Chapter 5

Modern diagnostic techniques

Dr Paul Johnson, Dr Tim Stinear, Prof. Françoise Portaels, Mr Karim Chamal, Dr Karen Dobos & Dr Harold King

Buruli ulcer is often diagnosed late, when treatment can be very difficult and frustrating. Confirmation by culture takes 6–8 weeks. Rapid diagnostic methods for *M. ulcerans* infection (i.e. skin and serological tests), as well as methods of rapid identification of the organism in clinical and environmental specimens would be a significant advance in the management of *M. ulcerans* infection. Screening to detect early infection could guide early intervention; at present, only early excision of the nodule is possible.

Polymerase chain reaction (PCR) in the diagnosis of *M. ulcerans* infection

All free-living organisms contain DNA molecules that encode the genetic information necessary for the structure, function and replication of that species. DNA usually exists as paired, very long, complementary molecules (double-stranded DNA) made of combinations of four different units or "bases" (A,G,C,T). The order of bases along the molecule encodes the genetic information. When DNA needs to be copied to allow a cell to replicate, double-stranded DNA is first made single-stranded by cellular enzymes. The single strand then acts as a blueprint or template. Because the bases A and T always pair with each other and not with G or C (and vice versa), the new or complementary strand is a perfect copy of the original, but in the reverse direction. When the two strands are again separated and the complementary strand is itself copied, the resultant molecule will contain the same information in the same direction as the original.

PCR is a method that artificially amplifies minute quantities of DNA to levels that can be easily detected in the laboratory. Most importantly, the sequence of the DNA that is amplified is determined by the sequence of the PCR "primers" – short sections of DNA that initiate PCR amplification. Since the primer sequences can be chosen, one can specify exactly which sequence of DNA will be amplified.

PCR has two main advantages over traditional methods that identify microorgansims by culture. Firstly, for slow-growing organisms such as mycobacteria, an accurate etiological diagnosis can be made in hours instead of weeks. Secondly, PCR may be very helpful when bacteria or viruses are not recoverable by culture, as in the case of leprosy.

PCR requires a test-tube, a supply of primer-pairs, an enzyme that is able to copy single-stranded DNA (e.g. *Taq* polymerase) and a supply of free bases that can be used to create the complementary strand (all commercially available). Purified DNA

(e.g. from a swab or tissue biopsy) is then added, and the mixture is "melted" by heating in a machine called a thermal cycler (heat makes double-stranded DNA become single-stranded). Once the primers have attached, *Taq* polymerase can begin to copy the rest of the template strand. The second primer is designed to attach at a different site on the template DNA strand; it initiates the creation of a new strand but in the opposite direction. Exponential amplification is achieved in the thermal cycler, by sequentially heating and cooling the PCR reaction mixture. Heating causes double-stranded DNA to melt and become single-stranded. Cooling allows new primers to bind to the single-stranded template DNA, and *Taq* polymerase can then generate new strands starting at the point where the primers attach (extension). Each cycle of extension and denaturation leads to a doubling of the amount of template DNA. As extension occurs from the two primers in opposite directions, and heating and cooling is repeated many times, the net result is the exponential amplification of the DNA sequence that lies between the two primer sequences. At the end of the reaction, a section of template DNA will have been amplified millions of times and can be readily detected by size using gel electrophoresis (PCR-positive). If DNA of the correct sequence was not present in the original sample, no amplification occurs (PCR-negative).

There are several PCR methods available that could increase the speed of diagnosis of *M. ulcerans* infection (*43*,*45*,*62*). PCR is relatively expensive, however, and is notorious for producing false-positive results in laboratories that lack experience with PCR. In high-prevalence regions such as West Africa, PCR may not be any more rapid than an accurate clinical case definition combined with a smear that shows acid-fast bacilli. In countries such as Australia, where the incidence is low, the great majority of patients who have nodules, papules or skin ulcers do not have *M. ulcerans* infection. In this situation, PCR is a quicker way of making the diagnosis with a high degree of confidence. The main advantage of PCR is that *M. ulcerans* infection can be diagnosed within 24 hours. Culture confirmation takes 6 weeks or more. PCR usefulness for mycobacterial infections is generally limited, however, and at present it is recommended that PCR is used as a rapid ancillary test, not as a replacement for culture and histology.

The PCR method recently developed in Australia targets a newly described DNA insertion sequence in *M. ulcerans*. When genomic *M. ulcerans* DNA is digested with the restriction enzyme *Alu*I, many 1109 base-pair fragments are obtained. These *Alu*I fragments have now been shown to be part of a larger 1293 base-pair repeated sequence that, by chance, happened to contain two *Alu*I restriction sites. This insertion sequence is repeated at least 50 times per genome. It has been identified in all Australian and African isolates of *M. ulcerans* tested to date and has not been found in at least 45 other mycobacterial species, including *M. marinum*, *M. leprae* and *M. tuberculosis*. The sequence has been named IS*2404* (Genbank accession number AF003002) (*62*,*63*).

The diagnostic PCR protocol consists of 3 phases (*62*):

1. heat and alkaline lysis (to release DNA from *M. ulcerans* cells);
2. extraction of total DNA from sample; and
3. PCR reaction to detect *M. ulcerans*-specific DNA in extracted total DNA (primers slightly modified from Ross et al.) (*42*).
   Primer 1: 5´-gat caa gcg ttc acg agt ga-3´
   Primer 2: 5´-ggc agt tac ttc act gca ca-3´

Suitable clinical specimens for PCR include material obtained with a dry swab and fresh tissue. Swabs are rubbed carefully but firmly around the undermined edge of the ulcer and the material obtained is washed off the swab by vortexing the tip of the swab in a small volume of distilled water. Fresh tissue specimens are diced with a sterile blade in a sterile dish and then resuspended in distilled water. Great care must be taken to keep the sample preparation, PCR master-mix preparation and agarose gel areas of the laboratory separate in order to prevent cross-contamination. It is advisable to include multiple negative controls on every PCR run. All results must be discarded if any negative control is positive. To control for inhibition, each PCR is performed in duplicate. The second tube is "spiked" with approximately 100 molecules of purified *M. ulcerans* DNA. If this spiked positive control tests negative, the PCR reaction is being inhibited. Inhibition in clinical specimens can often be overcome by repeating the PCR using a 1:10 dilution of the extracted DNA sample.

In the past, all presumed positive PCR results were checked by Southern blot, using an internal probe based on IS*2404*. However, a PCR product of the correct size that did not hybridize with the probe was rarely identified. Current practice is to rely on comparison of the size of the PCR product from unknown samples, with the size of the product obtained with the positive control. If the two PCR products (positive control and unknown sample) align precisely, and the negative controls are negative, it may be concluded that the unknown sample is positive for *M. ulcerans*. It is recommended that new laboratories use Southern blotting or an equivalent method of verification to establish that the PCR product is the correct sequence.

To date, it has been established that PCR has a specificity of 100% and a sensitivity of 96% compared with culture. As with all microbiological methods, sensitivity depends heavily on the quality and representativeness of the specimen that is received by the laboratory.

Recent studies have shown that swabs taken from a patient with a strongly smear-positive *M. ulcerans* infection remained positive by PCR for up to 3 weeks when the swabs were stored dry in plastic containers out of direct sunlight at room temperature (approximately 22 °C). It is recommended, however, that samples be processed within 48 hours of their arrival in the laboratory. Some samples may contain very few organisms, and a degree of reduction in organism numbers over time is to be expected.

PCR for detecting *M. ulcerans* in environmental samples

There is strong epidemiological evidence that *M. ulcerans* is an environmental mycobacterium, although it has never been successfully cultured from any environmental site. As PCR is not inhibited by the presence of culturable organisms, it has the potential to overcome this problem. Unfortunately, PCR is exquisitely sensitive to inhibition by many compounds such as humic and fulvic acids, which are ubiquitous in the environment and are not removed by standard DNA extraction protocols. The first confirmation that *M. ulcerans* was present in environmental water samples was obtained in 1997 (*42*), by combining the highly sensitive and specific IS*2404* PCR with a method that separated sample DNA from naturally occurring inhibitors of PCR.

Three different strategies have now been used to overcome inhibition in environmental samples from *M. ulcerans* endemic regions. The first of these is gel chromatography. Environmental water samples are concentrated and subjected to homogenization with glass beads, followed by heat and alkaline lysis to release DNA. Total extracted DNA is then run through gel chromatography columns that separate DNA from contaminants on the basis of size (*62*). Although relatively simple, the method is cumbersome and time-consuming. The second method uses paramagnetic beads linked to *M. ulcerans* antibodies to capture whole cells and separate them from contaminants in a magnetic field (immunomagnetic separation) (*43*). Antibodies are raised in laboratory animals. Captured cells are washed to remove inhibitors and then DNA is released by standard methods prior to PCR. The third approach also uses paramagnetic beads, but here the beads are linked to *M. ulcerans*-specific oligonucleotide probes, which capture IS*2404* DNA that has been released from *M. ulcerans* by homogenization and alkaline lysis. The immobilized DNA is washed to remove inhibitors and used directly as a template for IS*2404* PCR. The latter two methods each have limitations and advantages, but offer superior detection sensitivity and are less time-consuming than gel chromatography.

An important consideration with any of these methods is quality assurance. The high sensitivity of the IS*2404* PCR means that inadvertent contamination may lead to false-positive results. It is essential that appropriate quality assurance measures are implemented if these types of assay are being attempted. Safeguards include physical barriers in the laboratory, such as separate work areas for each stage of the assay, and frequent use of negative controls within a batch so that contamination can be rapidly detected. It is important that negative controls are used all the way through the extraction and PCR process. Even experienced workers have intermittent problems with contamination.

DNA fingerprinting techniques for M. ulcerans

Molecular typing methods may be categorized into three broad groups on the basis of the type of macromolecules targeted for sub-typing, i.e. methods based on fatty acids, proteins and nucleic acids. Actually, the genotypic typing methods (DNA fingerprinting) that evaluate differences at the DNA level are used more commonly and have emerged as revolutionary tools for epidemiological studies.

The use of DNA fingerprinting for the identification of *M. tuberculosis* has greatly improved understanding of the epidemiology of tuberculosis: transmission routes of different strains have been recognized (*64*); outbreaks of multidrug-resistant strains have been detected early; and the relative importance of reinfection versus reactivation can now be elucidated (*65*).

Various molecular methods for fingerprinting of *M. ulcerans* are now being developed to facilitate studies on the epidemiology of Buruli ulcer.

DNA sequencing

Direct comparison of genomic DNA sequences of bacterial strains is the best means of quantitatively determining whether two strains are similar or different. Portaels et al. have analysed the 3´-terminal region of the 16S rRNA gene sequence of 17 strains of *M. ulcerans* from Africa, Australia and America (*57*). This analysis has revealed three subgroups that vary according to the continent of origin. More recently, a fourth subgroup

was discovered in China and Japan confirming the existence of an Asian type (Faber WR et al., unpublished data, 1999).

Restriction fragments length polymorphism (RFLP)

RFLP based on insertion sequences

Insertion sequences (IS) are mobile genetic elements that are usually present in numerous copies within a bacterial genome. These elements can be used as probes, and because the number and location of IS elements vary, each strain will have a unique banding pattern. Recently, molecular analysis of *M. ulcerans* has revealed two new insertion sequences: IS*2404* and IS*2606* (*63*). Southern blot analysis to detect IS*2404* and IS*2606* shows inconclusive RFLP patterns between different strains. Due to the high number of copies of both elements, the banding patterns are difficult to interpret, limiting the value of the Southern blot method to type *M .ulcerans* isolates (*63*).

RFLP based on pTBN12 plasmid.

Jackson et al. have used pTBN12, a well defined plasmid, as a probe with *Alu*I restriction fragments (*66*). The probe was able to distinguish 11 RFLP patterns.

PCR typing methods

PCR is another molecular method that has become increasingly important for epidemiological studies. The technique detects and amplifies small amounts of DNA; 10–100 copies of the templates are enough to perform DNA amplification. Thus, PCR can be used to type organisms that grow slowly on laboratory media, such as *M. tuberculosis* (*67*). PCR also can be used to detect and type pathogens in patients whose cultures are negative because they have been treated. Moreover, PCR can be used to amplify the DNA from organisms that are present in tissues preserved in formalin (*68*).

PCR of repetitive chromosomal elements (Rep-PCR)

Rep-PCR is a modification of the PCR technique that is more suitable for epidemiological purposes than conventional PCR. In this case, the primers are directed towards repetitive chromosomal elements such as IS*6110* in *M. tuberculosis* and the ERIC sequence in other bacteria (*66*). In *M. ulcerans*, the genomic sequence between the IS*2404* elements has been amplified. The profiles produced by this technique categorized the strains into three subgroups related to the three different endemic regions (Africa, Australia and North America).

Ribotyping

This method involves amplification of a known sequence cut by restriction enzymes, and compares restriction fragments of amplified DNA from different strains. Using this technique, the *M. ulcerans* genome has been found to produce three different restriction profiles related to the origin of the strains.

Pulsed field gel electrophoresis (PFGE)

PFGE permits the generation of simplified chromosomal restriction fragment patterns without having to resort to probe hybridization methods. In this method, restriction enzymes that cut DNA infrequently are used to generate large fragments of chromosomal DNA, which are then separated by special electrophoretic procedures. Preliminary results obtained in collaboration with Dr M. Picardeau of the Pasteur Institute, Paris showed that *M. ulcerans* genomes produce three different profiles according to the three geographical origins of the strains (Type I: Africa, Type II: Australia and Type III: North America).

Amplified fragment length polymorphism (AFLP)

The AFLP technique is based on the selective PCR amplification of restriction fragments from a total digest of genomic DNA (*69*). This technique involves three steps: restriction of DNA and ligation of oligonucleotides and adaptors; selective amplification of sets of restriction fragments; and gel analysis of the amplification fragments. Typically 50–100 restriction fragments are amplified and detected on denaturing polyacrylamide gel. Preliminary results using the radioactive method have shown the presence of two clones related to two different regions of Benin. An alternative method using non-radiolabelled components is currently being developed in collaboration with Professor J. Swings at the University of Ghent, Belgium.

Conclusion and perspectives

To be of use as an epidemiological tool, a typing system must give an unambiguous result for each isolate (typeability), give the same result each time the same isolate is tested (reproducibility) and differentiate epidemiologically unrelated strains (discriminatory power). A comparison of the different methods used in the molecular epidemiological studies of *M. ulcerans* is summarized in Table 2.

In conclusion, given the promising results obtained with AFLP (excellent typeability and reproducibility as well as good discriminatory power) efforts should be concentrated on concentrate on the AFLP techniques to apply it to strains isolated from the same patient at different periods of time, to strains from patients belonging to the same family, to strains originating from different villages and departments, and to strains from patients with different clinical forms of the disease.

Table 2. Fingerprinting techniques used for *M. ulcerans*

Genotypic method	Typeability	Reproducibility	Discriminatory power
RFLP	Excellent	Unknown	Good: with pTBN12; non-interpretable profiles with IS*2404* and IS*2606*
DNA sequencing	Excellent	Excellent	Four profiles related to the four geographical regions (Africa, Asia, Australia, North America)
Ribotyping	Excellent	Excellent	Limited to three profiles (Africa, Australia, North America)
PFGE	Excellent	Excellent	Same as ribotyping
Rep-PCR	Excellent	Unknown	Same as ribotyping
AFLP	Excellent	Excellent	Good differentiation between strains from the same region

Candidate antigens for the serodiagnosis of Buruli ulcer disease

Very little information is known about the host immune response to *M. ulcerans* during infection. Nonetheless, several observations relevant to both humoral and cell-mediated immunity have been reported. Convalescent patients rarely become reinfected with *M. ulcerans*, suggesting that there is a protective immune response from prior disease (*70*). In some cases, a delayed hypersensitivity response has been observed on subcutaneous injection with either *M. ulcerans* or *M. tuberculosis* purified protein derivative, indicating that a cell-mediated immune response can persist during and after infection (*7,71*).

In an attempt to more fully characterize the humoral immune response to *M. ulcerans* infection, Dobos et al. tested 62 serum samples from a well characterized case series of Buruli ulcer patients from West Africa for antibodies to *M. ulcerans* culture filtrate (CF) (*7*). For this study, CF was prepared in a serum- and protein-free medium, allowing direct analysis of the constituents actively secreted by *M. ulcerans*. Buruli ulcer patients with active disease were found to produce an antibody response to several different *M. ulcerans* antigens (Fig. 3 A, B). In contrast, serum sample from people

without Buruli ulcer residing in the endemic area produced little to no antibody in response to the CF antigens (Fig. 3 C). Interestingly, three proteins, with apparent molecular masses of 70, 28/26 and 5 kA, were identified that demonstrated strong positive antibody responses in a large number of serum samples from Buruli ulcer patients; these proteins lacked antibody reactivity in control samples. Serum samples from tuberculosis patients from Atlanta, Georgia, USA (where no Buruli ulcer has been reported) demonstrated very low cross-reactivity when tested against the *M. ulcerans* CF, with the exception of a few samples from patients with an antibody response to a common mycobacterial antigen (Fig 3 C). This antigen was found to be the super oxide dismutase (SOD) of *M. ulcerans*, and has been shown to share homology with the characterized *M. tuberculosis* SOD (*43*). In contrast, samples from Buruli ulcer patients did not react specifically against *M. tuberculosis* CF proteins, suggesting that this response could be diagnostic for *M. ulcerans* infection in areas where tuberculosis is endemic.

These studies suggest that patients with Buruli ulcer generate a humoral immune response specific to *M. ulcerans*. Further studies are now being conducted to examine a broad spectrum of patient sera for antibodies to *M. ulcerans* CF and other subcellular components of *M. ulcerans* to identify specific serodiagnostic antigens. These antigens will then be tested in an experimental ELISA to assess the sensitivity and specificity of this assay using serum samples from Buruli ulcer patients from endemic areas.

Figure 3. Preliminary serological work for the diagnosis of Buruli ulcer.