Modern Sensitive Techniques for the Detection of *Mycobacterium leprae**

When Shepard reported that the leprosy bacillus could be grown in the foot pads of mice in 1960,[1] it introduced a new era and dimension to research and the understanding of disease processes in leprosy. Recent advances in molecular biology appear to be on the brink of offering highly technical and sensitive implements for the diagnosis of leprosy. It would appear that some 30 years of research and, hopefully, the consequent management of leprosy is on the brink of another quantum leap forward. At the moment extensive applications can be envisaged for research, but applications in the field for disease control would appear to be some way off. The World Health Organization (WHO) has set up trials in some centers with a view to the possibility of adapting some of these interesting high-technology tools for use in the field.[2]

This essay discusses these recent developments together with the implications they hold for the advancement of clinical understanding and treatment of leprosy.

Historical perspective. Since the discovery of the leprosy bacillus[3] by G. H. A. Hansen in 1873 (a remarkable achievement in its day due to the prevalent moral and religious beliefs associated with the disease), scientific knowledge of leprosy has progressed slowly in the main. However several important individual steps have given extra impetus.

Since that time tests for the organism have involved complex and time-consuming histological and microscopical techniques. The complex nature of the infective process and the unpredictable response to the infection by the host have all contributed to the slow progress. The practical aspects of identifying and culturing the leprosy bacillus have also contributed to the slow progress. Two major advances in the past 30 years have maintained momentum in research into leprosy. However, apart from information on the development of resistance to drugs already available, their impact on the management of the disease in the field has been limited.

The mouse foot pad model for *Mycobacterium leprae* infection developed by Shepard was important since it provided the start for laboratory based research into leprosy. It offered a means of assessing the growth of the leprosy bacillus. Growth in the foot pads of mice is an indicator of viability; it is not quantitative on its own. Quantification of viability is determined by the proportional bactericidal test.[4] Although only limited multiplication occurs in the mouse foot pad, it allows evaluation of chemotherapeutic regimens;[5] it also serves as the basic model for testing the efficacy of potential vaccine preparations. The use of nude mice, which are congenitally athymic,[6–9]

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* This review, written in 1991 by Peter R. Ayliffe while a student at St. George's Hospital Medical School, London, shared first prize in the annual competition sponsored by the British Leprosy Relief Association (LEPRA) for essays on various aspects of leprosy. We take pleasure in publishing this review. The author's present address is: Peter R. Ayliffe, 4 Dahomey Road, Streatham, London SW16 6ND, U.K.

The JOURNAL will also publish the other essay sharing LEPRA's 1991 first prize in its December 1992 issue.

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provided an animal model of lepromatous leprosy that considerably expanded the knowledge and defined the importance of cell-mediated immunity (CMI) in host resistance to leprosy, and provided models that more closely mimicked the clinical findings seen in lepromatous leprosy.

Kirchheimer and Storrs in 1971[10] identified the nine-banded armadillo as a susceptible host for the growth of *M. leprae*. The armadillo model provided for the first time large quantities of homogenous bacilli for immunological, immunotherapeutic and immunoprophylactic studies.

Important in research and clinical applications over the years has been the Ridley-Jopling classification.[11] This is an important test in the clinical setting for determining the response to treatment. Over the years it has provided a reliable and reproducible assessment of disease activity based on the Ziehl-Neelsen staining of histological specimens. Specimens are classified by a bacterial index, where the number of acid-fast bacilli (AFB) in the biopsy is assessed on a logarithmic scale of 0 to 6+, and this information helps to place the patient on the scale within the classification. The morphological index relates the number of solid-staining bacilli to the total number of AFB. This is important in assessment of the viability of bacilli in the biopsy, since some organisms may be fragmented or stain unevenly, and this is used in monitoring the efficacy of treatment. Other characteristics, such as type of immune cells present in the biopsy sample and the nature of the skin-test response, are also important in classification.

Obstacles to advances in research and clinical progress. Although the discovery of the bacillus is nearly 120 years old, *M. leprae* remains uncultivatable. Human biopsies have served as the only source of the leprosy bacillus. These crude preparations yielded inadequate numbers of organisms for most studies, and the lack of reliable methods for quantitating viable organisms prevented standardization of different preparations. It seemed that every step forward was associated with a despairing obstacle. Despite the dedication of researchers, these predicaments have contributed to the lack of progress and impeded the necessary advances in the field. The study of the epidemiology of leprosy has been particularly hampered by the lack of sufficiently reliable quick and efficient tests. Knowledge of the disease in this area remains particularly scanty.

It has been known for a long time that people exposed to leprosy do not necessarily catch the disease. The majority of people exposed effectively resist infection with *M. leprae* even in highly endemic areas. It is now thought that as many as 200 individuals become infected with *M. leprae* for each case that is detected or develops overt disease.[12] In those people unable to mount an adequate immune response to infection with *M. leprae*, the incubation period varies; usually in the range of 2 to 4 years but it may be 30 to 40 years.[13–15] The histopathology of the early lesion in leprosy (usually a hypopigmented macule) may be that of nonspecific, chronic dermatitis. The presence of acid-fast bacilli or infiltrate in nerve bundles is diagnostic, but indeterminate leprosy remains diagnostically difficult with a variable course. In approximately 75% of patients the disease heals spontaneously; some cases remain indeterminate for a prolonged period of time, and some progress to one of the established forms of the disease.

Immunochemical research combined with extensive ultrastructural analysis of *M. leprae* cell walls and associated component

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molecules has demonstrated a surface to the bacilli essentially bereft of proteins and polypeptides.[16–18] The major obstacle to the study of *M. leprae* stems from the inability to culture the organism *in vitro,* limiting workers to bacteria grown experimentally in infected mice, rats, or armadillos. These relatively low numbers of organisms consequently yield only small amounts of purified proteins; this severely limits analysis.

The practical clinical immunology of leprosy is at present based almost entirely on reactivity to intradermal skin tests with lepromin, a heat-killed suspension of *M. leprae* originally obtained from homogenized human lepromas[19] now prepared from infected armadillo tissue. The response to lepromin has no real diagnostic value because individual patients' immune responses vary immensely. However, it does indicate the immune capability of any individual to *M. leprae,* and has a prognostic value in clinical practice.[20] Typically, a positive reaction is biphasic. An early reaction[21] at 24 to 48 hours (Fernandez reaction) is a delayed-type hypersensitivity reaction (probably to soluble protein antigens in the preparation) and occurs in tuberculoid leprosy as well as contacts or healthy individuals sensitized to *M. leprae* or who are sensitized to crossreacting antigens from other mycobacteria. The late (Mitsuda) reaction to lepromin is measured at 21 days, and represents the induction of acquired cell-mediated immunity to *M. leprae,* manifested by an epitheloid cell granuloma reaction.[22] Positive Mitsuda reactions are seen in patients with tuberculoid leprosy. Weakly positive cases may help in the diagnosis of borderline disease. In lepromatous leprosy, no response is seen, indicating the absence of cell-mediated immunity to *M. leprae.* Even after many years of chemotherapy, the lepromin test remains negative in lepromatous leprosy.[23]

Need for sensitive tests

Research applications. Prior to the application of monoclonal antibodies and recombinant DNA techniques, immunochemical approaches for the study of proteins in *M. leprae* were tedious and highly complex. Most of the early work was performed by the immunoprecipitation of protein and other antigens with polyclonal antisera in agarose gels by two-dimensional immunoelectrophoresis.[24] Despite sophisticated analysis of these profiles, little has been learned about the chemical nature of the antigens contributing to these various immunoprecipitates.

Diagnostic tools. Established leprosy illustrates a continuous spectrum of disease from localized, self-healing, granulomatous lesions with very few demonstrable bacilli to a widespread, progressive, anergic disease with massive numbers of *M. leprae.*[25] Borderline leprosy encompasses those types of the disease between lepromatous leprosy and tuberculoid leprosy. A patient with borderline leprosy can develop clinical, bacteriological, and histopathological features of more tuberculoid disease with time; this is called upgrading. As in tuberculoid leprosy, the signs and symptoms of borderline leprosy tend to be due to a mixture of bacterial proliferation and the immunological host response. The numbers of AFB in lesions vary from undetectable to 1 AFB to 100 AFB in any oil immersion microscopic field.[26] Sensitive tests to detect infection with

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the lepromatous bacilli will, it is envisaged, help in the prediction of clinical outcome.

Sensitive and specific tests have the potential for early diagnosis, detection of subclinical infection and the monitoring or predicting of the disease outcome of individual patients, and the response to chemotherapy and drug screening.

Monitoring response to treatment. Better tests would provide rapid and reliably sensitive means in multibacillary patients of distinguishing between relapse due to reactivation of residual leprosy bacilli and relapse due to reinfection with a different strain of bacillus.[27, 28] More sensitive tests would also be helpful in defining a cure for leprosy. It is known that many mycobacteria in both leprosy and tuberculosis remain metabolically inactive for considerable periods of time and, as such, are not killed by antibiotics. These inactive bacteria, referred to as persisters, can reactivate many years later. Thus, any test which can detect them will be very useful clinically.

Development of drug resistance. One of the most promising applications of specific tests could be to discriminate between living and nonliving leprosy bacilli in tissue and blood specimens.[29, 30] Potential applications in this area are very exciting and would be useful in determining and monitoring the development of drug resistance (an increasingly worrisome problem in the treatment of leprosy previously only achieved by injecting *M. leprae* from patients into foot pads of mice, feeding the mice the drugs, and following the course of this localized infection). This is a procedure that is tedious and time consuming; and development of significant results might take months. The procedure is costly in materials and resources, so much so that realistically the idea could only be entertained in the research laboratory. Furthermore, an effective clinical test would have tremendous applications for sensitivity testing of *M. leprae* prior to initiating chemotherapeutic measures in the field.

Epidemiology and targeting resources. More sensitive tests have important possibilities in epidemiological studies; specific sensitive tests will find applications in monitoring the course of control measures in endemic areas. More fundamental research can be focused on the immunological mechanisms that contribute to the protective as well as the pathological aspects of host responses to *M. leprae.* Eventually, exploitation of this fundamental knowledge should allow development of an effective vaccine.

A number of research groups have found molecular biological techniques useful for distinguishing between the different variant strains of *M. tuberculosis.* If similar methods could be used to subtype different strains of *M. leprae,* as many people hope and as it seems likely due to the extraordinary sensitivity of these techniques, they could turn out to be very useful tools in epidemiological studies.

New strategies and recent discoveries

Many new advances have been made in the last few years in the study of infectious diseases at the biochemical and molecular biological levels. With the inherent difficulties in studying the leprosy bacillus, much effort has been put into adapting many of these techniques to research into leprosy. Furthermore, it would appear that molecular biology is all set to enter the diagnostic microbiology laboratory.[2] By looking at specific unique components of *M. leprae,* it may be possible to circumvent the problems associated with the growing and studying of the leprosy bacillus.

Immunology. Infection with *M. leprae* is characterized by a broad spectrum of host responses; there is great variability in the histopathology and clinical course of the infection. There is also a spectrum in the patient's humoral and cell-mediated immune response. Although specific reactivity of lymphocytes can routinely be demonstrated *in vitro* in peripheral blood, the reactivity of the cells in the leprosy lesions is probably more relevant to the cell-mediated immune

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response of the host to *M. leprae*. Modlin, *et al.* have investigated the cellular makeup of the various lesions in leprosy, using immunopathological techniques,[31-33] and their results show that the T4/T8 ratio is altered and the relative number of helper-T cells is markedly lower (0.5:1) in lepromatous lesions compared to a normal 2:1 ratio in peripheral blood. More importantly, they showed that the cells are arranged in a distinct architecture within the epithelioid granulomatous lesions. It is difficult to see the development of these recent findings into clinically useful tests, but many of these experimental findings will be useful in assessing the response of patients to various antigenic preparations in the quest for a vaccine that will be effective against *M. leprae*.

Extensive chemical analysis has shown the cell wall of *M. leprae* to be a highly complex, lipid-rich, macromolecular structure.[17] Some cell-wall components are unique to mycobacterial cells, and are found in most of the different species, for instance, peptidoglycan is present in all mycobacteria with only minor variations. The most notable advances in the immunology of leprosy have come from the isolation and characterization of two *M. leprae* antigens—PGL-I and LAM-B.

In the study of *M. leprae* an important cell-wall-associated glycolipid molecule has been of considerable interest—phenolic glycolipid I (PGL-I)—shown to be species specific and immunogenic during infections with *M. leprae*.[34, 35] In a series of experiments, Brennan and co-workers established the chemical structure and the immunological specificity of PGL-I.[36, 37] The general structure of PGL-I can be described as a trisaccharide moiety linked to a phthiocerol lipid core through a phenolic group.

The discovery of PGL-I in armadillo-derived *M. leprae*[36, 38-40] was important since this is immunologically specific to the leprosy bacillus and has subsequently been shown to be located on the surface of the organism by electron microscopic and immunochemical studies. This unique trisaccharide structure accumulates in the tissues of the armadillo in quantities equal to half the total weight of the leprosy bacilli present.[38, 40] The ability to produce purified preparations of antigens specific to *M. leprae* allowed immunologists to use advanced techniques to further the knowledge of the immunology of leprosy.

PGL-I appears to be associated with the outer surface of *M. leprae*[41] and, significantly, has been isolated from both purified bacteria and *M. leprae*-infected tissues in relatively high concentrations.[38] PGL-I may act as a virulence factor in infection. The application of these studies in clinically monitoring the response to chemotherapy has already been demonstrated.[42] The assay

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of whole cell antigens of *M. leprae* in the serodiagnosis of leprosy has always been complicated by the extensive crossreactions among mycobacterial species. Changes in patients' sera may give early indications of change in disease status, and a sensitive test to detect the changing levels and type of antibody response could, therefore, be of value in managing patients. Specific, synthetic antigenic components of PGL-I of *M. leprae* can be used in an enzyme-linked immunosorbent assay of patients' sera; this may extend the knowledge of the immune response to leprosy with exciting implications in the development of immunotherapeutic and immunoprophylactic treatments.[43]

Arabinose- and mannose-containing phosphorylated lipopolysaccharides from *M. leprae* have been isolated and characterized.[44] A detailed study by Hunter, *et al.*[44] established that the serologically active component, lipoarabinomannan B (LAM-B), is acetylated, contains substituents of phosphatidylinositol, and may be membrane associated. LAM-B has proven to be highly immunogenic in most mycobacterial infections, inducing strong humoral antibody responses. Since LAM-B is a common antigen among mycobacteria, it cannot be used effectively for serological tests to detect early infection with *M. leprae.* However, there is evidence to suggest that high levels of anti-LAM-B are associated with high bacterial loads in leprosy.[45] This may prove to be a useful test for estimating bacterial clearance during antimicrobial chemotherapy by monitoring serum antibody levels to LAM-B.

Another helpful advance with significant results was the construction of a genomic library in bacteriophage λgt11, which was designed for regulated expression of *M. leprae* proteins in *Escherichia coli.*[46] So far, five protein antigens of *M. leprae* have been expressed in *E. coli* and analyzed in detail.[46, 47] Cloned segments of *M. leprae* DNA from the λgt11 library have provided DNA for sequencing of two proteins (18 and 65 kDa) from *M. leprae,* both of which have been shown to produce a humoral immune response subsequent to vaccination with *M. leprae.*[48, 49] Similarly, work with T-cell clones in tissue cultures should provide information on epitopes recognized by T cells on these and other proteins, allowing for detailed immunochemical analysis of proteins important in the immunological interactions of *M. leprae* and the host. DNA-derived peptide sequences will provide the reagents needed to analyze the relationships of antigenic peptides and class II major histocompatibility complex (MHC II) molecules on antigen-presenting cells. Such studies will be very helpful in unraveling the molecular aspects to the triggering of reactive T cells in response to *M. leprae,* important in mediating protective cell-mediated immunity.

The use of epitope libraries[50, 51] for the molecular cloning and precise identification

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levels in lepromatous patients. Lepr. Rev. **59** (1988) 127–135.

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of T-cell and B-cell subsets is the latest major advance that is likely to have implications throughout the field of immunology. If and when epitope library studies are used in the study of leprosy, they will provide the facility to look at millions of short peptide sequences that can be surveyed for their tight binding to antibodies, receptors or other binding proteins. The library consists of a vast mixture of filamentous phage clones, each displaying one peptide sequence on the virion surface. Any peptide that shows affinity to the protein being investigated can be purified and propagated using *E. coli,* and then sequenced by looking at the coding region in the viral DNA. It will be possible to investigate the peptide MHC II interaction in leprosy and the pairing of a large number of epitopes with their functional restriction elements and, thus, to study T-cell interactions in great detail.

Molecular biology. Original ideas in science usually have more far-reaching effects than is initially envisaged, often providing new insight on unresolved questions or alternative approaches to circumvent long standing problems in basic and applied research. Such is the outcome of the development of DNA amplification by the polymerase chain reaction (PCR) first described by Mullis in California.[52] His invention was stunningly simple and yet awesome in its potential. Research applications of this technique of DNA amplification using commercially available equipment and reagents have already transformed molecular biology.[53]

The major strength of PCR is the ability of the reaction to produce incredibly large amounts of specific DNA sequences, using two oligonucleotide primers that hybridize to opposite strands and flank the region of interest in the target DNA. A repetitive series of cycles involving template denaturation, primer annealing, and the extension of the annealed primers by DNA polymerase, results in the exponential accumulation of a specific fragment whose termini are defined by the 5′ ends of the primers. Because the products of one cycle can serve as templates in the next, the number of DNA copies of the target approximately doubles at every amplification cycle. Thus 20 cycles of PCR yield about a million-fold (2^{20}) amplification.[54, 55] With appropriate hybridization for the detection of the target DNA sequence, PCR has the theoretical capability of detecting one target sequence in a sample preparation.[56, 57]

The introduction of the thermostable DNA polymerase (*Taq* polymerase) transformed PCR into a simple and robust reaction which can be automated by a thermal cycling device, instead of having to add fresh enzyme at every cycle when using the heat-labile *E. coli* DNA polymerase.[53] The reaction components (template, primers, *Taq* polymerase, deoxyribonucleotides, and buffer) are all easily available, and all can be assembled within the reaction tube and the amplification reaction carried out by simply cycling the temperature. The specificity and yield of the PCR could be increased by changing the reaction parameters, for example primer, Mg^{2+} concentration as well as the temperature cycling profile.

The specificity of the PCR can be analyzed by carrying out gel electrophoresis on the product, evaluating the production of the target fragment, and comparing this to the other products of the reaction.[58, 59] The initial PCR method using the heat-labile en-

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zyme was not very specific; although a specific target fragment could be amplified up to a million-fold, most of what was synthesized was not the target sequence. By cloning a β-globin amplification reaction and screening the individual clones with a β-globin probe to detect the target sequence and, with one of the primers, to detect any amplified sequence, the specificity of the PCR was estimated to be ~1%.[59] The use of the *Taq* polymerase not only simplified the PCR procedure, it significantly increased the specificity and the overall yield. *Taq* polymerase also allows the amplification of much longer fragments, up to 10 Kb (with some reduction in efficiency); whereas using the Klenow fragment of *E. coli* DNA polymerase allows amplification of less than 400 base pairs.[60] The higher optimum temperature of around 75°C for *Taq* polymerase allows for a higher temperature to anneal the primers and extension; this increases the overall stringency of the procedure and minimizes the extension of mismatched primers. This increase in specificity results in an improved yield of the amplified target fragment by reducing the competition for enzymes and primers by nontarget products at each cycle.

The development of a highly specific PCR test is dependent upon the selection of an appropriate target sequence.[54–56, 61] DNA sequence data from which specific PCR tests are based come from recombinant DNA libraries for *M. leprae*[62, 63] and other mycobacteria. Genetic studies have detailed numerous DNA sequences suitable for developing species-specific PCR tests.[64, 65] Three well-defined antigenic proteins have regularly been used so far in PCR. Two of the proteins (18 kDa and 36 kDa) were found exclusively in *M. leprae*,[66–68] while the third protein (65 kDa) has been shown to be a highly conserved heat-shock protein thought to be expressed in all prokaryotes. PCR tests have been developed based on the amplification of species-specific target regions of the genes encoding these proteins. Thus, 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. It is not clear at this point which variation will be most universally applied to PCR.

Sensitivity requirements of PCR tests to detect *M. leprae* designed so far mean that they must surpass detection levels achieved by conventional standard microscopic observation (10^4AFB/ml)[69, 70] and maintain specificity. Ideally, this should be directly from clinical specimens without other manipulations.

Mycobacterial identification tests developed from nucleic-acid hybridization techniques preceded PCR; they have become important tools for the identification of mycobacterial groups.[71–74] These tests are de-

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signed for identification of the organisms when sufficient quantities can be grown *in vitro*, and cannot be used directly for detection from clinical specimens. The Clark-Curtiss group developed a DNA hybridization assay for the identification of *M. leprae* in biological tissues, the theoretical lower limit of sensitivity was 4×10^3 for detecting the bacillus.[75] The DNA probe used in the test contained a repetitive sequence specific to *M. leprae*, enhancing the theoretical sensitivity. However, tests showed the practical lower limit of sensitivity for detecting *M. leprae* in the skin of multibacillary patients was 1×10^5.

PCR tests with or without the use of DNA hybridization for quality control are challenging direct DNA hybridization assays for the detection of mycobacteria producing similar levels of specificity with claims of increased levels of sensitivity. No study using PCR to date has reported a sensitivity level worse that 100 organisms, and claims have been made for the technique being able to detect a single *M. leprae* bacillus. Most reports show lower levels of detection in the presence of prokaryotic[60] or eukaryotic[59, 60, 76] DNA as well as normal, uninfected human skin biopsies.[59] Even if the detection limit of PCR proved to be a thousand times poorer in clinical use compared to controlled research laboratory conditions, it would still be far more sensitive than any other available test. The utility of these assays for improving current diagnostic criteria for leprosy remains undefined.

Conclusions

Problems: criteria for tests to be useful. Many problems have been unearthed by the rapid advances in scientific knowledge of the leprosy bacillus in the last few years, and it seems that for every advance there is still a new obstacle around the corner. At the moment, the full capabilities of most of these techniques have only been briefly touched on, and advances in this area are self fueling in their possible implications. However specific problems exist, so much so with immunological tests that it is difficult to see them as being of much use outside the research laboratory. For a new test to be of any use in the clinical setting, it must be better or cheaper than methods currently available. Apart from very specific and limited applications, the diagnostic value of serological tests for leprosy would appear to be limited. Leprosy diagnosis is usually not difficult to establish in most cases by the traditional methods of clinical examination together with skin smears and histopathology when needed. Indeed, in most cases, the histological examination of lesions using the microscope is very sensitive, more so than many of the immunological tests so far offered. At the moment, sensitivity levels of immunological tests may be appropriate for research purposes when experimental infections are studied. If one organism can be detected per microliter of test sample, when this is applied to the clinical situation a 70 kg patient would have to be infected by 70×10^6 organisms, assuming erroneously that the bacteria were uniformly spread throughout the body before it could be detected (this compares with infection by about 10^{13} bacteria in the far-advanced lepromatous patient). However, currently available tests using traditional techniques do not give information on the viability of the organism since dead bacteria will be stained positive together with live organisms. Although this is not a problem in the diagnosis, it is a problem in the management of patients infected with *M. leprae* resistant to drugs, for example.

The varied immune responses of patients infected with *M. leprae* severely limit the value of immunological tests. In the study

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be Douglas, *et al.*[42] there was no correlation between quantitative changes in antibody levels measured by immunodiffusion with values obtained by ELISA. In individual patients periods of abnormally high levels of one class of antibody could be associated with normal values for others; this varied with time and unfortunately did not necessarily correlate with antileprosy chemotherapy. Touw, *et al.*[77] also reported elevated levels of immunoglobulins with no correlation with bacillary load or other clinical parameters in patients they studied.

The ELISA for PGL-I has a sensitivity of only about 30% for paucibacillary leprosy, which does not make it a useful field test for the early stages of paucibacillary leprosy. Even if the specificity were 98%, and even in a population with a theoretical prevalence rate of 1% (ten times higher than most endemic countries), for every thousand tests carried out PGL-I serology would detect only 3 of the 10 patients who actually has leprosy if the test was used "blind" with no additional clinical information. At the same time, the test would "identify" 20 people without leprosy as being infected with *M. leprae.*

Antigenic analysis of proteins is only the first step in investigating the complex immune interactions subsequent to the infection of humans with *M. leprae.* Although immunogenic proteins might provide suitable components for the development of vaccines against leprosy, their relevance to the immune response remains unanswered. Detailed studies are needed to define the B- and T-cell stimulatory capacity of these molecules. This will require large amounts of purified proteins which are not obtainable by extraction from native purified bacilli.

The extraction of DNA from *M. leprae* is notoriously difficult since the cell is refractory to most cell lysis techniques. It has been estimated that only 20% of the genomic capability of a microorganism is represented when applying detailed phenotypic analyses. Thus, major areas of the genome, possibly encoding important functional or regulatory genes not demonstrable as quantifiable phenotypic characteristics, may go undetected. Furthermore, when extracting DNA from *M. leprae* the number of DNA containing, and thus potentially viable, organisms represents only a small fraction of the total sample.[78]

The major problem with PCR at the moment is the occurrence of false-positive reactions due to contamination of samples or reagents with DNA. This is not usually due to biological contamination with test organisms, but is often due to the phenomenon of product carry over due to minute quantities of DNA created in previous stages contaminating the reaction mix, and they are amplified by the PCR to give a false-positive result or raise control background levels, reducing sensitivity. To avoid the problems of contamination, strict aseptic techniques must be used in handling PCR reagents and DNA samples. A laboratory design implementing physical separation of certain procedural aspects of PCR is essential. Apart from the use of strict asepsis, specialized equipment and regular cleaning in detergents and hydrochloric acid are necessary. Negative controls, excluding analyte DNA, must be tested alongside the unknown sample being targeted to monitor contamination of buffers and PCR reagents.

Despite contamination being a problem in some laboratories, this technique is still in its early days. There is already a list of relatively simple measures to reduce the risk of false-positives, with new ideas being proposed all the time.[29, 61, 62, 79, 80] A procedural variation of the standard PCR, called nested PCR, has been suggested as a way to reduce false-positive results produced by the contamination of samples by the products of previous amplifications.[81] In the nested PCR

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technique, two sets of primers are used for amplification. The first pair of primers (outside primers) amplifies a portion of the genome, and the nested primers (inside primers) direct the amplification of sequences containing the product. 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.[84]

Quantifying the results of PCR is very difficult at the moment. Although the final product of a PCR amplification can be assessed quantitatively, relating this 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. It is difficult to compare amplification between unknown concentrations of sample DNA and known concentrations of control DNA.[82–84] An error can also be introduced in reading the results, since it is possible to get signals on the gel electrophoresis from segments of DNA that are of the same length as the target. This can be prevented by using specific radiolabeled DNA hybridization probes, a problem in the study of other mycobacteria. In reviewing the literature many studies using PCR do not use any methods of internal control, such as this use of specific probes.

It is likely that free DNA from *M. leprae* is present in infected tissues, and this is likely to confound standardization of PCR tests. Portions of DNA at varying degrees of disintegration will be capable of providing suitable template DNA for PCR. It has been shown[84] that the removal of *M. leprae* from fresh, human biopsy homogenates by centrifugation can yield bacilli-free supernatants capable of generating strong PCR signals. This is further evidence that PCR may not be a useful test for assessing the viability of *M. leprae,* and the development of mRNA PCR is likely to be the solution to this problem.

The transfer of PCR technology from the research laboratory to the clinical diagnostic laboratory is still a long way off. The cost of using PCR is likely to fall progressively. Before the time arrives that it could be considered economically viable for use in the field, several technical obstacles will have to be overcome, including test formats, reducing contamination and the use of simple nonradioactive binding assays. Also, the cost is quite likely to fall in the future as the patent runs out and open commercial competition starts. At the moment the patent is held by one company, Perkin-Elmer-Cetus. Already a PCR kit is in use in the U.S.A., marketed by IDEXX for the detection of *M. paratuberculosis.*[85] It is used to diagnose Johne's disease in cattle by the disease reference laboratory.

Future possibilities: research toys or clinical tools? With the emergence of ever-increasing numbers of dapsone-resistant *M. leprae* infections, the importance of monitoring chemotherapy has been established. Chemotherapy has been further compromised by poor compliance, especially by light-skinned patients treated with clofazimine, the necessity of daily treatment, and by toxic side effects. Newer drugs, such as rifampin, may alleviate some of these problems, but the need for careful monitoring is still very important. A sensitive assay which can detect quantitatively and specifically active, viable *M. leprae* could reflect the bacterial load and, hence, the clinical progression of the disease.

Studies have been carried out using changes in patients' sera[42, 77] using enzyme-linked immunosorbent assays of immunoglobulin levels in response to specific antigens (especially PGL-I) and whole cell antigens of *M. leprae* in patients treated with

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various antimycobacterial agents. Many of these studies showed potential in the application of ELISA techniques to the monitoring of individual patient's antibody responses during the course of treatment. They may prove to be useful in measuring the effectiveness of chemotherapy. This certainly is a tremendous advance on previous methods of analyzing material for viability studies that involved the inoculation of a considerable number of mice and following them up for a least a year, as in the proportional bactericidal test of Colston, *et al.*[4]

A rapid and sensitive technique for assessing growth and metabolism has been described by Franzblau,[86] but it does not seem to have been widely used. This test (BACTEC 460 test) would appear to be particularly suitable for assessing the susceptibility of *M. leprae* to antimicrobial drugs *in vitro* and can give results in 1 week. The system uses a radiorespirometric method of assessing cell metabolism. Nude-mouse-derived *M. leprae* are grown in a ^{14}C palmitic acid labeled medium and the production of $^{14}CO_2$ is measured by using a Buddemeyer-type detection device. The measurement of CO_2 gives the test quantitative capabilities and Franzblau suggests that since activity can be readily detected with 10^6 bacilli, the test has potential for use in clinical susceptibility testing. PCR techniques will probably eventually make this area of study even more simple and speedier. Immunological tests are fraught with problems and, as yet, are very disappointing since the assay of PGL-I is highly specific but unfortunately its sensitivity is very poor at the moment.

The development of simple, sensitive PCR tests to detect *M. leprae* using specific DNA sequences as primers is a major development in leprosy research. Immediate applications with the existing technology may include epidemiological studies to determine the distribution of *M. leprae* in the various subpopulations in endemic areas and within the surrounding environment.[84] PCR could give useful information on the transmission of the disease and the nature of infective reservoirs. Studies are needed in order to establish the accuracy and relevance of information obtained by new molecular biological techniques correlated to clinical disease.

Other immediate applications for PCR may include complementing current clinical and histopathological diagnostic criteria for leprosy if useful information from PCR can be obtained on the relationship of targeted DNA sequences in PCR to the current classification of leprosy, as proposed by Ridley and Jopling, and currently used for clinical and research purposes.[11] This will be particularly useful when signs and symptoms are equivocal, such as often is the case in indeterminate leprosy and in suspected cases where no AFB can be detected in tissue samples. PCR could also be used in the archival analysis of paraffin-embedded samples, since samples of this type may be suitable for PCR[87, 88] but unsuitable for microbiological assessment by conventional means. Preliminary studies indicate that not all fixatives are good for maintaining DNA integrity for PCR based on the *M. leprae* 18-kDa protein gene.[84] Studies still remain to be performed to define the appropriate fixatives for clinical tissues so that both histological analysis and testing by PCR can be performed. A large multicenter study using the PCR based on the selective amplification of a 530-bp fragment of the gene, encoding the proline-rich antigen of *M. leprae,* was applied on sections of variously fixed or frozen biopsy samples from patients with leprosy resident in Malawi, Pakistan, Thailand, Belgium and The Netherlands. A simple procedure for the extraction of DNA from *M. leprae* in clinical specimens which provided a suitable template DNA for amplification was developed. Previously, DNA extraction involved purification and an elaborate enzymatic treatment in many stages. The simplified method uses the ex-

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[88] Lai-Goldman, M., Lai, E. and Grody, W. W. Detection of human immunodeficiency virus (HIV) infection in formalin-fixed, paraffin-embedded tissues by DNA amplification. Nucl. Acids Res. **16** (1988) 8191.

traction of DNA from clinical samples with the enzyme proteinase *K* and the detergent Tween without any further DNA purification. This simplified technique showed no loss in amplification efficacy with subsequent PCR using heat-stable *Taq* polymerase for the specific detection of *M. leprae*.[89] Simpler techniques may prove to be equally as effective: boiling has been successfully used to extract DNA from other mycobacteria in clinical samples.

Further, if PCR is to be a useful tool in the management of leprosy correct protocols for the handling of tissues in the field will need to be established. No doubt there will be a rush of clinical testing kits on the market in the not too distant future. But before these techniques can be usefully applied to the field, preliminary tests on clinical material and cost effective analysis of their value as leprosy control tools must be established.

Studies need to be performed to determine the relationship between the 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 the management of the disease. The monitoring of more highly labile bacterial components may fulfill this role better. As has already been mentioned, DNA is a very stable and resistant molecule and despite even fixation with Formalin can be detected by PCR. It is not likely to be a good indicator of viability since it obviously is present in dead bacilli. The monitoring of highly labile components of bacterial activity will probably be far more sensitive in establishing the viability of *M. leprae* in clinical biopsy specimens and, thus, more reliable in assessing the response of patients to treatment. Many workers are looking into the possibility of using components associated with transcription and translation, for example, mRNA. This is particularly appropriate since the half-life of mRNA is ten minutes at the most in the prokaryotic cell. In using mRNA as the target for analysis, a complementary strand of DNA is made by using reverse transcriptase before amplification using the PCR.

One of the special features that PCR should bring to leprosy research is the level of accuracy in diagnosis. Since *M. leprae*-specific sequences of DNA have been characterized, these can be used to differentiate *M. leprae* from other species. Tests looking for AFB in biopsy specimens as used previously in the clinical diagnosis of difficult or ambiguous cases had no specificity. Should stable isolates containing specific DNA or RNA sequences be characterized, as they have been for other human pathogens,[90, 91] PCR tests could be developed and used for taxonomic purposes with realistic possibilities in the study of disease transmission.

One of the biggest claims made for the future of PCR is that it could be used in detecting drug resistance. It is conceivable that sequences related to drug resistance or sensitivity could be characterized and used for developing PCR tests for use in clinically monitoring resistance in individual patients. For PCR to be used for this requires identification and sequencing of drug-resistant genes and, also, that these genes have specific sequences suitable for primers. For instance, it is known that a point mutation in the gyrase gene gives resistance to the quinolones, but this is probably not the only mechanism since changes in the cell membrane, for instance, could confer drug resistance. With such a small percentage of the bacterial genome being expressed, it may be some time before the appropriate target sequences for PCR can be found.

Already immunological tests have been applied to this problem but PCR should offer the potential for greater sensitivity.[92]

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[89] deWit, M. Y., Faber, W. R., Kreig, W. R. Application of a polymerase chain reaction for the detection of *Mycobacterium leprae* in skin tissues. J. Clin. Microbiol. **29** (1991) 906–910.

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Combining a PCR test for drug resistance with an *M. leprae*-specific test may provide simultaneous analysis of pathogen identification and drug sensitivity testing directly from infected host tissues. New approaches are needed for studying larger areas of all bacterial genomes and particularly noncultivable bacteria, such as *M. leprae*. Analysis of DNA restriction fragments after restriction endonuclease digestion of genomic DNA and the related techniques of fragment length polymorphism analysis hold promise. As a more complete understanding of the *M. leprae* genome and its related functional capacity develops, it is anticipated that most areas of study related to leprosy will be enriched and, thereby, advance the understanding of *M. leprae,* its pathogenicity, and the response of the human to its infection.

It is difficult to say with any certainty at all that any of the sensitive techniques reviewed in this essay will become part of the routine clinical management of leprosy, but they certainly seem to address many of the problems with the study of the disease. Indeed, modern techniques are very sensitive, very useful, and are beginning to be used more widely as research tools. Serological tests based on the detection of the *M. leprae*-specific antigen PGL-I have been in use for some time. A Japanese firm has even developed a kit to detect this antibody. They believe that PGL-I serology could clinch the diagnosis in suspected leprosy, detect subclinical infection with *M. leprae,* and possibly indicate the disease outcome in individuals.

Things are not so simple in the field, and expensive serological tests will probably be of limited use. In the vast majority of leprosy cases, is not difficult to diagnose by the traditional clinical examination, with or without skin smears and histopathology. Serology for leprosy therefore has a limited diagnostic value; furthermore PGL-I serology has a low level of sensitivity of around 30% (i.e., 70% false negatives) for paucibacillary leprosy.

It can be argued that the treatment of leprosy is no longer a scientific problem but a logistical one. Antibacterial chemotherapy for leprosy, in general, has been both available and adequate since the early 1940s.[93] The problems in disease control have come from inadequacies in health care delivery systems due to the inherent mountainous obstacles with the disease being endemic in some of the world's most geographically remote and economically poor nations. It is also a feature of the disease that it carries a huge social stigma which results in poor utilization of health care resources that are provided, since patients do not want to be labeled with a condition unacceptable to their culture.

After all, leprosy is treatable. The development of resistance to drugs, especially dapsone, is a worrying problem, but modern sensitive tests will probably play a very important role in the field for the management of this. They are already proving invaluable in the research laboratory, but if they are to be of any use at all in the diagnostic microbiological laboratory they must be easy and cheap to use. Therefore, they must be relevant to the areas in which leprosy is endemic. To steal a phrase from the marketing world, they must be "user friendly." The most likely users need sensitive tests that are cheap, easy to use, and simple for screening vast numbers of patients.

—Peter R. Ayliffe

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romatous leprosy (BL-LL) patients during their first two to four years of DDS treatment. Int. J. Lepr. **50** (1985) 271–281.

[93] Faget, G. H., Pogge, R. C., Johansen, F. A., Dinan, J. F., Prejean, B. M. and Eccles, C. G. The Promin treatment of leprosy: a progress report. Public Health Rep. **58** (1943) 1729–1741.