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Polymerase Chain Reaction and Leprosy

Revolutionary developments in science, by definition, have far-reaching effects on many fields, often providing new insight on unresolved questions or alternative approaches to circumvent long-standing obstacles to basic and applied research. Such is the case with the discovery of the polymerase chain reaction (PCR). Since the discovery of PCR by K. Mullis and associates[1, 2] in 1987, it has become an important tool for many scientists studying deoxyribonucleic acid (DNA). The major strength of PCR is the ability of the reaction to produce incredibly large amounts of DNA of defined length and sequence from small quantities of DNA through enzymatic amplification using reasonably inexpensive equipment and reagents.[3, 4]

Specific DNA amplification of this magnitude has created a new approach for detecting and identifying pathogenic microorganisms using defined gene segments for amplification. Of particular interest to workers studying leprosy, tuberculosis and other mycobacterial diseases has been the potential for PCR to simplify the detection of the etiologic agents involved in these diseases. Assuming PCR can meet this goal and remain cost-effective, it may eventually aid in the diagnosis of these diseases and provide a new approach for studying various epidemiologic aspects of these important human diseases. The purpose of this editorial is to review the current literature associated with the application of PCR to the study of pathogenic mycobacteria with primary emphasis on the potential of this technique for the study of leprosy.

PCR methodology

PCR takes advantage of the normal molecular mechanisms associated with enzymatic replication of DNA.[3] Reactants needed to perform PCR can be obtained commercially and include positive and negative strand priming oligonucleotides (primers), deoxyribonucleotides (dNTPS), and DNA polymerase. The final ingredient is the template DNA (e.g., bacterial chromosomal DNA) to which the other components bind and produce multiple copies of a defined DNA segment (target). DNA amplification is based on the repetition of three steps: denaturation, annealing, and primer extension. In the denaturation step, the double-stranded template DNA is separated into single strands by heating. This is followed by the annealing step, in which positive and negative strand primers bind to complementary DNA sequences flanking the "target" region of the template DNA. In the primer extension step, primers, in the presence of *Thermus aquaticus* (*Taq*) DNA polymerase, initiate synthesis of two new strands complementary to the original strands. Repetitive temperature cycling of the reaction mixture can result in greater than a million-fold amplification of a desired gene segment within a few hours.[3, 4] With appropriate detection methods (hybridization), this technique has the theoretical capability of detecting one target sequence in a sample preparation.[1, 5]

Rationale for PCR in diagnosis of leprosy. Routine diagnosis of leprosy is based on clinical observation of the patient's dermatologic condition and associated neurologic sequelae. Histologic examination of skin lesions confirms the clinical picture when intraneural acid-fast bacilli (AFB) and/or granulomatous destruction of the nerve are present. These procedures are perfectly suitable for the diagnosis of most forms of leprosy, but can suffer from a lack of sen-

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sitivity and specificity when the clinical or histopathologic findings are equivocal. For example, histologic examination of tissues cannot differentiate one AFB from another, and the practical lower limit of detection by microscopic observation is approximately 10^4 AFB/ml.[6,7] When no AFB are seen on histologic examination, or if the inflammatory response in the tissues is nonspecific and intraneural inflammation is absent, a definitive diagnosis of leprosy cannot be made. Since two major strengths of PCR are sensitivity and specificity, it is possible that PCR or other DNA amplification procedures may find a role in this area, providing a rapid method with extremely high sensitivity and specificity for detecting small numbers of *Mycobacterium leprae* present in biological specimens.

Specificity. Standard microbiologic identification tests require specific knowledge of the genotypic or phenotypic characteristics of microorganisms. Identification tests based on PCR are no different, and require DNA sequence data from which specific tests are developed.[1-4] With the establishment of recombinant DNA libraries for *M. leprae*,[8,9] *M. tuberculosis*,[10] and other mycobacteria, genetic studies have detailed numerous DNA sequences suitable for developing species-specific PCR tests.[11-16] Indeed, species- or genus-specific PCR tests have now been reported for *M. leprae*,[17-21] *M. tuberculosis*,[22-25] *M. avium-intracellulare* complex,[24,26] *M. fortuitum*, and *M. paratuberculosis*.[24]

Selection of the appropriate DNA "target" for amplification is very important for

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the development of a highly specific PCR test. *M. leprae*-specific PCR tests have been developed from DNA sequences encoding three well-defined antigenic proteins. Two of the proteins (18-kDa and 36-kDa) were found almost exclusively in *M. leprae,*[14, 27, 28] while the third protein (65-kDa) has been shown to be a highly conserved heat-shock protein and thought to be expressed in all prokaryotes as a homolog of the GroEL protein of *Escherichia coli.*[29, 30] PCR tests have been developed based on the amplification of species-specific "target" regions of the gene for these proteins or by use of a second "nested" amplification of a common gene sequence to produce the *M. leprae*-specific product. A fourth PCR test for *M. leprae* was developed by Woods and Cole[18] based on an *M. leprae*-specific repetitive DNA sequence found at approximately 20 sites in the *M. leprae* genome.[13, 31–33] While specificity analysis for most of these PCR tests was not exhaustive, most studies included DNA from appropriate mycobacteria, DNA from related and unrelated bacterial genera (often found in association with tissues taken for clinical diagnosis of leprosy), and DNA from eukaryotic sources including man, mouse, and the nine-banded armadillo. Taken together, the above data indicate that species-specific PCR tests do not have to be limited to *M. leprae*-specific genes but can be developed from sequences found within a highly conserved gene within the mycobacterial genus containing species-specific regions. Since mutations in species-specific DNA regions of either common or specific genes could lead to false-negative results by PCR, it is not clear at this point which approach will provide a more universally applicable PCR test. However, in light of the apparent low degree of the genetic diversity of isolates of *M. leprae,* this may not prove to be a serious problem.[13, 31–33]

Sensitivity. Sensitivity requirements for PCR tests designed to detect mycobacterial pathogens must surpass detection levels of standard microscopic observation (10^4/ml) and maintain specificity. Ideally, pathogen detection and identification should be made directly from patient specimens obviating the need for cultivation. This is particularly relevant to the identification of *M. leprae,* but is also important for identifying slowly growing pathogens such as *M. tuberculosis* and *M. avium.*

Mycobacterial identification tests developed from nucleic acid hybridization methods preceded PCR, and have become important tools for the specific identification of mycobacterial groups such as the *M. tuberculosis*[34, 35] and *M. avium* complexes.[36, 37] These assays, however, are designed

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[37] Saito, H., Tomioka, H., Soto, K., Tasaka, H., Tsukamura, M., Kuzi, F. and Asano, K. Identification and partial characterization of *Mycobacterium avium* and *Mycobacterium intracellulare* by using DNA probes. J. Clin. Microbiol. **27** (1989) 994–997.

for identification of the organisms upon achieving a prescribed degree of growth *in vitro,* not allowing direct detection from biologic specimens. A DNA hybridization assay developed for the identification of *M. leprae* from biological tissues was reported by Clark-Curtiss' group and showed a theoretical lower limit of approximately 4×10^3 *M. leprae.*[13] The DNA probe used in the assay contained an *M. leprae*-specific repetitive sequence, enhancing the theoretical sensitivity for detecting *M. leprae* DNA. However, tests showed that the practical lower limit of the assay for detecting *M. leprae* from multibacillary patients' skin biopsies was approximately 1×10^5 bacilli. While DNA hybridization assays are highly specific, improved levels of sensitivity will be necessary to detect microorganisms from biological samples routinely.

PCR-based assays with or without DNA hybridization are challenging direct DNA hybridization assays for mycobacterial detection with similar levels of specificity and increased levels of sensitivity. For example, all PCR tests for *M. leprae* reported thus far can detect between 1 to 100 bacilli. Most of these reports measured lower detection limits in the presence of excess prokaryotic[20] or eukaryotic[19, 20, 24] DNA as well as normal, uninfected human skin biopsies.[19] While the detection limits of these assays are impressive and provide new ways to detect *M. leprae,* the utility of these assays for improving current diagnostic criteria for leprosy remains undefined.

Sample preparation for PCR. Sample preparation for PCR should be simple, inexpensive, and safe, while maintaining the integrity of the DNA for subsequent analysis. Physical barriers, such as standard microbiologic hoods and screw-cap test tubes, should be used throughout sample preparation of infectious material to protect the worker and to reduce the possibility of contaminating the samples. Disruption of infected tissues using a Mickle homogenizer and glass beads provides *M. leprae* suspensions suitable for extraction of DNA.[19, 20] Enzymatic digestion as well as mechanical breakage of the cell wall (with or without heating) have been used to release DNA from *M. leprae* for subsequent PCR.[17–21] Standard techniques for DNA extraction and concentration have been used and found effective.[17, 19, 21] When large numbers of *M. leprae* are present, a few cycles of freezing and thawing are sufficient to release adequate amounts of DNA for PCR.[18] It should be noted, however, that extraction of DNA from proteins and other defined contaminants is preferable, particularly when low numbers of *M. leprae* are present in tissues (personal observation).

Pitfalls. A major concern for laboratories using PCR is the contamination of samples or reagents with DNA from either the test organisms or the amplified product.[38] The presence of minute quantities of DNA from either source can produce false-positives or raise control background levels reducing sensitivity. It has been estimated that 25,000 molecules of PCR product can be found in one aerosol droplet originating from a previous PCR amplification.[4] Accordingly, aseptic techniques suitable for containment of microbial contaminants have been recommended for handling PCR reagents and DNA samples.[4] Most laboratories have found that this level of containment without other special precautions is not sufficient to avoid spurious contamination. For example, laboratory design implementing physical separation of certain procedural aspects of PCR is essential. At a minimum, these should include separate areas for set up, cycling, and product analysis. In addition, workers need to use positive displacement pipettes, or standard micropipettes with aerosol-containment tips, to avoid contaminating the pipetting instrument. Regular soaking of pipetting devices in detergents, followed by 1 N HCl and autoclaving is recommended where appropriate. Finally, negative controls, excluding analyte DNA, must be tested along with unknown samples to monitor contamination of buffers and PCR reagents.

A procedural variation of the standard PCR, called nested PCR, has been suggested as a way to reduce false-positive results due to contamination of samples by the end products of prior amplifications.[2] In the nested PCR approach, two sets of primers are selected for amplification purposes. The

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first pair of primers (outside primers) amplifies a portion of the genome, and the two inside primers (nested primers) direct the amplification of sequences contained with the product produced by the first set of primers. The number of cycles required for each pair of primers is much smaller (15–25 cycles) than that required for high levels of sensitivity using a single set of primers (30–50 cycles), so that background or nonspecific amplification should be reduced.

Another area needing further definition is the quantitative aspects of PCR. Although the final product of a PCR amplification can be quantitated, relating that figure to the absolute number of microorganisms present in a given sample can be difficult. Quantitative PCR tests have been developed and rely on either internal or external controls for standardization. Both approaches suffer from problems inherent with comparing amplification efficiencies between unknown concentrations of sample DNA and known concentrations of control DNA. However, studies analyzing these aspects are being explored.[39, 40]

Confounding standardization of PCR tests designed to quantitate *M. leprae* in infected tissues is the possibility of finding free *M. leprae* DNA in host tissues. It is likely that free DNA is present in infected host tissues as a result of death and decomposition of the bacilli. DNA at varying degrees of disintegration should be present and a portion of these molecules will be capable of providing suitable template DNA for PCR amplification. In fact, we have shown that removal of *M. leprae* from fresh human biopsy homogenates by centrifugation can yield bacilli-free supernatants capable of generating strong PCR signals. This finding begs the question of whether a direct relationship exists between the viability of *M. leprae* in tissue and a positive PCR test result. Well-controlled studies in both experimental animals and leprosy patients, under strict antimicrobial therapy, need to be done to define this relationship and thereby place PCR results in proper perspective.

The cost of PCR could conceivably be a problem for widespread clinical use, particularly in developing countries. At the current price of approximately $3.00–$3.50 per test, PCR will remain a research tool used to answer specific research questions. Transfer of this technology to clinical laboratories would require several technological advancements, including test formats minimizing the potential for contamination, and the use of simple, nonradioactive binding assays. Finally, the applicability of PCR for clinical use must await further testing to establish its usefulness, if any, in diagnosis and patient monitoring.

Future

The development of simple, sensitive PCR tests to detect *M. leprae* using specific DNA sequences is a major development in leprosy research. Immediate applications with the existing technology may include epidemiologic studies to determine the distribution of *M. leprae* in the various segments of endemic populations and the surrounding environment, as well as studies to correlate PCR results with various forms of clinical disease.

Other immediate applications for PCR may include complementing current clinical and histopathologic diagnostic criteria for leprosy, particularly when signs and symptoms are equivocal, such as can be the case in indeterminate leprosy and in suspected cases where no AFB can be detected in tissue samples. Another application may be to study archival, paraffin-embedded samples since samples of this type are suitable for PCR.[41, 42] Preliminary studies however, indicate that buffered formalin is not a good fixative for maintaining DNA integrity for the PCR based on the *M. leprae* 18-

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kDa protein gene (personal observation). Accordingly, studies need to be done to define appropriate fixatives which will allow proper fixation of tissues for histologic analysis and for testing by PCR.

Studies need to be performed to determine the relationship between viability of *M. leprae* and PCR. If PCR results can be shown to reflect the viability status of *M. leprae,* then the potential of PCR for monitoring antileprosy drug therapy and to differentiate reaction from relapse of the disease in paucibacillary patients may be an important advance in disease management. Alternatively, monitoring highly labile bacterial components associated with transcription and translation, for example, mRNA and rRNA, may provide another option for judging the viability of *M. leprae.*

Thus far, *M. leprae*-specific sequences have been characterized for PCR which can be used to differentiate *M. leprae* from other species. Should stable, isolate-specific sequences be characterized, as they have for other human pathogens,[43, 44] PCR tests could be developed and used for taxonomic purposes and possibly for studying disease transmission.

Finally, it is conceivable that sequences related to drug resistance or sensitivity could be characterized and used for developing PCR tests to detect the same.[44] Combining a PCR test for drug resistance with an *M. leprae*-specific PCR test may provide simultaneous analysis of pathogen identification and drug sensitivity testing directly from infected host tissues.

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