

## APPLICATIONS OF THE POLYMERASE CHAIN REACTION TO INFECTIOUS DISEASE DIAGNOSIS

Richard Frothingham, MD

The polymerase chain reaction (PCR) was first reported in 1985.<sup>1</sup> Kary Mullis recently received the Nobel Prize for this discovery. PCR is a simple, rapid, and powerful method of gene amplification, with many potential applications to the diagnosis and management of infectious diseases. This review will discuss the principles behind PCR, the strengths and limitations of the method, and guidelines for using PCR in clinical diagnosis. Clinical applications of PCR will be discussed in relationship to two specific infectious diseases of importance to Saudi Arabia (tuberculosis and hepatitis C).

### PCR Methodology

#### Materials

PCR is an *in vitro* method for specific DNA amplification. The ingredients are remarkably simple: target DNA, a polymerase, the four DNA nucleotides, two primers, and a reaction buffer. Target DNA is the DNA which will be amplified by PCR. For infectious disease diagnosis, target DNA may be derived from a microbial culture or a clinical specimen. The polymerase is an enzyme which copies DNA, normally as part of replication. The polymerase uses one strand of DNA as a template to synthesize a new complementary strand, using the four DNA nucleotides as building blocks. The two primers are synthetic fragments of single-stranded DNA (oligonucleotides), typically about 15 to 25 bases long. Each primer is complementary to a region on one strand of the target DNA (the recognition site). Complementary base-pairing between each primer and target DNA provides the specificity of PCR. PCR itself is inexpensive. The ingredients for a typical 50  $\mu$ L reaction

volume cost less than US \$1.00. The actual costs of performing PCR will be higher, including equipment, sample preparation, positive and negative controls, detection of PCR products, and labor.

#### Temperature Cycles

The ingredients listed above are mixed in a total volume of about 50  $\mu$ L. They are then subjected to multiple temperature cycles, each of which theoretically doubles the amount of target DNA present. Each cycle includes three steps (denaturation, annealing, and extension), as shown in Figure 1. First, double-stranded DNA is separated into two single strands by heating the tube to 90°C-95°C (denaturation). The mixture is then cooled (typically to 35°C-60°C), allowing the primers to attach to complementary regions of target DNA (annealing). Primer attachment is specific dependent on base-pairing rules. Nucleotide A pairs with T, C pairs with G, etc. The mixture is then heated to the optimal temperature of the DNA polymerase (typically 72°C). Wherever the DNA polymerase finds a primer attached to a strand of DNA, it synthesizes new DNA. The primer is extended to form a new strand of DNA in a manner analogous to DNA replication (extension). The recognition sites of the two primers face each other. Extension from one primer generates the recognition site of the other primer, allowing it to attach on the next cycle. After extension, the mixture is heated to separate the newly formed DNA into two strands, beginning the next cycle.

#### Equipment

The reaction mixture is rapidly heated and cooled in a specialized piece of equipment called a temperature cycler. These are available from a variety of manufacturers in various formats, costing US \$2000 to \$15,000. Current models of temperature cyclers can complete 30 to 40 amplification cycles in about two hours.

#### Reverse Transcriptase PCR

PCR can also be applied to the amplification of RNA,

From the Infectious Disease Section, Durham Veterans Affairs Medical Center and the Division of Infectious Diseases, Department of Medicine, Duke University Medical Center, Durham, North Carolina, USA.

Address reprint requests and correspondence to Dr. Frothingham: Veterans Affairs Medical Center, 508 Fulton Street, Building 4, Durham, NC 27705 USA.

Accepted for publication 25 May 1996. Received 25 February 1996.

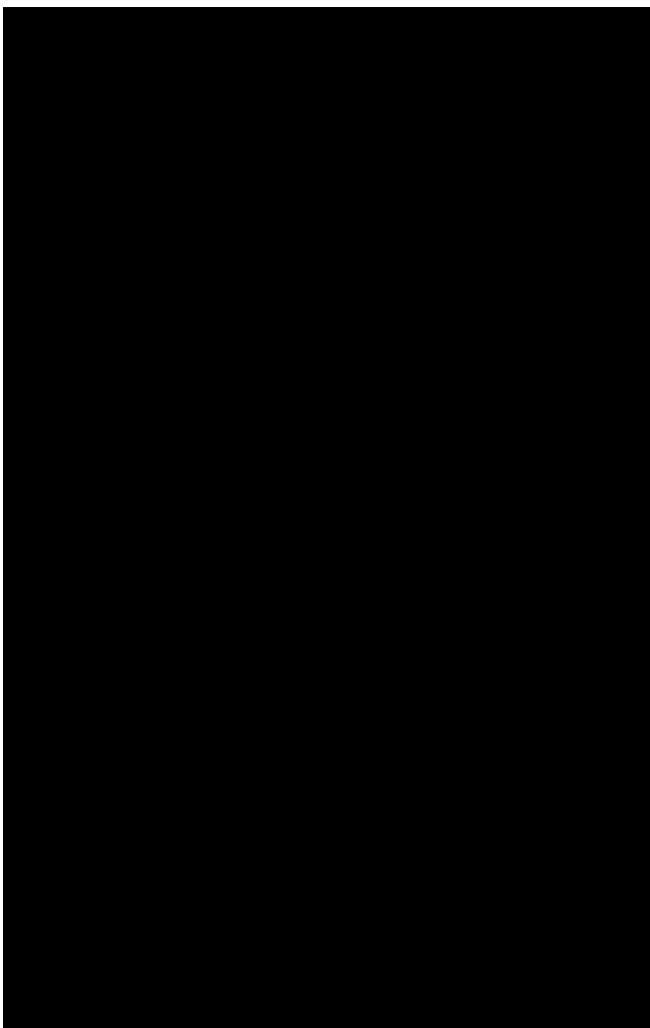


FIGURE 1. The polymerase chain reaction (PCR). Double-stranded DNA is separated into two single strands by heat (denaturation). The mixture is cooled, allowing the primers to attach to complementary regions of target DNA (annealing). The primers are then extended by the DNA polymerase to form a new strand of DNA (extension). The amount of target DNA is theoretically doubled during each cycle.

a process referred to as reverse transcriptase PCR (RT-PCR). RT-PCR is similar to regular PCR, with the addition of an initial step in which DNA is synthesized from the RNA target using an enzyme called a reverse transcriptase. A wide variety of RNA molecules have been used in RT-PCR, including ribosomal RNA, messenger RNA, and genomic viral RNA. The following discussion will refer generically to nucleic acid targets, including both DNA and RNA.

### Power and Limitations of PCR

#### *Power of PCR*

PCR theoretically provides a doubling of target DNA in each cycle. In practice, amplification by a factor  $10^6$  to  $10^8$  is readily achieved in 30 to 40 cycles. PCR is

extremely sensitive. Under artificial laboratory conditions, it is possible to detect a single nucleic acid copy. In clinical practice, the threshold of detection is generally 10 to 1000 nucleic acid copies. PCR yields rapid results, typically in one to two days in a clinical setting. It is applicable to a wide variety of clinical, pathological, or forensic specimens. DNA is a very stable molecule, and PCR has been successfully applied to formalin-fixed tissue, inactivated bacterial cultures, and archeological specimens. PCR often provides simultaneous detection and characterization of microbes, based on analysis of the amplified DNA sequence between the two primers.

#### *Versatility of PCR*

The specificity of PCR is based on the sequence of the two primers. This means that any segment of genomic DNA or RNA is a potential target for a PCR diagnostic assay. Bacterial genomes contain several million bases, and viral genomes contain tens of thousands of bases, so there are usually many potential targets to choose from. An ideal target sequence should be found in all strains of the microbe of interest, but not found in any other microbe. For a properly-defined species, many such sequences will normally be available. The versatility of PCR can be contrasted with a limitation of serologic methods. If the particular antigens expressed on the surface of a given microbe do not provide the desired sensitivity and specificity, then serologic diagnosis may not be possible.

#### *Contamination in PCR*

Carryover contamination is the most important problem for diagnostic PCR. A typical PCR reaction produces millions to billions of copies of the amplified DNA segment. These PCR products are ideal sources of target DNA for future reactions. They can be transferred accidentally to a new sample or can contaminate PCR reagents. PCR products are quite stable in the laboratory environment, resisting drying and even autoclaving. Once a laboratory becomes contaminated by PCR products from previous reactions, it is difficult to eliminate the problem. Various precautions can reduce carryover contamination. Normally, all of the steps of PCR preparation, including sample preparation and reagent mixing, should be done in a separate room from PCR product analysis using separate equipment. Special PCR reagents have been used to create modified products which can then be inactivated in subsequent reactions. Ultraviolet light can be used to inactivate DNA in PCR reagents. All these methods have limitations.<sup>2</sup> In addition to carryover contamination, cross-contamination between samples is a potential problem for PCR, just as it is for routine cultures.

#### *Sample Preparation*

PCR itself is quite simple, but sample preparation can be laborious. The goals of sample preparation include the release of nucleic acid (DNA or RNA), concentration of the nucleic acid to a small volume for PCR, and removal of inhibitors of PCR. Inhibitors of PCR are naturally occurring substances which reduce the efficiency of PCR, and which are often present in clinical samples. When the specimen contains a large amount of target nucleic acid, sample preparation is trivial. For example, a bacterial colony can be suspended in appropriate buffer, boiled, and added directly to the PCR mixture. But sample preparation is more difficult in most clinical specimens, particularly when a large volume specimen must be processed and only a few microbes are present. Complex protocols are often required, and these increase the risk of cross-contamination between specimens.

#### *Other Limitations of PCR*

PCR detects the presence or absence of a particular nucleic acid target. It will only detect a microbe if its nucleic acid is present in the particular specimen. PCR detects nucleic acids from living or dead microbes. This must be recognized if PCR is used to monitor response to therapy. PCR provides at most nucleic acid sequence information. PCR can be used to screen for drug resistance mutations, but it does not provide direct antibiotic susceptibility data.

#### *Negative Controls in Diagnostic PCR*

Appropriate controls are necessary when PCR is used diagnostically. These include negative controls, positive controls, and specificity controls. Negative controls (no target DNA) are needed to detect contamination. Contamination can occur during sample preparation or reagent mixing, so negative controls need to be processed in parallel with clinical samples. Negative controls should be interspersed among the samples to detect cross-contamination from sample to sample. Contamination is frequently intermittent; a sufficient number of negative controls must be included to detect low rates of contamination. If a false-positive rate of 1% would be clinically unacceptable, then 100 negative controls are needed. Most published studies have not included a sufficient number of negative controls.

#### *Positive Controls in Diagnostic PCR*

Positive controls include a small number of target DNA copies. Positive controls are needed to ensure efficient release of target DNA from microbes, to guard against loss of DNA during sample processing, and to identify the presence of inhibitors (natural substances sometimes present in clinical samples which reduce PCR efficiency). Positive controls should be processed in parallel with clinical specimens. Clinical specimens vary in the

presence of inhibitors of PCR, and it may be necessary to add an internal positive control for each sample. The internal positive controls have the same recognition sites as the target DNA, but are designed with some difference in the internal sequence. Amplification of the internal positive controls can be distinguished from that of the real target DNA.

#### *Specificity Controls*

Specificity controls are needed to determine the range of target DNAs which will be amplified by the PCR assay. For assays designed to detect microbes in clinical samples, human DNA samples must be tested to ensure that the PCR primers do not recognize a human DNA target by chance. Related microbes must be tested to determine the range of species/strains which will be amplified. Specificity controls are needed only once, when a new PCR assay is designed. Negative and positive controls must be included every time that samples are processed, and should be processed simultaneously with the clinical samples.

### **Diagnostic Goals for PCR**

PCR has been used for three broad categories of diagnostic goals, namely detection, characterization, and quantitation. The practical advantages and limitations depend upon the goal to which PCR is applied.

#### *Detection (Is the target nucleic acid present in the specimen?)*

This is the most difficult goal of PCR, especially when the number of microbes in the specimen is low. PCR is conducted under conditions of high sensitivity. Many temperature cycles are used, or a nested protocol is used in which the products from the first reaction are re-amplified with a second set of primers. This makes PCR for detection especially prone to carryover contamination. Sample preparation may be laborious, as there is an attempt to process as large a specimen volume as possible. Inhibitors of PCR occur naturally in many clinical samples, and are a major limitation. Numerous positive and negative controls must be included as described above.

#### *Characterization (Which variant of the target nucleic acid is present?)*

Variants are identified based on the nucleic acid sequence between the two PCR primers. Many techniques can be used to detect variable sequences, including length polymorphism, changes in restriction sites, and direct DNA sequencing. This is often the easiest type of PCR to apply clinically. Ample quantities of nucleic acid target may be present in the specimen, either an already grown bacterial or viral culture or a clinical sample with large numbers of microbes. Goals may include rapid detection

of drug resistance mutations, assignment of strains to clinically meaningful phylogenetic groups, or epidemiological tracing. Examples will be discussed below.

*Quantitation (How many copies of the target nucleic acid are present?)*

Quantitative PCR has primarily been applied to chronic viral infections, especially hepatitis C virus (HCV) and human immunodeficiency virus (HIV) infections. The level of viremia has prognostic implications, and has been used to demonstrate response to antiviral drugs. PCR is quite sensitive, but it is not inherently quantitative. The amount of the final PCR product is usually similar from an initial sample containing 10 or 10,000 copies. This limitation can be overcome by serial dilution of the clinical sample until no target DNA is detected, or by the addition of synthetic competitor DNA molecules. The competitor molecules have regions complementary to the two primers, but differ in some way from the natural target (e.g., a different length). By comparing the amount of the natural and competitor PCR products, a rough estimation of the number of target molecules in the sample is possible.

*Ideal Applications for PCR*

PCR has been applied in the research setting to hundreds of microbes, and has yielded important insights into pathogenesis and epidemiology of many infectious diseases. For clinical purposes, PCR-based diagnostic tests are best applied when the following conditions are fulfilled: 1) The results of the test will make a clear clinical difference. A therapy will be given or withheld based on the results of PCR. 2) Routine culture methods are limited because the microbe cannot be grown (e.g., *Mycobacterium leprae*, HCV), grows slowly (e.g., *M. tuberculosis*), or is difficult to culture (e.g., *Brucella* species, HIV). 3) There is an accessible clinical specimen which contains large numbers of microbes (e.g., blood for HCV or HIV).

*Specific Applications of Diagnostic PCR*

PCR has been useful in a variety of chronic virus infections (HIV, HCV, hepatitis B virus, human papillomavirus, cytomegalovirus). PCR has been crucial for the detection of HIV infection in neonates, since maternal antibodies complicate serologic diagnosis. Quantitation of HIV and HCV viremia by PCR has important prognostic implications, and has been used to monitor response to drug therapy. PCR is useful for the rapid diagnosis of pulmonary infections in immunocompromised hosts, particularly for cytomegalovirus and *Pneumocystis carinii*.<sup>3</sup>

A complete discussion of potential PCR applications is beyond the scope of this review. Two microbes will be discussed in detail (*M. tuberculosis* and HCV) for which

the application of PCR is particularly promising. Both of these microbes are prevalent in Saudi Arabia and cause substantial morbidity and mortality. They illustrate the principles involved in application of diagnostic PCR to other microbes.

## Tuberculosis

*Tuberculosis in Saudi Arabia*

Approximately 1.7 billion humans, or about one-third of the world population,<sup>4</sup> are infected with *Mycobacterium tuberculosis*. Tuberculosis is the leading cause of adult death due to an infectious agent.<sup>4</sup> There are nine million tuberculosis cases per year, resulting in three million deaths worldwide.<sup>4</sup> The annual risk of tuberculosis infection is determined by the rate of skin test conversion in unvaccinated children. Saudi Arabia has a moderate rate of 0.5% per year, much lower than the rate in sub-Saharan Africa (about 2%), but much higher than the rate in Europe (about 0.1%).<sup>4,5</sup> Similarly, Saudi Arabia has a moderate incidence of active tuberculosis cases, about 30 per 100,000.<sup>5</sup>

*Diagnostic Challenges in Tuberculosis*

*M. tuberculosis* is a hardy bacterium, and it is readily cultured when it is present in a clinical specimen. There are two major limitations to the diagnosis of tuberculosis by culture: 1) *M. tuberculosis* grows extremely slowly, leading to delays in species identification and drug susceptibility testing. In some recent outbreaks of multidrug-resistant tuberculosis, the results of drug susceptibility testing were not available until after most patients had already died. Using the best available culture methods, species identification typically takes about two weeks from specimen submission, and drug susceptibility testing an additional two weeks. This is not fast enough for some patients, particularly those with HIV infection and multidrug-resistant strains of *M. tuberculosis*. 2) The available clinical specimens may contain few or no organisms, or may contain organisms only intermittently.

There has been tremendous enthusiasm for the application of PCR to tuberculosis diagnosis. A recent literature search revealed over 400 publications on the subject. Particular attention has focused on rapid detection of *M. tuberculosis* in sputum specimens, with the goal of providing the diagnosis of tuberculosis more rapidly than by routine culture.

*PCR for Detection of M. Tuberculosis in Sputum*

PCR has been evaluated in numerous studies for the detection of *M. tuberculosis* in sputum specimens.<sup>6-10</sup> Specimens are typically tested simultaneously by PCR, acid-fast smear, and routine mycobacterial culture. The

overall sensitivity of PCR has been quite variable among studies, ranging from 58% to 100%. Sensitivity is much higher in smear-positive specimens (95% to 100%) than smear-negative specimens (46 to 63%). Many published studies have reported problems with false-positive PCR results, at rates ranging from 0.8% to 30%.<sup>10</sup> False-positive PCR results are especially problematic when the rate of true-positive cultures is low. If the true rate of positive cultures is 5%, then a 1% false-positive PCR rate means that one out of six positive PCR results are false-positive. A false-positive PCR result for tuberculosis may have significant consequences (e.g., toxic drugs, unnecessary isolation, psychological trauma, and neglect of alternate diagnoses).

Reported studies suffer from the inevitable difficulty in defining the gold standard. It is well recognized that sputum cultures are intermittently positive in pulmonary tuberculosis. Clinicians typically culture at least three sputum specimens in the hope that at least one will be positive for *M. tuberculosis*. It is difficult to calculate sensitivity and specificity when some sputum specimens from a given patient are positive by culture alone and other specimens are positive by PCR alone. The variations may be due entirely to sampling variations.<sup>7</sup> Furthermore, some cases of tuberculosis have negative cultures and are diagnosed on the basis of typical clinical features and a good response to antituberculous medications. In a recent CDC survey, 17% of total cases reported were culture-negative.<sup>11</sup> Some authors have attempted to construct clinical definitions of tuberculosis, based on the combination of smears, cultures, pathology, clinical histories, and response to medications. They have then used the clinical definitions to calculate the sensitivity and specificity of PCR.<sup>6,9,10</sup>

#### Proficiency Testing

Noordhoek et al. recently published a blinded comparison of the PCR detection of *M. tuberculosis* by seven laboratories.<sup>12</sup> They added known numbers of *M. bovis* BCG cells to sputum, saliva, and water samples. This tuberculosis vaccine strain was used for safety. Identical sets of 200 samples each were sent to the seven laboratories, each of which had experience in the use of PCR for *M. tuberculosis* detection. The results varied widely among the laboratories. False positives were a problem for all seven laboratories. Three of the seven laboratories reported at least 20% of the negative samples as positive. Only one laboratory reported zero false-positives. However, that laboratory reported "no result" for half of the specimens when their negative controls revealed a contamination problem.

The sensitivity of all the laboratories fell below expectations. Several laboratories were able to detect 5000 bacteria/mL (1000 bacteria in a 0.2 mL specimen) in the

majority of specimens (70% to 90%). None of the labs reliably detected 500 bacteria/mL. The BCG vaccine strain used in this study contained only one copy of the target DNA, while wild type *M. tuberculosis* strains often contain five to 20 copies. PCR might be 10-fold more sensitive for detection of *M. tuberculosis* than for BCG. Based on these results, the authors estimated that PCR might detect as few as 500 *M. tuberculosis* bacteria/mL. That would be somewhat more sensitive than the AFB smear, but not nearly as sensitive as culture. A number of technical problems were identified in the various laboratories, such as the lack of negative and positive controls at all steps. Most of the labs did not assay the full specimen volume (only 0.2 mL!) in a single PCR reaction. This is a major limitation, since it is known that culture yield for *M. tuberculosis* increases with increasing sample volumes. A sputum volume of at least 5 mL is recommended for mycobacterial culture.

#### Other Applications of PCR for *M. Tuberculosis* Detection

The diagnosis of tuberculous meningitis and pleuritis has been difficult due to the low yield of bacterial cultures. Several small studies have applied PCR to these diagnoses with encouraging results. For tuberculous meningitis, the sensitivity has ranged from 75% to 100%.<sup>13</sup> The sensitivity for pleural tuberculosis in one study was 81%.<sup>14</sup> Published studies are limited by small numbers, the difficulty of clinical diagnosis, and in some cases, unacceptable rates of false positives.<sup>14,15</sup> PCR seems especially useful in the diagnosis of tuberculous meningitis in AIDS patients, possibly due to larger numbers of bacteria in these patients.<sup>16</sup> Two recent reports have described amplification of *M. tuberculosis* from the buffy coats of peripheral blood of patients with pulmonary tuberculosis.<sup>17,18</sup> This approach appears to be more useful in patients with HIV infection than in immunocompetent patients. Confirmatory studies are needed.

#### Value for Clinical Tuberculosis Care

It has been assumed that more rapid detection of *M. tuberculosis* by PCR would translate into improved clinical care. However, few studies have addressed this point specifically. Clinicians appear to be reasonably accurate in identifying patients with pulmonary tuberculosis based on clinical features and the initial acid-fast smear. Empiric antituberculous therapy is often begun long before culture results are obtained, so the clinical benefit of early diagnosis by PCR may be minimal for some patients. In one small report, 10 patients were eventually found to have positive *M. tuberculosis* cultures. Eight of the 10 patients were started on tuberculosis therapy at the time of specimen submission, and the other two patients were begun on therapy 10 and 13 days later.<sup>19</sup> There are

probably subsets of patients where a more rapid PCR-based diagnosis will be clinically important, possibly including the following: patients with negative smears but high likelihood of disease, immunosuppressed patients in whom treatment delay is particularly hazardous, patients with relative contraindications to empiric antituberculous treatment, and patients with suspected tuberculosis meningitis, pericarditis, or pleuritis. The benefits of rapid PCR-based tuberculosis diagnosis must always be balanced against the risks of a false-positive diagnosis.<sup>20</sup>

#### *PCR for Characterization of M. Tuberculosis*

Because of the slow growth of mycobacteria, species identification and drug susceptibility determination can also be delayed. PCR has been used to characterize mycobacteria which have already been grown in culture. These assays begin with a large amount of target DNA from the bacterial culture. Sensitivity and specificity are excellent when proper controls are used. The *M. tuberculosis* complex (including *M. tuberculosis*, *M. bovis*, *M. microti*, and *M. africanum*) can be reliably distinguished from nontuberculous mycobacteria.<sup>19,21</sup> Multiple species of mycobacteria can be identified by PCR followed by restriction enzyme analysis of the PCR products.<sup>22,23</sup> PCR assays have been designed to identify particular strains of importance, such as the multidrug-resistant *M. tuberculosis* strain W or the vaccine strain *M. bovis* BCG.<sup>24,25</sup> Many investigators have pursued the goal of differentiating *M. tuberculosis* strains by PCR for epidemiological tracing. This goal has not yet been fully achieved, largely due to the high degree of DNA sequence conservation among strains.<sup>26</sup>

#### *PCR for rapid M. Tuberculosis Drug Susceptibility Testing*

Rapid identification of *M. tuberculosis* drug susceptibility is an important goal. Early appropriate therapy reduces the spread of tuberculosis and improves clinical outcome. Edlin et al. described 18 AIDS patients with multidrug-resistant *M. tuberculosis* infection.<sup>27</sup> Those who received appropriate therapy within two weeks of diagnosis had an 86% survival, compared to an 18% survival among those who did not ( $P = 0.01$ ). Turett et al. studied 34 similar patients.<sup>28</sup> The 23 patients who received appropriate therapy within four weeks had a 65% one-year survival, but the 11 patients who did not receive such therapy had a median survival of only 19 days. Current susceptibility tests are not rapid enough for these patients, typically requiring four weeks from the time a specimen is submitted.

Two rapid PCR-based assays for rifampin resistance have been described recently.<sup>29,30</sup> The assays are based on detection of mutations in a small segment of the *rpoB* gene which cause virtually all rifampin resistance in *M. tuberculosis*. Both assays are sensitive and specific, and

can be applied to smear-positive specimens or early positive cultures. Work is underway to develop similar assays for isoniazid and other drugs. The variety of mutations responsible for resistance to some drugs has posed a practical limitation. However, new PCR-based methods are available for simultaneous detection of multiple mutations. Examples of these new methods include multiplex PCR, heteroduplex analysis, and single strand conformation polymorphism.<sup>25,29,30</sup> It may soon be possible to screen isolates simultaneously for resistance to several first-line antituberculous medications using rapid PCR-based assays.

PCR is not yet ready for clinical application for the routine detection of *M. tuberculosis* in smear-negative sputum samples.<sup>31</sup> PCR can be reliably applied for rapid species identification and for rapid screening for rifampin resistance in smear-positive sputum specimens or early positive cultures.

## **Hepatitis C Virus**

### *Hepatitis C in Saudi Arabia*

Hepatitis C virus (HCV) infection is prevalent in Saudi Arabia, and is a major cause of liver disease. Between 1.2% and 3.5% of Saudi blood donors are HCV-seropositive.<sup>32-34</sup> These rates are much higher than those seen in European countries. An extremely high 18% seropositivity rate was found in Egyptians who donate blood in Saudi Arabia.<sup>33</sup> About 30% of Saudis with chronic liver disease and 30% of Saudis with hepatocellular carcinoma are HCV-seropositive.<sup>34</sup> Reports of successful treatment of HCV infection with interferon have increased interest in applications of PCR.

### *HCV Diagnosis*

Available tests for HCV infection are limited. Initial serologic tests for HCV had poor sensitivity. Second- and third-generation serologic tests have improved sensitivity, but are still not completely dependable.<sup>35</sup> HCV RNA is readily detected in serum using RT-PCR. Viremic patients typically have very high viral titers. In one study, 93% of patients had at least 100,000 HCV-RNA molecules per mL of serum.<sup>36</sup> Given the large amount of viral RNA present, one would predict that PCR for HCV detection should be a reliable test.

PCR has been applied to the diagnosis of HCV infection in a variety of clinical settings.<sup>35,37</sup> HCV can be detected as early as one week after infection, and PCR can be used to detect HCV infection during the "window" period between infection and seroconversion. HCV PCR is useful for detecting HCV in seronegative individuals with liver disease. It can be used to confirm maternal to fetal spread of HCV. HCV PCR may be useful in the evaluation of seropositive individuals as candidates for interferon or

other therapies. A portion of HCV-seropositive patients are negative by HCV PCR, and may have resolved their infections. PCR-negative individuals have lower serum transaminase concentrations and less histologic activity on liver biopsies.<sup>38</sup> Long-term follow-up studies are needed, but it may be reasonable to withhold therapy from patients with negative HCV PCR results.

#### *HCV Quantitation*

The amount of HCV viremia can be determined by either quantitative PCR or a branched-DNA assay (not discussed here). PCR is more sensitive and is quantitative over a wider range of viral titers, but it is technically more difficult.<sup>36</sup> High-titer viremia is correlated with an advanced disease stage.<sup>39</sup> The prognostic value of HCV quantitation awaits prospective studies, but the level of viremia may be useful in selecting candidates for therapy. Quantitative HCV PCR also appears to be useful in monitoring the response to therapy.<sup>36</sup>

#### *HCV Characterization*

Various HCV genotypes have been identified based on sequence analysis of amplified HCV RNA. It appears that there is a strong correlation between the HCV genotype and the response to therapy. Long-term response is common for genotypes 2 and 3, but unusual for genotype 1.<sup>40,41</sup> HCV genotypes can be determined quickly by PCR followed by restriction enzyme analysis. There is substantial geographic variation in genotypes, with genotype 1 being most common worldwide.<sup>42</sup> However, most Egyptians working in Saudi Arabia have genotype 4, which is rarely found outside of the Middle East. Information is limited about the distribution of HCV genotypes in Saudi nationals, but both genotypes 1 and 4 have been reported.<sup>43</sup> HCV genotype determination by PCR will be useful in identifying patients most likely to benefit from therapy.

#### *Proficiency Testing in HCV PCR*

A multicenter collaborative study was recently conducted to determine the reliability of PCR for HCV detection in various laboratories.<sup>44</sup> The design was similar to that described above for tuberculosis. Coded aliquotted human serum samples were tested by 31 HCV research laboratories. Each lab received 10 samples (six negative sera, three strongly positive sera, one weakly positive serum) and two dilution series ( $10^{-2}$  through  $10^{-7}$  dilutions of strongly positive sera). Reliability varied greatly among the labs. Overall, 89% of the negative samples were correctly reported as negative, and 96% of the strong positive samples were correctly identified. Ten of the 31 labs correctly identified all 10 samples, and 11 labs identified all but the single weak positive correctly. Fifteen labs correctly characterized both dilution series,

although the quantitative titer estimates varied greatly. Five of 31 labs performed flawlessly in identification of all ten samples and both dilution series. It appears that HCV detection and quantitation is feasible for clinical application. However, not all labs are currently proficient.

#### **Novel Nonculturable Microbes**

PCR has been used to identify novel microbes as the causes of human disease. PCR primers were directed at DNA targets which are conserved among a variety of microbial species, such as conserved regions of the ribosomal RNA gene, which are found in all bacteria, but not in humans. Using this approach, the causative bacteria for bacillary angiomatosis and Whipple's disease have been identified.<sup>45,46</sup> In both diseases, bacteria could be seen in histologic sections, but could not be grown in culture. Attempts to amplify bacterial DNA from sarcoidosis specimens have been less encouraging.

#### **Summary**

PCR is a powerful tool for research and diagnosis. PCR is useful for detection, quantitation, and characterization of a variety of microbes. It is most applicable when there is a compelling clinical question to be answered and when there are numerous microbes present in an accessible clinical specimen. Although the PCR method is simple, diagnostic PCR is a major undertaking, requiring rigorous precautions against contamination, multiple negative and positive controls, validated sample processing, and proficiency testing. Diagnostic PCR may be best carried out by laboratories which handle a large number of specimens. Comparative studies have demonstrated major variations in proficiency among experienced laboratories. Proficiency testing should be required before the results of PCR-based assays are used for clinical care.

#### **Acknowledgments**

I thank Drs. Michael Ellis and Majid Halim for the invitation to give a series of lectures at King Faisal Specialist Hospital and Research Centre in February and March, 1996. These lectures provided the impetus for this review. This work was supported by the Saudi-US Universities Project, US Public Health Service grant AI35230, the Durham Veterans Affairs Medical Center's Research Center on AIDS and HIV Infection, and the Department of Veterans Affairs.

#### **References**

1. Saiki RK, Scharf S, Faloona F, et al. Enzymatic amplification of  $\beta$ -globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. *Science* 1985;230:1350-4.
2. Frothingham R, Blichington RB, Lee DH, Greene RC, Wilson KH. UV absorption complicates PCR decontamination. *BioTechniques* 1992;13:208-10.
3. Schluger NW, Rom WN. The polymerase chain reaction in the diagnosis and evaluation of pulmonary infections. *Am J Respir Crit Care Med* 1995;152:11-6.
4. Raviglione MC, Snider DE, Kochi A. Global epidemiology of tuberculosis. *JAMA* 1995;273:220-6.
5. Al-Kassimi FA. Review of tuberculosis in Saudi Arabia. *Saudi Med J* 1994;15:192-5.
6. Chin DP, Yajko DM, Hadley WK, et al. Clinical utility of a commercial test based on the polymerase chain reaction for detecting *Mycobacterium tuberculosis* in respiratory specimens. *Am J Respir Crit Care Med* 1995;151:1872-7.
7. Clarridge III JE, Shawar RM, Shinnick TM, Plikaytis BB. Large-scale use of polymerase chain reaction for detection of *Mycobacterium tuberculosis* in a routine mycobacteriology laboratory. *J Clin Microbiol* 1993;31:2049-56.
8. Nolte FS, Metchock B, McGowan Jr JE, et al. Direct detection of *Mycobacterium tuberculosis* in sputum by polymerase chain reaction and DNA hybridization. *J Clin Microbiol* 1993;31:1777-82.
9. Schirm J, Oostendorp LAB, Mulder JG. Comparison of Amplicor, in-house PCR, and conventional culture for detection of *Mycobacterium tuberculosis* in clinical samples. *J Clin Microbiol* 1995;33:3221-4.
10. Schluger NW, Kinney D, Harkin TJ, Rom WN. Clinical utility of the polymerase chain reaction in the diagnosis of infections due to *Mycobacterium tuberculosis*. *Chest* 1994;105:1116-21.
11. Bloch AB, Cauthen GM, Onorato IM, et al. Nationwide survey of drug-resistant tuberculosis in the United States. *JAMA* 1994;271:665-71.
12. Noordhoek GT, Kolk AH, Bjuene G, et al. Sensitivity and specificity of PCR for detection of *Mycobacterium tuberculosis*: a blind comparison study among seven laboratories. *J Clin Microbiol* 1994;32:277-84.
13. Kaneko K, Miyatake T, Tsuji S. Tuberculous meningitis [letter]. *Neurology* 1995;45:1425-6.
14. de Wit D, Maartens G, Steyn L. A comparative study of the polymerase chain reaction and conventional procedures for the diagnosis of tuberculous pleural effusion. *Tubercle Lung Dis* 1992;73:262-7.
15. Lee BW, Tan JAMA, Wong SC, et al. DNA amplification by the polymerase chain reaction for the rapid diagnosis of tuberculous meningitis. Comparison of protocols involving three mycobacterial DNA sequences, IS6110, 65 kDa antigen, and MPB64. *J Neurol Sci* 1994;123:173-9.
16. Fogueira L, Delgado R, Palenque E, Noriega AR. Polymerase chain reaction for rapid diagnosis of tuberculous meningitis in AIDS patients. *Neurology* 1994;44:1336-8.
17. Rolfs A, Beige J, Finckh U, et al. Amplification of *Mycobacterium tuberculosis* from peripheral blood. *J Clin Microbiol* 1995;33:3312-4.
18. Schluger NW, Condos R, Lewis S, Rom WN. Amplification of DNA of *Mycobacterium tuberculosis* from peripheral blood of patients with pulmonary tuberculosis. *Lancet* 1994;344:232-3.
19. Morris A, Reller L, Devlin B. Clinical usefulness of detecting growth of *Mycobacterium tuberculosis* in positive Bactec phials using PCR [letter]. *J Clin Pathol* 1994;47:190-1.
20. Macher A, Goosby E. PCR and the misdiagnosis of active tuberculosis [letter]. *N Engl J Med* 1995;332:128-9.
21. Cormican MG, Barry T, Gannon F, Flynn J. Use of polymerase chain reaction for early identification of *Mycobacterium tuberculosis* in positive cultures. *J Clin Pathol* 1992;45:601-4.
22. Plikaytis BB, Plikaytis BD, Yakus MA, et al. Differentiation of slowly growing *Mycobacterium* species, including *Mycobacterium tuberculosis* by gene amplification and restriction fragment length polymorphism analysis. *J Clin Microbiol* 1992;30:1815-22.
23. Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* 1993;31:175-8.
24. Frothingham R. Differentiation of strains in *Mycobacterium tuberculosis* complex by DNA sequence polymorphisms, including rapid identification of *M. bovis* BCG. *J Clin Microbiol* 1995;33:840-4.
25. Plikaytis BB, Marden JL, Crawford JT, Woodley CL, Butler WR, Shinnick TM. Multiplex PCR assay specific for the multidrug-resistant strain W of *Mycobacterium tuberculosis*. *J Clin Microbiol* 1994;32:1542-6.
26. Frothingham R. Discrimination of *Mycobacterium tuberculosis* strains by PCR [letter]. *J Clin Microbiol* 1995;33:2801.
27. Edlin BR, Tokars JI, Grieco MH, et al. An outbreak of multidrug-resistant tuberculosis among hospitalized patients with the acquired immunodeficiency syndrome. *N Engl J Med* 1992;326:1514-21.
28. Turett GS, Telzak EE, Torian LV, et al. Improved outcomes for patients with multidrug-resistant tuberculosis. *Clin Infect Dis* 1995;21:1238-44.
29. Telenti A, Imboden P, Marchesi F, et al. Detection of rifampicin-resistance mutations in *Mycobacterium tuberculosis*. *Lancet* 1993;341:647-50.
30. Williams DL, Waguespack C, Eisenach K, et al. Characterization of rifampin resistance in pathogenic mycobacteria. *Antimicrob Agents Chemother* 1994;38:2380-86.
31. Centers for Diseases Control and Prevention. Diagnosis of tuberculosis by nucleic acid amplification methods applied to clinical specimens. *MMWR* 1993;42:686.
32. Bakir TMF. Age-specific prevalence of antibody to hepatitis C virus among the Saudi population. *Saud Med J* 1992;13:321-4.
33. Fathalla SE, Al-Jama AA, Badawy MS, et al. Prevalence of hepatitis C virus infection in the Eastern Province of Saudi Arabia by RE-DNA second generation and supplemental EIA tests. *Saudi Med J* 1994;15:281-5.
34. Al-Karawi MA, Shariq S, Mohamed ARES, Saeed AA, Ahmed AMM. Hepatitis C virus infection in chronic liver disease and hepatocellular carcinoma in Saudi Arabia. *J Gastroenterol Hepatol* 1992;7:237-9.
35. Tedeschi V, Seeff LB. Diagnostic tests for hepatitis C: where are we now? *Ann Intern Med* 1995;123:383-5.
36. Gretch DR, dela Rosa C, Carithers Jr RL, Willson RA, Williams B, Corey L. Assessment of hepatitis C viremia using molecular amplification technologies: correlations and clinical implications. *Ann Intern Med* 1995;123:321-9.
37. Brechot C. Polymerase chain reaction for the diagnosis of viral hepatitis B and C. *Gut* 1993;34:S39-S44.
38. Shakil AO, Conry-Cantilena C, Alter HJ, et al. Volunteer blood donors with antibody to hepatitis C virus: clinical, biochemical, virologic, and histologic features. *Ann Intern Med* 1995;123:330-7.
39. Gretch D, Corey L, Wilson J, et al. Assessment of hepatitis C virus RNA levels by quantitative competitive RNA polymerase chain reaction: high-level viremia correlates with advanced stages of disease. *J Infect Dis* 1994;169:1219-25.
40. Chemello L, Alberti A, Rose K, Simmonds P. Hepatitis C serotype and response to interferon therapy [letter]. *N Engl J Med* 1994;330:143.
41. Pontisso P, Gerotto M, Chemello L, et al. Hepatitis C virus genotypes HCV-1a and HCV-1b: the clinical point of view [letter]. *J Infect Dis* 1995;171:760.
42. McOmish F, Yap PL, Dow BC, et al. Geographical distribution of hepatitis C virus genotypes in blood donors: an international collaborative survey. *J Clin Microbiol* 1994;32:884-92.
43. Saeed AA, Al-Admawi AM, Rankin D. Hepatitis C virus RNA and types in Saudi blood donors. *Saudi Med J* 1994;15:87-8.
44. Zaaier HL, Cuyper HTM, Reesink HW, Winkel IN, Gerken G, Lelie PN. Reliability of polymerase chain reaction for detection of hepatitis C virus. *Lancet* 1993;341:722-4.
45. Relman DA, Loutit JS, Schmidt TM, Falkow S, Tompkins LS. The agent of bacillary angiomatosis: an approach to the identification of uncultured pathogens. *N Engl J Med* 1990;323:1573-80.
46. Wilson KH, Blichington R, Frothingham R, Wilson JAP. Phylogeny of the Whipple's-disease-associated bacterium. *Lancet* 1991;338:474-5.