

BACK TO BASICS



A review of the scientific foundations of current clinical practice

Molecular Tools in the Diagnosis and Management of Infectious Diseases

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Introduction

Molecular diagnostic tools and detection methods such as nucleic acid amplification are being used increasingly in the clinical microbiology laboratory to enhance the identification of microbial pathogens and to assist physicians in the diagnosis and management of a variety of infectious diseases. The principle of this technique is to detect and amplify a unique gene of the microorganism. This speeds the identification and reporting of microbial pathogens without reliance on their phenotypic characteristics.

Effective clinical management of infectious diseases depends primarily on accurate identification of the causative pathogen. The early recognition of an infectious agent allows clinicians to make sound therapeutic decisions and avoid the indiscriminate use of antibiotics, which ultimately favors the development of antimicrobial resistance.

To attempt to make nucleic acid amplification techniques (NAATs) understandable, we discuss the limitations of conventional diagnostic methods and how NAATs and polymerase chain reaction (PCR), in particular, can overcome these limitations.

Conventional Clinical Microbiology

Microbiology laboratories have developed many dependable ap-

proaches for detecting and characterizing pathogens within a reasonable time frame. These methods include microscopy, culture, antigen detection, immunoserology, and more recently, nucleic acid probes. Conventional identification of microbial pathogens relies on discerning the phenotypic characteristics of the organisms. Bacterial metabolic characteristics, fungal conidiogenesis, parasitic morphology, and viral cytopathic effect are some of the phenotypic characteristics used commonly. Unfortunately, phenotypic characteristics often are not sufficiently discriminatory for strain differentiation. DNA probes, which identify nucleic acid sequences by hybridization, have replaced biochemical reactions and morphology for definitive identification of some infectious agents. Probes are used to identify slowly growing mycobacteria and to detect fastidious organisms such as *Chlamydia trachomatis* and *Neisseria gonorrhoeae*. DNA probes offer somewhat greater specificity than do immunoassays, although like immunoassays, they are not sufficiently sensitive to replace conventional culture in most cases.

Limitations of Conventional Diagnostics

Although conventional diagnostic methods are dependable, most approaches have limitations.

Culture

Certain microbes require special media (eg, Lowenstein-Jensen selective media for *Mycobacterium tuberculosis* and other mycobacteria and Martin-Lewis media for *N gonorrhoeae*); others require special conditions (medi-

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cally important anaerobes require an atmosphere devoid of oxygen and media containing reducing substances such as cysteine). Cultivable microbes may fail to grow after exposure to antimicrobials or poor specimen handling. Certain pathogens, such as *Bordetella pertussis*, *Bartonella henselae*, and *Legionella pneumophila*, are fastidious and, consequently, difficult to isolate. Others, such as *M leprae*, *Treponema pallidum*, and *Pneumocystis carinii*, cannot be cultivated in vitro. Viruses such as human papillomavirus, hepatitis C virus, and hepatitis B virus also resist cultivation. The detection of *M tuberculosis* requires prolonged periods of cultivation, and empiric therapy frequently is initiated pending the accurate identification.

Direct Antigen Detection

The direct detection of microbial antigens in clinical specimens is an essential component of diagnostic microbiology. These tests demonstrate the reaction of an antibody that has a target antigen in the clinical specimen. Some of the methods used for microbial antigen detection include immunofluorescence (IF), radioimmunoassay (RIA), and enzyme-linked immunosorbent assay (ELISA), which largely has replaced RIA. With the advent of highly specific monoclonal antibodies, immunoassays provide technical simplicity, specificity, and rapidity.

Antigen detection methods provide diagnostic information within a few hours for viruses such as respiratory syncytial virus (RSV), influenza, rotavirus, herpes simplex virus, cytomegalovirus, hepatitis B virus, and human immunodeficiency virus (HIV). The rapid diagnosis of bacterial antigens such as group A beta-hemolytic streptococci in pharyngeal specimens has a significant impact on management. Antigen detection as-

says for *Giardia* sp and *Cryptosporidium* sp have replaced the traditional ova and parasite examination in many laboratories.

The sensitivity and specificity of antigen detection tests vary according to the test method and the pathogen antigen. Despite their rapidity and simplicity, these tests often have relatively poorer sensitivities, resulting in low negative predictive values. In such cases, a negative result requires further confirmatory testing by conventional cultures.

Nucleic Acid Detection and Amplification

Over the past decade, nucleic acid amplification has emerged as a diagnostic strategy that competes successfully with biologic amplification (culture) and, in some settings, significantly improves microbial detection and characterization by increasing sensitivity further while retaining relatively high specificity. This results in a significant impact on the diagnosis and management of infectious diseases. Nucleic acid amplification also provides a sensitive alternative for the diagnosis of noncultivable or slowly growing pathogens.

Several strategies for the amplification of nucleic acid have been described, including amplification of target nucleic acid (eg, PCR and transcription-mediated amplification), amplification of nucleic acid probe (eg, ligase chain reaction), and signal amplification (eg, branched-probe DNA assay). As these molecular methods become more widely available, physicians need to understand their clinical applications, interpretation, potential advantages, and limitations. In this article we focus on the PCR and its applications because it currently is the molecular diagnostic tool used most widely.

PCR

PCR exploits the ability of the polymerase enzyme to synthesize DNA from the four precursor molecules (adenine, guanine, cytosine, and thymine) and reproduce the genetic material of any organism in essentially unlimited quantities to use for detection of any organism containing nucleic acid. The primary principle of PCR is exponential amplification of a target DNA or RNA sequence, resulting in the generation of millions of identical copies of DNA fragments starting from just a few copies. The reaction can be completed in less than 1 hour. The essential reagents required for nucleic acid amplification are: 1) template: the sequence of nucleic acid (DNA or RNA) that is to be amplified; 2) nucleotides: (adenine, guanine, cytosine, and thymine) building blocks needed for making the product DNA; 3) primer: a short sequence of nucleotides complementary to template nucleic acid; 4) *Taq* DNA polymerase: a heat-stable enzyme that makes a new complementary copy of the template nucleic acid by adding nucleotides to the annealed primer; and 5) reverse transcriptase: an enzyme that converts RNA into a complementary DNA copy (cDNA) for reverse transcriptase (RT)-PCR.

PCR is based on the ability of the thermostable *Taq* DNA polymerase to make a new complementary copy of the template DNA sequence. Initially, the nucleic acid (DNA or RNA) is extracted from the clinical specimen. The basic technique of PCR includes repeated cycles of heating and cooling to amplify the nucleic acid sequence (DNA fragments) (Figure). Each cycle consists of three steps: 1) DNA denaturation, in which heat (95°C) is used to separate the extracted double-stranded DNA into single strands; 2) annealing, in which cooling to about 55°C

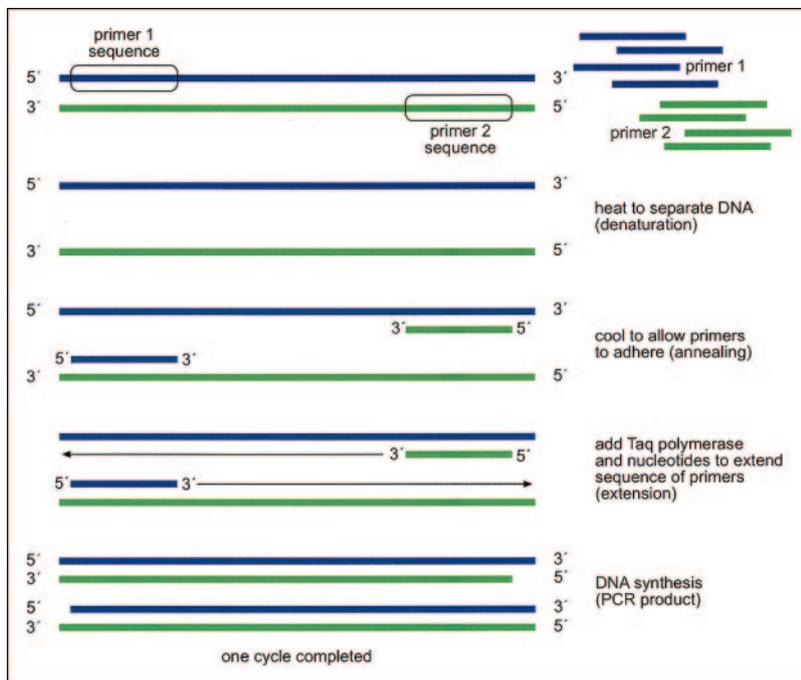


Figure. Schematic representation of the polymerase chain reaction.

allows specifically designed primers to adhere or anneal to their complementary template sequence; and 3) extension, in which the thermostable enzyme (*Taq* DNA polymerase) and nucleotides are added to extend the sequence between the primers. Consequently, at the end of each cycle a new DNA fragment (product DNA) complementary to the template DNA is created, and the quantity of PCR product is doubled. The amplified template (target DNA) is usually a 100- to 400-base pair and represents a small but unique portion of the gene. The entire procedure is carried out in a programmable instrument that has the ability to change to the different temperatures required for repeated PCR cycles. Generally, the performance of 30 to 40 thermal cycles results in the generation of greater than 1 billion copies of product DNA.

(RT)-PCR

(RT)-PCR is a modification of the standard PCR that was developed to

amplify RNA templates. Because *Taq* polymerase uses only DNA as a template, the use of PCR to amplify RNA requires the inclusion of a reverse transcription step. The major steps of the (RT)-PCR assay are isolation of RNA, reverse transcription of RNA into cDNA, and amplification of the cDNA by PCR. In this process, the RNA template is converted initially to cDNA, which then can be amplified by standard PCR methods. (RT)-PCR has played an important role in the identification of RNA-containing viruses, including HIV, hepatitis C virus, enteroviruses, RSV, influenza viruses, and parainfluenza viruses. (RT)-PCR also can be applied to detecting messenger RNA.

The evaluation of bacterial 16S ribosomal RNA (16S rRNA) is another significant contribution of (RT)-PCR. Several segments of bacterial 16S rRNA gene are highly conserved in almost all bacterial species. Other segments are more variable and, thus, more species-specific. PCR amplification of the 16S rRNA

sequence has identified specifically the noncultivable *Tropheryma whippelii* and *B henselae*, which are the causative agents for Whipple disease and bacillary angiomatosis, respectively.

PCR Product Identification and Confirmation

Following target amplification, the PCR product DNA can be detected by a variety of methods. The methods used play an important role in defining the sensitivity and specificity of the assay.

PCR has been applied successfully to the diagnosis of virtually all classes of microorganisms. Organisms that are detected commonly by NAAT are listed in the Table.

Although PCR is a powerful tool that has extreme rapidity, sensitivity, and specificity in diagnosing infectious diseases, it may have potential technical difficulties. These include false-positive reactions due to the amplification of contaminating DNA and false-negative reactions due to the presence of PCR inhibitors interfering with the nucleic acid amplification.

Quantitative PCR and Real-time PCR

PCR and other amplification methods have been adapted to measure the amount of target nucleic acid present in clinical specimens. The most accurate of these methods is the real-time PCR. With specially designed thermocyclers, attached optical systems, and labeled probes, the amplification process is monitored in each tube as it occurs. A labeled probe added to the PCR mix confirms and quantifies the PCR product as it is being generated (in real-time). No additional postamplification detection or confirmation systems are required, thereby shortening the effective assay time markedly.

Table. Microorganisms Commonly Detected in Clinical Specimens by Nucleic Acid Amplification Methods for the Diagnosis of Laboratory Infectious Diseases

Organism	Specimen	Clinical Application	Comments
<i>Bordetella pertussis</i>	Nasopharyngeal swab	Diagnosis	Significantly more sensitive than culture and antigen detection by FA
<i>Chlamydia trachomatis</i>	Genital tract	Diagnosis	10% to 30% more sensitive than culture; United States Food and Drug Administration (FDA)-approved*
Cytomegalovirus	CSF, blood, ocular fluid, amniotic fluid	Diagnosis of CNS infection, systemic infection, retinitis, congenital infection; monitoring of preemptive therapy	Approximately twice as sensitive as culture; FDA-approved*
Epstein-Barr virus	CSF, blood	Diagnosis of AIDS-associated primary CNS lymphoma, PTLD	Quantitative measures used to monitor PTLD
Enteroviruses (coxsackie, ECHO, and polio)	CSF, blood	Diagnosis of encephalitis, meningitis	10% to 30% more sensitive than culture
Hepatitis B virus	Blood	Monitoring of therapeutic efficacy	Routine culture unavailable
Hepatitis C virus	Blood	Diagnosis of active infection, monitoring of therapeutic efficacy	Routine culture unavailable; FDA-approved*
Human immunodeficiency virus (RNA)	Blood	RNA: prognosis and monitoring of therapeutic efficacy	Several FDA-approved* quantitative RNA assays are available
Herpes simplex virus	CSF, blood, ocular fluid	Diagnosis of encephalitis, meningitis, acute retinal necrosis	Method of choice for CNS disease and acute retinal necrosis
<i>Mycobacterium tuberculosis</i>	Sputum	Diagnosis in conjunction with culture	Same-day diagnosis in >90% of smear-positive sputum specimens and ~50% sensitive for smear-negative specimens; FDA-approved*
<i>Neisseria gonorrhoeae</i>	Genital tract specimen	Diagnosis	FDA-approved*
<i>Toxoplasma gondii</i>	CSF, amniotic fluid, ocular fluid, tissue, blood, BAL	Diagnosis of <i>Toxoplasma</i> encephalitis, retinitis, congenital infection	Routine culture unavailable
Varicella zoster virus	CSF, ocular fluid, dermal lesions	Diagnosis of encephalitis, myelitis, congenital infection	Significantly more sensitive than cultures in all settings.

FA=fluorescent antibody, CSF=cerebrospinal fluid, CNS=central nervous system, AIDS=acquired immunodeficiency syndrome, PTLD=posttransplantation lymphoproliferative disorder; BAL=bronchoalveolar lavage

*United States Food and Drug Administration/FDA-Approved Molecular Diagnostic tests (<http://www.ampweb.org/>).

Adapted from Graman PS, Menegus MA. Microbiology laboratory tests. In: Betts RF, Chapman SW, Pen RL, eds. *A Practical Approach To Infectious Diseases*. Philadelphia, Pa: Lippincott Williams & Wilkins; 2003:929–956.

Clinical Applications

Case 1

A 19-year-old Caucasian male presented to the emergency department with fever and altered mental status. He was combative and had difficulty speaking. After the initial evaluation,

he developed a seizure that started with shaking of the left upper extremity and progressed to become generalized. Computed tomography showed no lesions or hydrocephalus. Cerebrospinal fluid (CSF) analysis demonstrated a white blood cell count of 250 cells/mm³

(12% polymorphonuclears, 78% lymphocytes), red blood cell count of 560 cells/mm³, protein of 85 mg/dL, glucose of 75 mg/dL (4.2 mmol/L), and a negative Gram stain.

PCR detection of HSV DNA in the CSF has become the method of

choice for the diagnosis of HSV encephalitis (HSE). Historically, the diagnosis of HSE required a brain biopsy with cell culture because the virus rarely can be detected by routine culture of the CSF. The sensitivity and specificity of HSV PCR for the diagnosis of HSE are estimated to be 96% and 99%, respectively, thus negating the need for brain biopsy.

Although the early initiation of acyclovir improves the prognosis for patients who have HSE, PCR as an adjunct to careful clinical assessment also may be used to achieve early diagnosis and minimize the unnecessary use of acyclovir. However, it must be emphasized that although used increasingly, PCR assays for herpes simplex DNA currently are not standardized, and the accuracy and reliability may vary widely among laboratories. Therefore, careful clinical assessment prevails.

Case 2

A 4-year-old African-American boy presented in August with a 2-day history of fever and headache. On the day of the presentation, he began to complain of neck pain. On physical examination, he had a temperature of 102.7°F (39.3°C), otherwise normal vital signs, mild photophobia, positive Brudzinski and Kernig signs, no rashes, and intact neurologic examination findings. A complete blood count showed a white blood cell count of 9,300 cells/mm³ with 45% polymorphonuclears and 40% lymphocytes. Analysis of the CSF revealed a white blood cell count of 75 cells/mm³ with 72% neutrophils, 8% lymphocytes, and 20% monocytes; protein of 22 mg/dL; glucose of 60 mg/dL (3.3 mmol/L); and a negative Gram stain. Intravenous ceftriaxone was started. Blood, urine, and CSF bacterial cultures were negative. He was discharged on the fourth hospital day. After dis-

charge, his CSF viral cultures became positive for enterovirus.

Aseptic meningitis refers to clinical and laboratory evidence of meningeal inflammation in patients who have negative bacterial CSF cultures. Aseptic meningitis affects an estimated 30,000 to 50,000 individuals each year in the United States. Viral infections are the most common cause, with enteroviruses accounting for approximately 85% of the cases. Occasionally, bacteria such as *Borrelia burgdorferi* may cause aseptic meningitis; noninfectious causes such as drug-induced aseptic meningitis rarely are reported following ibuprofen ingestion. In the past, identification of a specific agent has been difficult; 30% to 50% of the cases remained of an undetermined cause. Compared with cell culture, the traditional “gold standard,” PCR of CSF for detection of enteroviruses exhibits sensitivities of 86% to 100% and specificities of 92% to 100%. (RT)-PCR currently is superior to cell culture in terms of speed and sensitivity for the detection of enteroviruses in infants and children. Recent reports indicate that (RT)-PCR could have an impact on the clinical management of patients infected with an enterovirus, resulting in earlier hospital discharge, fewer diagnostic tests, decreased antibiotic use, and increased diagnostic certainty.

Case 3

A 16-year-old HIV-positive girl acquired hepatitis C virus (HCV) (genotype 1a) following a contaminated intravenous immune globulin transfusion. She was otherwise healthy, compliant with her antiretroviral therapy, and had undetectable HIV plasma viral loads. Her liver transaminase concentrations were chronically elevated. A liver biopsy was performed to determine the risk of disease progression.

Subsequently, combination therapy with pegylated interferon (peginterferon) plus ribavirin was initiated. HCV plasma viral loads were monitored by real-time quantitative PCR. A 2 log₁₀ drop in viral load was not realized within 6 months of initiating therapy. In keeping with 2002 National Institutes of Health guidelines regarding the management of HCV infection, treatment was discontinued.

In this case, (RT)-PCR identified the HCV infection, provided quantification to monitor therapy, and permitted genotyping of the HCV as a prognostic indicator. Six distinct HCV genotypes and multiple subtypes have been identified. In the United States and Western Europe, genotypes 1a and 1b are the most common, followed by genotypes 2 and 3. Knowledge of the genotype is crucial because it has predictive value in terms of the response to antiviral therapy, with better responses associated with genotypes 2 and 3 and poorer response with genotype 1. The recent development of effective regimens, including interferon and ribavirin, has increased the usefulness of HCV RNA loads in treatment follow-up and in defining patients who require therapy.

NAATs have been modified to determine viral loads. Viral load testing now is being used routinely to assess prognosis and monitor response to therapy for patients who have infection with HIV, hepatitis B virus, HCV, and cytomegalovirus. When used with other markers, such as CD4 cell count, plasma HIV viral load is an early and accurate marker of disease progression and a decisive factor in the initiation or modification of antiretroviral medications. Estimation of cytomegalovirus or Epstein-Barr virus viral loads may mark and predict the onset of reactivation. This allows better identifica-

tion of patients at high-risk for cytomegalovirus- or Epstein-Barr virus-related diseases and is especially useful in immunocompromised individuals.

Conclusion

PCR and related NAATs now are used widely by clinical microbiologists for diagnostic purposes (eg, *Chlamydia*, HCV, and HIV infection in the newborn) as well as for monitoring of therapeutic efficacy (HIV and HCV viral loads assays). In addition, molecular methods are gaining acceptance in identifying resistance genes (eg, *mecA* gene in methicillin-resistance staphylococci),

detecting viral mutations associated with drug resistance (eg, HIV, cytomegalovirus, HSV), and characterizing organisms for epidemiologic purposes. Molecular methods already are an important part of our daily lives, and their role is bound to expand significantly within the next few years. Therefore, it is important to understand the strengths and weaknesses of molecular testing methods and to stay abreast of developments in this rapidly evolving field.

Suggested Reading

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In Brief

Azole Antifungal Agents

Update on Antifungal Agents. Wellington M, Gigliotti F. *Pediatr Infect Dis J*. 2001;20:993–995

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Antifungal Agents: Mode of Action, Mechanisms of Resistance, and Correlation of These Mechanisms With Bacterial Resistance. Ghannoum MA, Rice LB. *Clin Microbiol Rev*. 1999;12: 501–517

Azole antifungals consist of two primary classes: imidazoles and triazoles. Both classes are fungistatic agents and share similar mechanisms of action. The

azoles interfere with the synthesis and permeability of fungal cell membranes by inhibiting cytochrome P450-dependent 14- α -sterol demethylase. This enzyme is required for the synthesis of ergosterol, the major sterol of most fungal cell membranes. The loss of cell membrane integrity inhibits cell division by causing intracellular swelling and interferes with cellular adhesion and epithelial penetration by allowing the protrusion of cytoplasm through the membrane. Currently available imidazoles include clotrimazole, miconazole, ketoconazole, econazole, and butoconazole. The triazoles used most widely are fluconazole, itraconazole, and terconazole.

Among the imidazoles, ketoconazole gained widespread popularity after its release as the only available oral agent for the treatment of systemic fungal infections. Its usefulness has been overtaken since by the new triazoles, but

ketoconazole remains effective as a second-line agent against certain candidal species, histoplasmosis, blastomycosis, coccidioidomycosis, and paracoccidioidomycosis. Itraconazole and fluconazole offer less drug-related toxicity and have been found to be more effective in the treatment of immunocompromised patients who have systemic fungal infections.

The absorption of oral ketoconazole depends on an acid pH. Antacids, histamine₂ blockers, and foods that neutralize gastric acid may interfere with drug solubility. Ketoconazole is 99% serum protein-bound, with drug penetration to the epidermis, skin appendages, synovial fluid, lung, saliva, and vaginal secretions. Penetration into cerebrospinal fluid is poor, thus limiting the drug's effectiveness against meningeal infections. Ketoconazole is metabolized in the liver. Dose adjustments are not required for patients who have

renal insufficiency, but many clinically significant drug interactions have been reported, thus restricting its use.

The most common adverse reactions to ketoconazole are dose-dependent anorexia, nausea, and vomiting, which occur in 20% of users. Patients also may experience rash or pruritus (2% to 4%), alopecia (8%), and diarrhea. By interfering with steroid biosynthesis, ketoconazole may contribute to the development of menstrual irregularities, gynecomastia, impotence, and a decrease in adrenocorticotropic hormone-stimulated adrenal cortisol production. Approximately 5% to 10% of patients develop increases in serum transaminases, with symptomatic hepatitis occurring much less frequently (~1:100,000 patients). If it must be used, the ketoconazole dose should be adjusted for patients who have significant hepatic dysfunction.

The primary triazoles, fluconazole and itraconazole, are now the first-line agents for the treatment of a variety of fungal infections, including oropharyngeal and esophageal candidiasis, for which they largely have replaced ketoconazole.

Fluconazole is highly absorbable from the gastrointestinal tract regardless of the gastric environment. In fact, oral fluconazole produces therapeutic drug levels as well as the intravenous preparation does. It is effective against oral, esophageal, and vaginal candidiasis; ringworm; histoplasmosis; mucormycosis; cryptococcosis; and coccidioidomycosis. Fluconazole is distributed widely throughout the body, penetrating well into saliva, urine, vaginal secretions, liver, skin, eye, and brain. Its excellent penetration into cerebrospinal fluid makes fluconazole useful in the treatment of cryptococcal and coccidioid meningitis, usually in conjunction

with amphotericin B. Fluconazole is excreted largely through the kidneys, thus requiring dose adjustment for patients who have renal compromise. Nausea (4%), vomiting (2%), and headache (2%) are its most common adverse effects, along with rash (2%), abdominal pain (2%), and diarrhea (1.5%). Reversible alopecia has been reported, as has hepatotoxicity, limiting its use by patients who have liver insufficiency.

Like fluconazole, itraconazole is available orally and intravenously and is metabolized predominately by the liver. Although the two drugs share a similar mechanism of action, itraconazole has a broader spectrum of activity. In addition to its use as an alternative to fluconazole, it has activity against blastomycosis and paracoccidioidomycosis, as well as subungual onychomycosis, sporotrichosis, tinea corporis, and tinea versicolor. Itraconazole penetrates poorly into the cerebrospinal fluid, limiting its effectiveness against infections involving the central nervous system. Common adverse effects include nausea and vomiting (10%), hypertriglyceridemia (9%), hypokalemia (6%), and rash (2%). Prolonged use has been associated with adrenal insufficiency and hypertension, and chemical phlebitis is a risk with the intravenous preparation.

With the increasing use of the imidazoles and triazoles, resistant organisms have emerged, prompting the development of new antifungal agents. Voriconazole, a second-generation triazole, inhibits both 14- α -sterol and 24-methylene-dihydrostanosterol demethylation, giving it a broader coverage than its predecessors, including activity against invasive aspergillosis and non-*albicans* candidal species. It has good bioavailability both intravenously and orally. Reversible visual disturbances occur in as many as 30% of

patients who use voriconazole. Because it is metabolized in the liver, it poses a risk for hepatotoxicity.

In addition to the azoles, other classes of antifungal agents are coming into use. Caspofungin is the first of the echinocandins to be released. It inhibits beta-1,3-glucan synthase, which is critical for the formation of the fungal cell wall. Not well absorbed from the gastrointestinal tract, caspofungin is available only for intravenous use. It is particularly useful against refractory aspergillosis and as an adjunct to amphotericin B. Caspofungin is fungicidal and has relatively few adverse effects. Some patients experience fever, infusion site reactions, or headache. Serum transaminases may become elevated during use and should be monitored in patients who have hepatic insufficiency.

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Comment: Back in 1998, commenting on an *In Brief* about amphotericin, I expressed my dismay that so old an agent, with all its unpleasant adverse effects and serious toxicity, remained the drug of choice for almost every serious fungal infection a pediatrician might have to treat. I said, "Somehow, in the age of nth generation cephalosporins, monobactams, quinolones, and ever newer macrolides, we should have available a 'kinder' antifungal agent." We are not yet ready to send amphotericin into retirement, but that day may be approaching.

*Henry M. Adam, MD
Editor, In Brief*

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