

# 5

## Analytical methods for the detection of waterborne and environmental pathogenic mycobacteria

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### 5.1 INTRODUCTION

This chapter reviews the state-of-the-art methods for the detection of pathogenic mycobacteria in the environment. It provides a concise review of the literature surrounding this aspect of mycobacteriology.

Some unusual features define the genus *Mycobacterium*. These include a waxy cell wall made up of long-chain mycolic acids and DNA with a high G+C content. However, these shared characteristics (discussed in Chapter 4) belie a high degree of inter-species heterogeneity. Widely varying growth rates, specific growth requirements and differing natural ecologies are three examples of this heterogeneity (see Chapter 2). It has important consequences for the detection of mycobacterial pathogens in the

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environment because it means that general mycobacterial methods must often be tailored to detect specific pathogens. In mycobacteriology there are no standard methods.

Mycobacterial diagnostics have benefited enormously from the many recent advances in biotechnology. These advances have had the greatest impact at the level of isolate identification and characterization. Techniques such as IMS, PCR and automated DNA sequencing are now beginning to form the foundation for modern mycobacterial analytical methods. However, to a large extent one of the most important steps in analysis - the isolation in culture of mycobacteria from the environment - has not significantly changed since the 1950s and 1960s when methods for chemical treatment of samples to remove background microorganisms were first implemented. This lack of progress is unfortunate as there are serious issues surrounding the speed and sensitivity of these culture methods. Furthermore, the power of the molecular tools now available for modern diagnostics is largely dependent on the availability of isolates in pure culture. One could pessimistically summarize this situation as being akin to a present day Formula One racing car powered by a 19<sup>th</sup> century steam engine.

Nevertheless, by using these flawed techniques over 70 mycobacterial species have been formally recognized and the study of these isolates by molecular genetic methods has shed considerable light on the population structure of the genus mycobacteria and is dramatically improving our understanding of the relationship between pathogenic and non-pathogenic mycobacteria. An important consequence of this work is the discovery of genetic markers of virulence. These markers are discussed later in this chapter (and in Chapter 4) and they are the basis for modern molecular detection methods.

The search for genetic markers has been greatly assisted with the advent of whole-genome sequencing projects (i.e. determining the complete DNA sequence of an organism). As of September 2002, four mycobacterial genomes have been fully sequenced (a cumulative total of 17 000 000 bp) and there are a further seven currently in progress (<http://wit.integratedgenomics.com/GOLD/>). These projects are a fundamental resource for unlocking the pathways to pathogenesis among the mycobacteria and in so doing they are revolutionizing mycobacteriology. The full diagnostic potential afforded by whole genome sequences is still yet to be realized but significant advances in this area have already begun. Comparative genomic analysis of the different members within the MTB complex has permitted identification of a panel of DNA markers that unequivocally differentiate each sub-species within this very homogeneous complex (Brosch *et al.* 2002). Comparisons between complete mycobacterial genome sequences have also highlighted the mechanisms and the significant extent to which some members of the genus can evolve. DNA insertions, deletions and point mutations are important drivers of genome change. IS in particular mediate many changes within mycobacterial genomes and some of these changes can

have profound effects on virulence (see McAdam (2000) for a review of mycobacterial IS). IS are short stretches of non-essential DNA, around 1.5kb in size, often repeated many times throughout a genome. They encode a transposase, an enzyme that permits the sequence to copy itself. In this respect IS are said to be mobile DNA elements. IS have been shown to move between different bacteria and thus they are a means for the exchange of DNA between bacteria. But despite this potential to jump across so-called species boundaries some mycobacterial IS have proved to be useful species and sub-species markers. Examples include IS1245 and IS1311 for MAC, IS900 for MAP and IS2404 for *M. ulcerans*. As IS copy themselves within the genome they can produce a unique and heritable distribution pattern for a particular strain. Over time this can lead to inter-strain IS variation and it is this variation that has been extensively exploited for the development of molecular fingerprinting methods. These methods are discussed in section 5.2.4.

Our improved understanding of both mycobacterial genetics and ecology has had obvious implications for those undertaking surveillance studies for these bacteria. Analysis of only grab samples of water is unlikely to be sufficient to reflect the mycobacterial composition of the greater environment from which the water came, nor will such analyses reflect the inherent risk of mycobacterial infection from that environment. A holistic approach to environmental surveillance is required that draws on a detailed knowledge of the biology and ecology of the mycobacteria.

Their ubiquitous presence means it is a relatively simple affair to find environmental mycobacteria per se but it is the specific detection of a particular mycobacterial pathogen that can require a comprehensive understanding of its biology and ecology, with subsequent modification or development of the detection methodology. All this knowledge of the organism must be supported by good epidemiological data. The recent finding of *M. ulcerans* in the salivary glands of aquatic insects is a useful example of the application of these principles. (Refer to Chapter 8 for a discussion of Buruli Ulcer). For 50 years the environmental source of *M. ulcerans* was unknown despite very good epidemiological evidence linking it to aquatic environments (Barker 1973). In 1997 an *M. ulcerans* specific IS was identified, named IS2404, and a highly sensitive PCR test was developed based on this sequence (Ross *et al.* 1997a). This test circumvented the need to culture *M. ulcerans* as a means of demonstrating its presence in the environment. The new PCR was applied to water and plant samples collected from *M. ulcerans* endemic and non-endemic areas. PCR-positive samples were obtained only in the endemic regions (Roberts & Hirst 1997; Ross *et al.* 1997; Stinear *et al.* 2000). This was the first direct evidence of *M. ulcerans* in the environment. Subsequent studies using the same DNA marker found *M. ulcerans* PCR-positive samples in aquatic insects collected in West Africa (Portaels *et al.* 1999). Most recently, in a series of experiments that modelled and tracked the fate of *M. ulcerans* in aquatic insects, Marsollier and co-workers succeeded

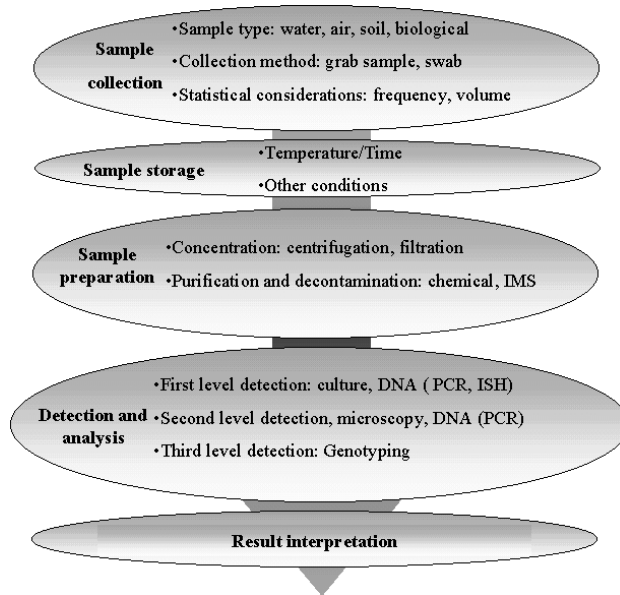
in culturing *M. ulcerans* from the salivary glands of aquatic insects captured from endemic areas in the Ivory Coast (Marsollier *et al.* 2002).

In summary, mycobacteria are not all the same and the efficiency of the many methods used for their detection is highly variable. A good knowledge of the biology and ecology of a target organism using the best available technology is essential to maximize the chance of detection.

## 5.2 THE ANALYSIS PROCESS

From the perspective of the water industry there are two general requirements of a microbiological analytical method, namely to be able to provide data for routine surveillance (e.g. as part of risk assessments or catchment management programmes) and to be able to respond quickly to a public health emergency. Each scenario will require a different approach to analysis.

Analysis is a multi-step process and the reader is referred to *Handbook of Water Analysis and Standard Methods* for a more general introduction to this topic. A schematic of the analysis pathway for environmental mycobacteria is given in Figure 5.1. It has been depicted as five distinct phases: (i) sample collection; (ii) sample storage; (iii) sample preparation; (iv) detection; (v) result interpretation.



**Fig. 5.1** Flow chart of the analysis process

The following sections review the various methods that have been developed for mycobacterial analysis of the environment. A summary of methods has been made from a representative selection of recent reports that have described the isolation of a range of both slow and rapid growing mycobacteria from various environmental sources (Table 5.1). The summary has been constructed following the criteria outlined in Figure 5.1.

**Table 5.1** Methods recently used for the isolation of mycobacteria from the environment

Sample type	Method Summary	Reference
Water (raw and treated): surface and groundwater sources. Biofilms: pipes, water meters	Sampling: 300 ml water samples, 4 cm <sup>2</sup> biofilm samples. Concentration: centrifugation. Decontamination: CPC, 0.005%, 30 min. Isolation: 7H10 agar (OADC), 37 °C. Detection: representational colony selection, ZN staining, sub-culture to purity, 16SrRNA PCR and sequencing	(Falkinham <i>et al.</i> 2001)
Water (raw and treated): surface and groundwater sources	Sampling: 1000 ml water samples. Concentration: filtration. Decontamination: SDS/NaOH. Isolation: Lowenstein-Jensen, 37 °C. Detection: ZN staining, sub-culture to purity, 16SrRNA PCR and sequencing, <i>hsp65</i> PRA and sequencing	(Le Dantec <i>et al.</i> 2002)
Water: hospital and consumer taps	Sampling: 500 ml water samples. Concentration: 0.45 µm filtration. Decontamination: CPC, 0.005% 15 min. Isolation: BACTEC and Lowenstein-Jensen, 37 °C. Detection: AFB staining, sub-culture, biochemical ID,	(Peters <i>et al.</i> 1995)
Water: various sources	Sampling: 25 ml water samples. Concentration: centrifugation. Decontamination: none used. Isolation: Tsukamura minimal-Tween80-cycloheximide agar, 37 °C. Detection: AFB staining, sub-culture to purity, DNA probes for MAC.	(von Reyn <i>et al.</i> 1993)
Water (raw and treated): cold taps, hot taps, ice, showers, bottled water	Sampling: 500 ml samples. Concentration: 0.45 µm filtration. Decontamination: CPC. Isolation: 7H10 agar (cycloheximide), 37 °C, 5% CO <sub>2</sub> . Detection: colony morphology, sub-culture, 16SrRNA PCR and sequencing	(Covert <i>et al.</i> 1999)
Swimming pools: water, biofilms	Sampling: 500 ml samples, swabs (20 cm <sup>2</sup> ). Concentration: 0.45 µm filtration. Decontamination: CPC 0.04%, 30 min. Isolation: 7H10 agar, 30 °C, 5% CO <sub>2</sub> . Detection: colony morph, sub-culture, biochemical ID	(Leoni <i>et al.</i> 1999)
Water (raw and treated)	Concentration: 0.45 µm filtration. Decontamination: CPC 0.05% - CPC 0.005% 30 min. Isolation: Lowenstein-Jensen, 30 °C. Detection: sub-culture based on colony type on 7H10 agar, TLC and <i>hsp65</i> PRA.	(Neumann <i>et al.</i> 1997)
Hospital tap water	Sampling: 200 ml samples. Concentration: centrifugation. Decontamination: not specified. Isolation: Lowenstein-Jensen, 37 °C. Detection: AFB, sub-culture, <i>hsp65</i> PRA	(Chang <i>et al.</i> 2002)
Surface water, vegetation, soil	Concentration: centrifugation, filtration. Decontamination: IMS, CPC 0.005% 30 min, Isolation: Brown & Buckle agar, 30 °C, microaerophilic. Detection: subculture, AFB, 16SrRNA PCR sequencing	(Stinear <i>et al.</i> 2000)

### 5.2.1 Sampling

The objective of sampling is to obtain a sample from a particular environment that can be said to represent that environment. Given the stochastic nature of bacterial populations how can this be achieved? There is no simple answer to this question. Sampling demands at least that the researcher clearly define the question(s) being asked in any investigation. Consideration must be given to both spatial and temporal aspects of a particular environment. Thus the issues of sampling frequency and sample type will vary with each situation. Sampling an aquatic environment requires collection of more than just bulk water samples. The habitats of mycobacteria are diverse (refer to Chapter 3 for a review of the different sources of mycobacteria). As an example of this diversity, a recent survey of several drinking-water distribution systems has shown that pipe biofilms are probably the most significant sources of mycobacteria within a drinking-water supply. Conservative concentration estimates of 4300 slow growing mycobacteria per cm<sup>2</sup> were reported (Falkinham *et al.* 2001). This study and others (Schulze-Röbbecke & Fischeder 1989), suggests that efforts to examine the risk posed by mycobacteria in drinking-water supplies should focus more heavily on pipe biofilms than bulk water samples. Biofilms can be sampled either by collecting swabs from which mycobacteria may be recovered by culture or DNA methods. Alternatively, the biofilm can be kept intact and a portion embedded. This portion can then be thin-sectioned and the mycobacteria analysed *in situ* using techniques discussed in section 5.2.4.1. Given the ubiquity of slow growing mycobacteria in both potable and hot water systems, research is increasingly focused on monitoring growth of these organisms using recirculating or by-pass systems. This parallels other trends in drinking-water microbiology research, where it is realized that accurate simulations of drinking-water systems are necessary to examine pathogen survival and proliferation in biofilms and to implement control methods.

Considerable research efforts are in place to optimize these simulations. The most accurate simulations are those involving removable coupons that are placed in by-pass systems plumbed directly into a hot or cold water supply. The primary method of sampling is through removal of coupons and either direct DNA extraction or embedding followed by cryosectioning and imaging with fluorescent labelled antibodies.

Developed at Montana State University, the cryosectioning technique has been widely applied to imaging mixed species biofilms (Yu *et al.* 1994; Murga *et al.* 1995). Its application to dual species biofilms (*Pseudomonas aeruginosa* and *M. avium*) grown in cold water recirculating systems has

shown preferential survival of *M. avium* at the metal coupon-biofilm interface (Ford 1999). Research is now focused on optimized ISH methods to visualize mycobacterial species in biofilms (refer to section 5.2.4.1).

Methods have also been described for sampling aerosols (Wendt *et al.* 1980). The following list is a summary of potential mycobacterial environments with references to reports concerning sampling each of them. In short, everything must be considered as a potential mycobacterial habitat:

- aerosols (Wendt *et al.* 1980)
- air/water interface (Falkinham 2003)
- biofilms (Schulze-Röbbecke & Fischeder 1989)
- insect populations (Marsollier *et al.* 2002)
- animal populations (Fischer *et al.* 2000)
- vegetation (Stinear *et al.* 2000)
- soil (Iivanainen *et al.* 1999b)
- food (Argueta *et al.* 2000)
- water (refer to Table 5.1)

### 5.2.2 Sample storage

Several studies have been performed to examine the effects of different storage conditions on the viability of mycobacteria collected from the environment. Iivanainen *et al.* (1995) demonstrated that long-term storage at  $-80\text{ }^{\circ}\text{C}$  of crude cell-concentrates obtained from water samples without the addition of cryoprotectant reduced levels of background bacteria and enhanced the recovery of mycobacteria. However, it is important to understand something of the physiology of individual mycobacteria as some species show significant changes to their cell wall or lose viability as a consequence of freezing (Thoen *et al.* 1977; Silva *et al.* 1989).

### 5.2.3 Sample preparation

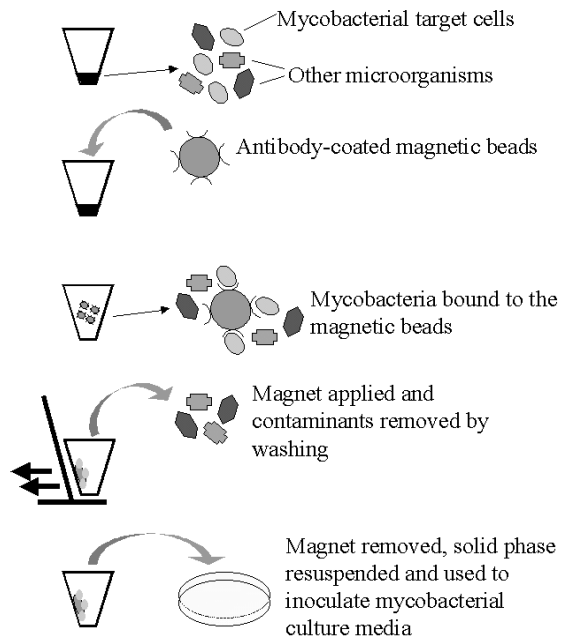
This step incorporates sample concentration, decontamination and purification. It is usual to analyse sample volumes up to one millilitre. If the concentration of mycobacteria in a given sample is expected to be low, as is often the case with treated water samples, then it may be necessary to analyse up to 1000 ml sample volumes. Either filtration through  $0.45\text{ }\mu\text{m}$  membranes or centrifugation methods are employed to concentrate mycobacterial cells in samples of this volume prior to analysis.

Sample decontamination methods have been designed to remove or reduce the non-mycobacterial microbial flora in a sample. These techniques utilize the relative resistance of mycobacteria to chemical treatments such as

combinations of acid/alkali or detergents. Several studies have been conducted to try and determine the optimum decontamination method. There is no clear consensus as to the optimum method; however, treatment with the detergent CPC (du Moulin & Stottmeier 1978; Fischeder *et al.* 1991) has repeatedly been shown to promote the recovery of a wide range of mycobacteria with low rates of background contamination (Table 5.1). The comparative efficacy of agents such as CPC and NaOH is greatly influenced by the contact time and concentrations used (Hunter *et al.* 2001). Thus it may be necessary for investigators to test empirically the relative resistance of different mycobacteria under authentic test conditions before commencing any environmental analyses (i.e. conduct seeding experiments to determine the best decontamination regime in a given sample matrix for a given group of mycobacteria).

Sometimes it is insufficient just to reduce the background microflora. To enable the detection of mycobacterial pathogens such as MAP and *M. ulcerans* in the environment it has been necessary to develop methods that very specifically enrich for target cells. A recent development has been IMS. This technique uses 1-2  $\mu\text{m}$  paramagnetic beads coated with anti-mycobacterial antibodies. These antibody-coated beads are incubated with a sample concentrate, typically in a 1.5 ml tube. The beads specifically bind mycobacterial cells. A magnet is then applied to the tube and a complex of magnetic beads and mycobacterial cells form on the side of the tube. This complex can then be decontaminated by a low concentration CPC treatment and then used as the inoculum in mycobacterial culture media or subjected to a diagnostic PCR. This method has been used successfully to isolate *M. ulcerans* in culture from the environment (Marsollier *et al.* 2002). It has also been used to detect *M. tuberculosis*, *M. avium* and MAP (Li *et al.* 1996; Mazurek *et al.* 1996; Grant *et al.* 1998; Mason *et al.* 2001). The general approach is summarized in Figure 5.2. There are variations on this method; for example, "indirect capture" where the antibodies are first incubated with the sample before the addition of the magnetic beads. The success of IMS is dependent on the specificity of the anti-mycobacterial antibodies used to coat the magnetic beads. Improvements here will see the more widespread application of IMS in mycobacterial diagnostics.

Future decontamination protocols may focus on the use of free-living aquatic amoebae as a means for purifying and isolating mycobacteria as they are natural hosts for many mycobacteria (Steinert *et al.* 1998).



**Fig. 5.2** The basic principle of IMS

## 5.2.4 Detection

Detection methods can be subdivided into three categories. First level detection refers to those methods involved with primary isolation; second level detection refers to methods that provide genus and species identification; and third level detection refers to methods used for sub-species discrimination and molecular epidemiology.

### 5.2.4.1 Detection (first level)

There are three general approaches for the detection of mycobacteria in the environment.

The first approach is to identify the mycobacteria in a sample by culture enrichment. That is, take a sample, perform some pre-treatments (such as those described above) to try and remove other microorganisms and then enrich for

the mycobacteria using artificial media. Mycobacterial-like colonies can then be confirmed using a number of techniques. These types of methods are said to be quantitative because the output is cfu per volume analysed.

There have been several comparisons of different media for the recovery of mycobacteria from the environment. The conclusions from different studies are difficult to compare and often contradictory. An extensive and recent review of the literature in this field concluded that, depending on the mycobacterial species under investigation, multiple types of media should be used and incubation conditions should be varied, such as medium pH, temperature and atmospheric CO<sub>2</sub> concentration (Hunter *et al.* 2001). In general, combinations of egg-based (e.g. Lowenstein-Jensen agar slopes) and defined salt media (Middlebrook 7H10 agar with OADC supplement), with lower incubation temperatures (around 32 °C) seem to promote the recovery of the widest range of mycobacteria. More information regarding different mycobacterial culture media can be found in Brown & McNeil (2003). To suppress fungal overgrowth and other microbial overgrowth, agents such as cycloheximide or malachite green are often added to the media. Antibiotics, such as a mixture of polymyxin B, amphotericin B, nalidixic acid, trimethoprim and azlocillin (PANTA supplement), are frequently added to liquid media. It is regularly used in clinical mycobacteriology in conjunction with the BACTEC medium (Becton Dickinson, Sparks, Maryland, USA). For the recovery of some mycobacterial species it is necessary to use media containing specific growth factors such as mycobactin, a siderophore required for the growth of MAP.

It is well recognized that culture enrichment methods for mycobacteria are inefficient and are hindered by many factors. The differences in growth rates between mycobacteria in artificial media are very large. Rapid growers such as *M. smegmatis* double in 2 hours compared to slow growers such as *M. ulcerans* that double in 48 hours or, at the extreme of the spectrum of growth rates, *M. leprae* which does not grow at all in axenic culture. Thus a major issue for culture methods is the overgrowth of slowly growing mycobacteria by faster growing mycobacteria or by other microorganisms. Chemical decontamination methods were developed to reduce overgrowth problems but these methods are necessarily harsh and there are always a percentage of mycobacterial cells destroyed during the decontamination process. Mycobacterial reduction levels of 1-2 log are not uncommon (Dundee *et al.* 2001; Le Dantec *et al.* 2002a). There are also no selective media for mycobacteria in the same way that there are for members of the enterobacteriaceae. There have been some modifications made to try and improve selectivity for particular species such as MAC (George & Falkinham 1986) but several method comparisons have suggested that less selective methods perform better (Neumann *et al.* 1997). In addition to the problems of overgrowth there are always the issues of mycobacteria in a viable but non-culturable state (Kazda 2000). Furthermore, colony confirmation is both subjective and laborious. So even though one obtains a numerical result there

are so many accumulated uncertainties that it is difficult to define these methods as quantitative. Despite these problems a wide range of known (*M. gordonae*, *M. chelonae*, *M. gastri*, *M. kansasii*, *M. fortuitum*, *M. avium*, *M. flavescens*, *M. malmoense*, *M. xenopi*, *M. mucogenicum*, *M. intracellulare*, *M. peregrinum*, *M. scrofulaceum*, *M. shimoidei*, *M. szulgai*, *M. haemophilum*, *M. terrae*, *M. abscessus*, *M. aurum*, *M. phlei*, *M. marinum*, *M. sphagni*, *M. farcinogenes*, *M. nonchromogenicum* and *M. hodleri*) and many unidentified mycobacterial species have been recovered by the techniques reviewed in Table 5.1, suggesting that these methods have some merit.

An alternative approach, which overcomes these problems of culture isolation, is to use direct DNA detection methods. These are relatively rapid techniques that indicate the presence of mycobacteria by detecting signature mycobacterial DNA sequences without a prior culture enrichment step. Result turnaround times can be hours instead of weeks or months as is often the case with culture methods. The general approach is to take a sample, extract all DNA present in that sample and then identify in that pool of DNA, specific mycobacterial sequences. However, the concentration of mycobacterial DNA in environmental samples is too low to be detected directly, so PCR has been used to amplify target mycobacterial DNA sequences to detectable levels (Wang *et al.* 1996; Stinear *et al.* 2000; Marsollier *et al.* 2002).

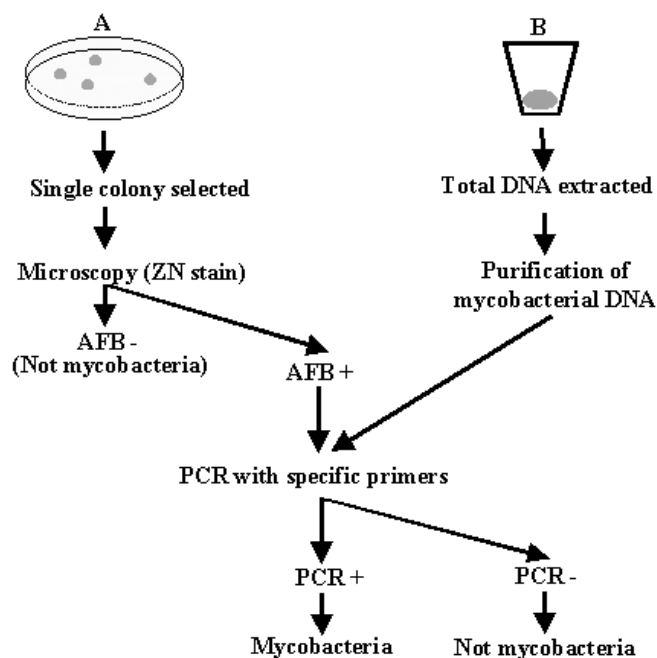
A critical point in this type of analysis is the primary DNA extraction step. The mycobacterial cell wall is a lipid-rich envelope that resists common bacterial cell lysis treatments such as NaOH/SDS combinations. Optimal protocols for extraction of mycobacterial nucleic acids have been developed recently and shown to permit the detection of less than 10 bacterial cells in complex sample matrices such as soil and faeces (Stinear *et al.* 2000; Bull *et al.* 2003a). These methods use high-speed cell disintegrators to disrupt mycobacterial cells and liberate the nucleic acids. Stinear *et al.* (2001) have published a detailed protocol for the detection of mycobacterial DNA in plant, water and soil samples.

Several years ago PCR screening of environmental samples was dogged by problems of poor sensitivity due to inhibition of the Taq polymerase by ubiquitous environmental compounds, such as humic and fulvic acids. PCR inhibition is no longer the significant issue it was as there are several technologies now available to remove these contaminants (Rochelle 2001). However, direct DNA detection methods have their limitations. No isolate is recovered and one can only detect short stretches of DNA, thus the amount of genetic information obtained is limited. There is also the issue of detecting dead cells or remnant DNA sequences. Direct DNA detection does have the advantage of giving an absolute indication of the presence or absence of mycobacteria in a given sample and can be quantitative when used in conjunction with a most probable number format (Stinear *et al.* 2000) or with quantitative PCR (MacGregor *et al.* 1999).

The third approach to first level detection is ISH. A sample is immobilized (such as a biofilm sample) and then probed for mycobacteria using either antibodies (Naser *et al.* 2002), or short DNA or RNA sequences (oligonucleotide probes) that have been labelled fluorescently or by some other means. The samples are then visualized by epifluorescence microscopy. By careful design of the probes one can detect at the genus level or at the species level. These methods have the advantage of allowing one to visualize the mycobacteria *in situ*. These techniques work very well for other bacteria and show great promise for mycobacteria but they are still under extensive development. ISH with oligonucleotide probes for the detection of mycobacteria show poor detection sensitivity (Stender *et al.* 1999a). This is thought to be due to problems with the poor penetrability of the hydrophilic probes through the lipid-rich mycobacterial cell wall. Some recent studies have shown successful ISH detection of mycobacteria with peptide nucleic acid probes (hydrophobic, DNA analogue probes) (Stender *et al.* 1999; Zerbi *et al.* 2001).

#### 5.2.4.2 Detection (second level)

This is the primary identification step and ten years ago this category would have included combinations of techniques such as microscopy, biochemical ID methods or cell wall lipid analysis by HPLC. Nowadays, these techniques, with the exception of microscopy, have been almost completely replaced by molecular genetic methods. Unlike the other techniques, genetic methods are independent of growth rate and independent of phenotype. Assuming appropriate DNA sequences are analysed then one can identify all known mycobacterial species and readily discern the presence of new ones. Thus, genetic methods offer greater speed, accuracy and precision. A basic flow chart of this process is given in Figure 5.3, linking PCR-based identification of mycobacterial DNA sequences with the culture enrichment and the direct DNA isolation methods. After obtaining pure culture isolates by culture enrichment, each colony is subjected to ZN stain, which is a stain for cell wall acid fastness. This is a rapid and cost-effective screen with a high negative predictive value that eliminates many non-mycobacteria. ZN-positive isolates can then be screened for mycobacterial DNA sequences by PCR. For direct DNA detection methods, the purified DNA is subjected directly to PCR. It is common at this stage to use mycobacterial genus-specific primers based on the 16SrRNA gene (Boddinghaus *et al.* 1990a) or on the *hsp65* gene (Plikaytis *et al.* 1992). The type of PCR performed is dependent on the goals of the investigation. It may be a PCR designed to detect a particular pathogen, such IS900 to detect MAP, or a genus-level PCR to detect all mycobacteria, or a combination of PCR tests.



**Fig. 5.3** Flow chart for the second level detection of mycobacteria following (A) culture isolation or (B) direct DNA detection

#### 5.2.4.2.1 Genus and species identification

The 16SrRNA gene is the most widely used target for genus and species level identification. The complete DNA sequence of this gene (approximately 1500 bp) has been extensively studied and compared among many organisms. Sequences have been identified that are conserved among all mycobacteria but differ to those in other bacteria. These sequences have been used for genus level identification; usually by amplification of a 1030 bp product (Boddinghaus *et al.* 1990a). The detection of this product indicates the presence of mycobacteria. The region of the 16SrRNA gene amplified encompasses two hypervariable regions, A and B. By subjecting the 1030 bp PCR to nucleotide sequence analysis and then comparing the resulting sequences of the two regions to those obtained from reference strains, an isolate can be readily classified or associated with a species or species complex. There is now a large worldwide, searchable database of mycobacterial 16SrRNA gene sequences (<http://www.ncbi.nlm.nih.gov>). This

repository of sequences forms the basis for mycobacterial systematics. However, the mycobacteria are unusual in that, compared to other bacteria, there is a very high degree of sequence conservation of the 16SrRNA gene. Indeed, there are several examples of distinct species such as *M. gastri* and *M. kansasii*, and *M. ulcerans* and *M. marinum* that share identical 16SrRNA sequences through their A and B hypervariable regions. One very important consequence of this is that it is rarely possible to identify a mycobacterial isolate based on the 16SrRNA sequence alone, as sequence identity - whilst indicating an obviously close evolutionary relationship - does not infer species identity. Other conserved genes such as *hsp65* (Plikaytis *et al.* 1992), *rpoB* (Lee *et al.* 2000), *dnaJ* (Inyaku *et al.* 1993), *sodA* (Zolg, 1994), *gyrB* (Kasai *et al.* 2000) or intergenic regions such as the 16S-23S ITS (Roth *et al.* 1998) have been used as alternative diagnostic targets in an attempt to find sequences with greater polymorphism than the 16SrRNA gene.

The *hsp65* gene has proved to be a very useful target in this respect because, among many mycobacterial species and species complexes, it has shown a greater amount of sequence diversity than 16SrRNA sequences (Devallois *et al.* 1997). A very widely used test is *hsp65* PCR restriction enzyme analysis (*hsp65* PRA). The principle of this test is quite simple. A 439 bp fragment is amplified from the *hsp65* gene. The DNA sequence of the PCR product is then interrogated by restriction enzyme digestion with two enzymes, *Hae*III and *Bst*EII and the products of the digestion reaction are separated and visualized by gel electrophoresis. The pattern of bands obtained is then compared with the banding patterns obtained from reference strains. A website has been established that contains a database of *hsp65* PRA patterns to assist with species identification (<http://www.hospvd.ch/prasite>). Many mycobacteria, including *M. avium*, *M. fortuitum*, *M. gordonae*, *M. kansasii*, *M. abscessus*, *M. perigrinum* and *M. chelonae*, can be recognized by their unique banding pattern. In some cases there is sufficient diversity within this region to identify multiple alleles within a species (da Silva Rocha *et al.* 2002). PRA is a rapid and relatively inexpensive diagnostic tool. However, not all mycobacteria can be distinguished by this technique such as the *M. tuberculosis* complex, or *M. marinum* and *M. ulcerans*.

Modern automated DNA sequencing methods have made it possible to rapidly determine the nucleotide sequence of short stretches of DNA. One can rapidly sequence the 439 bp PCR product from the *hsp65* gene, even for a large number of isolates. This approach reveals all the potential nucleotide polymorphisms within this region and thus offers an even higher level of discrimination than PRA (Ringuet *et al.* 1999). In a recent study of 273 clinical and environmental MAC isolates, *hsp65* sequencing revealed 18 different *hsp65* alleles and 54 different polymorphic sites (Smole *et al.* 2002).

Apart from demonstrating the extraordinary genetic diversity among MAC isolates, this study also clearly demonstrated the power of DNA sequencing to unambiguously define the true relationship between isolates.

Single gene analyses such as these have their limitations, as the level of sequence conservation between some species is so high that there is insufficient intra-gene variation to provide discrimination. Again, as for *hsp65* PRA, *M. marinum* and *M. ulcerans* are examples of this category as a recent study of a large number of *M. marinum* fish isolates demonstrated (Ucko *et al.* 2002). MLST is a technique that analyses the nucleotide sequence of several genes. This resolves many of the issues surrounding discrimination between species. MLST is discussed in more detail in the following section, 5.2.4.3.5.

#### 5.2.4.2.2 Commercial tests for genus and species identification

Some of the DNA sequences described above have been used to develop commercial tests for rapid, species-level identification of mycobacteria. The INNO-LiPA Mycobacteria kit (Innogenetics, Ghent, Belgium) is a reverse line-blot hybridization assay that uses a panel of different probes, based on species-specific sequence variations in the 16S-23S ITS region, to identify several different mycobacteria (Suffys *et al.* 2001). The test is in a convenient format but there are several instances where the probes cross-hybridize with other species because of insufficient sequence variation or polymorphism within the ITS. Other commercial DNA-based detection kits include Accuprobe, a DNA probe system based on the detection of 16SrRNA molecules. The tests are performed directly on isolates obtained from either solid or liquid media. The test is simple and rapid but, as the probes are based on highly conserved sequences, cross-hybridization does occur and can give false positive results.

#### 5.2.4.2.3 Pathogen-specific PCR tests

Some DNA sequences, such as certain IS, have been shown to be markers for pathogenic strains or at least potentially pathogenic strains. The DNA targets DT1 and DT6 are often used to detect MAC as these sequences have been shown to be present in all members of the complex (Thierry *et al.* 1993) but absent from other species. A DNA fragment cloned in the plasmid p6123 has been shown to be a specific marker for *M. kansasii* (Yang *et al.* 1993; Picardeau *et al.* 1997a). IS2404 is a high copy number IS in *M. ulcerans* that is a highly specific and sensitive target for this species (Ross *et al.* 1997a). Similarly IS900 has been shown to be a very useful marker for the detection of MAP (Hermon-Taylor *et al.* 1990). Table 5.2 contains the primer sequences for these different PCR tests.

**Table 5.2** Primer sequences for mycobacterial pathogen-specific PCR tests

Species	Target	Primer	Sequence 5' – 3'	Size	Reference
<i>M. ulcerans</i>	IS2404	MU5	AGCGACCCAGTGGATTGGT	492 bp	(Stinear <i>et al.</i> 1999)
		MU6	CGGTGATCAAGCGTTCACGA		
MAC	DT1	AV6	ATGGCCGGGAGACGATCTATGC GGCGTAC	666 bp	(Devallois <i>et al.</i> 1997a)
		AV7	CGTTCGATCGCAGTTTGTGCAG CGCGTACA		
MAC	DT6	IN38	GAACGCCCGTTGGCTGGCCATT CACGAAGGAG	187 bp	(Devallois <i>et al.</i> 1997a)
		IN41	GCGCAACACGGTCGGACAGGC CTTCTCGA		
MAP (nested PCR) 2 <sup>nd</sup> round for TJ1/TJ2	IS900	TJ1	GCTGATCGCCTTGCTCAT	294 bp	(Bull <i>et al.</i> 2003a)
		TJ2	CGGGAGTTTGGTAGCCAGTA		
		TJ3	CAGCGGCTGCTTTATATTCC		
		TJ4	GGCACGGCTCTGTGTGTAGT		
<i>M. kansasii</i>	p6123	K1	GTGCCACACCGACGTTGC	268 bp	(Picardeau <i>et al.</i> 1997a)
		K2	GGTAGTGGGCTCGGATATGGA		

### 5.2.4.3 Detection (third level)

This section is concerned with the methods that are used to analyse strain variation. These methods are an important component of the techniques used to investigate the epidemiology of mycobacterial diseases. A significant research effort has been put into this area as people try to address the key question: “is this strain the pathogenic strain?” Traditional techniques such as biotyping, serotyping and phagotyping have now been replaced by DNA methods. The aim of these methods is to try and find regions of DNA difference between strains. For mycobacteria the following approaches have been used successfully.

#### 5.2.4.3.1. IS restriction fragment length polymorphism

As discussed earlier, IS are short stretches of DNA that have the ability to copy themselves in a random or semi-random fashion. This means that they are often present in multiple copies in a bacterial chromosome and the pattern of IS distribution can vary significantly from one strain to the next (see Chapter 4). By using restriction enzymes to specifically cut the bacterial chromosome into fragments, size-separating those fragments by gel electrophoresis, and then probing the fragments with a labelled copy of the IS, it is possible to obtain an IS banding pattern or fingerprint for that strain. This technique is used for several environmental mycobacteria. Normally, MAC isolates can be readily discriminated by RFLP

analysis using *IS1245* and *IS1311* (Guerrero *et al.* 1995), and standardized protocols have been developed (van Soolingen 1998). *M. xenopi* isolates have been typed using *IS1395*-RFLP but this species displays limited polymorphism (Picardeau *et al.* 1996). Significant polymorphism was discovered among *M. gordonae* strains typed using *IS1511/IS1512*-RFLP (Picardeau *et al.* 1997).

#### 5.2.4.3.2 Pulsed-field gel electrophoresis

PFGE is a technique that permits very large DNA fragments to be separated and visualized. Bacterial DNA specially prepared in agarose plugs to prevent shearing is digested with rare-cutting restriction enzymes. This results in a pool of 10-20 DNA fragments that are then separated using a special electrophoresis apparatus that size-separates DNA in an alternating electrical field. It is widely used in bacterial molecular epidemiology and especially in mycobacteriology. It does not require any prior knowledge of a particular strain, such as the presence of repetitive IS. It has been used to type many species of mycobacteria. The preparation of the agarose-embedded DNA can be problematic. Clumping of cells and subsequent difficulties in standardizing cell lysis efficiencies can lead to dramatic differences in DNA yield and quality between preparations. Recent advances have been made to try and tackle this problem (Hughes *et al.* 2001) by culturing the bacteria under constant, stirred agitation. A significant drawback of PFGE is that while it can reliably demonstrate strain relatedness, the converse is not true: large chromosome rearrangements can readily occur and produce very different PFGE profiles between otherwise highly related strains.

#### 5.2.4.3.3 Inter-insertion sequence polymerase chain reaction

Some mycobacterial species, such as MAC, contain multiple copies of different IS. Different strains have varying distances between the IS copies, depending on the pattern of distribution of each IS within that strain. Inter-IS PCR uses PCR to amplify between adjacent copies of different IS. Outward facing primers are designed to each IS type and a PCR is performed on genomic DNA extracted from the isolate. The resulting amplified DNA fragments are separated and visualized by gel electrophoresis. This method is rapid and simple to perform. It is used for genotype analysis of MAC by targeting *IS1245* and *IS1311* (Picardeau & Vincent 1996) and for *M. ulcerans* by targeting *IS2404* and *IS2606* (Stinear *et al.* 2000a). It also has the advantage of not requiring a high concentration or high quality DNA. Using this technique, it has been possible to genotype strains of *M. ulcerans* directly from tissue specimens (Stinear *et al.* 2000a).

#### 5.2.4.3.4 *Random amplified polymorphic DNA (RAPD)*

This technique uses short oligonucleotides of random sequence in a low stringency PCR reaction to produce a strain-specific pattern of PCR fragments after gel electrophoresis. It is a rapid test that, like PFGE, requires no prior knowledge of the strain. RAPD has been used relatively widely and has shown utility in outbreak investigations (Zhang *et al.* 2002). There are issues surrounding the reproducibility of this method but attempts have been made to try and standardize the procedure (Ramasoota *et al.* 2001).

#### 5.2.4.3.5 *Multi-locus sequence typing*

All the techniques discussed thus far in this section have one major limitation in common. They all rely on producing DNA bands on electrophoretic gels and then comparing the bands obtained with one strain with those obtained from another. Strain relatedness is inferred by noting the number of shared bands; the more shared bands, the more similar the strains and vice-versa. Electrophoresis is a difficult method to standardize as it is influenced by many parameters, such as sample quality, agarose quality, agarose concentration, buffer composition, running times, apparatus, voltage, etc. Even within the same laboratory it is difficult to compare the banding patterns obtained from one experiment with those obtained from the next. Genotyping methods are now moving away from these approaches and new techniques are being developed that offer high levels of discrimination, reproducibility and portability between laboratories.

MLST is a recently developed technique, widely used now for bacterial molecular epidemiological and population genetics studies (Clarke 2002). The technique is analogous to Multi-Locus Enzyme Electrophoresis except that the nucleotide sequences for the genes of housekeeping enzymes are determined rather than looking for differences in the electrophoretic mobility of the enzymes themselves. This technique identifies unique combinations of alleles. A strain displaying a unique allele combination is assigned a sequence type (analogous to a genotype).

The method is quite straightforward. As described for 16SrRNA and *hsp65* sequencing, DNA is extracted from a strain and then PCR is used to amplify specific gene sequences of approximately 500 bp. It is usual to select seven or more distinct loci. The more loci that are analysed, the greater the level of discrimination. The products are then subjected to nucleotide sequencing and then sequence comparisons are made using combinations of alignment and phylogenetic software. MLST has many advantages over other typing methods; in particular it provides unambiguous data that is readily standardized, easily transferred and compared between laboratories. MLST has not yet been widely used for the analysis of mycobacteria but one recent study, examining the relationship between *M. ulcerans* and *M. marinum*, demonstrated the power of this approach. MLST analysis at 7 loci

of 20 strains of *M. ulcerans* and 22 strains of *M. marinum* provided a means to discriminate not only between the species but also within the species (Stinear *et al.* 2000b). Future research must be directed toward developing a pan-mycobacterial MLST scheme. Such a scheme would be the basis for examining the fundamental relationship between all members of the genus.

### **5.3 KEY RESEARCH ISSUES**

The mass of information and information potential presented by the availability of whole genome sequences for the mycobacteria means that we will soon have access to very intimate details regarding the biology of the genus. Mycobacterial diagnosticians must be prepared to use this data to develop specific tests and testing strategies for specific mycobacteria. Information gleaned from genome analyses about the metabolic pathways of a particular species could be used to design better, more selective media or isolation conditions; for example, culturing under anaerobic conditions on a defined substrate medium. New technology such as high-throughput DNA sequencing will continue to promote rapid changes in laboratory method manuals. But traditional microbiology still has its place and must not be overlooked by the glamour of molecular methods. Research focused on environmental modelling of mycobacterial habitats has been shown to be a potent tool to understand the ecology of mycobacterial pathogens. The continuation of these types of studies, supported by the best available diagnostic technology and epidemiology will be the key to understanding and controlling the spread of PEM.