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Applications of the Polymerase Chain Reaction

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1. INTRODUCTION

During the past decade, the proliferation of polymerase chain reaction (PCR) applications has been phenomenal. Few of the biological sciences have failed to benefit from this amazingly versatile methodology. Today, PCR methods are being used by archeologists to identify dead royals; by water science chemists to detect pathogens in water supplies; by food scientists to genotype bacterial and yeast strains and to detect foodborne pathogens; by wildlife biologists to identify game animals confiscated from poachers; and by clinical laboratories to detect previously unrecognized infectious agents. The clinical applications of PCR methods are legion, and no single work can list them all. This chapter summarizes the technology, describes the major clinical applications of PCR, and discusses its limitations.

2. THE POLYMERASE CHAIN REACTION

Developed by researchers at the Cetus Corporation,[1–3] the polymerase chain reaction is an elegantly simple method for the *in vitro* synthesis and ampli-

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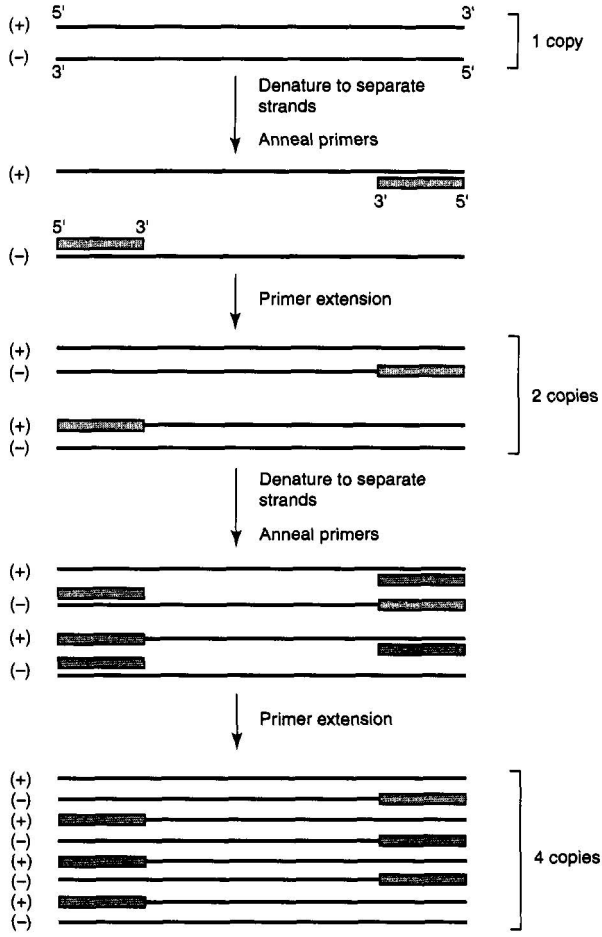

FIGURE 1. Diagram depicting the first two cycles of the polymerase chain reaction. Double-stranded target DNA is separated by heat denaturation. Two synthetic oligonucleotide primers anneal to the separated strands in the 5' to 3' orientation and flank the target DNA sequence. *Taq* DNA polymerase initiates new DNA synthesis at the 3' end of each primer, completing 1 cycle of amplification. After two cycles of synthesis, four copies of the original target DNA molecule have been generated.

fication of specific DNA sequences. To perform PCR, the nucleic acid sequences of an infectious agent must be known, and the target sequences must be unique to that organism (or a group of organisms) to be detected. Two oligonucleotides or primers, typically 15 to 30 bases long, are synthesized so that they are complementary to nucleic acid sequences flanking the region of interest in the target

DNA. In the assay, the primers are added to a reaction mixture containing the target nucleic acid, a heat-stable DNA polymerase, a defined solution of salts, and excess amounts of each of the four deoxynucleoside triphosphates. The mixture is subjected to repeated cycles of temperature changes. These thermal changes facilitate denaturation (94 ºC to 97 ºC) of the template DNA, annealing (55 ºC to 72 ºC) of the primers to the target DNA, and extension (72 ºC) of the primers so that the target DNA sequence is replicated (Fig. 1). When the reaction mixture is heated again to the denaturation temperature, both the original DNA and the newly synthesized DNA strand serve as templates for another round of DNA replication. Thus, the number of target DNA strands doubles with each cycle. PCR procedures for infectious agents typically include 20 to 40 thermal cycles. A single copy of the target sequence can be amplified 10^5- to 10^6-fold within 3 to 4 hours.

The target nucleic acid may be single- or double-stranded DNA or RNA. For RNA, a reverse transcriptase enzyme must be used to transcribe the RNA into cDNA before amplification. The amplified DNA is detected by one of many methods, including capillary electrophoresis, solid-phase or solution hybridization with appropriate detector probes, high-performance liquid chromatography, and agarose gel electrophoresis with direct visualization of nucleic acids stained with ethidium bromide or comparable reagents.

PCR is both sensitive and specific and is a relatively simple procedure to perform. Because PCR was the first widely used target amplification technology, a large number of clinical applications are available.[4-6]

3. CLINICAL APPLICATION OF THE POLYMERASE CHAIN REACTION

3.1. Early Detection of Infection

Early detection of infectious diseases is extremely important because early treatment often limits the extent of disease, reduces associated sequellae, and improves patient outcome. Early detection of infectious agents can also have a significant financial impact. For instance, early detection of enteroviral meningitis can reduce or eliminate unnecessary hospitalization and antibiotic usage. Early detection of sexually transmitted diseases can reduce the costs associated with pelvic inflammatory disease and the treatment of these infections as they are spread through the community.[7]

However, detecting early infections may be difficult by using traditional assays. Direct fluorescent antibody procedures and enzyme immunoassays (EIA) for detecting microbial antigens often do not have sufficient sensitivity to detect these early infections because the specimens often contain very few organisms or

infected cells. Culture methods provide a definitive diagnosis for many infections but culture results are usually not available for days or weeks after the specimens are collected. The enhanced sensitivity of PCR allows the laboratory to detect infectious agents early in the disease process. Because PCR can be completed in 8 to 48 hr, the test information is usually available within a clinically relevant time frame.

Serological testing can also be used to detect early infections. However, serologies are often unrewarding in early disease because agent-specific IgM may not be detectable at the time of presentation. In addition, the IgM response to infectious agents, such as *Toxoplasma gondii,* can persist for up to one year after the primary infection has resolved.[8] Thus, the presence of *T. gondii* IgM may not indicate an active infection. PCR can rapidly identify *T gondii* in amniotic fluids thereby providing a definitive diagnosis despite confusing serological patterns.

Seroconversion can also be used to document acute infections. For many infectious agents, the "window period" from infection to seroconversion is 7–21 days. This window period can be significantly longer with hepatitis C virus (HCV) where antibody responses may not be detectable until 12 or more weeks after the onset of hepatitis.[9] In contrast to serological tests, PCR methods usually detect HCV viremia 10–19 days after infection—well before the onset of hepatitis.[10]

3.2. Detecting Unculturable Infectious Agents

The use of PCR to detect unculturable infectious agents has significantly changed clinical medicine. Before PCR testing, infections caused by unculturable organisms were inferred on the basis of clinical presentation, tissue histopathology, and/or serological testing. Today, PCR detection of HCV virus and human papillomavirus (HPV) are the gold standards for identifying these agents in clinical specimens. PCR has also made significant contributions to our understanding of the natural history and pathology of these unculturable infectious agents. A representative list of unculturable agents detectable by PCR is shown in Table I.

Many other syndromes exist where an infectious etiology is suspected but the causative agent has not been isolated. The eventual identification of the pathogens associated with sarcoidosis, Kawasaki's disease, and type I diabetes mellitus will most likely be determined through the use of arbitrarily primed PCR and other molecular techniques.[11,12] Detecting and identifying these agents are the first steps toward their eventual control.

3.3. Detecting Slow Growing or Fastidious Agents

Some infectious agents grow slowly *in vitro* and detection using traditional culture methods can require days to weeks. Culture-based tests for slow-growing infectious agents, such as *Mycoplasma pneumoniae* and enteroviruses (Table 11), often

TABLE I
Unculturable Human Disease Agents Detected by PCR Methods

Infectious agent	Associated diseases
Astroviruses	Nosocomial and community-acquired gastroenteritis
Bartonella spp.	Bacillary angiomatosis and cat-scratch disease
Hepatitis B virus	Serum hepatitis, associated with hepatocellular carcinoma
Hepatitis C virus	Non-A, non-B hepatitis
Hepatitis D virus	Fulminant hepatitis in patients with hepatitis B coinfections
Hepatitis E virus	Epidemic or enterally transmitted, non-A, non-B hepatitis Associated with waterborne transmission
Hepatitis G virus	Associated with fulminant hepatitis
Human papillomavirus	Oral. skin, and anogenital warts, vaginitis, and cervical carcinoma
Norwalk agents	Sporadic community-acquired gastroenteritis
Whipple's disease bacterium	Systemic disease that usually involves the gastrointestinal tract and the mesentery and also produces intermittent arthralgia

have limited clinical utility because the laboratory results do not contribute to the initial diagnosis or treatment of the patient. In addition, delays associated with *Mycobacterium tuberculosis* cultures can significantly impede disease control efforts.[13] PCR and other amplification methods have significantly improved our ability to detect slow growing agents within a clinically relevant time frame. Rapid identification of infectious agents can also help limit the spread of these agents in the community.

Some infectious agents are fastidious (Table II), and isolating these agents requires special cells or media, special handling, and /or animal inoculation. Detecting these fastidious agents is beyond the capabilities of many hospital laboratories, and the delays in sending specimens to a reference laboratory limits the value of these tests for most practicing physicians. The increasing availability of PCR kits and reagent sets has allowed many hospitals to detect fastidious organisms, such as *T gondii* and Coxsackie A viruses, in a timely fashion and without animal inoculation.

3.4. Detecting Infectious Agents Dangerous to Grow

One of the problems in handling and cultivating infectious agents is the increased risk of acquiring laboratory-associated infections.[14] Laboratory workers are clearly at higher risk for acquiring *Coxiella burnetti*, *Brucella spp.*, *Francisella tularensis*, *Chlamydia psittaci*, and certain pathogenic fungi infections than the general population.[15] Although technical and scientific personnel account for most (83.2%) laboratory-acquired infections, janitors, dishwashers, and maintenance

TABLE II
Fastidious or Slow Growing Infectious Agents Detected by PCR

Agent	Associated disease(s)	Problems with isolation
Bartonella henselae	Cat-scratch disease	Does not grow on routine bacteriological media; growth is inhibited by sodium polyanethol-sulfonate (SPS); isolation requires >7 days
BK virus	Urethral stenosis in renal transplant patients and hemorrhagic cystitis in marrow transplant patients	Does not grow in most standard cell cultures; isolation requires weeks to months
Chlamydia pneumoniae	Pneumonia, associated with atherosclerotic heart disease	Cell culture can take 5 days
Coronaviruses	Colds, pneumonia, diarrhea, and necrotizing enterocolitis in newborns	Difficult to grow reproducibly in cell culture
Enteric adenoviruses	Diarrhea	Grows poorly or not at all in standard cell cultures
Enteroviruses	Upper respiratory disease, aseptic meningitis, myocarditis, enteritis, hand, foot, and mouth disease, hemorrhagic conjunctivitis	Not all enteroviruses grow in cell culture; virus isolation requires 2–14 days
Hepatitis A virus	Infectious hepatitis	Isolation methods can take up to 8 weeks
Human parvovirus B19	Erythema infectiosum (fifth disease), hydrops fetalis, chronic anemia, transient aplastic crisis	Does not grow in standard cell cultures; requires bone marrow explant cultures or erythrocyte precursor cultures
JC virus	Progressive multifocal leukoencephalopathy	Requires primary human fetal glial cultures; isolation requires weeks to months
Legionella pneumophila	Legionnaires' disease, community-acquired pneumonia	Fastidious organism; isolation requires 3–5 days
Mycobacterium tuberculosis	Tuberculosis	Slow growth; isolation requires up to 30 days
Mycoplasma pneumoniae	Atypical pneumonia and extrapulmonary manifestations	Culture requires 2–4 weeks
Mycoplasma hominis	Pelvic inflammatory disease, postabortal fever, postpartum fever, pylonephritis, involuntary infertility	Difficult to isolate; isolation procedures can take one week

TABLE II *(Continued)*

Agent	Associated disease(s)	Problems with isolation
Rubella virus	Rubella	Traditional isolation methods can take 2–6 weeks
Toxoplasma gondii	Toxoplasmosis	Isolationrequiresmouse inoculation; primary isolates rarely recovered in cell cultures

workers account for 13.7% and clerical workers account for 3.7% of infections in one study.[16]

PCR methods reduce the risks of cultivating these infectious agents because the organisms are usually inactivated during the nucleic acid extraction process and because the PCR products are normally not infectious. Although laboratory workers must still handle potentially infectious specimens, PCR can reduce the cutaneous, percutaneous, and aerosol exposures in handling infectious cultures. PCR also allows laboratories without biosafety level (BSL) 3 and 4 facilities to detect BSL-3 and BSL-4 agents in clinical (BSL-2) laboratories.

3.5. Detecting Nonviable Agents

The clinical laboratory is sometimes asked to detect infectious agents late in the course of disease or after the initiation of antimicrobial or antiviral therapies. Conventional culture methods usually produce negative results in these patients because most of the infectious agents are nonviable. The ability of PCR to detect a nonviable virus assists in diagnosing infection when other methods cannot. PCR also detects pathogens in dried blood spots, forensic specimens, and environmental samples where few, if any, viable organisms are present. Detecting infectious agents in immune complexes can help to elucidate the natural history of these infections and has improved our understanding of the immune response to infection.

The ability to detect nonviable infectious agents also presents problems in clinical medicine. Infectious agents killed by appropriate antibiotic usage may still be present at the sampling site and produce a positive result despite effective treatment. Therefore, PCR should not be used as a test of cure because killed organisms persist at some sites for weeks to months. In addition, Kaul *et al.*[17] found that 11 of 55 (20%) washes from sterile bronchoscopes contained residual human DNA and 2 of 55 (3.6%) bronchoscope washes contained *M. tuberculosis* DNA. These findings indicate that residual DNA persists in sterilized bronchoscopes and may be a source of false-positive PCR results.

3.6. Resolving Indeterminate Serologies

PCR methods are valuable for resolving indeterminate human immunodeficiency virus (HIV) and human T-lymphotrophic virus (HTLV) serologies.[18,19] Depending on the study cited, 2 to 49% of specimens that are positive for HIV antibody by enzyme immunoassay (EIA) may be indeterminate by Western blot.[20] 1 to 5% of these specimens are true positives, and these patients will develop antibodies to the *gag, pol,* and envelope proteins within 3 months.[21] Repeat testing in three months is usually sufficient to resolve these indeterminate western blot results. However, indeterminate Western blots are particularly troublesome during pregnancy because the obstetrician must determine whether to initiate antiretroviral therapy, thereby reducing the risk of transmitting HIV to the child.[22]

Detecting HIV infection of newborns born to HIV-seropositive mothers is difficult and time-consuming by culture methods. Antibody tests for HIV are not useful in these cases because these newborns possess maternal antibodies to HIV that may persist for 15 to 18 months after birth. PCR has proven extremely valuable for the early diagnosis of HIV infection in these children.[23]

3.7. Detecting Organisms Present in Low Numbers

Chlamydia trachomatis is the leading cause of sexually transmitted disease in the United States. More than four million new infections occur each year.[7] Left untreated, *Chlamydiae* ascends the female reproductive tract causing cervicitis, uteritis, and salpingitis. Nearly 50% of all pelvic inflammatory disease infections are caused by chlamydiae and one-fifth of these infections produce long-term complications, such as infertility and increased ectopic pregnancy rates.[7] Detecting and treating *C. trachomatis* infections is a formidable public heath challenge because most chlamydia-infected women and many men are asymptomatic and do not seek medical treatment.[27] Many of these asymptomatic individuals have low-level infections that are missed by traditional culture procedures.[7] PCR is significantly more sensitive than culture[24–26] and has largely replaced culture for screening asymptomatic women.

3.8. Small Specimen Volumes

PCR is especially well suited for detecting infectious agents in pediatric, forensic, ocular, and other specimens where tiny sample volumes are the norm. Small sample volumes severely limit the number of agents detectable by conventional methods. In contrast, multiple PCR procedures can be performed on these specimens. For example, five PCR tests (herpes simplex virus (HSV), cytomegalovirus (CMV), varicella-zoster virus (VZV), Epstein-Barr virus (EBV), and human herpesvirus 6) can be performed on a single 100 microliter intraocular

fluid specimen—a specimen volume barely sufficient to inoculate one tube or shell vial culture.[27] Multiplex PCR methods can further extend our ability to detect multiple agents from small volume specimens.

3.9. Predicting Antimicrobial/Antiviral Resistance

PCR-based genotypic assays have been developed for rapidly detecting genetic mutations that confer antimicrobial drug resistance. Specific bacterial or viral genes can be amplified by PCR and directly sequenced to identify alterations in the genome known to be associated with resistance to a given antibiotic or antiviral agent. Such assays have been used to detect specific mutations in the reverse transcriptase and protease genes of HIV-1 following the treatment of patients with antiretroviral drugs.[28,29] Clinical isolates of CMV have been screened by PCR to identify mutations in the UL97 phosphotransferase gene and the UL54 DNA polymerase gene that confer resistance to ganciclovir and/or foscarnet.[30] Genotypic analysis following PCR has also been used to detect multidrug resistant *M. tuberculosis*,[31] methicillin-resistant *Staphylococcus aureus*,[32] penicillinase-producing *Neisseria gonorrhoeae*,[33] and to identify antibacterial genes coding for extended-spectrum ß-lactamases,[34] aminoglycoside-modifying enzymes,[35] and erythromycin resistance.[36] For viruses and certain bacteria, using PCR to predict drug resistance offers speed and efficiency over phenotypic antimicrobial susceptibility assays in screening multiple isolates. Genotypic resistance assays can be cumbersome and technically demanding, however, and they are not routinely available in most clinical laboratories. These methods also have a disadvantage in that only known drug-resistant mutations are detected and phenotypic drug susceptibility assays are still required to identify drug-resistant microorganisms with novel resistance mutations.

3.10. Genotyping of Antigenically Identical Organisms

PCR has facilitated detecting and typing antigenically similar organisms and has allowed characterizing genetic variants of a given microbial agent. Such genetic analyses can provide useful information about the epidemiological and pathogenic behavior of microbes; detecting microbial colonization and spread within a given patient population; establishing phylogenetic relationships among organisms; recognizing genetic variants that may be resistant or refractile to antimicrobial drugs; and studying species identification and evolution.

Methods, such as arbitrarily primed PCR, interrepeat PCR, and random amplified polymorphic DNA PCR have been described for detecting genetic variations in a number of medically important bacteria, fungi, and parasites.[37,38] All of these techniques are based on the specific selection of primer annealing sites, which leads to the production of amplified fragments of different sizes that

can be visualized as fingerprints by gel electrophoresis. PCR fingerprinting[39] has been used to study species evolution, analyze population genetics, and identify new species of the parasites *Trypanosoma, Leishmania, Naeglaria, and Giardia.* This technique has been applied to epidemiological studies and the direct identification and species determination of fungi, such as *Aspergillus, Candida, and Histoplasma capsulatum.* Molecular typing and epidemiological analysis have also been performed on a number of medically relevant bacteria, including methicillin-resistant *S. aureus, Legionella pneumophila, Campylobacter spp.,* and *Escherichia coli strains* responsible for foodborne gastroenteritis, and *Helicobacter pylori.* Genetic information obtained about patient colonization and infection, outbreak delineation, and treatment failure may play a major role in managing and controlling infections with these organisms.

Viruses, such as HIV-1, HPV, and hepatitis viruses B (HBV) and HCV, demonstrate considerable nucleic acid sequence variability. The development of PCR-based genotyping assays for distinguishing genetic variants of these agents is clinically important for managing infected patients.[28,29,40–42] Viral genetic variation may affect the overall course of disease with these viruses and the responses of patients to antiviral therapy.

Failure of patients with HCV infection to respond to interferon-α therapy, for instance, is the result of the presence of a particular viral genotype. Infection with either genotype 1a or 1b is associated with a poor response to interferon treatment compared with infection with types 2a, 2b, or 3a. Also, the *in vivo* and *in vitro* resistance of HIV-1 to antiretroviral therapy is caused, in part, by the high spontaneous mutation rate in the viral genome during active replication of the virus and to the accumulation of multiple specific mutations in the coding regions of either the reverse transcriptase or the protease of the virus during prolonged therapy. More than 70 recognized HPV types produce epithelial warts of the skin and mucous membranes, but only a select number of these types cause lesions that progress to malignancy. HPV types 5 and 8 are associated with severe squamous cell carcinomas in patients with the rare skin disorder epidermodysplasia verruciformis, and HPV types 16, 18, 31, and 45 are associated with severe cervical dysplasia and the development of cervical carcinoma. HPV types 6 and 11 are rarely found in invasive cancers, but genetic variants of these types have an increased pathogenic potential which can lead to progressive cervical and respiratory cancers. Mutations in the precore/core gene of HBV in patients with hepatitis have led to the development of precore genetic variants that do not produce hepatitis B e antigen. These variants are thought to be associated with severe chronic active hepatitis or acute fulminant hepatitis. The development of mutations in the pre-S/S region of the surface antigen gene of HBV also results in the production of genetic variants capable of evading the immune response induced by HBV vaccines.

TABLE III
Identification of New Infectious Agents by PCR

Infectious agent	Associated disease
Bartonella spp.	Bacillary angiomatosis and cat-scratch disease
Ehrlichia chaffeensis	Human ehrlichiosis
Hepatitis C virus	Non-A, non-B hepatitis
Hepatitis G virus	Fulminant and chronic hepatitis
Human herpesvirus 8	All forms of Kaposi's sarcoma; AIDS-related body-cavity-based lymphomas; non—Kaposi's sarcoma skin lesions; Castleman's disease
Sin Nombre virus	Hantavirus pulmonary syndrome
Tropheryma whippelli	Whipple's disease—systemic disease that usually involves the gastrointestinal tract and the mesentary

3.11. Identifying New Infectious Agents

PCR enables the rapid detection and identification of previously unknown pathogens that cannot be grown in culture, that are present in too low numbers to be detected by more conventional laboratory methods, or are sufficiently different from previously characterized microbial agents. [11,43,44] The procedure relies on the use of primers to conserved or consensus sequences for initial microbial identification and then uses genus or species-specific primers and/or probes to further identify the organism of interest. In this way, diseases of unknown etiology and new microbial agents responsible for defined clinical syndromes are recognized and characterized. The impact of PCR on the association of newly described viral or bacterial agents with established infectious diseases can be seen in Table III

In 1990, DNA was extracted from bacillary angiomatosis lesions of AIDS patients and was analyzed using PCR and sequencing of conserved bacterial 16S ribosomal RNA (rRNA) to identify a unique bacterium.[45] Phylogenetic analysis of the amplified sequence showed that the agent is closely related to *Rochalimaea quintana,* the agent of trench fever. The organism was eventually classified as *Rochalimaea henselae* and later reclassified in the genus *Bartonella. B. henselae* is now recognized as a cause of bacillary angiomatosis and peliosis hepatitis in HIV-infected patients, and PCR has been used to help establish this organism as the primary etiologic agent of cat-scratch disease.[46] Because this organism is difficult to culture and detect by standard laboratory methods, PCR and other molecular techniques have played a major role in establishing this agent as the etiology of several important diseases.

Similarly, consensus sequence-based PCR was used to identify an organism

associated with Whipple's disease.[47] Although rod-shaped bacilli were identified histologically in Whipple's disease lesions many years ago, the suspected bacterium was never cultured or identified by conventional methods. With the advent of PCR, conserved regions of bacterial 16S rRNA were amplified and sequenced directly from infected tissues, and phylogenetic analysis characterized the agent as the actinomycete, *Tropheryma whippelli.*

PCR amplification and sequencing of bacterial 16S rRNA was used again in 1992 to identify a newly recognized organism from patients with ehrlichiosis.[48] Through phylogenetic analysis of the amplified sequence, it was shown that the organism is related but not identical to *Ehrlichia canis,* the cause of canine ehrlichiosis. Unlike *E. canis,* however, initial attempts to isolate the human agent were unsuccessful. This organism was later named *Ehrlichia chafeensis.*

An outbreak of unexplained acute pulmonary disease occurred in the southwestern United States in May 1993. Although clinical and epidemiological features suggested an infectious disease, no etiological agent was initially recovered. Cross-reactive antibodies to known hantaviruses were soon identified in sera from infected patients, but the antibody response suggested that the disease was caused by a previously unrecognized hantavirus. Conserved sequences of the G2 region of the M segment of known hantaviruses were used in a reverse transcriptase PCR of RNA extracted from diseased tissues to amplify genomic sequences of a hantavirus similar to Prospect Hill virus.[49] Sequence and phylogenetic analysis of the PCR products led to the discovery of a novel hantavirus called Sin Nombre virus. PCR and nucleotide sequence analysis also helped establish that this hantavirus could be maintained and transmitted by the deer mouse, *Peromyscus maniculatus.*

Unique DNA sequences of human herpesvirus type 8 (HHV-8) were first recognized using PCR and representational difference analysis.[50] The technique involves PCR amplification of small DNA fragments present in diseased tissue but absent from healthy tissue of the same patient. Now HHV-8 DNA has been detected by PCR in AIDS-associated and classic Kaposi's sarcoma and that occurring in HIV-negative homosexual men. PCR has also been used to detect HHV-8 DNA in AIDS-related body-cavity-based lymphomas, non-Kaposi's sarcoma skin lesions of transplant patients, Castleman's disease, peripheral blood lymphocytes of HIV-seropositive individuals with and without Kaposi's sarcoma, and prostate tissue and human semen of healthy immunocompetent individuals. PCR and representational difference analysis has also led to the identification of an HHV-8-infected established body-cavity B-lymphoma cell line and the subsequent development of an *in vitro* culture system for growing and characterizing the virus.[51]

Lastly, the molecular cloning of hepatitis C and G viruses has resulted in establishing sensitive PCR assays which have been used to detect these viruses and validate the importance of these agents as causes of chronic and fulminant non-A, non-B hepatitis.[52,53]

TABLE IV
Uses of Multiplex PCR to Detect Bacterial, Viral, or Parasitic Infections

Bacteria
Distinguish *Mycoplasma genitalium* from *Mycoplasma pneumoniae*
Molecular typing and epidemiological survey of *Clostridium perfringens*
Detection or differentiation of *Salmonella, Shigella,* or *Campylobacter* species in stools
Differentiation of enteropathogenic, enterotoxigenic, enteroinvasive, enterohemorrhagic, and enteroaggregative types of *Escherichia coli*
Simultaneous detection of *Actinobacillus actinomycetemcomitans* and *Porphyromonas gingivalis*
Detection of *Bordetella pertussis* strains in nasopharyngeal specimens
Detection of methicillin-resistant *Staphylococcus* species
Detection of *Chlamydia trachomatis Neiserria gonorrrhoeae, M. genitaliurn, Ureaplasma urealyticum* from patients with urethritis
Detection of *Hemophilus ducreyii* and *Treponema pallidum* in genital ulcerative disease (can also detect herpes simplex virus type 1 and 2 in the same reaction)
Detection and identification of *Yersinia* species
Rapid detection and differentiation of *Mycobacterium* species
Detection of multidrug resistant strains of *M. tuberculosis*
Detection of *H. influenzae* and *Streptococcus pneumoniae* in blood culture
Molecular typing of *H. influenzae*
Viruses
Detection of high- and low-risk types of human papillomaviruses
Simultaneous amplification of multiple HIV-1 gene sequences
Genotyping of hepatitis B, C, E, and G viruses
Detection and typing of human herpesviruses
Simultaneous amplification of respiratory synctial virus, influenza virus types A and B, and parainfluenza virus types 1, 2, and 3
Differentiation of human T lymphotrophic virus types I and II
Detection and differentiation of HIV-1 and hepatitis C virus from blood
Detection of adenovirus type 12, cytomegalovirus, and herpes simplex virus types from patients with celiac disease
Detection of human herpes virus type 6 and cytomegalovirus
Detection of adenovirus and herpes simplex virus types from eyes
Differentiation between polio and non-polio enteroviruses
Detection of herpes simplex virus types 1 and 2 in genital ulcerative disease (can also detect *H. ducreyii* and *Treponema pallidum* in same reaction)
Parasites
Differentiation of toxoplasmosis from AIDS-related central nervous system lymphoma

3.12. Simultaneously Detecting Multiple Agents

Multiplex PCR has been developed for simultaneously coamplifying two or more distinct nucleic acid targets in a single reaction tube. Unlike uniplex PCR, that detects only one gene target at a time, multiplex PCR is more practical and allows the development of diagnostic PCR panels for detecting multiple microbial pathogens from a single specimen. Multiplex PCR methods are especially useful

for differentiating microbial species or strains within a single assay. This method has also been used for gender screening, disease linkage analysis, genetic mapping and disease diagnosis, forensic studies, target quantitation, molecular typing, and epidemiological surveys.[54,55] The growing utility of multiplex PCR in detecting bacterial, viral, or parasitic infections is summarized in Table IV.

3.13. Quantitating Infectious Agents

The development of molecular assays to quantitate the levels of virus in infected patients may prove to be one of the most valuable tools to assess the progression of viral diseases, monitor the efficacy of antiviral therapy, predict treatment failure and the emergence of drug-resistant viruses, and to facilitate our understanding of the natural history and pathogenesis of certain viruses. Molecular methods, such as quantitative competitive PCR, reverse transcriptase-PCR, nucleic acid sequence-based amplification, and branched-chain technology are now available for accurately quantitating viral nucleic acids of HIV-1,[56] CMV,[57] EBV,[58] HBV,[59] and HCV.[60] Quantitative PCR assays include those based on externally amplified standards, limited dilutions of target sequences, or competitive amplification with internal standards. The incorporation of internal standards into quantitative PCR is the most informative with regard to controlling the many aspects of assay variability.

In HIV infections, quantitation of viral load is important because high levels of HIV-1 RNA have been related to vertical transmission of the virus from mother to fetus, faster progression to AIDS and death in both adult and pediatric patients, and the emergence of drug resistance during prolonged antiretroviral therapy.[61] Quantitative PCR studies with CMV have demonstrated that patients with active CMV disease have higher levels of CMV DNA and that a rapid rise in CMV DNA copy number correlates with symptoms and drug failure during treatment.[57] Monitoring the quantitative levels of HBV or HCV in patients with chronic hepatitis can assist in identifying those patients who will derive the most benefit from interferon-α therapy and for recognizing treatment failures and relapsing infections.[40,59,60] Using quantitative PCR it has recently been shown that EBV viral load increases several weeks before the development of post-transplant lymphoproliferative disorder (PTLD) in patients who have undergone solid organ transplantations.[58] Such information may be beneficial for preventing the severe morbidity and mortality associated with PTLD by providing more rapid and appropriate management of these patients.

4. PROBLEMS WITH POLYMERASE CHAIN REACTION TESTING

There are a number of technical and procedural issues that need to be considered when performing PCR. Variables that influence the utility of PCR

include the appropriate collection and transport of specimens, the nucleic acid target selected, the selection and design of suitable oligonucleotide primers and probes, optimization of specimen preparation and PCR amplification conditions, and the method chosen to detect the amplified product. No single PCR protocol can be applied to all situations, and great care must be taken in choosing the conditions that are right for the microorganism(s) to be detected.

One of the greatest strengths and a major weakness of PCR is its high sensitivity. The generation of millions of copies of DNA from a template sequence can lead to product carryover and cross-contamination of negative specimens with an amplified target. False-negative results can also occur due to interfering substances found in biological specimens. Appropriate processing of clinical specimens and stringent amplicon control measures can limit these problems.

Although PCR provides an exquisitely sensitive and specific method for detecting infectious agents, PCR will never completely replace other laboratory procedures. One reason for this prediction centers on the type of test requests received by virology and microbiology laboratories. PCR performs very well for agent-specific test requests but PCR is not cost-effective when screening for a large number of infectious agents. The presence of normal flora provides an additional level of complexity for microbiological test requests. In these cases, the microbiology laboratory must determine if there are infectious organisms present, and the laboratory must also determine if the presence of these organisms has medical significance. Such determinations cannot be made by current PCR methods.

Most PCR tests in existence today detect a single organism. Therefore, exclusive use of PCR will miss dual infections unless the laboratory is specifically instructed to look for multiple agents. Respiratory specimens often contain more than one virus, and significantly more specimens contain both bacterial and viral agents. Exclusive use of PCR also presumes that the ordering physician knows exactly which infectious agents may be causing the disease. This is clearly not the case because our laboratories detect HSV from approximately 30% of all specimens sent for VZV detection.

Because of its extreme specificity, PCR may not detect new (or even common) infectious agents present in the community. Unless the physician makes the request or the laboratorian decides to test for a given agent, both new and unusual infectious agents can be missed when using PCR methods.

The extreme sensitivity of PCR also makes it difficult to distinguish active infection from colonization. As a result, some qualitative PCR tests may be too sensitive for routine clinical use. For instance, a positive PCR test for *Enterococcus* in a urine specimen does not necessarily indicate that a woman has enterococcal disease. Many women asymptomatically shed enterococci in their urine and enterococcal disease is usually not suspected until there are 50,000 to 100,000 colony forming units/mL. Likewise, a positive PCR for *Pneumocystis carinii* in an immunocompetent patient with pneumonia does not indicate that the pneumonia is caused by *Pneumocystis* because most healthy adults are colonized by this organ-

ism. PCR alone also cannot distinguish viral reactivation from primary disease, especially in infections with herpesviruses. Therefore, results for herpesviruses should be interpreted with caution when PCR is used as the sole method for detecting herpesviral disease.

Cost is a major issue in every laboratory, but cost issues are especially acute in managed care environments and in laboratories that are downsizing to remain competitive. Many of these laboratories simply cannot afford to use PCR. Commercial PCR methods are capital-intensive and require dedicated equipment and laboratory space. In addition, the reagent and material costs for commercial PCR assays are 2–5 times the cost of bacterial cultures. The labor costs for PCR and culture are similar when performing a single test. However, labor costs of culture methods become insignificant when one considers that a trained microbiologist can rule out the presence of hundreds of different microorganisms for $2–$10 in labor costs. In contrast, the labor costs for ruling out the same number of organisms using antibody- or PCR-based tests is well over $3,000.

5. CONCLUSIONS

PCR has revolutionized our ability to detect infectious agents in clinical specimens. During the past decade, a large number of extremely sensitive and specific PCR methods have been developed for detecting these agents. Today, PCR is an integral and necessary component of diagnostic and research laboratories. PCR is especially valuable for detecting unculturable infectious agents, fastidious and slow-growing agents, and infectious agents that are dangerous to amplify biologically through culture methods. Quantitative PCR has improved our ability to monitor therapy, detect the development of drug resistance, and to predict the progression of certain infectious diseases. The availability of commercial kits and the continuous improvement and simplification of the technology and instrumentation have moved PCR from an esoteric methodology to a routine clinical tool.

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