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Congenital
and Perinatal
Infections

A Concise Guide to Diagnosis

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HUMANA PRESS • TOTOWA, NEW JERSEY
To the memory of my parents, Thelma and Haywood Hutto
I wish to acknowledge the dedication, patience, and hard work of Terri Hicks without whom this book would not have been possible.

—CH
A concise clinical reference that facilitates the diagnosis of intrauterine and perinatally acquired infections was the goal in creating the *Congenital and Perinatal Infections: A Concise Guide to Diagnosis*. Information about the natural history, management, and outcome of these infections is well detailed in many other sources and so has not been included. Rather, the focus of the book is diagnosis. The initial chapters provide general information about serological and nonserological assays that are used for the diagnosis of infections, and a chapter about the placenta includes details about histopathological findings that can be helpful with the diagnosis of congenital infections. The remainder of the book is devoted to the diagnosis of specific congenital and/or perinatal infections. As illustrated in the chapters about specific infections, the approach to diagnosis of a congenital or perinatally acquired infection in the neonate begins, when possible, with consideration and diagnosis of infection in the pregnant woman, knowledge of how the infection is transmitted, and the risk of that infection for the woman and her fetus or neonate. The possibility of congenital or perinatal infection in neonates is usually considered because of the diagnosis of, or concern about a specific infection in, a mother during pregnancy that can be transmitted to the neonate or because of clinical findings in the neonate at birth that suggest an infectious cause. Diagnosis is then made using both knowledge about the most appropriate assays for detection of that infection and the timing of these assays.

This book includes chapters about microorganisms that are both common and uncommon causes of congenital and perinatal infections. Some are considered infrequent causes of infection in the United States, but may be common in other areas of the world. With increasing global travel, even these less common infections must be considered at times, particularly in large urban areas. The book is not comprehensive in its coverage of microorganisms that have been reported to cause congenital and perinatal infections. It may, however, serve as a reminder to the clinician caring for pregnant women and their neonates of the increasing diversity of microorganisms that may be transmitted from a pregnant woman to her infant and result in congenital and perinatal infections.

It is hoped that the information provided in *Congenital and Perinatal Infections: A Concise Guide to Diagnosis* will be useful to all clinicians providing care to pregnant women and/or their infants for determining when women and their neonates are at risk for these infections and which assays are most appropriate to use for their diagnosis.

*Cecelia Hutto, MD*
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The Tools of Diagnosis
INTRODUCTION

Prior to the advent of highly sensitive antigen and nucleic acid detection methods, the diagnosis of acute infection was based on the measurement of the appearance of pathogen-specific antibodies, which most commonly were immunoglobulin G (IgG). A variety of assays were initially used, including complement fixation, hemagglutination, hemagglutination inhibition, immunofluorescence assays, radioimmunoassay (RIA), and eventually enzyme-linked immunosorbent assay (ELISA). Complement fixation, hemagglutination, hemagglutination inhibition, and immunofluorescence assays are usually titrated by assessing the highest dilution of serum that results in a positive response. In the case of RIA and ELISA, a quantitative value is determined at a single dilution of serum.

RIA quantitation is done by assessing counts per minute of an isotope (often $^{125}\text{I}$), which is used to label a pathogen-specific antibody. The scale of this quantitation can be on the order of several logs.

In the case of ELISA, a colorimetric change resulting from the action of an enzyme on a clear substrate is measured in a spectrophotometer as an optical density (OD), measured at a wavelength appropriate for the specific substrate. The OD reading is generally at a range of 0–2 or 3. These OD values are not linear but semiquantitative. Thus, changes in “positive” ELISA values are not accurate. Positive responses are generally those that exceed some cutoff value. This cutoff is often determined by performing the test in a noninfected population and by adding several (e.g., 3) OD standard deviation values to the mean OD value. For example, if the uninfected population has a mean OD of 0.2 and a standard deviation of 0.04, a cutoff of 0.2 and $3 \times 0.04$ would result in a value (0.32) that statistically represents the 99th percentile. Only 1% of uninfected individuals would have OD values that exceeded the cutoff.

This false-positivity rate, perhaps because of nonspecific antigen–antibody reactions, is acceptable in certain situations but not others, such as screening for human immunodeficiency virus (HIV) infection. In such cases, a more specific test (e.g., a Western blot assay) that assesses true interaction of antibody with microbe-specific
antigens is necessary to confirm infection. These shortcomings limit the usefulness of ELISAs to screening tests and not to changes in antibody present in the biologic fluid tested.

**DIAGNOSING INFECTION BY SEROLOGIC MEANS**

When there is no preinfection serologic IgG titer, the presence of significant changes (i.e., greater than fourfold increases or decreases of antibody for all tests except RIA and ELISA) within a 2- to 6-week period of time would constitute significant antibody production. Although this technology is reasonably specific and sensitive when dealing with adult infection, the ability to utilize this strategy in the diagnosis of congenital infections is compromised by the fact that the transplacental passage of IgG is highly efficient in the last months of gestation (1). Thus, a full-term newborn was likely to “inherit” titers of specific IgG comparable to what was present in the maternal circulation. Transport of the other immunoglobulin classes does not occur because the passage of antibody from the maternal side of the placenta to the infant side results from the expression of only IgG Fc receptors (FcγRIII or CD16) on the syncytiotrophoblast. Expression of these receptors begins at 3 months of gestation and increases during gestation. Bound IgG can then be internalized in the placental cell and eventually released into the fetal/infant circulation. Consequently, the measurement of pathogen-specific IgG antibody in the newborn can only be interpreted as a surrogate for evidence of maternal infection. Pediatricians and other health care providers are then left to turn to a tincture of time to utilize this information as a diagnostic tool. Three of the four IgG subclasses have half-lives of about 23 days (IgG3, which represents only 7% of the total IgG, has a half-life of 9 days). By waiting for several half-lives, maternal antibody would be expected to decline significantly. A stable titer of antibody for 6 or more months, which does not drop as expected, is presumably caused by intrinsic antibody production by the fetus or newborn.

When maternal titers of antibody are high (e.g., HIV-infected mothers may have anti-HIV titers that exceed 1 million), the time required for sufficient half-lives to pass to render an ELISA assay negative may take as long as 15–18 months. For example, an HIV ELISA test often is performed at a 1/100 dilution of serum. If the maternal titer is 4 million at birth, a 33,000 reduction in titer (which represents 15 half-lives or 15 × 23 = 345 days) would still yield a positive assay. In fact, the average time to deplete maternal HIV and hepatitis C antibody in the infant is about 9 months.

Even in an earlier era, when the only treatable congenital infections were syphilis and toxoplasmosis, waiting for several months to make a diagnosis of congenital infection was not tolerable for physicians and parents of affected or at-risk children. In the case of treponemal infection, the empiric treatment of all children born to mothers with serologic evidence of syphilis (positive serum rapid plasma reagin and fluorescent treponemal antibody-absorption tests) is sufficiently simple that actual diagnosis of infection in the child was not usually necessary for initiating therapy. Moreover, the utility of the strategy of testing cord blood and neonatal sera also appears to be inferior to maternal sera for detecting prenatal exposure to syphilis (2).

In contrast to the situation with congenital syphilis, therapy for congenital toxoplasmosis is very long and should not be offered to those without definitive infection. With the advent of potentially effective therapies for infections with rapidly replicating
agents such as cytomegalovirus (CMV) or HIV, the need to make a rapid diagnosis increased in importance. In the following sections, various strategies used to diagnose congenital infections, particularly those comprised by the TORCH (toxoplasmosis, other infections, rubella, CMV, and herpes simplex virus [HSV]) agents, are explored. It should become obvious that they all have shortcomings. As a consequence, and with the development of more rapid and specific nucleic acid detection methods, assays utilizing polymerase chain reaction amplification of specific pathogen-related nucleic acid has largely supplanted serologic methods.

**Nonspecific Assays**

Initial efforts were directed at the measurement of immunoglobulin classes IgM and IgA, which are produced by the fetus and do not cross the placenta. The simple measurement of total IgM in newborn serum has been applied to the diagnosis of congenital infection (3). Alford and colleagues were able to attribute a 40-fold increased risk of congenital syphilis, CMV, and toxoplasma infection in infants with elevated cord blood IgM (4). Unfortunately, the usefulness of this approach is limited by the fact that as many as 10% of “normal” newborns have levels this high. In addition, in a study of congenital rubella, cord blood IgM was not increased unless the infected child had three or more abnormalities (5).

Elevated cord blood IgA has also been used to test nonspecifically for possible congenital infection. In a study of congenital CMV infection, nine of nine infected infants had increased IgA in their cord sera (6). Unfortunately, 7% of normal newborn sera also had elevated IgA. As for the measurement of IgM in cord blood specimens, contamination with maternal blood is always a worry.

**Specific Assays**

The measurement of specific antibody might improve the specificity of diagnostic serology. Ideally, an assay that is both very sensitive and very specific could even be adopted as a neonatal screening tool to detect clinically inapparent infections that would escape detection. The measurement of pathogen-specific IgM would be a reasonable choice to fulfill these criteria. When this strategy was applied in a Massachusetts neonatal screening program for congenital toxoplasmosis, 100 of 635,000 infants tested had positive screening tests. Congenital toxoplasma infection was confirmed in 52 infants, 50 of whom were identified only through neonatal screening and not through initial clinical examination. However, after the serologic results became available, more detailed examinations revealed abnormalities of either the central nervous system or the retina in 19 of 48 infants evaluated (7). The ability to perform a variety of serologic assays on dried filter paper makes neonatal screening feasible (8–10).

**Maternal Screening**

In contrast to routine neonatal screening, more typically physicians first try to identify women who underwent primary infection with a pathogen such as toxoplasma, CMV, HSV, rubella virus, or parvovirus during pregnancy. This can be accomplished by screening early and late in pregnancy and comparing titers of IgG to specific pathogens or by measuring pathogen-specific IgM. In the case of CMV infection, specific IgM is produced in more than 75% of pregnant women within 8 weeks of seroconversion (11). Unfortunately, one may not have the luxury of having an early
natal or prenatal serum titer. IgM, although produced in acute infection, may also persist for long periods of time, as evidenced by detection of toxoplasma-specific IgM in 7% of pregnant women who were infected prenatally (12). IgM may persist for more than 1 year after acute infection (13). In addition, for viruses that cause chronic infection (i.e., the herpesviruses), exacerbations of latent infection or reinfection with another strain of virus may also result in IgM production. It is important to differentiate primary infection from secondary infection for some pathogens in that the former is associated with a higher likelihood of congenital infection (14).

To complicate matters even further, not all IgM measurements are alike. For example, detection of specific antitoxoplasma IgM by immunofluorescence is seen less often (e.g., in 25% of congenitally infected) than with a double sandwich ELISA or by a similar agglutination assay (15,16). The interpretation of test results requires knowledge about the sensitivity and specificity of each assay, including its performance within a given laboratory. This has provided an impetus to explore other avenues to differentiate new from old infection.

Some investigators have utilized IgG avidity as a tool to better specify primary from recurrent infection. Primary infection typically results in low-avidity antibody (i.e., antibody that does not bind strongly to antigen under extreme conditions); recurrent infection produces high-avidity antibody. In these assays, binding is often assessed in the presence of chaotropic (denaturing) agents, such as urea. When serum samples from 5 patients with recent primary toxoplasma infection were compared with those from 21 subjects with preexisting toxoplasma immunity, patients with primary infection exhibited a low avidity of toxoplasma-specific IgG, which persisted for several months after the onset of symptoms of toxoplasmosis. In contrast, all subjects with past immunity had a high avidity of toxoplasma-specific IgG (17). This assay has been evaluated in controversial serology results in a reference lab and has been found to be the best discriminator for the evaluation of acute toxoplasma infection (18). Another approach to differentiating remote from recent toxoplasmosis infection in a pregnant woman utilizes the fact that acute infection results in greater agglutination of acetone-fixed tachyzoites than with formalin-fixed tachyzoites (19). This differential agglutination assay is only available in the laboratory of Jack Remington (Toxolab@PAMF.org).

In one study of CMV infection during pregnancy, even in the presence of an IgM-positive result, a CMV IgG avidity index above 65% on serum obtained during the first trimester of pregnancy could reasonably be considered a good indicator of past CMV infection (20). In acute infections, avidity indices below 50% were seen. Often, combining IgM detection with IgG avidity results in better predictor of acute maternal infection (21,22). The predictive value of IgG avidity in one study was quite significant in that the determination of anti-CMV IgG avidity at 6- to 18-week gestation could identify all women who would have an infected fetus/newborn (100% sensitivity) (23). Antibody avidity is also useful when trying to diagnose congenital rubella late in the first year of life, when maternal IgG of possible high avidity has waned, leaving low-avidity IgG in the infant.

The ability to make neutralizing antibody to a pathogen is well correlated with antibody avidity. In the case of acute CMV infection in pregnancy, neutralizing antibody is not seen until 15 weeks after infection, but it persists thereafter (24). Consequently,
when there is lab expertise to perform this assay, the neutralization assay can be used as a reliable method for discriminating acute primary from previous or recurrent infection in a single serum sample.

Infant Testing

In theory, the measurement of IgM and IgA can be achieved by immunofluorescence when a specific pathogen-infected cell is available or by ELISA techniques when a relatively purified antigen is available. In practice, as indicated in the discussion of maternal screening, there are problems. A comparison of the characteristics of the various antibody classes in diagnostics is shown in Table 1.

In addition, because the immune system of the fetus is relatively immature, particularly early in gestation, the ability to make specific antibody in utero may be impaired. In congenital rubella infection prior to 10 weeks of gestation, no antibody production may occur (25). Fewer than 50% of fetuses with congenital parvovirus infection have measurable IgM (26), and less than 50% of fetal blood from toxoplasma-infected fetuses was IgM positive (27). Even if antibody production occurs, competition from the presence of high titers of maternal IgG antibodies in the serum or plasma may obscure the antigen and prevent binding of the child’s IgG or IgM (28). In addition, if there is rheumatoid factor (e.g., IgM anti-IgG) present in the pediatric serum, the detection of non-IgG antibodies binding to the pathogen antigen in the infant serum may represent antimaternal IgG (perhaps specific for the pathogen of interest) rather than actual antibody directed at the micro-organism itself. The rheumatoid factor may result from fetal immunization with maternal IgG allotypes.

To improve the specificity of IgM measurement in the child, a number of strategies have been employed. These include (1) chromatographic separation of IgG and IgM from serum (29); (2) the treatment of the serum with goat or rabbit anti-IgG antibodies (30); (3) mercaptoethanol treatment of sera to disrupt IgM (31); (4) pretreatment of the sera with staphylococcal protein A (32) (a protein to which all IgGs and some IgMs bind); (5) pretreatment of the sera with streptococcal protein G (33) (an even more selective IgG-binding protein); and (6) the use of antigen capture techniques (34). In this last assay, nonhuman anti-IgM antibody is used to capture human IgM. The specificity of this IgM is determined by adding a labeled microbial antigen, which can be detected by ELISA.

A different but related approach to the serologic diagnosis of congenital infection attempts to measure pathogen-specific IgA or IgE. Although IgM production may oc-
congenital and perinatal infections

Cur prior to the development of IgA, this should not be a factor when infection has occurred in utero. When pathogen-specific IgM and IgA were compared for sensitivity and specificity in the diagnosis of congenital toxoplasmosis, they were comparable, with a sensitivity of about 60% in neonatal blood specimens and excellent specificity (IgM 98%; IgA 100%) (35). In other studies, the sensitivity of IgA may either exceed that of IgM (36–38) or be less than what is seen with IgM (39,40). Even when used together, the measurement of IgM and IgA will still miss about 25% of children with congenital toxoplasmosis (41). Toxoplasma-specific IgE was detected in 86.6% of patients with toxoplasmic seroconversion, and it was produced as early as IgA and IgM (42). The relatively short kinetics of IgE (it is rarely detectable beyond 4 months) can be a useful tool to date the infection more precisely. For the diagnosis of congenital toxoplasmosis, specific IgE was detected less frequently than IgM or IgA (25 vs 67.3%). An immune capture IgE assay for toxoplasmosis has also been evaluated in congenitally infected infants (43).

The ability to measure IgM and IgA is still problematic as a diagnostic tool when trying to diagnose a congenitally infected child after 6 months of age. In this situation, these immunoglobulins that were present at birth may no longer be so. In this scenario, the ability to assess IgG avidity may yield useful information. In a study of congenital rubella infection, low-avidity specific IgG persisted in two children until age 23–31 months (44).

For many congenital infections, the ability to detect IgM or IgA in infected infants is considerably less sensitive than detection of pathogen-specific nucleic acid (45). However, for parvovirus, the sensitivity of serologic diagnosis may approach that of polymerase chain reaction positivity when newborn sera are tested (46). In some situations, such as diagnosing enterovirus infections, the number of different serologically unique strains capable of producing disease makes serologic testing impractical. In most studies, the application of serologic techniques to cord blood rather than to neonatal sera results in a decrease in specificity, perhaps because of contamination with maternal blood.

When perinatal infection occurs, such as HIV infection, production of either IgM or IgA is delayed for several months after infection (47–50). This virtually precludes the use of serology for the very early diagnosis of perinatal HIV infection.

In Vitro Antibody Production

A different approach to serologic diagnosis of newborn infection is to measure in vitro antibody production by lymphocytes isolated from the child and washed free of maternal antibody prior to culture. Specific antibody can be measured by Enzyme-linked ImmunoSPOT (ELISPOT), ELISA, or Western blot. In ELISPOT, lymphocytes are added to antigen-coated plastic or nitrocellulose and incubated for some period of time; after the cells are washed away, local antibody production is measured by detecting “spots,” resulting from adherent antibody, by enzymatic-colorimetric techniques (51). For the other two assays, the supernatants of cultured lymphocytes are added to wells or nitrocellulose strips containing antigens, much the way diluted sera are tested by conventional serology. The presence of color changes in the ELISA plates or the presence of bands consistent with known antigen determinants on strips from electrophoresed crude antigens is diagnostic of pediatric infection.
Such techniques have been utilized to diagnose HIV infection in young infants (52–55). Unfortunately, the production of antibody to HIV in infants is not usually seen until 3–6 months of age. Because viral replication or nucleic acid can be measured before 2 months of age, these techniques, as well as the simpler, more conventional measurement of serum IgM and IgA to HIV, have largely been abandoned as diagnostic tools.

**Antibodies to Neonate-Specific Antigens**

One largely unexplored serologic avenue for the diagnosis of congenital infections involves the demonstration of antibodies in congenitally infected fetuses or neonates to microbial antigens that are not present in maternal serum. This strategy was employed in a study of Chagas’ disease. In most cases, IgG specificities in the newborns mirrored those of their mothers, but congenitally infected newborns in addition had IgG antibodies to *Trypanosoma cruzii* antigens that were undetectable in their mothers. The new IgG specificities observed most frequently were against a shed acute phase antigen and less frequently against nine different parasite antigens (56).

Finally, serologic testing is also useful for screening children with evidence of potential congenital infection (e.g., children with mental retardation and chorioretinitis or chorioretinal scars) who test seronegative for traditional TORCH agents. In one study, lymphocytic choriomeningitis virus (LCMV) was responsible for visual loss in two of four children, secondary to chorioretinitis in a population of severely retarded children (57). The diagnosis was realized by detecting elevated levels of IgG to LCMV in these children, a finding also noted by a French study of two children with chorioretinal scars for whom LCMV was detected by ELISA and confirmed by Western immunoblotting (58).

**REFERENCES**


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**

Imunoassays 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 pneumophilia* 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 appear 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/μL (0.002% parasitemia).

**DFAs 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-
otially gained by working with specimens from patients infected with human immuno-
deficiency 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 hybridiza-
tion 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 prokary-
otic and eukaryotic micro-organisms. The 70S ribosome of prokaryotic cells is com-
posed 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 mi-
croorganisms 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 se-
quence 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 discrimi-
nation among members of the group. Reports have demonstrated the detection of sub-
species 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 $10^3$–$10^5$ copies of rRNA per cell, whereas fast-growing faculta-
tive 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 re-
leased 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 microorganisms 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 microorganisms 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 amplicon 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

<table>
<thead>
<tr>
<th>Bacteria</th>
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<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
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<tr>
<td><em>Streptococcus pyogenes</em></td>
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<tr>
<td><em>Streptococcus agalactiae</em></td>
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<tr>
<td><em>Enterococcus</em> species</td>
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<tr>
<td><em>Listeria monocytogenes</em></td>
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<tr>
<td><em>Corynebacterium diphtheriae</em></td>
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<tr>
<td><em>Mycobacterium tuberculosis</em></td>
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<tr>
<td><em>Haemophilus influenzae</em></td>
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<tr>
<td><em>Neisseria gonorrhoeae</em></td>
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<tr>
<td><em>Neisseria meningitides</em></td>
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<tr>
<td><em>Moraxella catarrhalis</em></td>
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<tr>
<td><em>Bordetella pertussis</em></td>
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<tr>
<td><em>Bordetella parapertussis</em></td>
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<tr>
<td><em>Mycoplasma pneumoniae</em></td>
</tr>
<tr>
<td><em>Mycoplasma genitalium</em></td>
</tr>
<tr>
<td><em>Chlamydia pneumoniae</em></td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em></td>
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<table>
<thead>
<tr>
<th>Fungi</th>
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<tbody>
<tr>
<td><em>Candida</em> species</td>
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<tr>
<td><em>Aspergillus fumigatus</em></td>
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<tr>
<td><em>Pneumocystis jiroveci</em></td>
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<th>Parasites</th>
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<tr>
<td><em>Plasmodium vivax</em></td>
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<td><em>Plasmodium falciparum</em></td>
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<tr>
<td><em>Plasmodium malariae</em></td>
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<tr>
<td><em>Plasmodium ovale</em></td>
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<tr>
<td><em>Toxoplasma gondii</em></td>
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<td><em>Trichomonas vaginalis</em></td>
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<tr>
<th>Viruses</th>
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<tr>
<td><em>Herpes simplex</em> virus</td>
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<tr>
<td><em>Varicella-zoster</em> virus</td>
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<tr>
<td><em>Cytomegalovirus</em></td>
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<tr>
<td><em>Epstein-Barr virus</em></td>
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<tr>
<td><em>Parvovirus B19</em></td>
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<tr>
<td><em>Influenza</em> A virus</td>
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<tr>
<td><em>Influenza</em> B virus</td>
</tr>
<tr>
<td><em>Respiratory syncytial</em> virus</td>
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<tr>
<td><em>Adenovirus</em></td>
</tr>
<tr>
<td><em>Human metapneumovirus</em></td>
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<tr>
<td><em>Severe acute respiratory</em> syndrome virus</td>
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<tr>
<td><em>HIV-1 virus</em></td>
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<td><em>HIV-2 virus</em></td>
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<tr>
<td><em>Enteroviruses</em></td>
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<tr>
<td><em>Hepatitis</em> B virus</td>
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<tr>
<td><em>Hepatitis</em> C virus</td>
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