similar to the previous panel, each column indicates a pathogen target, and each row represents a sample. The numbers in the table are MFI values obtained from a Luminex 100 machine. Positive results are indicated in bold.

Molecular Differential Diagnostic System for Human Papillomavirus Typing

Human papillomavirus (HPV) is known to cause cervical cancer, the second most common cancer among women worldwide. In the United States, HPV is responsible for approximately 13,000 new cases of cancer and 4500 deaths each year. A Pap test only indicates the possibility of cervical cancer. For more direct disease diagnosis, the MDD system for HPV Typing I accurately detects different HPV subtypes. When detected early, cervical cancer is one of the most successfully treated cancers.

Type-specific DNA diagnosis is important for disease prevention, prognosis, and treatment. Until now, an efficient method for HPV typing was not available. The FDA-approved Digene HPV DNA diagnostic product can only identify HPV infections as high-risk or low-risk groups. Coinfection by multiple HPV types is likely to occur in more than 30% of HPV patients (Swan et al., 2005, personal communication). Certain combinations of these co-infections may be more prone to cause cancer than others. Information regarding HPV co-infections can be an essential part of determining treatment options and immunotherapy. The Genaco product identifies the 25 most common HPV subtypes, including 21 high-risk HPV types (16, 18, 31, 33, 35, 45, 51, 52, 56, 58, 59, 68, 26, 53, 66, 67, 69, 70, 73, and 82). It also detects four low-risk HPV types (6, 11, 42, 44). Table 27.13 shows sample data. DNA samples were provided by Drs. Unger and Swan from the CDC. HPV types in each sample were confirmed by standard sequencing method.

Typically, a single pair of degenerate primers is used in PCR to amplify the conserved L1 gene region (Yoshikawa et al., 1991; Kornegay et al., 2001; Wallace et al., 2005). With the Templex technology, Genaco MDD system for HPV Typing I includes more than 100 primers in the multiplex reaction for the amplification of type-specific sequences from the E6 and E7 genes region. The last column in Table 27.14 is the internal positive control (IPC) that detects for a human gene located on the X chromosome.

Molecular Differential Diagnostic System for Encephalitis

Encephalitis is most often caused by viral infections, including herpesviruses and arboviruses, and is common among children, the elderly, and those with

TABLE 27.11. Detection results of the HAI I system.

		Detectable pathogens														
No.	Samples	ABM	STP	STPM	SPYG	SPN	PMA	KPN	ENTFLS	ENTFCM	ECL	EBC	CPN	MPN	PROM	INT
1	*A. baumannii*	**4291**	53	44	47	48	42	59	46	50	44	50	46	48	57	52
2	*S. aureus (MRSA)*	67	**2519**	**3315**	56	36	49	63	64	46	42	64	54	75	53	55
3	*S. aureus*	43	**2134**	55	42	35	40	45	28	60	41	41	37	49	36	39
4	*S. pyogenes*	54	47	58	**3705**	34	45	45	45	52	33	48	46	47	53	48
5	*S. pneumoniae*	53	38	43	28	**5803**	46	47	34	37	34	45	25	50	47	46
6	*P. aeruginosa*	64	46	42	77	43	**1042**	46	34	51	31	20	39	38	45	38
7	*K. pneumoniae*	76	50	48	57	65	50	**1424**	49	51	30	49	51	52	60	**4029**
8	*E. faecalis*	45	40	42	48	43	41	46	**3311**	40	32	31	35	25	54	50
9	*E. faecium*	60	40	52	49	44	49	46	35	**1623**	44	43	32	64	48	41
10	*E. coli*	65	54	35	48	40	47	42	38	44	**4087**	44	45	36	30	45
11	*E. cloacae*	60	41	42	36	41	45	49	39	41	42	**1043**	40	37	55	46
12	*C. pneumoniae*	56	47	43	40	39	42	48	40	38	37	32	**3398**	49	42	48
13	*M. pneumoniae*	54	51	40	41	41	23	31	38	38	22	35	35	**4203**	53	22
14	*P. mirabilis*	52	38	28	35	46	35	24	46	26	51	32	32	45	**2953**	45
15	PCR blank	57	38	35	49	38	44	43	40	35	33	32	36	47	52	47
	Cutoff	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250

*Boldface indicate values above cut-off.

TABLE 27.12. Detection results of the HAI II system.

No.	Samples	Detectable pathogens									
		H.inf	C.alb	C.trop	C.para	C.kru	C.glab	Asper.	Rhizo	Mucor	Fusa
1	*H. influenzae*	**925**	24	17	26	27	25	23	21	22	39
2	*C. albicans*	49	**655**	47	52	47	59	49	50	33	42
3	*C. tropicalis*	46	63	**3519**	55	48	46	59	43	30	62
4	*C. parapsilosis*	59	49	36	**2716**	33	47	41	44	28	58
5	*C. krusei*	43	38	26	41	**4221**	49	47	39	41	40
6	*C. glabrata*	57	46	42	41	31	**3010**	43	32	38	28
7	*A. flavus*	44	30	22	29	26	44	**1037**	31	26	**792**
8	*A. niger*	36	38	39	25	40	40	**670**	41	42	**536**
9	*R. microsporus*	32	39	33	38	28	43	41	**507**	28	31
10	*M. hiemalis*	51	54	42	46	81	81	61	51	**629**	39
11	*F. tricinctum*	59	58	49	59	77	64	101	58	57	**3337**
12	PCR blank	52	41	31	46	43	45	50	42	36	40
	Cutoff	250	250	250	250	250	250	250	250	250	250

*Boldface indicate values above cut-off.

compromised immune systems. Today, there is an increasing concern over the threat of an encephalitis outbreak. In fall 1999, the first recognized outbreak of West Nile virus (WNV) in the United States occurred in New York City. Initially, it took weeks before the pathogen was identified. The spread of the virus in 2002 resulted in the largest arboviral outbreak ever recorded in the Western Hemisphere. WNV activity was reported in 44 U.S. states and in 5 Canadian provinces, and the outbreak resulted in more than 4500 human cases and 288 deaths.

The course of this illness is unpredictable. Generally, the initial symptoms are flu-like, and, with other methods, diagnosis is difficult and often only successful with the onset of full-blown encephalitis. Therefore, quick and accurate diagnosis is crucial. Genaco has developed a molecular differential diagnostic system for viral encephalitis that can help pinpoint the source of the infection and provide physicians and public health officials with a better means of managing this disease.

Tables 27.14 and 27.15 show the pathogen list and some sample data. Samples were obtained from American Type Culture Collection (ATCC).

Molecular Differential Diagnostic System for Food-Borne and Diarrheal Diseases

Food-borne and diarrheal diseases are the most common infectious diseases. An estimated 76 million cases of food-borne disease occur each year in the United States. The CDC estimates that, annually, there are 325,000 hospitalizations and 5000 deaths related to food-borne diseases. The CDC established the Food-borne Diseases Active Surveillance Network (FoodNet) to serve as the principal food-borne disease component of their Emerging Infections Program (EIP). Pathogens actively monitored by FoodNet include *Salmonella, Shigella, Campylobacter, E. coli* O157, *Listeria, Yersinia,* and *Vibrio*.

TABLE 27.13. Detection of 25 HPV types with MD^2 system.

Samples	T16	T18	T26	T31	T33	T35	T39	T45	T51	T52	T53	T56	T58	T59	T66	T67	T68	T69	T70	T73	T82	T6	T11	T42	T44	IPC
11209 (16)	**261**	40	32	38	50	43	35	36	28	29	30	36	35	40	32	35	44	40	27	50	31	35	38	44	32	**1807**
12156 (18)	33	**1536**	20	31	33	28	36	60	16	38	23	31	30	22	25	32	27	37	28	22	29	25	39	21	27	**1406**
12107 (18,35,51,26,82)	33	**928**	**3441**	31	49	**1024**	30	28	**583**	35	29	72	36	34	27	32	33	15	22	29	**4104**	43	40	68	37	**1267**
12334 (31)	23	28	17	**178**	38	41	36	32	38	37	31	32	25	34	26	27	26	30	240	24	32	32	38	30	32	**1557**
12472 (33,39)	35	46	34	45	**1564**	42	**283**	29	46	38	28	45	40	40	29	39	40	41	38	29	35	27	36	33	38	**1325**
11743 (35)	38	22	25	30	35	**515**	31	29	30	36	27	29	28	28	19	32	27	33	44	32	30	28	37	27	34	**794**
11612 (39)	32	30	21	37	51	33	**557**	35	33	38	29	28	36	43	34	37	29	32	31	29	21	31	41	27	32	**762**
12665 (45)	28	76	31	37	33	31	41	**2247**	32	35	34	30	31	23	42	29	28	26	27	18	32	31	37	30	28	**1445**
10342 (51)	37	41	23	102	40	44	30	30	**1293**	37	32	32	27	26	27	21	29	31	41	29	29	34	40	36	33	**843**
11234 (52)	32	28	**869**	25	38	32	32	24	30	**2028**	28	22	23	21	19	27	26	23	40	40	19	37	33	31	23	**1179**
12458 (53)	31	40	32	27	42	42	30	29	46	38	**347**	25	33	35	26	25	30	36	34	23	40	49	42	43	35	**819**
10308 (56)	42	57	25	29	46	36	32	29	39	53	31	**2806**	35	26	27	29	34	23	37	34	37	37	42	36	32	**1713**
11241 (45,58)	41	35	22	30	36	29	27	**279**	27	26	32	28	**2365**	25	23	22	31	29	33	20	36	101	38	27	34	**970**
11734 (59,44)	38	34	28	46	42	39	39	30	25	28	28	26	33	**1202**	25	28	26	31	29	37	35	30	40	35	**321**	**1438**
11837 (56,66)	36	64	25	40	36	41	35	32	36	41	27	**1330**	40	26	**2349**	22	24	12	31	59	42	39	47	42	35	**1628**
10787 (67)	22	43	28	31	47	35	27	44	37	36	27	26	32	42	30	**3448**	30	43	29	22	36	38	29	37	30	**600**
12506 (68)	42	30	21	30	31	38	42	28	27	24	34	29	29	16	21	37	**1305**	33	40	18	33	36	32	35	26	**1200**
12113 (18,56,69)	38	**1277**	21	38	172	32	28	42	23	35	37	**2418**	30	34	32	28	28	**2898**	44	98	25	24	40	26	32	**1332**
11214 (70)	15	37	17	19	29	32	22	19	19	17	35	14	20	13	17	11	17	12	**1856**	23	26	32	29	18	82	**1736**
12601 (73)	35	35	19	33	36	32	27	20	23	21	29	24	35	23	24	28	27	22	38	**1108**	27	30	35	29	29	**832**
11797 (82)	43	31	31	23	43	35	30	29	32	174	18	32	32	24	26	26	32	21	34	40	**4025**	25	26	26	27	**1356**
12929 (6)	25	42	35	30	41	33	33	35	31	35	25	29	30	28	40	35	35	19	40	33	28	**3430**	34	40	39	**357**
12361 (11)	20	23	29	24	47	30	27	20	28	29	34	23	32	34	23	27	29	30	18	37	11	29	**480**	32	29	**536**
11220 (56,42)	30	31	28	27	44	40	37	16	31	32	16	**769**	29	29	24	21	30	20	27	18	21	177	24	**1493**	22	**1411**
10269 (44)	25	31	23	24	36	31	28	27	29	30	32	35	32	25	30	26	31	17	42	41	36	189	43	40	**2970**	**1030**
PCR blank	29	28	23	15	42	31	24	20	21	27	32	24	33	22	18	25	25	23	28	18	20	34	31	25	23	23
Cutoff	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250	250

*Boldface indicate values above cut-off.

TABLE 27.14. List of pathogens amplified and detected by the Encephalitis I system.

Pathogen	Targeted gene	Target symbol
Dengue type 1	envelope	Den1
Dengue type 2	envelope	Den2
Dengue type 3	envelope	Den3
Dengue type 4	envelope	Den4
Coxsackie viruses & echovirus	5′ UTR	CVEV
Rhinoviruses	5′ UTR	RhV
Herpes simplex virus 1	UL5	HSV1
Herpes simplex virus 2	UL5	HSV2
Japanese encephalitis virus	NS	JE
West Nile virus	NS	WN

Tables 27.16 and 27.17 display the pathogen list and some sample data. DNA samples were extracted from ATCC strains.

Molecular Differential Diagnostic System for TB Drug-Resistance Mutations

According to the World Health Organization (WHO), tuberculosis affects 7.5 million people and kills as many as 2.5 million people each year, making TB the leading cause of death by an infectious disease. TB is extremely prevalent among people with AIDS. According to the WHO, *Mycobacterium tuberculosis* infects 33% of the entire human population. Approximately 10% of newly identified cases have resistance to at least one anti-tuberculosis drug. The statistics for previously treated cases indicate that the average occurrence of resistance toward at least one drug is 18%, with some areas, such as Kazakhstan, presenting drug resistance in as many as 82% of its cases. Quick diagnosis of TB infection and identification of drug resistance is essential for disease control and treatment.

TABLE 27.15. Detection results of the Encephalitis I system.

No.	Sample	Den1	Den2	Den3	Den4	CVEV	RhV	HSV1	HSV2	JE	WN
1	Dengue 1	**674**	18	32	19	23	29	13	20	21	27
2	Dengue 2	23	**1048**	22	20	19	28	30	13	17	25
3	Dengue 3	9	26	**687**	30	25	11	13	18	16	36
4	Dengue 4	27	18	35	**907**	27	22	33	27	23	27
5	Echovirus 30	24	17	37	20	**3125**	23	24	23	12	54
6	Rhinovirus	11	4	27	16	12	**1766**	8	21	15	36
7	HSV1	31	35	33	29	30	27	**958**	49	23	31
8	HSV2	21	30	23	19	41	26	33	**970**	19	28
9	JE	32	20	33	32	26	31	30	26	**605**	41
10	RT-PCR blank	18	9	27	27	16	20	16	28	29	33
	Cutoff	250	250	250	250	250	250	250	250	250	250

TABLE 27.16. List of pathogens identified by the Food-borne and Diarrheal Diseases system.

Pathogen	Targeted gene	Target symbol
E. coli (0157)	rfbE	ECLI1
E. coli (EPEC)	eae	ECLI2
Salmonella	invA	SALM
C. jejuni	ceuE	CAMP
Listeria	actA	LIST
Shigella (all four subgroups)	ipaD	SHIG1
Shigella flexneri 2a	ipaH	SHIG2
Yersinia (pathogenic serotypes)	ail	YERS

Table 27.18 shows the 25 mutations that can be detected with the Genaco MDD system for TB drug-resistance mutations. Table 27.19 shows some results of allelic differentiation. For each allele within the locus, the MFI is obtained and the allelic specific MFI percentage is calculated. In general, a positive result is determined if the allelic specific MFI percentage is above 25%. For other loci with less alleles, the cutoffs could be higher.

Molecular Differential Diagnostic System for Gram-Positive Cocci in Clusters

Rapid identification of methicillin-resistant *Staphylococcus aureus* (MRSA) is critical for the effective treatment of patients and to control the spread of the pathogen (Farr et al., 2001). An ideal MDD test should be able to distinguish coagulase-positive *Staphylococcus* (*Staphylococcus aureus*) from coagulase-negative *Staphylococcus* (CoNS); methicillin-sensitive *Staphylococcus aureus* (MSSA) from MRSA; and hospital-acquired MRSA (HA-MRSA) from community-acquired MRSA (CA-MRSA). An added benefit would be if such a test could identify some of the most common drug-resistance genes in the same assay.

Multiplex PCR assays have been developed to perform such molecular differential diagnosis. For example, Sakai et al. in 2004 reported the use of real-time PCR

TABLE 27.17. Example results of Food-borne and Diarrheal Diseases system.

No.	Sample	ECLI1	ECLI2	SALM	CAMP	LIST	SHIG1	SHIG2	YERS
1	*E. coli*	0	**2874**	38	48	74	37	0	0
2	*E. coli*	**4162**	**3035**	20	75	31	43	29	55
3	*Salmonella*	92	44	**3779**	75	34	43	32	27
4	*C. jejuni*	115	40	24	**2636**	37	40	34	19
5	*Listeria* 4b	142	49	33	86	**3199**	68	65	82
6	*Shigella*	136	69	45	78	51	**2635**	**2887**	50
7	*Yersinia*	107	59	50	73	56	48	34	**2883**
8	Blank	110	57	39	69	48	43	29	26
	Cutoff	250	250	250	250	250	250	250	250

*Boldface indicate values above cut-off.

TABLE 27.18. Detectable mutations that responsible for TB drug resistance.

Drug	Target gene	Codon change	AA changes
Isoniazid	katG	AGC -> ACC	S315T
	katG	AGC -> AAC	S315N
	katG	AGC -> ATC	S315I
	katG	AGC -> AGG	S315R
	katG	AGC -> GGC	S315G
	inhA	C->T @-15	N/A
	kasA	GGT -> AGT	G269S
	ndh	CGC -> TGC	R268H
Rifampin	rpoB	DGAC	DD@516
	rpoB	GAC -> GTC	D516V
	rpoB	CAC -> TAC	H526Y
	rpoB	CAC -> GAC	H526D
	rpoB	CAC -> CGC	H526R
	rpoB	CAC -> CCC	H526P
	rpoB	TCG -> TGG	S531W
	rpoB	TCG -> TTG	S531L
Streptomycin	rpsL	AAG -> AGG	K43R
	rrs	491C-T	
	rrs	512C-T	
	rrs	513A-T	
	rrs	516C-T	
Ethambutol	embB	ATG -> ACA	M306I
	embB	ATG -> ACC	M306I
	embB	ATG -> ACT	M306I
	embB	ATG -> GTG	M306V

for the simultaneous detection of *Staphylococcus aureus* and coagulase-negative *Staphylococci* in positive blood cultures. Louie et al. (2002) reported the development of a multiplex PCR assay that identifies three genes (*nuc, mecA,* and bacterial 16S rRNA genes) for the differentiation of MSSA and MRSA. Samples were collected from blood culture bottles, and PCR products were analyzed using gel electrophoresis. Francois et al. (2004) described a multiplex PCR assay that can discriminate between CA-MRSA and HA-MRSA. Strommenger et al. (2003) reported the development of a multiplex PCR assay for simultaneous detection of nine clinically relevant antibiotic resistance genes in *Staphylococcus aureus*.

Table 27.20 lists the molecular targets in an MDD system that Genaco recently developed. Using Templex technology to amplify 18 molecular targets, the system can identify and differentiate among CoNS (identify four most common CoNSs), MRCoNS (methicillin-resistant CoNS), MSSA, MRSA, CA-MRSA, and HA-MRSA. In addition, the system can also detect six common drug-resistance genes (*mecA, aacA, ermA, ermC, tetM*, and *tetK*).

Table 27.21 shows the results of some sample data.

TABLE 27.19. Results of the TB drug-resistance mutation detection.

Sample	katG315w	(%)	katG315m	(%)	katG315Gm	(%)	katG315Im	(%)	katG315Nm	(%)	katG315Rm	(%)	Total	(%)
1	**3370**	**48**	611	9	1391	20	442	6	553	8	581	8	6948	100
2	**3919**	**46**	765	9	1706	20	612	7	728	9	787	9	8517	100
3	**3066**	**46**	578	9	1395	21	459	7	603	9	618	9	6719	100
4	**3211**	**46**	606	9	1449	21	455	7	596	9	622	9	6938	100
5	1490	21	**2991**	**42**	299	4	913	13	1366	19	94	1	7152	100
6	1608	21	**3108**	**41**	296	4	1035	14	1425	19	88	1	7560	100
7	1505	19	**3394**	**44**	253	3	1090	14	1419	18	96	1	7757	100
8	1485	20	**3308**	**44**	244	3	1034	14	1338	18	95	1	7504	100
9	524	16	58	2	**2453**	**76**	60	2	76	2	64	2	3234	100
10	1670	24	1371	20	313	5	**2553**	**37**	860	12	121	2	6888	100
11	1306	21	608	10	209	3	1139	18	**2861**	**46**	84	1	6206	100
12	1856	21	976	11	413	5	1597	18	**3725**	**43**	155	2	8721	100
13	575	16	57	2	159	4	41	1	50	1	**2675**	**75**	3557	100
Blk	43	18	37	16	24	10	42	18	35	15	56	24	236	100
Blk	48	17	38	14	49	18	41	15	48	17	51	19	275	100

TABLE 27.20. List of gene targets identified with the GPCC system.

Pathogens	Classification	tuf1	tuf2	tuf3	tuf4	tuf5	nuc	mecA	ccrBI	ccrBII	ccrBIII	ccrBIV	pvl	aacA	ermA	ermC	tetM	tetK	IDS
Coagulase-negative *Staphylococcus*	S. epidermidis	+		+															+
	S. haemolyticus		+	+															+
	S. homins			+	+														+
	S. lugdunensis			+		+													+
	CoNS			+															+
	MRCoNS	±	±	+	±	±		+											+
Staphylococcus aureus	MSSA						+												+
	MRSA						+	+	±	±	±	±	±						+
	HA-MRSA						+	+	±	±	±								+
	CA-MRSA						+	+				+	+						+
Class	**Resistance**																		
Penicillin	Methicillin							+											+
	Oxacillin							+											+
Aminoglycoside	Gentamicin													+					+
	Arbekacin													+					+
	Tobramycin													+					+
	Kanamycin													+					+
	Amikacin													+					+
Macrolide–lincosamide–streptogramin	Erythromycin														+	+			+
	Clindamycin														+	+			+
	Quinupristin–dalfopristin														+	+			+
Tetracycline	Tetracycline																+	+	+
	Minocycline																+	+	+

+, must be positive; ±, may be positive.

TABLE 27.21. Example data for the GPCC system.

Sample	nuc	mecA	ccrBI	ccrBII	ccrBIII	ccrBIV	pvl	ermA	ermC	tetM	tetK	aacA	tuf1	tuf2	tuf3	tuf4	tuf5	IDS	Results
1	116	73	77	80	74	82	114	123	83	79	78	52	**1331**	229	**2306**	63	55	92	CoNS (*S. epidermidis*)
2	153	**2875**	129	70	75	82	63	87	70	74	77	56	87	111	**1451**	66	96	95	MR-CoNS
3	80	**2693**	71	85	81	**2102**	65	102	**4016**	73	78	43	**1780**	128	**1468**	56	78	97	MR-CoNS, ermR (*S. epidermidis*)
4	**1953**	56	48	47	58	54	54	60	52	49	41	38	38	**2487**	128	49	58	67	MSSA
5	**891**	62	57	46	50	54	50	**1392**	51	58	51	26	47	**1099**	53	37	68	61	MSSA, ermR
6	**1115**	**1985**	93	**900**	85	93	79	**2418**	96	87	98	42	62	**1625**	104	95	84	119	HA-MRSA, ermR
7	**643**	**2296**	**1759**	62	65	70	48	77	68	**3416**	**2601**	54	55	**1335**	94	65	76	79	HA-MRSA, tetR
8	**478**	**1840**	103	109	**3584**	108	91	**1924**	100	**3134**	117	169	94	**763**	111	95	52	130	HA-MRSA, ermR, tetR
9	**655**	**1470**	65	66	60	**468**	58	80	**4755**	75	70	42	51	**1097**	80	52	75	73	CA-MRSA, ermR
10	**382**	**1392**	84	113	87	**375**	**268**	114	90	87	**1361**	55	74	**1185**	119	101	49	113	CA-MRSA, pvl+, tetR
NC	69	87	86	85	82	79	66	104	80	79	95	47	73	84	89	81	67	111	Negative control
PC	92	93	82	99	79	83	58	99	79	80	85	64	66	98	102	77	88	**4801**	Positive control

*Boldface indicate values above cut-off.

Benefits and Impact: Delivering Value Through Reducing Cost and Saving Lives

The advances of genomic technology have changed the way we define diseases by transforming the definition from a phenotypic, syndromatic description of clinical presentations to a genotypic, molecular classification of underlying causes. Molecular differential diagnosis has become the hallmark of the 21st century medical practice.

Every infectious disease starts with an invasion by a microorganism's genetic material into the human body. The expression of pathogen genes inside human cells can interrupt normal cellular function and induce systematic responses or clinical syndromes. The goal for infectious disease molecular differential diagnosis is to investigate all possible causes of a common clinical syndrome and identify the offending pathogen. To achieve this goal, we need a multiplex technology that uses one sample, one test, one technician, one machine, and a small amount of time to obtain multiple answers.

Molecular differential diagnosis is necessary for controlling an outbreak, such as avian flu or SARS. A simple mathematical model shows that if one person contracts avian flu, it will likely be transfered to three additional people in four days. After 32 days, there will be 6561 people sick with the disease, increasing the probability that the disease will spread to even more people worldwide. After 4 weeks, it is virtually unstoppable. By this model, 4 weeks is the window of opportunity for public health professionals to contain a disease. With an early and accurate differential diagnosis, infected patients can be identified, isolated, and treated. In addition, the general population can be informed and protected.

The multiplexing capability of MDDs can also benefit diseases prevention by providing the most complete epidemic information. The application of an HPV or influenza-A typing assay, for example, will help to determine which strains or types should be targeted by vaccines.

Antibiotic treatment depends even more on molecular differential diagnosis. With MDDs, we can identify not only the pathogen but also the drug resistance. This information is critical for aiding physicians in selecting the right treatment options for patients. With MDDs, personalized medicine becomes a reality. MDDs also directly link diagnosis with treatment. With proper diagnosis, older, inexpensive antibiotics may also still be used to treat patients effectively. Under prolonged exposure to antibiotics, bacteria often acquire resistance capabilities by gaining additional genes to modify or inhibit a drug's effectiveness. Also, bacteria may mutate a gene to avoid being targeted by a drug. As a trade-off, these genetic changes may be associated with reduced survival fitness (Wichelhaus et al. 2002). Therefore, when the selective pressure is removed by withdrawing the antibiotic from the market, the bacteria may revert back to wild-type status and regain survival potential. This may once more render the bacteria vulnerable to the old antibiotic. So, instead of constantly battling bacteria by developing ever more expensive antibiotics, it may make sense to invest in "battlefield intelligence" (i.e., a molecular

differential diagnostic tool) to better guide the treatment. Resistance is caused by the misuse of antibiotics, and lack of proper diagnosis is the reason for such misuse. Therefore, accurate diagnosis could reduce resistance while improving treatment.

MDDs are an exciting method that brings revolutionary changes to many aspects of medical practice, especially to infectious diseases management. First, it changes the way an infectious disease doctor treats a patient. Instead of waiting days for culture results, a doctor can now act based on a comprehensive molecular diagnosis. Instead of guessing what may be the offending pathogen, a doctor can identify the microorganism with confidence. Instead of ordering the blood cultures to gain knowledge for future "empirical" treatment, a doctor can prescribe the test to seek immediate solutions. Instead of offering antibiotics to put families or parents (and sometimes the doctor) at ease, a doctor can now provide the accurate treatment to improve a patient's condition.

Second, MDDs will change the way hospitals operate. Hospitals can implement MDDs as an active surveillance measure to prevent HAIs. Many studies have shown that active surveillance, plus patient isolation, is one of the most effective methods to reduce HAIs (Farr, and Bellingan, 2004). Regularly scheduled surveys of critical environments (such as the ICU), instruments, and health care providers will raise the level of awareness and identify problems early. When an outbreak of HAI occurs, MDDs can quickly identify the source of an infection, helping health care providers determine which patients should be isolated to prevent the spread of the microorganisms. In an increasing number of states, hospitals are required to publish their rate of HAI. The rate is calculated based on discharge records. However, some patients may be misclassified as having a HAI because they were asymptomatic carriers before being admitted to the hospital. MDDs can help hospitals better identify, control, and report HAIs, thereby lessening their liability. MDDs can help reduce costs, shorten hospital stays, and improve the quality of care while protecting profits.

Third, MDDs will lead to many changes in the health care industry. Health care spending in the United States has grown rapidly over the past few decades—from $27 billion in 1960 to $900 billion in 1993 to $1.8 trillion in 2004 (Heffler et al., 2004). Depending on how you measure it, the health care industry represents between 15% and 16% of the gross domestic product. Traditionally, these financial activities occurred in three subcategories: providers (such as hospitals, nursing homes, and diagnostic laboratories), payers (such as insurance companies), and life sciences (such as biotechnology and pharmaceuticals). The cost of developing a new drug can be as high as $800 million (Adams and Brantner, 2004). That cost is passed on from the life science sector to the payers and then to the providers. Therefore, the rising tide lifts the boats, and every sector's expenditures increase. How could MDDs help in this situation? They can help by allowing the three health care sectors to work with each other instead of against each other.

In the life science sector, biotech companies with MDD technologies can work with pharmaceutical companies to develop pharmacogenomic or theranostic solutions. This kind of collaboration will improve treatment outcome without

significantly increasing development cost. Instead of developing blockbuster drugs that are one-size-fits-all, more effective treatment can be obtained by using an MDD to tailor the treatment options to the patient's needs. MDDs will make drugs smarter by providing a genotype-based targeting system.

For payers in the health care industry, MDDs will change the risk calculation equations used by the insurance companies, such as health maintenance organizations (HMOs) and preferred provider organizations (PPOs). The health care payers make money by managing "risk capitals" associated with health care services. Reducing costs and risks will directly result in increased revenue. Hallin et al. (2003) studied the clinical impact of a PCR assay for identification of MRSA directly from blood cultures. They found that, on average, results were available about 39 h earlier than with the culture method, and about 25% of the treatments were modified after molecular differential diagnosis. MDDs could provide faster, accurate diagnosis that directly influences the clinical outcome and reduces the risks and costs associated with traditional diagnostic methods.

For health care providers, the benefit of MDD is even more apparent. An MDD could help doctors make the right treatment decisions much sooner, thereby shortening the patients' hospital stay and improving the overall quality of care.

Fourth, MDDs will bring about societal changes. Society is threatened by emerging infectious diseases, including many drug resistant "super bugs." The global economy, with its traveling professionals, makes the spread of diseases much faster. Rising costs make quality health care more difficult to manage. The cost of developing new antibiotics is too high and the process is too slow. We have been promised a better system and have been awaiting the arrival of MDDs for a long time. Now that the technology has finally arrived, we must maximize its utility and benefit.

Finally, MDDs offer all of the benefits needed for patient care, at once. Using current culture methods, patients and physicians often need to wait for days before a result is available. Singleplex molecular analyses are labor intensive, expensive, and often inconclusive. A powerful multiplex technology like Templex provides a fast answer that leads to a faster recovery. The ultimate value of MDDs is found in its ability to save lives.

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Pathogen Detection in the Genomic Era

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Introduction

In the 21st century, one of the greatest challenges to public health and clinical microbiologists is the rapid detection and identification of emerging and reemerging pathogens. Complex factors such as genetic variation in the host and pathogen, environmental changes, population pressures, and global travel can all influence the emergence of infectious diseases. The SARS epidemic of 2003 highlighted the potential of an emerging pathogen to spread globally in a very short time frame (Peruski and Peruski, 2003). The diagnostics of such infectious diseases has been greatly affected in the past 20 years. No longer is cultivation and microscopy the only means of detecting infectious agents. With the introduction of molecular diagnostics, the ability to detect minute amounts of microbial nucleic acids in clinical specimens has revolutionized clinical microbiology. In particular, the utility of PCR allows the detection and quantitation of specific agents in a matter of hours. PCR sequencing of specific segments of nucleic acid allows for the determination of specific drug resistance that now aids in guiding viral therapies.

In 1876, a German physician named Robert Koch was in a race to discover the causative agent of a disease that was destroying cattle and sheep in Europe. What Koch found was a rod-shaped bacteria in the blood of the dead cattle, which is known today as *Bacillus anthracis*. He was able to culture the bacteria on nutrients and then inject these cultured bacteria back into a healthy animal. The once healthy animal feel ill and died. Koch was able to again isolate the bacteria from the blood of the dead animal. By performing these series of experiments, Koch was able to demonstrate that an infectious disease, anthrax, was caused by a specific infectious agent (*B. anthracis*). This technique was again used to demonstrate that *Mycobacterium tuberculosis* was the causative agent of tuberculosis. Thus, Koch's series of experiments, known today as Koch's postulates, has provided the foundation for the study of the etiology of infectious diseases.

Although Koch's postulates have been very useful in determining the cause of many bacterial infections, there are times when these principles do not apply, for example, when an organism is uncultivable on artificial medium. There are also situations when a number of different pathogens can cause similar clinical symptoms or when a single pathogen can cause several pathologies. The application of molecular diagnostics can be very useful to overcoming these situations because these techniques allow for the rapid detection of fastidious, uncultivable, or unknown agents. Using universal primers or multiplex systems, a panel of organism targets can be used to screen for in any clinical specimen. Molecular methods are a widely used tool for epidemiological fingerprinting of isolates that are important to public health. Through the use of sequence-based identification, strain typing, and specific markers, comprehensive isolate fingerprinting can be used for the tracking and control of disease.

In 1995, scientists at the institute for genomic research (TIGR) unveiled the first two complete DNA sequences of the bacterial genomes *Haemophilus influenzae* and *Mycoplasma genitalium*. This was followed in 1996 by the first complete genome sequencing of an archaea, *Methanococcus jannaschii*. This scientific achievement was made possible through the use of automated sequencing equipment. Since 1995, TIGR and others have completed the genome sequence of many pathogens as well as a number of microorganisms of environmental relevance. Comparison of genomes can pinpoint differences between virulent and avirulent medically important pathogens. At the species level, genome comparisons can provide information about host or tissue specificity. On an evolutionary level, comparison of genomes can help to reveal the origins of microbial life (Doolittle, 2002).

Viral genomes are smaller and can mutate faster than bacterial genomes. Current technology is already tapping into the use of viral genomes for guiding drug therapies. Implementation of viral load testing and specific nucleic acid sequencing provides physicians with valuable information regarding the clinical response of patients on antiviral therapy and emergence of antiviral drug resistance (Smith et al., 2004).

The foundation for the study of biological processes at the protein level is being driven by the rapid progress in genomics. The identification and characterization of proteins expressed in cells (microbial and host) under different cellular states is a growing area of interest. Messenger RNA is often spliced in different ways to code for different proteins; simply knowing a gene sequence and its transcriptional expression is not enough to understand critical protein functions. The study of proteomics is the next frontier in understanding genomic functionality. The goal of proteomics is to define and characterize the complete set of proteins (the "proteome") in an organism, tissue, or cell and determine their spatial and temporal variation. It is being increasingly applied to the study of various microbial processes (e.g., host and pathogen interactions). Proteomics holds great promise for enhancing our knowledge of how a cell functions under various conditions, thus it may allow for breakthroughs in new generations of diagnostics, antimicrobial agents, and vaccine candidates.

Culture Confirmation and Tissue Pathogen Detection by Direct *In Situ* Hybridization

Though *in situ* hybridization (ISH) is typically performed in histology rather than clinical microbiology laboratories, it can provide extremely useful information to clinical microbiologists. Several pathogen targets have been used for direct hybridization to nucleic acid probes *in situ*. Bacterial targets include *Helicobacter pylori* (Makristathis et al., 2004), and *Legionella* spp. (Hayden et al., 2001b). Yeast forms of the dimorphic fungi (Hayden et al., 2001a), and molds such as *Aspergillus* spp., *Fusarium* spp., and *Pseudoallesheria* spp. (Hayden et al., 2002, 2003) have also proven to be useful as ISH targets. Typically, *in situ* hybridization is chosen when it is useful for the pathogen to be identified in association with intact cells or tissue, but branched DNA probes have been used to identify the presence of human papilloma virus (HPV) and the gene expression signal from HPV mRNA (Kenny et al., 2002). As a method for culture confirmation, PNA FISH (peptide nucleic acid fluorescent *in situ* hybridization) has been used to identify *Staphylococcus aureus* (Stender, 2003) and coagulase-negative staphylococci from positive blood cultures (AdvanDx, Woburn, MA, USA). Although not an *in situ* technique, a hybridization protection assay using an rRNA probe matrix has been used for rapid identification of bacteria and fungi from routine blood cultures (Marlowe et al., 2003). In this study, Enterobacteriaceae, *Pseudomonas aeruginosa*, other Gram-negative bacteria, *Staphylococcus aureus*, coagulase-negative staphylococci, streptococci, enterococci, other Gram-positive bacteria, anaerobes, and yeast were successfully identified using hybridization methods that proved to be both sensitive (100%) and specific (96%).

Specimen Automation: Nucleic Acid Extraction and Molecular Diagnostics

Efficient extraction and preparation of a specimen for nucleic acid analysis is critical to the quality of molecular testing results. During the past decade, improvements in novel and convenient extraction schemes have allowed the routine integration of molecular testing in many clinical diagnostic laboratories. More recently, the evolution of extraction systems into an automated format allows for the common practice of molecular testing. Several reviews serve as a reference for specific details of extraction chemistries and real-time instruments (Wolk et al., 2001; Wolk and Persing, 2002).

Specimen preparation automation can be divided into three categories: (i) nucleic acid extraction, (ii) specimen processing/PCR assay set-up, and (iii) general liquid handing systems. Some systems combine part or all of these categories into one system (Table 28.1). Many companies developed these automated systems so they can be integrated with their real-time PCR instruments for complete automation of extraction, sample processing, and amplification/detection.

TABLE 28.1. Automated specimen processing and real-time instruments.

Manufacturer	Instrument	Throughput	Web site	Extraction	Amplification preparation	General/liquid handing	Amplification/ detection
Abbott	m1000	48/run	www.abbottdiagnostics.com	×	×		
ABI	ABI Prism 6100 Nucleic acid PrepStation	96/run	www.appliedbiosystems.com	×			
	ABI Prism 6700 automated Nucleic acid workstation	192/run		×	×		
	ABI Prism 7000 Detection System	96/run					×
	ABI Prism 7300 Detection System	96/run					×
	ABI Prism 7500 Detection System	96/run					×
	ABI Prism 7900 HT Detection System	384/run					×
Beckman Coulter	Biomek FX Laboratory Automation Workstation		www.beckman.com	×	×	×	
	Biomek NX Laboratory Automation Workstation			×	×	×	
	Biomek 2000 Laboratory Automation Workstation			×	×	×	
	Biomek 3000 Laboratory Automation System			×	×	×	
Beckton Dickinson	BD Viper Sample Processor	550/8 h	www.bd.com	×	×		
Biomerieux	Nucleisense Extractor	10/run	www.biomerieux.com	×			
	EasyMAG	24/run					
	NucliSens miniMAG	12/run					×
	Easy Q	48/run					
Bio-Rad Laboratories	iCycler iQ	96/run	www.bio-rad.com				×
	iCycler iQ5	96/run					×
	MiniOpticon	48/run					×
	Opticon 2	96/run					×
	Chromo 4	96/run					×

Cephid	Smart Cycler 1600	16/run	www.cepheid.com					×
	Smart Cycler 3200	32/run						×
	Smart Cycler 4800	48/run						×
	Smart Cycler 6400	64/run						×
	Smart Cycler 8000	80/run						×
	Smart Cycler 9600	96/run						×
	GeneXpert	1/cartridge		×	×			×
Corbett Research	x-tractor gene	96/run	www.corbettresearch.com	×				
	CAS-1200 liquid handing system	96/run			×			
	Gradient Palm-Cycler	96-384/run						×
	Rotor-Gene 3000	36-72/run						×
Gen-Probe	Tigirs DTS	1000/12 h	www.gen-probe.com	×	×			×
Hamilton	MICROLAB STAR^{LET}		www.hamiltonrobotics.com	×	×	×		
Qiagen	Biorobot EZ1 workstation	6/run	www.qiagen.com	×				
	Biorobot M48 workstation	48/run		×				
	Biorobot M96 workstation	96/run		×				
	Biorobot MDx workstation	96/run		×	×			
	Biorobot 9604 workstation	192/run		×	×			
Roche	MagNA Lyser Instrument	16/run	www.roche-applied-science.com					
	MagNA Pure Compact System	8/run	www.roche-diagnostics.com	×				
	MagNA Pure LC System	32/run		×	×			
	Light Cycler	32/run						×
	Cobas Ampliprep	72/run		×	×			
	Cobas TaqMan 48	48/run						×
Stratagene	Mx3000P	96/run	www.mx3000p.com					×
	Mx4000	96/run	www.mx4000.com					×
Tecan	Freedom EVO clinical		www.tecan.com	×	×	×		

Automated Systems: Extraction Without Amplification

Qiagen (Qiagen, Valencia, CA, USA) has six different robotic systems for clinical research applications to extract nucleic acids from a variety of specimens. Other robotic systems are available for additional research applications. These automated extraction systems can be used with a variety of PCR and real-time PCR instruments for nucleic acid detection. The systems offered for clinical use are designed to accommodate laboratories depending on their specimen volume needs. The BioRobot EZ1 workstation can purify nucleic acids from 1 to 6 samples using prefilled EZ1 DNA and RNA kits that are available in cards. The BioRobot M48 and M96 can process up to 48 and 96 samples per run, respectively. These two systems use the MagAttract magnetic particle technology for DNA extraction and ultraviolet lights to decontaminate surfaces between runs. The BioRobot MDx workstation can process 8 to 96 samples and is equipped with vacuum processing to eliminate the need for centrifugation that was present with the earlier BioRobot 9604. The MDx system uses bar coding for specimen tracking. High-throughput testing is optimal with the BioRobot 9604, which is equipped to run QIAmp protocols for the extraction of DNA and RNA and has barcode specimen tracking. The Qiagen BioRobot has been evaluated for both DNA and RNA viruses and the extraction efficiency found to be equivalent to manual methods (Espy et al., 2001; Knepp et al., 2003; Forman and Valsamakis, 2004; Xu et al., 2004).

Automated Systems: Extraction with Amplification

Roche (Roche Molecular Biochemical, Indianapolis, IN, USA) has three automated instruments. The MagNA Lyser Instrument is an automated tissue homogenization unit that can be combined with their automated nucleic acid extractors. For nucleic acids, there is the MagNA Pure Compact and the MagNA Pure LC. The MagNA Pure Compact has a small footprint and can extract nucleic acids from 1 to 8 samples, using 2 different compact nucleic acid kits for small and large volumes. The MagNA Pure LC can process up to 32 samples in 1 to 3 h. The LC model automates both extraction and PCR set-up for the LightCycler capillaries, COBAS A-rings, and 96-well plates or tubes. The MagNA Pure LC has been evaluated with bacteria, parasites, fungi, DNA viruses, and RNA viruses; it was found to be equivalent to manual methods (Espy et al., 2001; Loeffler et al., 2002; Wolk et al., 2002; Germer et al., 2003; Holzl et al., 2003; Knepp et al., 2003; Leb et al., 2004; Lee et al., 2003; Cook et al., 2004; Dalesio et al., 2004; Muller et al., 2004; Tang et al., 2005). The COBAS Ampliprep is an automated system for the Cobas Amplicor analyzer and is not available in the United States except for research purposes. The COBAS Ampliprep has been reported in the literature to be suitable for routine testing with decreased hands-on time (Gartner et al., 2004; Stelzl et al., 2004). The next-generation COBAS, the COBAS TaqMan 48, is now available and has the capacity to run two different assays with individual PCR profiles. The LightCycler 2.0 system is also available, which allows 6-dye channel detection instead of the previous 4-dye channel detection.

Biomerieux (Biomerieux, Marcy l'Etoile, France) has three systems for nucleic acid extraction: the Nuclisens Extractor, which uses silica dioxide Boom extraction protocol (Boom et al., 1990), and the NucliSens easyMAG and the NucliSens miniMAG, which both use magnetic silica. The Nuclisens Extractor is an automated closed system. It has been compared to Qiagen manual extraction and found to be efficient for extraction of viral DNA and RNA (Gobbers et al., 2001). The easyMAG is an automated magnetic silica extraction system that will extract 24 samples in less than an hour. The miniMAG is a manual system that can extract 12 samples in about 35 min and offers a low-cost extraction system to a smaller molecular laboratory. Recent studies have suggested that the miniMAG consistently provides high yields of low titer of target (Tang et al., 2005). These systems are designed to extract both DNA and RNA for use with either Nucleic Acid Sequence-Based Identification (NASBA) or PCR amplification technologies. The NucliSens Easy Q can also be coupled with these extraction methods for detection with real-time NASBA. The miniMAG has been used with the EasyQ real-time NASBA HIV assay to accurately quantify HIV over a six-log range.

Applied Biosystems (ABI; Foster City, CA, USA) has two nucleic acid extraction systems, which use proprietary flow-through chemistry. The ABI PRISM 6100 nucleic acid prep station can program up to 300 defined extraction protocols. The ABI PRISM 6700 Automated Nucleic Acid Workstation is a fully automated system that extracts nucleic acid, prepares the PCR reactions, and initiates the reverse transcription step of RT-PCR for up to 96 samples. The ABI Prism 6700 was used to automate the large-scale surveillance of West Nile virus during outbreaks in the northeastern United States (Shi et al., 2001). This workstation can interface with the existing ABI PCR instruments, the ABI PRISM 7900HT, 7500, 7300, 7000, and GeneAmp 5700.

Corbett Research (distributed by Phenix Research Products, Hayward, CA, USA) offers two separate automated instruments, the X-Tractor Gene for RNA/DNA extraction and the CAS-1200 liquid handling system for set-up of both standard and real-time PCR reactions. The CAS-1200 can be configured to support 96-well plates or the Rotor-Gene well rotors for use with the Rotor-Gene 3000, a four-channel real-time PCR fluorescent detector.

Becton Dickinson (BD, Franklin Lakes, NJ, USA) has an automated sample processor for the BDProbeTec ET system. The BD Viper Sample Processor is designed to automate sample processing with high-volume testing of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* (>500 samples/8-h shift). Gen-Probe (Gen-Probe Incorporated, San Diego, CA, USA) has launched the TIGRIS, which is a fully automated instrument for high-volume testing (1000 samples/12-h shift). The TIGRIS incorporates Gen-Probe's target capture technology for sample processing, Transcription-mediated Amplification (TMA) amplification, and Hybridization Protection Assay (HPA) detection into one instrument. Currently, the TIGRIS is limited to use with Gen-Probe's APTIMA *N. gonorrhoeae*/*C. trachomatis* assay and Procleix HIV/HCV blood screening assay. Gen-Probe also offers the DTS 800 and 1600 systems (manufactured by Tecan, Research Triangle Park, NC, USA), which automate the liquid handling of other various diagnostic kits. Abbott (Abbott

Laboratories, Abbott Park, IL, USA) is developing an automated sample preparation workstation. The m1000 is designed to process 48 samples in 2 h using a magnetic microparticle–based protocol and has been reported by Abbott to be integrated with the ABI PRISM 7000 detection systems.

Liquid Handling Systems

Beckman Coulter, Hamilton, and Tecan all offer robotic liquid handling systems, which offer flexibility in molecular diagnostic labs. Beckman Coulter (Fullerton, CA, USA) has available several liquid handling systems that can be used to extract nucleic acids, set up PCR and sequencing reactions, as well as immuno-detection assays. These systems include the Biomek NX, Biomek FX, Biomek 3000, and Biomek 2000. The Hamilton MICROLAB STAR^{let} (Hamilton Company, Reno, NV, USA) is a benchtop workstation for assay automation that can be used for nucleic acid extraction and PCR set-up. The vector software and various accessories allow the user to customize protocols to laboratory needs. Tecan supports the Freedom EVO PCR workstation, which also offers flexible software and accessories to allow the user to customize protocols to their needs.

Other Real-Time PCR Systems

Cepheid, Bio-Rad, and Stratagene all offer real-time PCR systems that could be combined with many automated systems. The Cepheid SmartCycler (Cepheid, Sunnyvale, CA, USA) offers flexible real-time PCR detection that allows multiple low sample number tests to be performed simultaneously or with up to 96 independently programmable reaction cells for higher throughput. Cepheid has announced plans to launch the GeneXpert system, which would be a real-time system with integrated sample preparation, amplification, and detection in a single-use cartridge that contains lyophilized reagents. Bio-Rad (Hercules, CA, USA) offers real-time detectors in the iCycler and now the iQ5 for a multiplex of up to five fluorophores. Additionally, they offer the MiniOpticon, a two-color 48-well system, the Opticon II, a two-color 96-well system, and the Chromo4, a four-color 96-well system (formerly MJ Research). The Stratagene (Stratagene, San Diego, CA, USA) systems include the Mx4000 and Mx3000P.

Extraction Summary

Although there have been many advances in the automation of nucleic acid extraction, sample processing, and amplification, there have been very few studies comparing the various automated systems. The limited studies available have demonstrated that the automated systems are comparable to each other as well as to manual methods (Espy et al., 2001; Knepp et al., 2003). Automation limits the risk of contamination, decreases hands-on time, and reduces repetitive-motion disorder among technicians. Ultimately, the decision to implement automation

into a molecular lab depends on cost, specimen-testing volume, and laboratory workflow.

Molecular Typing and Epidemiology

The use of molecular techniques in epidemiological studies is important for rapid, focused disease detection and for implementation of infection control measures. Molecular typing has become a valuable tool in defining disease source(s), determining the transmission modalities, and tracking outbreaks (Diekema et al., 2003; Soll et al., 2003). Pulse-field gel electrophoresis (PGFE) is considered the reference method for comparative purposes, although various methods are used (Tenover et al., 1995; Olive and Bean, 1999b; Goering, 2000b; Weller, 2000b). Although PGFE is considered a reliable, discriminatory, and reproducible method, it is expensive and time consuming, making this technique difficult for hospitals to use as a routine form of support for infection-control practitioners in outbreak investigations (Weller, 2000a; Shopsin and Kreiswirth, 2001; Stranden et al., 2003). Due to testing conditions, there is inherent gel-to-gel variability with PGFE, which makes it difficult to compare inter-institutional electrophoresis results. Public health laboratories have standardized PFGE for use in outbreak investigations, and digital imaging has provided some standardization and cross-gel comparative properties; still, the technique is relatively cumbersome and not practical in most hospital laboratories. With recent CDC and JCAHO recommendations to use molecular testing as the gold standard in investigation of hospital-acquired infections, hospitals are faced with difficult choices, referring specimens to reference laboratories or state public health laboratories for testing or investment in hospital-based infrastructure to support genotyping. Currently, there is a gap in our ability to provide results of molecular epidemiology in the hands of most clinical microbiologists and infection-control practitioners.

Though numerous bacterial genotyping methods have emerged for use in outbreak investigations and molecular epidemiology, few are completely standardized or practical. Among them, PCR-based amplification with subsequent typing (Rademaker and Savelkoul, 2004), genotyping via variable number tandem repeats (VNTR) (Mathema and Kreiswirth, 2004), multilocus sequence typing (MLST) (Hanage et al., 2004), and automated ribotyping (Pfaller and Hollis, 2004) provide options. Although all are useful tools,varying in their strengths and limitations (Tenover et al. 1997; Goering, 2000a), two methods appear to have promise for translation into a routine clinical laboratory practice: rep-PCR and ribotyping.

Repetitive-element sequence-based PCR (rep-PCR) is a DNA fingerprinting method that has been successfully used as a rapid molecular tool for outbreak investigation of oxacillin resistant *S. aureus* (ORSA) (Versalovic et al., 1991; Del Vecchio et al., 1995; van der Zee et al., 1999; Deplano et al., 2000) and has successfully demonstrated discriminatory typing for organisms such as *Salmonella typhi, Escherichia coli*, and *Bacillus* species (Olive and Bean, 1999a). The rep-PCR

method exploits the presence of repetitive elements dispersed throughout the chromosomes of ORSA and other organisms. Repetitive elements have different and specific positions within the genome among different strains of microorganisms. Primers, designed to amplify genetic regions between these repetitive elements, allow for generation of PCR amplification products of various sizes (Woods et al., 1993). Electrophoresis of the differently sized products, derived from different organisms strains, produces unique gel-banding pattern fingerprints, by which the strains can be compared and differentiated. Recent advancements and improvements to commercial rep-PCR fragment analysis using capillary electrophoresis (Bacterial Bar Codes, Inc., Atlanta, CA, USA) enables standardized and reproducible performance of rep-PCR and may provide a promising option for a rapid and cost-effective outbreak investigation in hospitals and communities.

Molecular Detection of Drug Resistance

Rapid and accurate determination of drug susceptibility of a clinical isolate can be useful for various aspects of patient therapy. The presence of resistance markers can also help distinguish ambiguous break points associated with susceptibility testing. Well-characterized resistant genes can be used to monitor their epidemiological spread in the community or hospital. Despite the fact that there still remains much to learn about these markers, the application of molecular diagnostic methods to detect drug resistance is evolving as a routine practice for some laboratories.

The use of molecular methods to detect resistance can be applied to bacteria, viruses, and fungi. The advantage of using molecular tests is that they do not rely on time-consuming incubations or media-dependent expression. Thus, educated choices for therapy can be initiated early in diagnosis to impact patient outcomes, particularly with slow-growing organisms such as *Mycobacterium* spp. (Inderleid and Pfyffer, 2003).

Antimicrobial resistance genes among bacteria include resistance for β-lactams, aminocylitols, aminoglycosides, chloramphenicol, fluoroquinolones, glycopeptides, isoniazids, macrolides, mupicurin, rifampin, sulfonamids, tetracyclines, and trimethaprim (Fluit et al., 2001; Rasheed and Tenover, 2003; Tenover and Rasheed, 2004). For a list of PCR primers used to target such resistance markers, see Tenover and Rasheed (2004).

Perhaps the most well-documented applied use of markers for bacteria are those of oxacillin-resistant *Staphylococcus aureus* (ORSA, formerly MRSA) and vancomycin-resistant enterococcus (VRE). The *mecA* gene mediates oxacillin resistance in most ORSA, and the *vanA* and *vanB* genes primarily mediate acquired vancomycin resistance in VRE. Commercially available tests for ORSA include latex agglutination tests for PBP2a (the product of *mecA)*, cycle probe technology, and PCR for the detection of *mecA* in *S. aureus*. PCR has been used to detect and track both ORSA and VRE (Clark et al.,1993; Gordts et al. 1995; Aarestrup et al., 1996; Satake et al., 1997; Hussain et al., 2000; Padiglione et al., 2000; Grisold et al., 2002; Jonas et al., 2002; Louie et al., 2002; Maes et al., 2002; Francois

et al., 2003; Strommenger et al., 2003; Sloan et al., 2004), A review by Diekema et al. (2004) highlights the fact that together, ORSA and VRE are the two most important resistant pathogens in U.S. hospitals, and their rapid detection remains a need. Antimicrobial resistance is continuing to increase worldwide. With active surveillance and proper isolation of infected patients, use of rapid PCR technology could play an important role in identifying carriers upon hospital admission and aide in the prevention and control efforts for ORSA and VRE. For routine use of these tests, not only would they have to be sensitive and specific, but they would also have to be cost effective with proven infection prevention studies.

The detection of viral mutations associated with drug resistance has been well documented. Examples include polymerase and protease inhibitors with human immunodeficiency virus (HIV), acyclovir and penciclovir resistance in herpes simplex virus, acyclovir resistance in varicella-zoster virus, ganciclovir resistance in cytomegalovirus, famciclovir and lamivudune resistance with hepatitis B virus, and amantidine resistance with influenza A (Shafer and Chou, 2003). Viral mutations are most commonly detected by direct sequencing of the specific viral reading frames, which encode the proteins that are targeted by currently available antiviral drugs. Genotypic resistance testing to mange HIV-1–infected patients is widely used by physicians.

There are currently two commercially available FDA-cleared sequencing assays that include reagent kits and software. These two kits are the Truegene HIV-1 genotyping kit and OpenGene DNA–sequencing system, (Bayer Corp, Tarrytown, NY, USA) and ViroSeq HIV-1 genotyping system (Applied Biosystems, Foster City, CA, USA). Genotype testing requires a skilled laboratory, which is proficient in sequencing, alignments, editing, mutation detection, and interpretation of sequences.

Commercially available line probe assays (LiPA), a reverse hybridization method, allow laboratories that are proficient in PCR and have limited sequencing capabilities to detect mutations without the need to sequence (Descamps et al., 1998; Schmit et al., 1998). Comparison studies of these HIV-1 viral genotyping methods have proved to be reliable and accurate. However, the LiPA is designed to identify known primary mutations associated with high-level drug resistance; direct sequencing can detect more and new mutations (Erali et al., 2001; Hanna and D'Aquila, 2001; Grant et al., 2003; Caliendo and Yen-Lieberman, 2004).

Rapid detection of antifungal resistance is useful, primarily due to the increase in fungal infections among immunocompromised patients. Current antifungal assays rely on fungal susceptibility testing, which is dependent on growth. The practical application of antifungal molecular testing is yet to be seen, as there is still much to learn about the genetic markers that mediate resistance. The genetic information needed to examine fungal resistance at the molecular level is much more complex than that of viruses, such as HIV-1, and could involve the evaluation of fungal gene expression. For a review of molecular mechanisms of antifungal resistance, see Edlind (2004).

The full potential of molecular diagnostics for drug-resistance testing in microbiology has not been reached, and its application is still in its infancy. As the

molecular mechanisms of antimicrobial resistance are described, newer technologies will enhance the utility of marker testing. Microarray technology has the promise to impact the rapid and accurate detection of multiple mutations associated with resistant bacteria, mycobacteria, viruses, and fungi. As with all molecular diagnostics, laboratories that perform molecular resistance testing need to ensure quality control of specimens. Currently, there is still a need to cultivate organisms for further testing of other antimicrobials or typing for epidemiological studies. Thus, it important to retain specimens or inoculate a culture until the laboratory can be sure a result is negative and the specimen can be discarded (Diekema et al., 2004). Until the full potential of drug-resistant markers is understood, rapid molecular antimicrobial testing must still be combined with traditional microbial cultivation.

Microbial Proteomics in Pathogen Detection

Though in its infancy, proteomic technology has the potential to play a key role in the future of clinical microbiology diagnostics as techniques become more rapid, affordable, and the list of applicable biomarkers expands. Mass spectroscopy (MS) and 2-D gel electrophoresis are the 2 common techniques in microbial proteomics (Douglas, 2004). In 2-D gel electrophoresis, proteins are first separated by their isoelectric point (pI) in glass tubes (Bjellqvist et al., 1982). Gels are then removed from glass and placed horizontally on top of polyacrylamide slab gels, and polyacrylamide gel electrophoresis (PAGE) further separates proteins with similar charges by their size (molecular weight). Gel electrophoresis is a simple method to catalogue microbial proteins grown under different conditions and disease states. A mass spectrometer can take proteins from PAGE and further separate them by producing charged particles (ions) (Shevchenko et al., 1996). The mass spectrometer differentially moves ionized molecules, separated by their mass-to-charge (m/z) ratio, through a vacuum by means of an electromagnetic field. For the sake of discussion, if one assumes that each component of the mixture has a different molecular weight, then the mass spectrum contains unique "peaks" for each compound that is present. For more information about the different types of mass spectroscopy, refer to Douglas (2004).

A few reports have begun to surface in the clinical microbiology literature and describe how proteomic methods may impact laboratories in the future. In one report, matrix-assisted laser desorption/ionization time-of-flight mass spectroscopy (MALDI-TOF-MS) was used to rapidly identify fungal proteins that evoked a specific human immune response, which may prove to be linked to active infection and outcome (Pitarch et al., 2004). In another study, MALDI-TOF-MS, gelelectrophoresis, and tandem mass spectrometry were used to identify intra-amniotic proteins, which could lead to discovery of novel human biomarkers for human intra-amniotic infection (Gravett et al., 2004). Ultimately, these tools will help to elucidate the interaction of proteins with protein precursors, DNA, and mRNA to add to the understanding of pathogenesis and disease. Out of this understanding,

novel biomarkers for early detection of disease or disease outcomes are expected to occur.

One emerging technology, the Luminex xMap System, can identify multiple immune proteins, like serotype-specific antibodies, in a single well or tube multiplex format. It has been used to identify multiple immune proteins (Jones et al., 2002) and bacterial DNA (Dunbar et al., 2003), but routine applications in the clinical laboratory will require further translational.

Although mass spectrometry is typically used to identify proteins, high-performance mass spectrometry has recently been adapted and developed for use in conjunction with PCR for rapid identification and strain typing of emerging pathogens, such as *Bacillus anthracis* and coronavirus, among others (Van Ert et al., 2004; Ecker et al., 2005; Sampath et al., 2005)

Conclusion

Over the past 10 years, molecular methods have emerged as essential tools in the clinical microbiology workplace. Identification of bacterial, viral, fungal, and parasitic genomes drives the emergence of new technologies to identify pathogens more rapidly, but limitations in the single target approach still exist. Miniaturization of robotics and automation will allow even small health care facilities to implement molecular methods. New discoveries in the human genome, linking disease susceptibility with infection, or human mutations with antibiotic utility, will certainly continue to impact the clinical laboratory of the next 10 years, broadening the scope of clinical microbiology from that of detection to include prediction, via protein or nucleic acid targets. Finally, miniaturization of techniques such as mass spectroscopy of PCR amplicon could change the entire face of clinical microbiology, in that multiple pathogens could be amplified or simultaneously detected and genotyped to allow rapid detection of many emerging pathogens and other infections.

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