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STATE-OF-THE-ART CLINICAL ARTICLE

Application of Polymerase Chain Reaction to the Diagnosis of Infectious Diseases

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PCR and other sequence-based microbial detection methods, once considered to be only research tools, are being used increasingly in the clinical microbiology laboratory. As this technology expands into the clinical arena, clinicians will need to learn its advantages and limitations so that sound judgments can be made. Astute clinicians know that results of blood culture reports, whether positive or negative, must be interpreted using an understanding of the test employed and an assessment of the clinical scenario. Similarly, infectious diseases practitioners will need to expand their understanding of PCR-based diagnostics so that these powerful tests are used appropriately.

It is our goal to make PCR-based diagnostics understandable to clinicians. We will point out the limitations of conventional diagnostic methods for infectious diseases, discuss the advantages and limitations of PCR-based methods, and mention some current and future applications of this technology. We will not discuss every current or pending application of PCR to diagnostic microbiology; the reader is referred to other publications for additional details [1–3]. We emphasize the principles behind PCR-based diagnosis, and acknowledge a research-oriented bias in our viewpoint.

The limitations of existing diagnostic methods and the potential of PCR-based detection and identification methods are demonstrated by a case from Stanford University Medical Center.

A Case of Meningitis

A 53-year-old woman was seen in the emergency department with a 5-hour history of severe headache and depressed

mental status. On physical examination, she had a fever (temperature to 38.6°C), nuchal rigidity, and no evidence of a rash. Multiple generalized seizures were noted. Therapy with ceftriaxone and vancomycin was instituted in the emergency department for empiric treatment of meningitis, and a CT scan of the brain was obtained prior to a lumbar puncture. Blood cultures were obtained after antibiotics had been started, because of difficulty with phlebotomy. The lumbar puncture demonstrated cloudy CSF with an elevated pressure, a WBC count of 17,500 cells/mm³ (93% neutrophils), a protein level of 756 mg/dL, and a glucose level of 41 mg/dL (serum glucose level, 209 mg/dL).

Examination of a gram-stained smear of the CSF was reported to show many WBCs and many gram-negative diplococci, suggesting the diagnosis of meningococcal meningitis. The patient ran a day care center, and the county health department was notified so that antibiotic prophylaxis could be started for case contacts. The patient's vancomycin was discontinued, and penicillin was added to the ceftriaxone therapy for optimal coverage of *Neisseria meningitidis*.

On the second hospital day, the gram stain of the CSF was reviewed. The laboratory concluded that the gram-negative organisms seen on the smear had a coccobacillary morphology more consistent with *Haemophilus influenzae* than with *N. meningitidis*. The penicillin was discontinued and ceftriaxone was continued. The patient's condition improved and she was discharged on the fifth day to complete a course of outpatient intravenous ceftriaxone. All blood and CSF cultures were negative. Latex agglutination tests performed on the CSF were negative for *Haemophilus* group b, *Streptococcus pneumoniae*, group B *Streptococcus*, and *N. meningitidis* antigens.

The etiology of this patient's meningitis was not confirmed using conventional methods of cultivation and antigen detection. To identify the bacterium responsible for this patient's illness, we used a sequence-based approach in our laboratory. A sample of the patient's CSF was centrifuged to concentrate bacteria, and the pellet was digested to liberate bacterial DNA. This DNA was used as template in a broad-range PCR assay designed to copy enzymatically (amplify) a portion of the bacterial 16S rRNA gene in vitro using a thermostable DNA polymerase. Oligonucleotide primers complementary to broadly conserved regions of the 16S rRNA gene were used to amplify segments of the gene that also contained variable, phylogenetically informative DNA sequence(s) (figure 1). The

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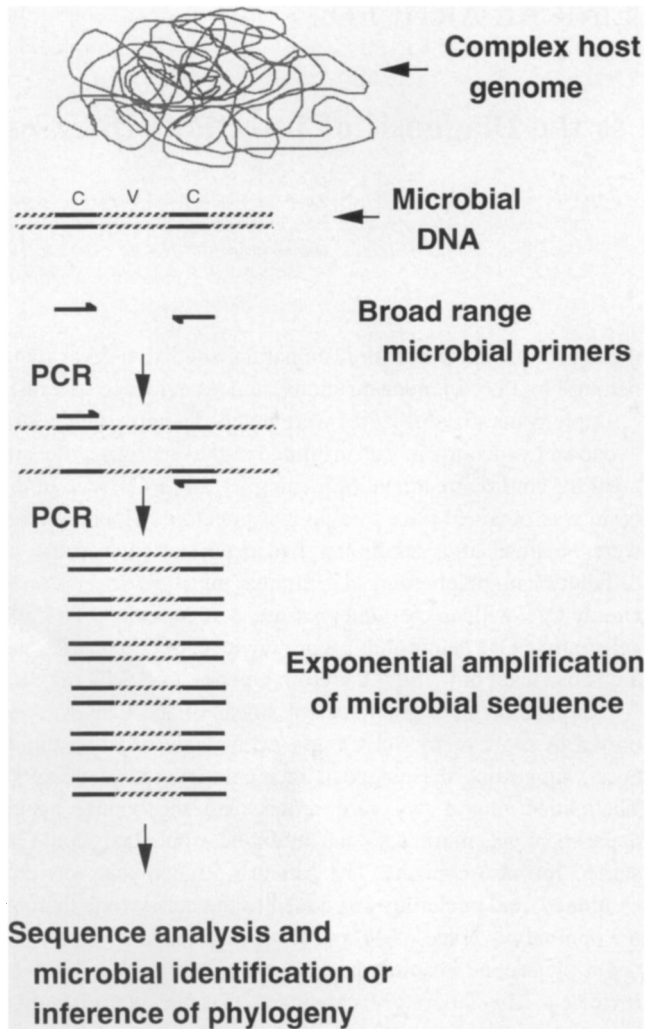


Figure 1. Diagram depicting a broad-range consensus PCR approach to microbial detection and identification. C = conserved, V = variable regions of microbial DNA. Primers anneal to conserved regions of microbial DNA, and the polymerase generates new DNA strands, including the variable region that may contain a phylogenetically informative sequence.

sequence of the amplified product was then determined using an automated DNA sequencer, and aligned with other known 16S rRNA gene sequences from bacteria present in the Genbank and ARB databases (ARB, Technical University, Munich). Because most bacterial genera and species have a unique 16S rRNA gene sequence, this sequence serves as a molecular fingerprint, and can be used to identify an unknown agent or infer its evolutionary relationships with other previously characterized organisms. In our assay, samples of CSF and water used as negative controls did not produce a PCR product, indicating lack of bacterial DNA template. On the other hand, CSF from the patient with meningitis produced a PCR product. Sequencing and phylogenetic analysis showed that the organism present in the patient's CSF matched that of *H. influenzae* biotype III [4].

Why did the conventional microbiological diagnostic methods fail in this case? Although microscopy (e.g., gram staining) may suggest an etiologic agent, it rarely provides definitive evidence of infection due to a particular species. In this case, microscopy initially misidentified the organism as consistent with *N. meningitidis*. Fortunately, the clinicians continued broad spectrum antibiotics while awaiting culture confirmation of the organism, and therefore continued to treat for the *H. influenzae* responsible for this patient's meningitis. Had ceftriaxone been stopped and penicillin used alone, the outcome may have been distinctly less favorable. Clearly the microscopic morphology of organisms may be misleading, with conclusions influenced by the training and subjective interpretation of the microscopist. In other cases, the number of organisms may be too low for visual detection by microscopy. The failure of CSF and blood cultures to provide a diagnosis in this case can be ascribed to the use of antibiotics before obtaining the culture samples. In the setting of meningitis, where the rapid initiation of antibiotics is paramount, this scenario is not unusual, especially when a lumbar puncture is delayed because a head CT is ordered. However, there are cases of bacterial meningitis in which cultures fail to yield the organism, even without the institution of antibiotics, further demonstrating the limitations of culture-based technology. The CSF latex agglutination test for *H. influenzae* group b antigen was negative in this case, probably because the responsible *Haemophilus* species did not possess group b antigen, as has been noted for other biotype III isolates.

Limitations of Conventional Diagnostic Methods

Cultivation

For more than a century, the standard diagnostic test in infectious diseases has been in vitro cultivation using artificial media. Even today, clinical microbiology laboratories devote a majority of their effort towards cultivation of clinical samples, which is a testament to the continued utility of cultivation technology. For microbes that are easily tamed in the petri dish, the sensitivity, specificity, ability to determine antibiotic susceptibility and other clinically relevant behavioral characteristics, and intrinsic amplification of cultivation make this approach attractive. Certain microbes may require special culture media and conditions, so failure to consider these microbes may yield negative culture results. Cultivable microbes may also fail to grow after exposure to antibiotics or after poor sample handling, rendering a culture-independent approach valuable in some circumstances. Similarly, cell culture can be used to detect some viruses and intracellular microbes, but the cost, labor, and time required for this approach beg for better diagnostic methods.

In contrast, other microbes are not so easily tamed in the laboratory. Certain pathogens such as *Bartonella henselae* are fastidious, and other human pathogens such as *Mycobacterium*

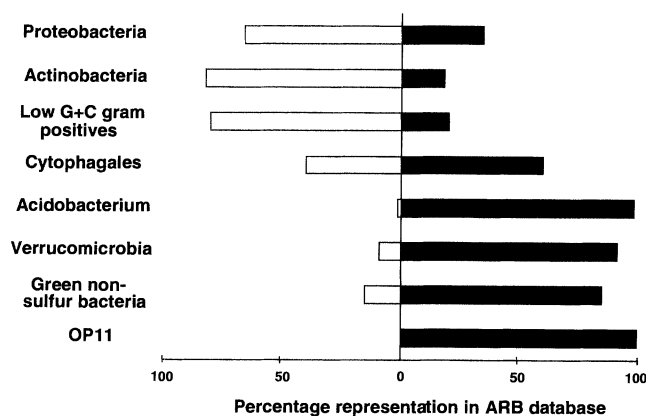


Figure 2. Percentage of cultivated and uncultivated bacteria from several selected bacterial divisions present in the ARB sequence database. From [5] (used with permission).

leprae and *Treponema pallidum* continue to defy our attempts at cultivation on artificial media. Efforts to grow viruses such as human papillomavirus and hepatitis C virus in cell culture have been equally frustrating. Why do these microbes resist our cultivation attempts? Answers to this question remain obscure. However, a more appropriate question may be “why are we successful in getting so many human pathogens to grow in the laboratory?”

It seems less surprising that there are culture-resistant human pathogens when one considers the situation in environmental microbiology. It is estimated that <1% of the bacteria present on earth have been described to date using cultivation technology. When environmental niches are sampled to determine the bacterial census, sequence-based techniques usually reveal large numbers of microbes that fail to grow using standard cultivation techniques; these organisms tend to be previously uncharacterized. Figure 2 illustrates the relatively high percentage of uncultivated bacteria present in several selected cosmopolitan bacterial divisions, even in those divisions such as the proteobacteria, actinobacteria, and low G+C gram positives that contain known human pathogens. Of 36 bacterial divisions noted by Hugenholtz, Goebel, and Pace in their review, 13 divisions are composed entirely of uncultivated organisms [5]. Therefore, we should be mindful of the limitations of cultivation technology, and should not be surprised when sequence-based methods reveal novel microbes associated with human disease.

Other pathogens, such as mycobacteria and fungi, will grow in the laboratory but may require prolonged periods of cultivation. In many cases, the delay between obtaining a culture and the generation of a result necessitates empiric antibiotic therapy, sometimes lasting for months. For these slow-growing microbes, a cultivation-independent method would offer the potential for rapid diagnosis. There are several potential advantages to a speedy diagnosis. First, one might reduce the use of empiric antibiotics, which in turn could help reduce antibi-

otic selection pressures and the emergence of antibiotic resistance. Second, with more specifically directed antimicrobial therapy, it is likely that antibiotic costs would drop and clinical outcomes would improve. These issues need to be addressed with careful studies.

Another problem with cultivation in the laboratory is that certain organisms constitute a health threat to laboratory workers who attempt to propagate them. Organisms such as *Francisella tularensis* and *Coccidioides immitis* are notorious causes of outbreaks among workers in the clinical microbiology laboratory. These highly infectious microbes must be handled in biological safety hoods or sent out to reference laboratories where such facilities exist. Unfortunately, these microbes are sometimes isolated from patients who are not suspected of harboring such highly infectious pathogens, and, therefore, appropriate precautions are not used. A sequence-based diagnostic method could identify these hazardous microbes without risk, since samples can be treated to kill microbes while preserving nucleic acid for analysis.

Finally, successful cultivation of a microbe does not necessarily imply successful identification of the microbe. Organisms isolated on artificial media must still be identified, traditionally by using phenotypic tests such as the ability to metabolize sugars or growth in the presence of certain chemicals or antibiotics. Although usually successful, these phenotypic tests have limited discriminatory power in identifying microbes, the results for a given microbe may vary depending on the state of the organism, and they are not always reproducible and they are usually nonquantitative. For instance, the cell wall composition of an organism may vary depending on the selection of growth media.

Serology

Serologic assays based on the detection of host-derived antibodies or microbe-derived antigens have several limitations. Serologic detection of antibodies may not be helpful in the very acute stage of illness, because the host may not have time to mount an antibody response. For rapidly evolving diseases, the host may succumb to infection before antibodies can be produced. The immunocompromised host may never mount an appropriate antibody response to infection, again limiting the utility of serologic assays. Detection of microbial antigens requires a relatively large microbial burden, which limits assay sensitivity. Unlike cultivation, which detects broad groups of microbes, serologic assays must be ordered individually and target narrow groups of organisms. In addition, serologic assays require specific and reliable antisera or antigens, which may not be available. If the clinician does not think of the correct serologic test to order, the diagnosis is not made.

Microscopy/Histology

The direct detection of microbes in tissues or fluids by microscopy has limited sensitivity and specificity. A relatively

large number of microbes must be present before they will be visible by microscopy (e.g., 10,000 bacteria per milliliter of fluid). Even if the organisms are present in sufficiently high concentrations, one must use the appropriate stains and conditions (e.g., darkfield for treponemes) to make them visible. As with serology, if one fails to consider a particular microbe, then one may miss that organism when using standard techniques. For instance, one will have difficulty visualizing *B. henselae* with a tissue gram stain, but may see the organism with a Warthin-Starry silver stain or with immunohistochemical methods. The limited specificity of microscopy reflects our meager ability to speciate organisms based on morphology. To identify microbes by direct examination, one is dependent on the training and experience of the microscopist, the correct choice of stain and illumination, and the presence of large numbers of organisms. These multiple variables conspire to make direct examination a poor diagnostic test in many situations.

In our case of meningitis, all three conventional diagnostic methods failed to identify the responsible organism. Is this just an isolated case, or is there a problem with our diagnostic armamentarium? Pneumonia is the most common infectious cause of death in the United States, with 4 million cases per year [6, 7]. No etiologic agent can be identified in >35% of cases of community-acquired pneumonia when using conventional diagnostic methods such as cultivation and serology. Better diagnostic methods are needed.

PCR

PCR is an enzyme-driven process for replicating DNA *in vitro*. Using this technology, one is capable of turning a few molecules of DNA into large quantities. Why is it useful to have large amounts of microbial DNA available for study? The levels of microbial DNA present in clinical samples are frequently too low for meaningful manipulation and measurement. PCR can produce sufficient amounts of DNA so that microbes can be detected and identified. Because each unique microbe has a unique complement of DNA (or RNA), DNA can function as a molecular fingerprint to help identify microbes. Certain DNA sequences (e.g., bacterial 16S rDNA) are particularly informative, allowing one to distinguish most microbes from one another.

In the PCR, a segment of DNA is copied *in vitro* by a thermostable DNA polymerase enzyme in the presence of buffer, magnesium, deoxyribonucleoside triphosphates, and primers. Oligonucleotide primers complementary to regions on the coding and the noncoding strand of the DNA template are responsible for specificity in the reaction, determining which region of DNA becomes amplified. As the primers anneal to their complementary regions of DNA, DNA polymerases attach to the primer-template complexes and extend the DNA strands, producing a copy of the DNA. Each copy of DNA may

then serve as another template for further amplification. Multiple rounds of heating and cooling of the reaction mixture in a thermal cycler produce rounds of melting of double-stranded DNA, annealing of primer to single-stranded templates, and extension of DNA strands, to produce a logarithmic increase in DNA. In the ideal scenario, the primers chosen in the PCR are specific for a particular microbial gene, and hence do not amplify nonspecific targets such as human genes. Theoretically, one could start with a single copy of the target microbial gene present in the reaction, and generate billions of copies of DNA from that gene.

Although PCR is the best known and most widely used nucleic acid amplification technology, there are other amplification technologies in use. These technologies include the transcription-based amplification system, strand displacement amplification, ligase chain reaction, and Q β replicase system. In addition, there are methods such as branched DNA technology that do not amplify the DNA, yet can detect low levels of DNA via signal amplification from a probe. We will not discuss these other techniques further; the reader is referred to other sources for more in-depth information [3].

There are several approaches for using PCR to detect microbial DNA. The simplest approach is specific PCR. Here, one designs primers that are complementary to a DNA target that is specific for the microbe being assayed. For instance, by selecting unique regions of the Whipple bacillus' 16S rRNA gene, one can create primers that will amplify only the 16S rRNA gene from the Whipple bacillus, *Tropheryma whippelii*.

In contrast, with broad-range PCR one attempts to detect a broader group of organisms by designing primers that are complementary to conserved regions of a particular gene that are shared by a given taxonomic group. For instance, one could design primers that are complementary to regions of the 16S rRNA gene that are shared by most members of the bacterial domain, with the intention of using the more variable regions of the amplified sequence for identification or phylogenetic assessment [8]. In this situation, one would expect to amplify any bacterial 16S rDNA present in the reaction. Between the extremes of specific and domain-wide PCR is a large middle ground of taxon-restricted PCR. Here, one designs primers that are complementary to conserved regions of a gene from a particular group of organisms; either the primers are not complementary to the same gene segment in other microbes outside the group or the distribution of the gene itself is limited to that group. For instance, primers have been designed that will amplify a segment of the DNA polymerase gene from all members of the herpesvirus family, but will not amplify DNA polymerase genes from other viral families.

Another variation of PCR is multiplexing, in which multiple specific PCR assays are run simultaneously in the same reaction tube to test for multiple different DNA templates. In multiplex PCR, several sets of primers are added to the reaction in order to generate several different PCR products. For in-

stance, one could have a PCR assay designed to detect bacterial DNA in CSF that uses five different specific PCR reactions in one tube, with primer pairs directed toward *S. pneumoniae*, *N. meningitidis*, *H. influenzae*, *Listeria monocytogenes*, and the group B *Streptococcus*. In such an assay, some method of postamplification analysis is needed to determine which organism is represented in a positive reaction. If the amplification products differ in size, then gel electrophoresis will provide an initial idea of which PCR reaction(s) took place. This approach is sometimes hampered by interference between primers within the same reaction.

Nesting of PCR increases assay sensitivity and can increase specificity as well. In nested PCR, one uses the product of a primary PCR reaction as template in a second PCR reaction. The first PCR reaction amplifies a microbial DNA target using primers complementary to the organism or group of organisms being assayed. In the second round, a sample of the first PCR is added to fresh reaction mixture for a second PCR using a set of primers that anneal to regions of the same gene, but at sites internal to the previous priming sites. For instance, the first round reaction may produce a 400-bp product, and the second round may produce a 200-bp product that is a subset of the 400-bp product. (In hemi-nested PCR, one primer from the first round is used in the second round reaction as well.) Increased sensitivity is obtained because the target is enriched in the first round of PCR, with subsequent dilution of other DNA and inhibitors. Additional specificity results from the set of specific primers employed in the second round. Even if nonspecific amplification occurs in round one, the nonspecific amplification product will probably not participate as template in the second round because it is unlikely to have regions of DNA complementary to the second set of specific primers. The problem with nested PCR is that it is highly prone to contamination with amplification products, and thus must be performed with extreme care and interpreted with even greater caution. The usual efforts to inactivate amplification products in order to prevent contamination do not work with nested PCR because one needs to use amplifiable template from the first round in round two. Opening PCR reaction tubes after round one and transferring amplification products to new tubes are conducive to contamination.

How does one detect an RNA target, such as rRNA or a segment from the genome of an RNA virus? A modification of PCR called reverse transcriptase PCR (RT-PCR) can be used. In RT-PCR, an RNA template is the initial target, and reverse transcriptase creates a complementary DNA copy of the RNA. Oligonucleotide primers catalyze conversion of a specific segment of RNA into DNA. Once the DNA template forms, it can be amplified as in standard PCR.

Confirmation and Identification of PCR Products

After completing a PCR, one must determine if the intended PCR amplification product was generated. The most commonly

used method for detecting PCR products in the research lab has been gel electrophoresis. The contents of the PCR are loaded into an agarose or acrylamide gel, an electrical gradient is applied through a buffer solution, and the products migrate through the gel matrix. The amplification products migrate through the gel according to size, with smaller products traveling farther in the gel because they experience less resistance. When DNA fragments of known size are run in the same gel (as size standards), the size of the PCR amplification products can be estimated after the DNA is visualized (e.g., using ethidium bromide staining and illumination with ultraviolet light). A given set of primers should generate a PCR product of a particular size, and the appearance of an amplification product of the appropriate size in a gel is consistent with a positive PCR. Unfortunately, there are examples in which a PCR product of the appropriate size is generated, but the product is not the intended amplification product. This occurs because of mispriming, in which the primers anneal to sites in the genome (human or microbial) other than the intended target sequences, and generate a PCR product that happens to be similar in size to the intended product.

Because nonspecific amplification products may be generated in a PCR, the identity of the products should be confirmed. Confirmatory methods include sequencing of the amplification product, annealing of a specific oligonucleotide probe to a region of the amplification product that spans the priming sites (e.g., Southern hybridization, slot blotting, probe ELISA, and hybridization protection assays), single-stranded conformational polymorphism analysis, or restriction enzyme cleavage of the amplification product (using an enzyme known to cut a specific sequence within the intended product) with gel electrophoresis of the digest (restriction fragment length polymorphism [RFLP] analysis). Although sequencing of the PCR product is the most rigorous method of confirming amplification product identity, it is also the most time consuming and laborious. Most commercial methods are likely to use an oligonucleotide probe in an ELISA format, as this will provide a rapid yet highly specific method of detecting a PCR product and confirming its identity. The TaqMan system (Perkin-Elmer Applied Biosystems, Foster City, CA) uses a fluorescently labeled probe to detect, confirm, and quantify the PCR product as it is being generated in real time (figure 3) [9]. This system obviates the need for postamplification detection and confirmation of product, and therefore reduces assay time. Probe-based confirmation methods also have the advantage of detecting small quantities of amplification products, and thus offer superior sensitivity.

Another method used to characterize and identify PCR products employs nucleotide sequences attached to solid supports, such as filters or glass slides. With so-called DNA chip technology, or high density DNA microarrays, one can quantitate and characterize fluorescently labeled mRNA or DNA by allowing it to hybridize to a complementary DNA sequence(s)

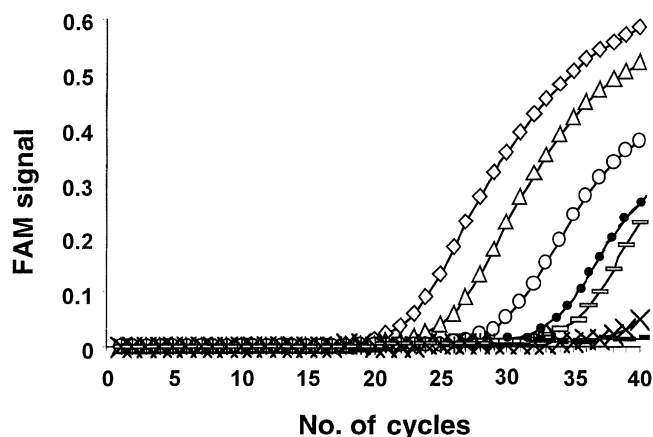


Figure 3. Real time detection of the human β -actin gene using TaqMan PCR reagents (PE Applied Biosystems, Foster City, CA) and a Smart Cycler high speed thermocycler (Cepheid, Sunnyvale, CA). *Y axis* = FAM (6-carboxyfluorescein) fluorescence intensity (volts), *x axis* = PCR cycle number. A fluorescent probe is released from the β -actin PCR product and detected during PCR. *Squares* = 1,000,000; *triangles* = 100,000; *open circles* = 10,000; *closed circles* = 1,000; *open bar* = 100; *X* = 10; *closed bar* = 0 gene copies. Higher amounts of starting target DNA require fewer PCR cycles before product is detected (courtesy of Linda Western, Cepheid).

anchored to the surface, and subsequent fluorescence scanning [10, 11]. Tens or hundreds of thousands of sequences (probes) can be placed within a surface area of 1 cm². DNA microarrays have been used to measure in a simultaneous, semiautomated fashion the mRNA expressed by all *Saccharomyces cerevisiae* genes, and ~20% of all expressed human genes. In another application not yet realized one could immobilize thousands of probes (e.g., 16S rDNA) for different bacterial divisions, genera, and species at a high density. Amplification products from clinical samples subjected to broad-range 16S rDNA PCR and incorporating a fluorophore could then be analyzed by comparative hybridization, using this chip to determine which bacterium or bacteria are present in the sample. The speed and power of DNA chip technology combined with PCR should not be underestimated.

Advantages of PCR Over Conventional Diagnostic Methods

PCR-based assays for the detection of microbial DNA can be extremely sensitive. Under the right conditions, one can amplify a single copy of a microbial DNA gene or gene fragment from a clinical sample and detect it. If the microbe contains multiple gene copies per organism, then one microbe may provide multiple targets for amplification. For instance, the bacterium *Escherichia coli* contains seven copies of the 16S rRNA gene per organism, so even a fraction of an *E. coli* (e.g., a lysed organism) may be detectable by PCR. Even greater assay sensitivity can be achieved by amplifying microbial

targets that are present in large numbers within a single microbe. One bacterium may contain thousands of 16S rRNA copies that are incorporated into ribosomes and can serve as targets in an RT-PCR assay. In one example, an RT-PCR assay was designed for the detection of *T. pallidum* 16S rRNA [12]. Using Southern hybridization to detect the PCR product, the investigators were able to detect 1/100 RNA equivalents of an organism in CSF. It may be possible that this RT-PCR assay could even detect a single organism that has lysed and liberated its rRNA into the CSF, although the half-life of rRNA in CSF is not known. Although studies of the clinical utility of this RT-PCR assay have not been published, the assay could revolutionize the diagnosis of neurosyphilis, since other diagnostic methods are insensitive (CSF VDRL) or too cumbersome (rabbit inoculation).

In addition to unrivaled sensitivity, PCR offers the potential for remarkable specificity. Specificity in PCR derives from the fact that each distinct microbe has unique DNA. One can design oligonucleotide primers complementary to these unique segments of DNA, so that only microbial DNA from the organism being sought is amplified. Alternatively, one can design oligonucleotide primers complementary to conserved regions of microbial DNA so as to detect more diverse groups of organisms. If the DNA amplified with this broad-range approach contains intervening segments of DNA that are unique to specific microbes, then these microbes can be identified using techniques such as sequencing or restriction enzyme analysis. An example of a phylogenetically informative gene that can be used for both organism-specific PCR as well as broad-range PCR is the bacterial 16S rRNA gene, as previously mentioned. When using sequence information to identify a microbe, one avoids the need to grow the organism or maintain the organism in a particular physiologic state for metabolic analysis. Although a microbe may switch certain metabolic traits on and off, leading to confusion when trying to identify the microbe using traditional phenotypic tests, the genetic fingerprint of the microbe remains fairly constant, offering a more reliable method of microbial identification.

Another advantage to PCR-based microbial detection and identification is speed. PCR can be completed in minutes to hours. Simple methods to confirm the identity of the amplification product can also be completed in minutes to hours. Therefore, a turnaround time of one day is not unrealistic for many types of assays. Cultivation of microbes tends to take hours to days for initial propagation, and hours to days for phenotypic diagnostic testing. Even a rapidly growing organism, such as *E. coli* in blood culture, requires days of laboratory time before definitive identification is made using cultivation methods with phenotypic testing. We are all familiar with cases in which a patient dies soon after an acute, nonspecific flu-like illness but before an organism can be grown in culture, such as with meningococcal sepsis. These cases remind us that for easily cultivatable organisms, culture is sometimes too slow to

be useful. This knowledge inevitably leads to the increased use of empiric antibiotics, even for illnesses that ultimately prove to be viral in origin, and to the emergence of antibiotic resistance. A more rapid and sensitive diagnostic test for infection, such as PCR, might reverse this trend toward empiricism if the correct microbes could be identified early in the infection, and if this test had a high negative predictive value [13].

Problems and Limitations of PCR

False Positives

Ironically, false positive reactions are the Achilles' heel of PCR and stem from its greatest strength, namely the incredible sensitivity of enzymatic amplification. False positive results occur because PCR may amplify "contaminating" DNA that finds its way into a sample, even when that DNA is present in infinitesimally small amounts. DNA contaminates samples through several means.

The most important means of contamination is through amplification product carryover. A single PCR can generate billions of DNA copies, each of which is capable of acting as target for a future PCR reaction. If even a submicroscopic portion of a positive amplification reaction gets into the environment where subsequent PCR reactions are mixed, then false positive reactions may ensue. PCR reagents, pipettes, pens, tubes, tube racks, hands, and door handles (almost any object) are capable of harboring or transmitting PCR amplification products. To reduce the risks of false positive reactions from amplification product carryover, laboratories are usually physically divided into pre-PCR and post-PCR rooms. Some laboratories also have a separate room for specimen digestion and processing. All materials and personnel are supposed to flow one way, from pre-PCR to post-PCR rooms. Thus, once a PCR reaction is set up in the pre-PCR area, it is moved to the post-PCR area where amplification and product analysis are performed. Materials are not allowed into the pre-PCR room unless they are new or have been decontaminated. Some labs have separate gowns or disposable gowns for each area.

Amplification product carryover contamination can also be eliminated or reduced by using some inactivation techniques. In one method, deoxyuridine triphosphate (dUTP) is used as a substrate in PCR instead of deoxythymidine triphosphate (dTTP). Before each PCR, the reaction mixtures are treated with the enzyme uracil *N*-glycosylase to render any contaminating (i.e., uracil containing) DNA incapable of amplification. The uracil *N*-glycosylase is then inactivated before proceeding with PCR. Thymidine remains intact in the sample DNA, and is used as template for new uracil-containing amplification products. In another method, the PCR reactions contain dTTP and isopropyl alcohol, and are treated with ultraviolet light after the amplification step [14]. Thymine dimers form between thymine bases, rendering the DNA incapable of further amplification. These methods do not work well for PCR products of ~100 bp or less in size.

One approach for monitoring amplification product carryover contamination in samples or solutions employs primers that contain additional signature oligonucleotides at the 5' end of each primer in order to make tagged amplification products. Bases at the 3' ends of the primers bind to their complementary bases in the target DNA, providing specificity in the PCR. The signature sequences at the 5' end become incorporated into PCR products but do not anneal to target. When a sample is positive, it can be re-tested in a PCR assay using primers that are complementary to the signature sequences. Amplification with the signature sequence primers proves that the sample contains previously amplified target, since native target does not contain this signature sequence and therefore will not amplify. Unfortunately, this method cannot monitor for episodic contamination from items such as gloves or pipettes, which may introduce amplification products into a reaction without directly contaminating the original sample or solutions. However, running samples in replicate and repeating PCR assays should reveal problems with episodic contamination.

False positive reactions may also be caused by intersample contamination. A clinical sample may have large quantities of target DNA present. When opening this sample, DNA may contaminate gloves or other items in the environment, leading to the inadvertent introduction of DNA into other PCR reactions where it is amplified. This problem can be minimized by changing gloves between handling of samples, duplicating sample analysis, and avoiding aerosol generation. This problem can be detected by interspersing negative control reactions with the test samples to see if these controls are positive. Amplification product inactivation will not control this problem because the contaminating DNA has not been previously amplified.

Another cause of false positive reactions occurs in the setting of broad-range PCR, e.g., when amplifying the bacterial 16S rRNA gene with consensus primers. With primers that are complementary to highly conserved bacterial 16S, or fungal 18S rDNA sequences and highly sensitive reaction conditions, one may detect microbial DNA uniformly. The negative controls are positive because the PCR reagents, such as water and *Taq* polymerase, contain small amounts of bacterial DNA. It is very difficult to eliminate all contaminating DNA, especially from the polymerase enzyme. If one looks carefully, it is possible to detect small fragments of bacterial 16S rDNA in many "sterile" solutions, such as water for injection, and uninoculated blood culture media [15]. Just because a solution is sterile does not mean that it is free of microbial DNA, only that no microbes can be cultivated. For highly sensitive, broad-range PCR applications, we may need to create a new standard for cleanliness in our reagents that measures microbial DNA instead of colony-forming units.

A more subtle problem with false positives may arise from the detection of small quantities of microbial DNA in clinical samples. If PCR is more sensitive than culture in some situa-

tions, then we may need to define what constitutes a “significant” level of microbial DNA for a given clinical situation. For instance, what does it mean if *S. pneumoniae* DNA is detected in a blood sample? Although the presence of pneumococcal DNA in blood would seem to suggest an invasive infection, this may not always be the case. In one study, when blood samples from healthy subjects were examined by PCR, 17% were positive for pneumococcal DNA (samples from children, not those from adults) [16]. As with other tests, clinical correlations will need to be made to see in what situations PCR offers an improvement in diagnostic capabilities, and to determine what levels of microbial DNA are significant for a given organism, site, and situation.

False Negatives

PCR assays for the detection of microbes may be falsely negative for several reasons. The sample may contain PCR inhibitors that interfere with amplification. Samples that have been shown to contain PCR inhibitory substances include blood (heme), blood culture media, urine, vitreous humor, and sputum. The PCR inhibitors must be diluted, removed, or inactivated in order to amplify any microbial DNA present. DNA purification methods help to remove many of these inhibitors, although some inhibitors persist when standard purification protocols are used. Samples may also be falsely negative because the digestion step has failed to release the microbial DNA present or because the DNA has been lost in the purification step. Microbes with thick cell walls, such as fungi or bacterial spores, may be difficult to break open and therefore may require additional mechanical or enzymatic lysis steps in order to liberate microbial DNA for amplification.

Amplification of a human gene can help monitor for PCR inhibitors and check the quality of the DNA present in a sample of human tissue subjected to PCR for the detection of microbes. For instance, if PCR using primers complementary to the human β -globin gene fails to yield a PCR product with a human tissue sample, then that sample is problematic. The problem sample should be checked for the presence of PCR inhibitors and DNA. If a tissue sample is β -globin PCR negative, then a negative result in a microbial DNA PCR assay has no meaning, since amplifiable DNA may not be present. If a sample is β -globin PCR positive but microbial DNA PCR negative, then the sample is more likely a “true” negative for microbial DNA. Obviously, this approach will not work with any procedure in which human DNA is removed (e.g., see sequence capture below and [17]). Some PCR kits contain an internal amplification standard that allows one to monitor inhibitory activity and test performance in each sample.

PCR-based assays may also be negative because of analysis of an inadequate sample volume. Large volumes of fluid can be cultivated, such as 10–20 mL of blood. On the other hand, sample volumes are usually very small with PCR, in the range

of 1–10 μ L, which are added to a reaction volume of 20–100 μ L. If one bacterium is present in 1 mL of blood, then cultivation of 10 mL of blood has a good chance of detecting the organism (assuming that the organism is cultivatable). PCR that is performed on purified DNA from a digest of 0.1 mL of the same blood concentrated to 10 μ L may not detect the organism, depending on the sensitivity of the assay and the number of gene copies of target present in the bacterium.

To increase the sensitivity of PCR assays, microbial DNA can be concentrated, such as with sequence capture PCR [17]. In this technique, microbial DNA is bound to complementary capture oligonucleotides, which in turn are bound to a solid support. Unbound DNA and inhibitory substances from the sample are washed away. The concentrated, purified microbial DNA is then used for PCR.

Sample Acquisition and Preparation

One should be mindful that the sample collection process can have a significant impact on the outcome of the PCR assay. Tissues and fluids should be refrigerated and rapidly processed, or stored frozen in order to preserve the DNA for amplification. Nucleases present in fluids can degrade DNA, so storage in a magnesium-free environment (e.g., with EDTA), at low temperatures, or in chaotropic solutions is helpful. In the field, where freezers are not available, tissues can be stored in ethanol or a chaotropic solution such as guanidine isothiocyanate. Fixation of tissues in formaldehyde and other pathological fixative solutions can damage DNA, particularly with prolonged fixation times. Fixation should be avoided if samples are being collected prospectively for PCR analysis.

When using broad-range PCR, such as with conserved 16S rDNA primers, one must be careful in selecting the tissue and anatomical site of acquisition. Broad-range bacterial PCR will detect normal bacterial flora, and thus should be applied to tissues that are usually free of bacteria such as blood, CSF, and brain. Broad-range PCR using tissues that are normally in contact with bacteria, such as the mouth, colon, and skin, makes interpretation of the results challenging because multiple PCR products may be generated. When a heterogeneous collection of PCR products is generated, these must be individually identified to sort out which sequence comes from a pathogen, and which sequence comes from a normal colonizer.

There are many protocols for sample digestion and DNA purification. Some digestion methods employ mechanical means such as freezing-thawing, sonication, agitation with glass beads or ceramic particles, or crushing with mortar and pestle. Other methods use chemical or enzymatic means to break open microbes, such as using chaotropes, detergents, proteases, or other enzymes active on microbial cell walls. Some methods or combinations work well for selected microbes, but there is presently no universal method optimized for digesting all microbes in all tissues. Similarly, certain DNA

purification protocols work well with certain samples. This is unfortunate because one would like to have a universal digestion and DNA purification protocol that one could use in the clinical microbiology lab for all samples destined for PCR. Semiautomated DNA and RNA purification systems are available for use in the clinical microbiology laboratory.

Cost

PCR is expensive. For example, our clinical microbiology laboratory charges \$125 for a herpes simplex virus (HSV) PCR assay using CSF. The cost of PCR reagents and equipment is substantial. The requirement for separate pre-PCR and post-PCR areas means that molecular microbiology laboratories use a disproportionate share of laboratory space. Furthermore, there are training costs associated with teaching microbiologists to perform these molecular diagnostic assays. Will PCR-based assays ever compete with traditional diagnostic methods such as cultivation and serology?

For certain diagnostic tests, such as HSV PCR for the diagnosis of encephalitis, PCR is currently more cost effective than previous diagnostic approaches (see below and [18]). The advantages to PCR-based tests, such as speed and sensitivity, may offset higher diagnostic costs by reducing hospitalization and treatment costs. However, these indirect cost advantages are difficult to quantify. As the budgets of clinical microbiology labs continue to shrink, administrators will look to the more easily quantifiable bottom line of the laboratory, and will demand that PCR-based assays be cost competitive with other diagnostic methods. The direct costs of PCR-based diagnostics will likely decrease as this technology becomes more refined. Some PCR assays such as the Chlamydia and *Neisseria gonorrhoeae* assays have direct costs in the \$8–10 range and are already cost competitive with culture technology. Miniaturization of PCR reactions and the use of high throughput robotics technology will likely lead to substantial cost reductions. Increased use of PCR-based methods may also reduce costs through competition and reduced labor costs. For certain microbes, PCR is the only diagnostic approach, and thus there is no basis for a cost comparison. For instance the Whipple bacillus, *T. whippelii*, cannot be detected using methods such as culture and serology, leaving PCR as the most definitive diagnostic test, although histology and electron microscopy of tissues may also suggest the diagnosis.

Antibiotic Susceptibility and Resistance

PCR amplification of phylogenetically informative sequences such as the 16S rRNA gene fails to provide data about the antibiotic susceptibility of the organism. One advantage of cultivation is that a susceptibility profile can usually be determined in order to help guide treatment. For organisms with stable antibiograms, this function is less important. For organ-

isms that are prone to developing resistance, the lack of susceptibility data is problematic. Yet PCR can play a role in determining antibiotic susceptibility. PCR assays have been designed for the detection of antibiotic resistance genes in microbes, such as the methicillin resistance gene (*mecA*) in *Staphylococcus aureus* and mutations in the rifampin resistance gene (*rpoB*) in *Mycobacterium tuberculosis*. Although the presence of a resistance gene in a microbe does not always imply expression of that gene and phenotypic resistance, its absence does imply a lack of resistance through that particular genetic mechanism. In the future, multiplex PCR or microarray technology may help to identify both the microbe and determine antibiotic susceptibility profiles in one reaction.

PCR in Practice: HSV PCR for the Diagnosis of Herpes Encephalitis

An excellent example of an organism-specific PCR-based assay is HSV PCR for the diagnosis of herpes simplex encephalitis (HSE). The previous gold standard for the diagnosis of HSE was brain biopsy with cell culture. The cost and morbidity of this diagnostic test were high, mostly related to the need for general anesthesia and craniotomy. The significant effort required to make the diagnosis by brain biopsy led some clinicians to treat patients empirically with acyclovir rather than pursue the diagnosis. Although there is little toxicity associated with acyclovir, the lack of a definitive diagnosis may have hindered further diagnostic evaluation of patients having other causes of encephalitis.

With HSV PCR, CSF is obtained from the patient by lumbar puncture and assayed, avoiding the need for the more invasive brain biopsy. CSF is added to a PCR reaction mixture containing primers that are complementary to regions in the DNA polymerase gene or the glycoprotein B gene of HSV-1 and HSV-2. The assay can detect about 20 gene copies of either herpesvirus. The presence of HSV DNA in the CSF of a patient with encephalitis is sufficient to make the diagnosis of HSE. This test is more rapid than culture and is sensitive and specific. It is less costly (considering that the costs of surgery and anesthesia run into thousands of dollars), less invasive, and produces less morbidity and mortality than does brain biopsy. Given the advantages of PCR for the diagnosis of HSE, there is now little reason to perform brain biopsies on patients suspected of having this diagnosis.

The HSV PCR assay provides an example of how difficult it can be to compare a new diagnostic test to a gold standard when the gold standard is not very golden. In one study that compared PCR to biopsy with culture, 53 of 54 biopsy-proven patients with HSE were also positive by PCR (98%) [18]. It is of interest that three of 47 biopsy negative patients were found to be PCR positive (6%). How does one interpret the results when a novel test (PCR) picks up more cases than the gold standard (brain biopsy)? Are the additional cases false positives

with the new diagnostic test, or false negatives with the gold standard? Review of the laboratory and clinical data from this study suggests that the positive PCR results in these biopsy negative patients are true positives that are due to errors in sample acquisition that led to negative culture results (e.g., placing the brain in formalin before culture). Because HSE can be a patchy process, biopsy may miss areas of involvement. If the three PCR positive but biopsy negative cases are considered to be true positives, then HSV PCR is the more sensitive test in this study. HSV PCR for the diagnosis of HSE will likely become the new gold standard.

Specific PCR Assays

A number of commercially available PCR assays have been designed for the detection of specific microbes [1, 2]. These assays use primers that are complementary to unique stretches of DNA present in a given microbe's genome. Assays are available for a variety of pathogens, including HIV, HSV, hepatitis B virus, hepatitis C virus, cytomegalovirus, enterovirus, *Chlamydia trachomatis*, *M. tuberculosis*, *Mycobacterium avium* complex, *T. whippelii*, and *Neisseria gonorrhoeae*. Three of these assays are discussed below.

RT-PCR is used to detect viral load in an assay of HIV RNA (MONITOR, Roche, Branchburg, NJ). The target is a segment of the HIV-1 *gag* gene. The assay normally detects as few as 50 gene copies per milliliter of plasma after ultracentrifugation of the sample to concentrate virions, and can be used to monitor the effectiveness of antiretroviral therapy. The PCR product binds to a probe-coated microwell plate, and a colorimetric assay quantitates the target. A modified target, called a quantitation standard, is added to the reaction so that HIV copy number can be determined. Plasma should not be collected in heparin, as it is a PCR inhibitor.

A PCR assay for *C. trachomatis* targets a segment of a cryptic plasmid, and is able to detect 10 plasmid copies, or 1 inclusion-forming unit (Amplicor, Roche). The assay is so sensitive that urine can be used to screen patients for infection, avoiding the need for more invasive examination, and thereby facilitating the acquisition of patients' samples. PCR is more sensitive than cell culture. A culture should still be obtained when collecting legal evidence, such as for cases of rape or child abuse, as PCR results may not be legally acceptable proof of infection. Endocervical and urethral swabs can also be tested. The assay does not detect plasmid-free variants of *C. trachomatis*, and urine should not be frozen, but stored in a refrigerator. Spermicide and surgical lubricant can act as PCR inhibitors, producing false negative reactions.

A PCR assay is available that detects a segment of the *T. whippelii* 16S rRNA gene for the diagnosis of Whipple's disease (Mayo Clinic, Rochester, MN). The assay can detect <100 copies/mL of sample fluid. PCR is more sensitive than histology for diagnosis. PCR is also more useful than histology

for monitoring response to antibiotic therapy because histologic resolution of intestinal lesions may take months to years, whereas PCR-based evidence of infection tends to correlate with disease resolution or relapse [19].

Broad-Range PCR

Bacteria isolated by cultivation can be identified using a commercially available broad-range 16S rDNA PCR assay with sequencing of the amplification product. This genotypic identification method was shown to be superior to other (phenotypic) methods of microbial identification when applied to a series of fastidious aerobic gram-negative bacilli [20]. However, broad-range PCR for the direct detection of microbial DNA in clinical specimens remains an experimental approach [8]. This approach is hobbled by the presence of contaminating DNA in PCR reagents, which prevents the use of very sensitive PCR conditions. In addition, when multiple organisms are present in a sample, direct sequencing of the amplification product cannot be performed because there are mixed amplification products. These multiple sequence types must be distinguished by methods such as cloning, single-stranded conformational polymorphism analysis, or group-specific probe hybridization. Broad-range consensus PCR with direct sequencing can be successfully applied to clinical samples that contain a single organism.

The gene targets that have been successfully used in broad-range consensus PCR assays for the identification and phylogenetic characterization of microbes include the small subunit ribosomal RNA genes (16S rDNA in prokaryotes, and 18S rDNA in eukaryotes), the citrate synthase gene, and heat shock protein genes. Phylogenetically informative gene targets should have regions of sequence conservation for the design of broad-range primers, and areas of sequence diversity to distinguish between organisms. The value of a gene target for broad-range PCR depends in part on the diversity and number of microbial sequence types from that target present in databases, as well as the reliability of that locus in reflecting organismal evolutionary history. For the small subunit rRNA gene, there are >9,000 sequences from different organisms present in databases, making this a useful gene for identifying a microbe or determining its close evolutionary neighbors. Primers have been described for broad-range bacterial or fungal PCR assays, but there are no primers that can detect all groups of viruses. There is too much sequence diversity in viral genes to design a broad-range viral consensus PCR assay. However, one can design primers complementary to conserved genes segments in certain viral families, such as the DNA polymerase gene of herpesviruses.

The Future of PCR: Technical Advances

Advances in nucleic acid amplification technology will make future diagnostic tests faster and less expensive [21]. PCR has

been miniaturized so that nanoliter quantities of sample are processed within a few minutes. For instance, high speed, continuous flow PCR has been performed on a glass microchip in which the sample is moved rapidly between thermostated temperature zones [22]. Using this microdevice, 20 cycles of PCR could be performed in as little as 90 seconds. Similarly, small-volume, rapid PCR has been performed in microcapillary tubes and micromachined silicon chip-based reaction chambers. The disadvantage of small volume PCR for the detection of microbes is that organisms present in low concentrations in a sample may be missed. This limitation can be overcome by sample preparation methods that concentrate microbial nucleic acids, or by continuous flow/multiple sampling methods that increase the volume of sample analyzed. The advantages of small-volume PCR include reduced cost from the use of fewer reagents, and the ability to analyze numerous aliquots from a clinical sample so that multiple tests can be performed on a limited volume of tissue or fluid.

Another advance in PCR-based diagnostics is real time detection of PCR products. For instance, the TaqMan system [9] uses a fluorescently labeled probe and the exonuclease activity of *Taq* polymerase to monitor the formation of product as it is being generated (figure 3). Real time detection methods can be combined with miniaturized, rapid PCR technologies. With currently available technology, it is possible to design a microchip or microcapillary PCR apparatus that can amplify, detect, and characterize a microbial DNA target within minutes.

Questions without Answers

The application of PCR to the detection of microbes in clinical samples raises several questions. How long does microbial DNA persist in tissues after disease resolution or antibiotic treatment? Does microbial DNA from some microbes persist in certain tissues or body fluids after viable organisms are gone? Does microbial DNA increase in the blood after lysis of organisms at a distant tissue site, such as with use of cell wall active antibiotics? Is the presence of bacterial RNA a better indicator of current infection with viable organisms than bacterial DNA? What is a clinically significant level of microbial DNA at a particular site? How can PCR distinguish between colonization, latent infection, active infection, and relapsing infection? Is microbial DNA routinely found in "sterile" sites sampled from normal individuals?

A few animal studies have attempted to address these questions. With a mouse model of Lyme disease, investigators were able to show by PCR that *Borrelia burgdorferi* DNA disappeared from tissues immediately after a 5-day antibiotic treatment course, and that the PCR results correlated with culture results [23]. This study suggests that the response to antibiotics for Lyme disease might be monitored by PCR. Similarly, investigators using a chinchilla model of otitis media found a

strong correlation between PCR results and culture results when animals were injected with viable *H. influenzae*. When animals were injected with purified bacterial DNA or with killed bacteria, the amplifiable DNA rapidly disappeared [24]. In a rabbit model of syphilis in which live or heat-killed *T. pallidum* was injected into the skin and testes, heat-killed treponemes were no longer detectable by PCR after 15–30 days, whereas viable organisms were detected by PCR for months [25]. These studies suggest that bacterial DNA is cleared from the tissue sites of animals after bacterial death, but that the DNA from different microbes may have different elimination kinetics at different sites.

Human studies looking at the persistence of microbial DNA by PCR are few, but include a study of pulmonary tuberculosis treatment that showed that sputum smears and cultures for *M. tuberculosis* convert to negative before PCR results, but that PCR results do correlate with clinical response to antibiotics and also can predict relapse. It is not clear if the persistent PCR signal seen in some of these patients is due to low levels of viable organisms, or due to amplifiable DNA from nonviable organisms [26]. A study of PCR for the detection of *T. pallidum* in CSF from patients with neurosyphilis suggests that bacterial DNA may persist for years after antibiotic treatment [27]. However, the episodically positive PCR results from this study may instead be due to false positive reactions, since the investigators used a nested PCR assay with its high potential for contamination. A study of HSV PCR for herpes encephalitis suggested that HSV DNA may persist in CSF for 2–3 weeks after initiation of effective treatment [18].

Answers to these questions will require clinicians and researchers to compare carefully PCR-based microbial detection methods with existing diagnostic methods. Only with further experience will the benefits and limitations of PCR become fully apparent.

Conclusions

PCR is a powerful technique that is increasingly applied to the diagnosis of infectious diseases. PCR-based assays detect microbial nucleic acid in clinical samples and do not require growth of the organism. PCR-based assays can be fast, sensitive, and specific, but may also be associated with technical problems such as false positive reactions due to sample contamination, and false negative reactions due to the presence of PCR inhibitors in the sample. As the cost of PCR reagents decline, and as the number of PCR-based applications increase, the clinical microbiology laboratory of the future will look increasingly like a molecular biology laboratory. Panels of PCR assays are likely to be developed, targeting microbes involved in specific syndromes such as pneumonia, meningitis, and diarrhea. Rather than having to grow an intact microbe, one will be able to "grow" a segment of its DNA, replacing culture media with PCR reaction mix, and the incubator with the

thermal cycler. PCR-based diagnostic tests offer clinicians a powerful new weapon to add to their quivers. We are long overdue for rapid, sensitive diagnostic tests in infectious diseases that allow clinicians to make sound judgments in real time. The diagnosis of Rocky Mountain spotted fever should be confirmed within hours of presentation, not days later at autopsy, or weeks later in convalescence. PCR and other molecular diagnostic methods hold the hope of making rapid diagnosis and directed therapy a reality.

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