

Nonserologic Assays for Detection of Bacteria and Other Nonviral Infections

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INTRODUCTION

Many of the old microbiology techniques continue to play an integral part in the laboratory diagnosis of infectious diseases. However, in the past several years advances in technology enabled the clinical microbiology laboratory to respond rapidly to the needs of patients and clinicians for the identification of possible infections. Three topic areas are discussed in this chapter. The first outlines specimen collection guidelines, various culture protocols for the isolation of organisms, identification protocols, and the value of antimicrobial susceptibility testing. The second broad category covers methods for the immunological detection of nonviral infectious processes. The final section introduces the expanding area of molecular microbiology.

The organisms that are considered neonatal pathogens include a vast array of bacteria, a few fungi, and parasites. This chapter does not focus on any specific organism or give guidelines for their identification in the laboratory, but rather paints a broad picture of what the practitioner should expect of the clinical microbiology laboratory.

CULTURE AND IDENTIFICATION PROCEDURES

Specimen Collection

The importance of proper specimen collection for the diagnosis of infectious diseases cannot be overstated. There are several excellent references that provide guidelines for specimen collection (1). Adherence should be to the following principles:

1. All specimens must be properly labeled with the patient's name and hospital number. Attached to each specimen must be a key-plated voucher bearing the same patient name, hospital number, and the name of the requesting physician. The requisition must also indicate the required test or tests, the source of the material, time of collection, plus the name of any particular organism suspected.
2. When possible, specimens should be obtained before antibiotics or other antimicrobial agents have been administered.
3. The specimen must be adequate in volume for desired tests. All specimens that are collected by swab must include two swabs if a Gram stain or other microscopy is requested.
4. Specimens must be received in a clean, sterile container and be sent to the laboratory

expeditiously or should be stored at a temperature that will not affect the growth of the pathogenic micro-organism. Use appropriate transport medium when required.

5. Avoid contamination from the indigenous bacterial flora.

Most of the reference guidelines available refer to specimen collection from older patients rather than the very young. For blood cultures, for example, most recommendations indicate that 10 mL blood must be collected for each bottle submitted to the laboratory for culture. Definitive criteria are not available for the very young. There are no clinical studies that address the collection of specimens in the neonate. It is important to get the best specimen as often as possible to submit for diagnostic procedures. All microbiologists bend the rules for this patient population, out of either empathy or the realization that “it’s all that you are going to get under certain conditions.” We also know from the literature that septic neonates often have very high bacterial loads. Therefore, the volume provided is not as important as proper collection techniques to avoid contamination in the blood culture bottle. The last point, the importance of proper collection techniques, is critical, particularly when the infected child is evaluated for suspected sepsis because of the frequent occurrence of blood cultures growing organisms that represent contamination associated with the collection procedure.

There is a saying that I heard for years (I do not know the original source): “Garbage in, garbage out.” Often abbreviated as GIGO, this is a famous computer axiom meaning that if invalid data are entered into a system, the resulting output will also be invalid. Although originally applied to computer software, the axiom holds true for all systems, including specimen collection of patient samples for microbiology culture.

Microscopy

The most important basic microbiology information for a patient often depends on a well-performed microscopic procedure. There are two parts to any microscopic procedure: The first and most critical is the preparation of the smear, and the second is the actual staining procedure. The cytocentrifuge is an excellent procedure for the preparation of smears from sterile fluids. It has been found that the sensitivity of the cytospin Gram stain from cerebrospinal fluid specimens equals or exceeds that for the traditional bacterial antigen detection. In addition, the cytocentrifuge may be used to prepare bronchoalveolar lavage and nasopharyngeal wash slides for subsequent staining with specific reagents for the detection of pathogenic organisms (e.g., respiratory viruses, *Bordetella pertussis*).

In addition to the Gram stain, the acridine orange stain is particularly useful in the rapid screening of normally sterile specimens in which few organisms may be present. It is also useful in the rapid examination of blood/buffy coat smears or preparations containing proteinaceous material in which differentiation of organisms from background material may be more difficult. Acridine orange is a fluorochromatic dye that binds to nucleic acids of bacteria and other cells. Bacteria and fungi uniformly stain bright orange, whereas human epithelial and inflammatory cells and background debris stain pale green to yellow. The only drawback to this procedure is that a fluorescent microscope is needed to visualize the organisms.

Culture Procedures

The majority of organisms that are involved in infections in the neonate will grow on the common isolation media used in the laboratory. These media include a general

purpose broth medium and agar-based medium (usually supplemented with 5% sheep blood or horse blood), an enriched medium (chocolate agar), and a selective medium (MacConkey agar). Special media have been developed for the cultivation of many fastidious organisms; the laboratory needs to be advised of special requests to ensure optimal recovery of these organisms.

By far the most important patient specimens sent to the laboratory in neonates are for evaluation for sepsis (2). Group B streptococcus (*Streptococcus agalactiae*) and *Escherichia coli* continue to be the most common pathogens isolated from septic infants. Increased automation in the laboratory has facilitated the recovery of pathogenic microorganisms. Current blood culture instruments continuously monitor individual blood culture bottles for the growth of micro-organisms every 10–15 minutes. The method for detecting growth varies with the automated system in use, but they generally rely on the production of a gas or pressure changes within the bottle. Growth is indicated by a change in the slope of the growth curve for the product that is monitored. Because each bottle is monitored around the clock, these systems will detect a positive sample 1–1.5 days faster than manual systems. Most significant cultures are detected within 24 hours of incubation. In addition, the culture bottles have been refined to optimize the growth of organisms. Most experts agree that a blood-to-broth-medium ratio of 1:5–1:10 should be maintained for the optimal recovery of the micro-organism. Pediatric blood culture bottles have a smaller volume of broth medium and enriched medium to meet these criteria. Antibiotic-binding resins, activated charcoal, and other formulations are often added to the blood culture bottle to neutralize compounds that may inhibit or retard the growth of organisms.

Special Culture Procedures

For the isolation of fastidious organisms, selective media and methods are needed. The isolation of *Mycobacteria tuberculosis* requires an enriched medium that contains a number of antibiotics to inhibit rapidly growing commensal bacteria that may contaminate the specimen. An automated detection system, similar to the instrument used for blood cultures, is used for detection of these organisms. Likewise, *Mycoplasma hominis* and *Ureaplasma* spp require special media and cultivation methods for isolation. Because these organisms are sensitive to environmental conditions, appropriate transport media should be used to enhance their recovery from clinical material. In vitro growth of the organisms requires that the medium be supplemented with serum and other nutrients. These media are commercially available. The chlamydia species (*Chlamydia trachomatis* and *Chlamydia pneumoniae*) are rarely isolated in the laboratory because cell culture procedures are needed for cultivation. The majority of these infections are detected using molecular techniques (3,4).

Identification of Organisms

As with the monitoring systems for the detection of organisms, automated systems are rapidly displacing conventional methods for the identification and antibiotic susceptibility testing of bacteria and yeasts (5). These systems usually consist of miniature wells that contain biochemical substrates and varying dilutions of antibiotics. Most bacteria and yeasts that are involved in infections are reliably identified by these systems. The antimicrobial susceptibility testing profiles that are generated can be reported as the minimal inhibitory concentration of the drug or by the category of

susceptible, intermediate, or resistant. These data can be available within 8–10 hours for most of the rapidly growing micro-organisms. For those organisms that are not reliably identified in the systems, specialized identifications systems are available. In the future, newer molecular methods will make their way into the laboratory to achieve this goal (3–5); these methods in some instances will be faster than the conventional procedures for the identification of the organisms.

IMMUNOLOGICAL PROCEDURES

Immunoassays play a critical role in diagnostic microbiology (6). These assays belong to two major categories based on whether examining for the presence of antibodies (immunoglobulins) or a specific analyte associated with a micro-organism (antigens). Serologic methods for the diagnosis of infectious diseases are addressed in Chapter 1. In this section, I examine immunological assays used for the direct detection or identification of micro-organisms from clinical specimens or organisms isolated on a culture plate. Advances in antigen detection are parallel to the advances made in the development of monoclonal antibodies (characterized with respect to their specificities and binding affinities). The immunological methods used include agglutination tests for bacterial antigens, enzyme immunoassay (EIA) antigen tests, and direct immunofluorescence assays (DFAs) and indirect immunofluorescence assays.

Bacterial Agglutination Test

A bacterial agglutination test is available for the detection of *Streptococcus pneumoniae*, *S. agalactiae*, *Haemophilus influenzae* type B, and *Neisseria meningitidis*. The assay uses latex particles coated with specific antibodies to structural antigens and can detect soluble antigens in urine, cerebrospinal fluid (CSF), and serum. The sensitivity and specificity of these tests in urine samples is extremely unreliable, and the tests should not be used on this specimen. For sterile body fluids, the tests are highly sensitive and specific for *H. influenzae*, but their sensitivity for other bacteria is much lower, particularly for *N. meningitidis*. It is important to note that the sensitivity of this assay was essentially identical to Gram stain smears prepared using a cytospin preparation of the fluid. Most laboratories have discontinued the use of this test. Those laboratories that still offer the latex particle agglutination tests will usually perform the procedure only on patients with sufficient white blood cells in the CSF.

There are a number of agglutination tests for the identification of organisms that are isolated on culture plates. Most laboratories use this procedure for the identification of β -hemolytic streptococci (groups A, B, C, D, F, G), *Salmonella* and *Shigella* typing, and the identification of *E. coli* O157.

Immunoassay Detection of Antigen

EIA systems are performed in microwells, in test tubes, or on solid membranes and incorporate an enzyme-substrate indicator system. These tests play a prominent role in viral diagnostics for the detection of influenza virus, rotavirus, and respiratory syncytial virus. The most commonly used bacterial test is for the detection of group A streptococci in pharyngeal specimens. A number of waived tests and moderately complex tests are on the market; the best tests have a sensitivity and specificity around 90–95%. Newer antigen assays for the detection of *Legionella pneumophila* and *S. pneumoniae* in urine have been introduced.

Until now, the accepted laboratory practice for the diagnosis of malaria was the microscopic examination of Giemsa or- Wright-stained blood films. An EIA has been developed to detect a plasmodium antigen. For the detection of malaria in blood samples, the assay is designed to detect an antigen (histidine-rich protein-2) associated with malaria parasites (especially *Plasmodium falciparum* and *Plasmodium vivax*) or to detect plasmodium-associated lactate dehydrogenase or aldolase. The monoclonal antibodies against these markers are immobilized in the nitrocellulose matrix; blood lysates are allowed to migrate over the membrane and are captured. The complex is visualized by the addition of the second antibody. The test lines are located at specific points on the strip to aid in the interpretation of positive results. Each test strip also contains two internal process control dotted lines that appears as positive confirmation of procedure and reagent viabilities. When compared to microscopy and clinical history, the assay has a sensitivity and specificity of 96 and 99%, respectively, for histidine-rich protein-2 detection for *P. falciparum*, with discrepant results having less than 100 parasites/ μ L (0.002% parasitemia).

DFA and Indirect Immunofluorescence Assays

DFAs are commonly used in immunology, microbiology, and virology laboratories to directly detect the presence of micro-organisms. They are fast, easy to perform, and very specific but require well-trained personnel and a fluorescent microscope. An important advantage of this type of assay is that it provides results quickly. After fixation of the specimen on the slide, results are available within 1 hour. In the DFA procedure, clinical specimens such as nasal washes, sputum, CSF, or culture material are centrifuged using a cytocentrifuge and are fixed on glass slides (usually by heat or cold acetone fixation). The slides are then reacted directly with a specific antibody probe that is labeled with a fluorochrome (fluorescein isothiocyanate). After washing excess fluid from the slide, mounting oil and a coverslip are added, and the slides are examined using a fluorescence microscope.

The *C. trachomatis* DFA is an alternative method for detection of *C. trachomatis* in urogenital and rectal specimens, in conjunctival specimens for the differential diagnosis of acute conjunctivitis, and in nasopharyngeal specimens for the differential diagnosis of afebrile pneumonia or lower respiratory tract infections in infants. Because most laboratories are unable to culture this organism, this test is often the only option available for the rapid identification of this infection. Newer probe and amplification assays are more sensitive than DFA testing.

To increase sensitivity, indirect fluorescent antibody methods can be used to offer a more versatile application. In this method, a primary immunoglobulin G antibody (unlabeled) is reacted with the fixed clinical sample, which may contain the specific antigen on a microscope slide. After a wash to remove the primary antibody, a second fluorescent-labeled antibody with specificity to the primary immunoglobulin molecule is added to the slide.

MOLECULAR MICROBIOLOGY

Conventional culture and immunoassay are gradually giving way to molecular methods for detecting bacterial and viral pathogens in the clinical microbiology laboratory (3,4). These assays are becoming commercially available, and the technical staff of the laboratory are easily adapting to these procedures. The molecular expertise was ini-

tially gained by working with specimens from patients infected with human immunodeficiency virus (HIV). That experience has now evolved to include a broader range of infectious pathogens. In addition, the evolution of newer technical procedures has played an important role in the acceptance of the molecular techniques (3,4,7).

Four major improvements have occurred in recent years: (a) commercially available automated nucleic acid extraction devices; (b) improvements in nucleic acid hybridization assays, which will allow clinical microbiology laboratories to use hybridization assays for the detection of potential pathogens directly from clinical specimens or from isolated colonies grown in culture; (c) adoption of real-time polymerase chain reaction (PCR) cyclers by clinical laboratories, which allows huge time and labor savings; (d) new technology to provide clinical laboratories with the ability to sequence nucleic acids on a timely basis (8). With new gel electrophoresis equipment, the identity of most micro-organisms can be made available within 24–48 hours of isolation.

Nucleic Acid Probes

With the exception of viruses, ribosomes are an integral part of the cell of all prokaryotic and eukaryotic micro-organisms. The 70S ribosome of prokaryotic cells is composed of two subunits, which contain 16S and 23S ribosomal ribonucleic acid (rRNA). Likewise, the 80S ribosome of eukaryotic cells contains 18S and 28S rRNA. Research into the comparative ribosomal deoxyribonucleic acid (rDNA) gene sequences of microorganisms has been ongoing for the past 30 years and has become an accepted method for establishing phylogenetic relationships among species. Currently, *Bergey's Manual of Systematic Bacteriology* is undergoing revision based on 16S rRNA sequence comparisons (9). Likewise, the taxonomy of yeasts is undergoing a revision based on a comparative 26S rDNA sequence analysis.

The sequence information of these ribosomal genes has been used in the clinical laboratories for diagnostic purposes. They are well suited for this purpose for several reasons. There are both highly conserved and variable regions along the length of these molecules. The conserved area allows the identification of a specific organism (species specific) or class of organisms (genus specific); the variable regions allow for discrimination among members of the group. Reports have demonstrated the detection of subspecies of organisms based on subtle differences in the rRNA. In addition, rRNA is usually present in large quantities, allowing for greater sensitivity in the assay. The amount of rRNA relates to an organism's growth rate. Slow-growing bacteria, such as *M. tuberculosis*, have 10^2 – 10^3 copies of rRNA per cell, whereas fast-growing facultative anaerobic bacteria may have as many as 10^4 – 10^5 molecules.

There are many different assays on the market. The Gen-Probe System (Gen-Probe Inc., San Diego, CA) uses chemiluminescence-labeled, single-stranded DNA probes that are complementary to the rRNA of the target organisms. After the rRNA is released from the organisms, the labeled DNA probes combine with the rRNA of the target organisms to form stable DNA:RNA hybrids. The labeled DNA:RNA hybrids are separated from the nonhybridized probes and are measured in the luminometer. The test results are calculated as the difference between the response of the specimen and the mean response of the negative reference. This method has been in place for over a decade for the detection of pathogenic organisms directly from clinical samples (including *C trachomatis* and *Neisseria gonorrhoeae*) or the culture confirmation of organisms grown in the laboratory.

Another method used for detection and identification of micro-organisms is fluorescent *in situ* hybridization (4). These probes are short sequences of single-stranded DNA that are complementary to the DNA target sequences. These probes hybridize, or bind, to the complementary DNA and, because they are labeled with fluorescent tags, allow the direct visualization of the cell that contains the specific sequence. The technique has been employed in the histopathology laboratory to aid in the diagnosis and management of a variety of solid tumors and hematologic malignancies in the clinical setting. It has been used in the clinical microbiology laboratory for the detection of micro-organisms directly from clinical material or as a procedure for culture confirmation.

Peptide nucleic acid (PNA) probes mimic DNA in many aspects but differ in the basic backbone that is used to tie the nucleotide bases together. The backbone of the PNA probes is made up of repeating *N*-2-aminoethyl glycine units linked by amine bonds instead of the sugar phosphate backbone characteristic of DNA. The resulting structure of PNA molecules allows normal base pair formation; however, there is no electric charge in the backbone of the probe, which results in very fast and strong hybridization. In addition, these probes are more resistant to protease and nuclease degradation in a cell environment, which assists in minimizing any enzymatic attack during the hybridization process. All of these factors make hybridization assays that use PNA probes more robust than other probe hybridization protocols.

Amplification Methods

The year 2003 saw the celebration of the 50th anniversary of the discovery of the molecular structure of DNA by the collaborative work of Watson, Crick, Franklin, and Wilkins. Since that time, we have witnessed an astounding growth in the area of molecular biology. No less of an achievement was the invention of the PCR method by Mullis in 1983. Since the initial description of his method, PCR methods have become the standard against which newer procedures are compared.

Traditional PCR

The PCR method is used for the specific amplification of a targeted DNA or RNA sequence. For DNA targets, the double-stranded DNA is rendered single stranded by denaturing with heating to 95°C for 2–10 minutes. The reaction vessel is rapidly cooled to between 55 and 65°C in the presence of short (15–20 bases) complementary oligonucleotides (primers), which bracket the targeted DNA to be amplified. During this step, the primer oligonucleotides hybridize to the DNA target molecule. Once the primers are bound, the temperature is raised, and the Taq DNA polymerase enzyme adds nucleotides to the 3' end of the primer. This occurs on both strands of DNA, resulting in the production of a new copy of double-stranded DNA. The sequence of temperature changes is repeated for up to 45 cycles, resulting in the production of (at the theoretical maximum efficiency) up to 10^{13} copies from a single copy of template DNA.

For RNA molecules, the sequence of events is initiated by the reverse transcription of the target RNA (messenger RNA for certain viruses) to generate complementary DNA. Following this step, the reaction proceeds as for DNA molecules.

Traditional PCR requires that the amplified product be further manipulated in the laboratory for detection. This detection can be performed by electrophoretic separation of the amplican on agarose gel followed by visualization with a labeled probe or by hybridization of the product to a specific probe using an enzyme-labeled immunoassay protocol.

Table 1
Micro-Organisms Identified by Real-Time Amplification Protocols

Bacteria

Staphylococcus aureus
Streptococcus pyogenes
Streptococcus agalactiae
Enterococcus species
Listeria monocytogenes
Corynebacterium diphtheriae toxin
Mycobacterium tuberculosis
Haemophilus influenzae
Neisseria gonorrhoeae
Neisseria meningitidis
Moraxella catarrhalis
Bordetella pertussis
Bordetella parapertussis
Mycoplasma pneumoniae
Mycoplasma genitalium
Chlamydia pneumoniae
Chlamydia trachomatis

Fungi

Candida species
Aspergillus fumigatus
Pneumocystis jiroveci

Parasites

Plasmodium vivax
Plasmodium falciparum
Plasmodium malariae
Plasmodium ovale
Toxoplasma gondii
Trichomonas vaginalis

Viruses

Herpes simplex virus
Varicella-zoster virus
Cytomegalovirus
Epstein-Barr virus
Parvovirus B19
Influenza A virus
Influenza B virus
Respiratory syncytial virus
Adenovirus
Human metapneumovirus
Severe acute respiratory syndrome virus
HIV-1 virus
HIV-2 virus
Enteroviruses
Hepatitis B virus
Hepatitis C virus

Real-Time Amplification

In traditional PCR, the amplicans or products of the PCR reaction are detected after the completion of the PCR cycle. For real-time amplification, the products are detected as they are made. There are several methods available for the amplification of the microorganism, and the basic protocols differ in the method used for amplification (10–15). The amplification methods are PCR, nucleic acid sequence-based amplification, and transcription-mediated amplification. The real-time amplification systems are based on the detection and quantitation of a fluorescent reporter molecule in the system. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the amplification reaction during the exponential phase, in which the first significant increase in the amount of product correlates with the initial amount of target template. A list of organisms that have been detected by real-time methods is found in Table 1.

REFERENCES

1. Miller JM. A Guide to Specimen Management in Clinical Microbiology. Washington, DC: ASM Press, 1996.
2. Buttery JP. Blood cultures in newborns and children: optimising an everyday test. *Arch Dis Child Fetal Neonatal Ed* 2002;87:F25–F28.
3. Bankowski MJ. Real-time nucleic acid amplification in clinical microbiology. *Clin Microbiol Newslett* 2004;26:9–15.
4. Persing DH, Tenover FC, Versalovic J, et al., eds. *Molecular Microbiology: Diagnostic Principles and Practice*. Washington, DC: ASM Press, 2004.
5. Tang TW, Von Graevenitz A, Waddington MG, et al. Identification of coryneform bacterial isolates by ribosomal DNA sequence analysis. *J Clin Microbiol* 2000;38:1676–1678.
6. Murray PR, Baron EJ, Pfaller MA, Tenover FC, Tenover RH, eds. *Manual of Clinical Microbiology*, 7th ed. Washington, DC: ASM Press, 2003.
7. Farkas DH, Kaul KL, Wiedbrauk DL, Kiechle FL. Specimen collection and storage for diagnostic molecular pathology investigation. *Arch Pathol Lab Med* 1996;120:591–596.
8. Randhawa JS, Easton AJ. Demystified ... DNA nucleotide sequencing. *Mol Pathol* 1999;52:117–124.
9. Garrity GM, Boone DR, Castenholz RW (eds.). *Bergey's Manual of Systemic Bacteriology*, 2nd ed. New York, Springer-Verlag, 2001.
10. Leone G, van Schijndel H, van Gemen B, Kramer FR, Schoen CD. Molecular beacon probes combined with amplification by NASBA enable homogeneous, real-time detection of RNA. *Nucleic Acids Res* 1998;1:26:2150–2155.
11. Bergeron MG, Danbing M, Menard C, et al. Rapid detection of group B streptococci in pregnant women at delivery. *N Eng J Med* 2000;243:175–179.
12. Tang YW, Ellis NM, Hopkins MK, Smith DH, Dodge DE, Persing DH. Comparison of phenotypic and genotypic techniques for identification of unusual aerobic pathogenic Gram-negative bacilli. *J Clin Microbiol* 1998;36:3674–3679.
13. Kraus G, Cleary T, Miller N, et al. Rapid and specific detection of *Mycobacterium tuberculosis* using fluorogenic probes and real-time PCR. *Mol Cell Probes* 2001;15:375–383.
14. Moody A. Rapid diagnostic tests for malaria parasites. *Clin Microbiol Rev* 2002;5:66–78.
15. Cleary T, Roudel G, Casillas O, Miller N. Rapid and specific detection of *Mycobacterium tuberculosis* using the Smart-Cycler instrument and a fluorogenic probe. *J Clin Microbiol* 2003;41:4783–4786.



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