SECTION I

FUNDAMENTAL ASPECTS OF MYCOBACTERIAL IDENTIFICATION

Chapter 1 - INTRODUCTION

1.1. Objectives

Diagnostic mycobacteriology laboratories are challenged nowadays with increasing numbers of mycobacterial species being described. These laboratories need to be prepared to differentiate isolates to the species level in order to define the value of an isolate as a true pathogen or an environmental and potential contaminant. Mycobacterial species identification has been traditionally been based on a series of biochemical tests and phenotypic characteristics, like growth rate and pigmentation, that allow the classification of one particular strain to a group of well-defined mycobacteria. Although most of these tests are simple to perform and do not require sophisticated equipment, they are nonetheless, laborious and cumbersome to perform, delaying in many cases prompt and correct mycobacterial identification, which can be of serious consequences in the clinical field. Nevertheless, they remain and constitute the main procedure for identification of mycobacteria in clinical laboratories especially in low-resource settings.

In the last years the description of new mycobacterial species has increased due to recent advancements in mycobacterial identification and taxonomy, mostly based in a better knowledge of mycobacterial genetics. Genotypic taxonomy is typically based on the detection of highly conserved regions within the genome that harbor hypervariable sequences in which species-specific deletions, insertions, or replacements of single nucleotides are present. 16S rRNA-based mycobacterial identification has been for many years the primary technique for molecular taxonomic studies. Other techniques like the polymerase chain reaction restriction enzyme analysis (PRA) of the *hsp65* gene and more recently molecular identification based on polymorphisms on a fragment of the gene coding for the beta sub-unit of RNA polymerase (*rpoB*) are also contributing to this field.

To cope with this, mycobacterial diagnostic laboratories must be able to face this challenge by establishing a minimum set of tests that allow them to classify and correctly identify the most commonly found mycobacteria in clinical practice.

In this context and in the framework of the Concerted Action project "Improved diagnosis, drug resistance detection and control of tuberculosis in Latin America" supported by the European Commission (ICA4-CT-2001-10087), the present Manual for the Identification of Mycobacteria attempts to put in one place the basic techniques and procedures that constitute the classical phenotypic methods for the identification of mycobacteria, to-

gether with an approach to some recently introduced molecular techniques that may facilitate or shorten the time to make a correct identification. It constitutes a first attempt to link together traditional and modern identification technologies to be used in the clinical diagnostic laboratories.

1.2. Short history of mycobacteria classification

The classification of mycobacteria started in 1896 when Lehmann and Neumann proposed for the first time the genus *Mycobacterium* including *Mycobacterium tuberculosis* and *Mycobacterium leprae* placing the genus in the family Mycobacteriaceae order Actinomycetales and class Actinomycetes. The genus *Mycobacterium* is the only genus in the family Mycobacteriaceae, although there was a proposal to include *Nocardia* and *Rhodococcus* in this family (90, 205). For many years only *M. tuberculosis*, *M. bovis*, and *M. leprae* were consistently recognized as human pathogens. In 1935, Pinner applied the term "atypical acid-fast microorganisms" to isolates that caused human disease but could not be differentiated from *M. tuberculosis* on the basis of morphology, pigmentation, and virulence in animals (173).

In 1938, da Costa Cruz described and named *M. fortuitum* as a rapidly growing Mycobacterium species in a human post-inoculation abscess (43). A decade later MacCallum *et al.* described a serious skin disease caused by a very fastidious new *Mycobacterium* sp., later named as *M. ulcerans* (123). Several new descriptions of mycobacteria then followed, probably stimulated by the introduction of specific chemotherapy for tuberculosis. In 1949, Cuttino and McCabe described a fatal disseminated infection in a child caused by a newly recognized microorganism that they named "*Nocardia intracellularis*" (42) later renamed *M. intracellulare* by Runyon (190). In 1951, Norden and Linell (154) reported on a swimming pool granuloma caused by "*M. balnei*" later recognized as synonymous with the fish pathogen, *M. marinum*, that Aronson had described in 1926 (9). In 1956, Masson and Prissick described a pigmented *Mycobacterium* that caused cervical adenitis in children and later named by them as *M. scrofulaceum* (130). Then in 1953, Buhler and Pollak reported on a human pulmonary disease caused by a novel acid-fast organism that was later named *M. kansasii* (27).

These descriptions of newly recognized mycobacterial human pathogens were based on a small number of case reports. In the same period, Timpe and Runyon were systematically assembling a large collection of clinically isolated mycobacteria other than the tubercle bacilli. They applied the same term, "atypical," that Pinner had used to describe clinical isolates that did not appear to be the human tubercle bacilli.

As the emphasis shifted from the *M. tuberculosis*-oriented animal virulence tests to a more general approach of the genus *Mycobacterium*, selected biochemical tests began to be developed for some of the physiologic characteristics of these organisms. As different tests, techniques, and classification strategies proliferated in laboratories throughout the world, consolidation and integration of the voluminous information being generated became necessary. In this connection the International Working Group on Mycobacterial Taxonomy (IWGMT) established in 1967 played an important role by performing a series of cooperative studies using the recently developed principles of numerical taxonomy. The results of those studies consolidated the phenotypic taxonomy of mycobacteria (244). Over the last decade, technologies for the analysis of semantides allowed comparisons to be made between phenotypic classification and evolutionary relationships.

The ability of mycobacteria to synthesize mycolic acids that are high molecular weight β-hydroxy fatty acids with a long á-side chain allows its differentiation from the other taxa. Although historically defined as 'non-encapsulated' microorganisms, mycobacteria are now known to possess a capsule-like structure especially in slow- growing pathogenic mycobacteria (56). Also, although considered as obligate aerobes, some species and strains grow in microaerophilic conditions (163, 249). Mycobacteria include obligate parasites, saprophytes and intermediate forms, that can be isolated from clinical samples, water, soil and other environmental sources (247). The currently accepted minimal criteria for classifying bacteria in the genus *Mycobacterium* are: a) the acid-alcohol fastness, b) the presence of mycolic acids containing 60-90 carbons which by pyrolisis are cleaved to C22 to C26 fatty acid methyl esters, and c) a guanine plus cytosine (G+C) content of DNA of 61-71 mol % (118), the only exception being *Mycobacterium leprae* with a G+C content of 54 to 57 mol %. Phenotypic characteristics and biochemical tests have been used to classify mycobacteria into different species; more recently genotypic characteristics have been introduced as a tool for mycobacterial identification and classification (179). According to the latest List of Bacterial Names with standing in Nomenclature there are more than 100 recognized species in the genus Mycobacterium (61).

1.3. Users of the Manual

This Manual is mainly addressed to people working in clinical diagnostic laboratories who need to diagnose infections caused by mycobacteria. Since it is a result of an on-going collaboration among researchers in Europe and Latin America under a Concerted Action project supported by

the European Commission (ICA4-CT-2001- 10087) its first end-users will be laboratories in Latin America dealing with mycobacterial diagnosis. However, it is envisaged that it will be available to other interested parties beyond this consortium.

The Manual is divided in two sections. Section I presents fundamental aspects of mycobacterial identification. It is divided in chapters describing non-tuberculous mycobacteria (NTM) and their importance for human health, safety in the laboratory, identification of the *M. tuberculosis* complex, most frequently isolated NTM and their identification by molecular methods and by mycolic acid analysis. Most epidemiological references cited in this Manual are related to isolation of different species in Latin America and the Caribbean. Section II provides methodological procedures, including chapters dealing with practical approaches for phenotypic and genotypic differentiation of mycobacteria. It is complemented with practical tables and flowcharts to be used on the bench and a CD containing interactive and comprehensive tables for identification using phenotypic and genotypic methods.

Chapter 2 - NON-TUBERCULOUS MYCOBACTERIA AND THEIR IMPORTANCE FOR HUMAN HEALTH

Nontuberculous mycobacteria (NTM) are ubiquitous environmental microorganisms whose pathogenicity for humans and animals range from innocuous colonization to disease. Therefore, the determination of the clinical significance of a NTM detected in a clinical specimen is not always easy and requires meeting specific criteria (4). NTM may be recovered from natural and man-made environments. In both cases, biofilms may be important sources of NTM and may be responsible for pseudo- infections, pseudo-outbreaks as well as diseases and disease outbreaks. Rapid detection of pseudo-infections and diseases due to NTM is important and requires the use of molecular techniques. Eradication of sources of pseudo-infections and diseases due to NTM is not easy. In this chapter, the clinical significance of NTM, their origin, detection and eradication will be discussed.

2.1. Mycobacteria associated with disease in humans and animals

Mycobacteria may be divided into three groups on the basis of clinical criteria. The first group includes strict pathogens in man and animals i.e. members of the *M. tuberculosis* complex, *M. leprae, M. avium subsp. paratuberculosis,* and *M. lepraemurium*. These strict pathogens are generally not found in the environment. The second group consists of mycobacteria potentially pathogenic in man or animals. The majority are found everywhere in nature and may become pathogenic under special circumstances. They are, therefore, called ″opportunistic mycobacteria″ or even ″occasional pathogens″ to distinguish them from the strict pathogens. Some of them have exceptionally (e.g. *M. malmoense, M. ulcerans*) or never been isolated from the environment (e.g. *M. haemophilum*), although the epidemiological profiles of the diseases they cause suggest that they are present in nature. The third group consists of normally saprophytic species that are nonpathogenic or only exceptionally pathogenic.

The saprophytic and the potentially pathogenic species are often referred to as nontuberculous mycobacteria (NTM), paratuberculous, ″anonymous″, MOTT bacilli (for Mycobacteria Other Than Tuberculosis), or ″atypical″ mycobacteria. As Wayne (245) points out, they are actually ″typical″ bacteria of the genus *Mycobacterium* and are well known to taxonomists. They have been the subject of numerous taxonomic investigations, and the strains classified under each species form homogeneous groups that are easily identified in the laboratory. Their clinical manifestions, however, which

are often similar to those of tuberculosis, can be considered "atypical", since the etiological agent is not a member of the *M. tuberculosis* complex. The designation "nontuberculous mycobacteria" has generally been adopted although the discussion about appropriate terminology continues.

A classification of microorganisms based on risk groups has also been used to rank mycobacteria (36). It is based on the risk of different species in causing infection in man:

- Risk group I – Low risk of infection for both the individual and the community. Diseases never or rarely described in normal adults. The species are generally classified as rare pathogens.
- Risk group II – Moderate individual risk, disease with average severity existing in the community. The indicated species are generally classified as potential pathogens or opportunists.
- Risk group III – Risk of transmission by air; the disease after infection is severe and sometimes fatal. High risk for the individual, but moderate for the community. The indicated species are generally classified as strict pathogens.
- Risk group IV – High individual risk of infection and often fatal. High risk for the community. (No mycobacterial species are included in this risk-group). Example: Ebola virus.

Table 1 - Classification of mycobacterial species according to the risk of infection in humans

Risk group I		Risk group II	Risk group III
M. agri	*M. komossense*	*M. abscessus*	*M. tuberculosis*
M. aichiense	*M. lentiflavum*	*M. asiaticum*	*M. bovis*
M. alvei	*M. lepraemurium*	*M. avium*	*M. africanum*
M. aurum	*M. madagascariense*	*M. celatum*	*M. microti*
M. austroafricanum	*M. mageritense*	*M. chelonae*	*M. leprae*
M. branderi	*M. moriokaense*	*M. fortuitum*	
M. brumae	*M. mucogenicum*	*M. haemophilum*	
M. chitae	*M. neoaurum*	*M. intracellulare*	
M. chlorophenolicum	*M. nonchromogenicum*	*M. kansasii*	
M. chubuense	*M. obuense*	*M. malmoense*	
M. confluentis	*M. parafortuitum*	*M. genavense*	
M. conspicuum	*M. phlei*	*M. marinum*	
M. cookii	*M. porcinum*	*M. peregrinum*	
M. diernhoferi	*M. poriferae*	*M. paratuberculosis*	
M. duvalii	*M. pulveris*	*M. shimoidei*	
M. fallax	*M. rhodesiae*	*M. simiae*	
M. farcinogenes	*M. senegalense*	*M. scrofulaceum*	
M. flavescens	*M. smegmatis*	*M. szulgai*	
M. gadium	*M. sphagni*	*M. ulcerans*	
M. gastri	*M. terrae*	*M. xenopi*	
M. gilvum	*M. thermoresistibile*		
M. gordonae	*M. tokaiense*		
M. hassiacum	*M. triplex*		
M. hiberniae	*M. triviale*		
M. hodleri	*M. vaccae*		
M. interjectum			

Table 2 - List of species recently described (1997-2003), not yet classified (61)

M. bohemicum	*M. heidelbergense*	*M. palustre*
M. botniense	*M. holsaticum*	*M. pinnipedii*
M. caprae	*M. immunogenum*	*M. septicum*
M. doricum	*M. kubicae*	*M. shottsii*
M. elephantis	*M. lacus*	*M. tusciae*
M. frederiksbergense	*M. montefiorense*	*M. wolinsky*
M. goodii	*M. murale*	
M. heckeshornense	*M. novocastrense*	

2.2. Clinical significance of mycobacteria in pathological specimens

The strict pathogens identified in specimens are almost always responsible for disease. This is not the case for potentially pathogenic species or saprophytes, whose presence does not systematically lead to disease. The presence of an atypical mycobacterium in a clinical specimen can have three meanings:

1. The mycobacterium is the etiological agent of a mycobacteriosis, in which case microbiological diagnosis must be supported by clinical signs and repeated isolation of the mycobacterium from the same patient, or by a single isolation in the case of samples collected aseptically from closed lesions. Repeated positive direct examinations add evidence in favor of a mycobacterial infection.
2. The mycobacterium has colonized the sample but does not have any clinical significance. This frequently results from equipment (e.g. endoscopes) contaminated with mycobacteria, usually from tap water. This phenomenon is not rare and gives rise to what is called ″pseudoinfection″. Colonization of clinical specimens may also result from contact of the patient with environmental mycobacteria (e.g. contaminated water). This colonization can sometimes entail infection although that is not necessarily always the case. By definition, colonization means that there is no reaction from the host, while infection means that the host reacts by producing a skin test reaction or antibodies, but without any overt manifestion of disease. (255). Colonization as well as infection may be transient, intermittent, or prolonged.
3. The mycobaterium originates from contamination in the laboratory (e.g. decontamination solutions or contamination by another positive specimen). This is easy to verify; if the same mycobacterium is isolated from other samples handled on the same day, laboratory contamination is the explanation.

Clinicians should be aware of the fact that situations 2 and 3 occur more frequently than expected. It is important to make the distinction between colonization, infection and disease, and to refer to strict criteria in order to determine the clinial significance of a mycobacterium isolated from a pathological specimen. The American Thoracic Society has recommended standard criteria for the diagnosis and treatment of disease caused by NTM (4). Similarly, laboratory workers should also be aware that ″sample

to sample" contamination is possible. Moreover, all precautions should be taken in order to avoid contamination of clinical samples by environmental mycobacteria.

2.3. Mycobacteria in the environment

2.3.1. Mycobacteria isolated from natural and artificial environments

Mycobacteria can be found everywhere in nature and at all latitudes. They live mostly in soil but are also present in fresh and seawater. We are regularly in contact with these environmental mycobacteria, which we inhale or ingest. Consequently, colonization – temporary or permanent – of our respiratory or digestive tract by these organisms is rather common. They can also be found on the skin and in the stools of healthy subjects (174).

It is important to differentiate natural environments – which by definition have not been created by man – from human-influenced environments (urban water systems, showers, swimming pools, fish tanks, sewage, sludge, etc.).

Indeed, certain species are very common in nature, while others are mostly encountered in artificial, man- made environments.

Artificial environments are colonized by a considerable spectrum of mycobacterial species (35) some of which seem to live almost exclusively in man-made habitats. This is the case of *M. kansasii* and *M. xenopi* which to date have never been found in a natural environment but have frequently been isolated from water supplies and from sewage (223). Their natural reservoir is unknown (198). Some species are rarely found in natural conditions but frequently found in an artificial environment; this is the case of *M. marinum, M. chelonae* and *M. avium*.

As mentioned earlier (174), several studies on environmental mycobacteria suggest that *M. avium* is extremely common in natural habitats. The majority of these studies, however, associated *M. avium* with the MAC or MAI (*M. avium-intracellulare*) complexes, without having used genetic tests such as DNA probes (AccuProbe, Gen-Probe, San Diego, CA or Inno-LIPA Mycobacteria, Innogenetics, Ghent, Belgium) to identify this species.

Certain species, such as *M. gordonae*, are common both in natural and man-made environments.

The strict pathogens are isolated from the environment only after human or animal pollution; such is the case of *M. tuberculosis* sometimes encountered in waste water (223).

Finally, up to the present day, some species (e.g. *M. haemophilum, M. genavense*) have never been isolated from nature although the epidemiology of the diseases they cause suggests their presence in the environment.

2.3.2. Role of biofilms

Donlan (55) defines a biofilm as "an assemblage of microbial cells that is irreversibly associated with a surface and enclosed in a matrix". This matrix is primarily composed of polysaccharide material but other non-cellular materials (e.g. proteins) may also be found depending on the environment in which the biofilm has developed.

Biofilms may be important sources of environmental mycobacteria. However, the role of biofilms in protecting environmental mycobacteria against aggressive environmental factors, and in some cases promoting the growth of mycobacteria, is not well characterized.

Mycobacteria in biofilms found in human-influenced environments, in particular biofilms in drinking water systems, have been documented (63, 93, 199, 200). Biofilms formed in natural environments are less documented (128).

The high hydrophobicity of mycobacteria, and their antibiotic-, disinfectant-, and heavy metal resistance, allow them to form biofilms on a variety of organic surfaces (e.g. plastics, PVC, rubber, silicone, cellulose) and inorganic surfaces (e.g. copper, glass) (63, 200).

The presence of environmental mycobacteria in biofilms can therefore impact human health, as they may be responsible for contamination problems and mycobacterial diseases (see below).

2.4. Pseudo-infection

There are essentially two situations where pseudoinfections and sometimes pseudo-outbreaks or pseudo-epidemics can occur:

1. contamination during collection of the specimen (e.g. hospital contamination)
2. contamination during processing of the specimen (e.g. laboratory contamination)

In both situations, the contaminating species are not necessarily limited to NTM but may also involve members of the *M. tuberculosis* complex.

2.4.1. Contamination during collection of the specimens

Certain medical examinations and special procedures for collection of specimens require the use of sophisticated equipment e.g. flexible gastrointestinal endoscopes and bronchoscopes, which can be difficult to sterilize efficiently. They can contaminate the specimen being collected, therefore causing a pseudo-infection, without any clinical signs of the disease in the patient.

In other cases, however, these same instruments contaminated by mycobacteria from the environment and from other clinical specimens can be responsible for disease, and for the spread of mini-epidemics.

The most commonly reported cases of pseudo-epidemics in the literature are those caused by contamination of flexible endoscopes. These cases have been reviewed by Guimard and Portaels (81)

Among the NTM that contaminate endoscopes, *M. chelonae* is the most frequent. The contaminating species are not restricted to NTM but sometimes also involve *M. tuberculosis*. In general, the contamination of medical equipment by *M. tuberculosis* has its origin from patients, while that by NTM arises from tap water based solutions or reservoirs, and the automated cleaning machines used to disinfect the endoscopes (215). These machines are presumably contaminated because of the presence of a biofilm inside the machine itself (70). Despite published recommendations for the disinfection of bronchoscopes and gastrointestinal endoscopes (80, 215), many hospitals still maintain inconsistent practices for disinfection (e.g. rinsing medical equipment with non-sterile tap water after disinfection).

Although these pseudo-infections do not cause diseases, they frequently create diagnostic errors and lead to unnecessary therapeutic interventions (81).

2.4.2. Contamination during analysis of the specimen

It may happen that a specimen is contaminated during the course of its analysis in the laboratory. The most common occurrences are as follows:

1. specimen contamination by laboratory solutions: pH indicators, decontamination and antimicrobial solutions made up with non sterile water contaminated by naturally occurring mycobacteria. It is therefore absolutely essential to always sterilize by autoclave or by filtration all solutions employed for the isolation and culture of mycobacteria.

2. cross-contamination from a heavily infected specimen to other specimens that are being analyzed at the same time. In this case, for example, tubercle bacilli originating from a positive sample may contaminate a negative sample. According to Kanduma *et al.* (97), reports of laboratory cross-contamination rates range from 0.1 to 65%! In well regulated laboratories, these rates are around, or less than 1% (50, 188). Cross-contamination is more frequent when specimens are processed in batches. The cross-contaminations may result, for example, from pipettes having touched a positive specimen and contaminated subsequent specimens (68).

New rapid diagnostic methods using broth media (e.g. BACTEC®, MGIT®, BacT/Alert®) appear to have increased the possibility of laboratory cross-contamination, particularly in settings with a high TB incidence.

Internal quality control should be performed by each mycobacteriology laboratory to detect possible cross-contamination (168). This internal quality control is based on continuous monitoring of all results by comparing the yields of positive results in microscopy and cultures, and comparing the laboratory results with the clinical history of the patient (presence of clinical signs of TB).

Fingerprinting techniques should be used to confirm cross-contamination. A variety of methods of DNA fingerprintings are available for environmental mycobacteria (63) as well as for typing *M. tuberculosis* (97).

2.5. Iatrogenically and nosocomially acquired mycobacterial diseases

Post-injection abscesses generally due to contamination of the injected solutions are the most frequent iatrogenic and nosocomial infections caused by mycobacteria (174). *M. fortuitum, M. chelonae* and *M. abscessus* are well known pathogens causing sub-cutaneous or intramuscular abcesses at the site of injection or vaccination (17, 30, 229, 241, 243). Soft-tissue infection by *M. chelonae* and due to the use of jet injectors have also been reported (252).

M. chelonae, M. abscessus and *M. fortuitum* infections transmitted by mesotherapy (149), cosmetic interventions such as liposuction (133, 147) and by acupuncture have also been considered as an emerging problem (256).

M. chelonae has also been associated with specific medical procedures such as peritoneal dialyses (86) or hemodialysis (120). Reviglio et al. (184) described the first case of corneal infection by *M. chelonae* after LASIK (laser

in situ keratomileusis), a surgical procedure for correction of visual refraction problems. Holmes *et al.* (91) isolated *M. szulgai* in two cases of keratitis after LASIK and also from the ice machine used to refrigerate the solution that was used during surgery, and both isolates were identical. The air conditioning system has been implicated in one outbreak of post-LASIK keratitis caused by *M. chelonae* (71).

Due to inadequately sterilized surgical equipment or solutions, *M. chelonae, M. fortuitum* and *M. xenopi* have been implicated in surgical infections (10, 32, 52, 191, 214).

Cases of active tuberculosis and chest wall abscess due *M.chelonae* have been associated with bronchoscopy (164).

2.6. Diseases acquired through water supplies

NTM may be responsible for diseases that are not iatrogenically or nocosomially acquired but that are acquired after contact with infected water supplies. NTM survive and multiply inside biofilms in waterlines, swimming pools, whirlpools, footbaths and aquaria.

In general, infection occurs in patients who had prolonged contact with water supplies and/or with a history of skin injuries or micro-traumas.

M. marinum skin granulomas have been described in patients with a history of trauma and water/fish- related hobbies or occupations (6).

Recently, outbreaks of *M. fortuitum* and *M. chelonae* furunculosis have been reported among persons who had had footbaths and pedicures in nail salons. The isolates from footbaths and patients were indistinguishable using pulsed-field gel electrophoresis (210, 254). Shaving the legs with a razor before pedicure was a risk factor for infection (254) because it induces micro-traumas of the skin.

2.7. How to control pseudo-infection and infections due to NTM

Control of pseudo-infection and infections due to NTM depends on two main factors:

1. rapid detection of (pseudo)infections
2. eradication by appropriate disinfection.

2.7.1. Rapid detection

Rapid detection can be achieved by culturing the environmental isolates and the clinical isolates using specific culture media.

Some mycobacterial contaminants such as *M. abscessus* were isolated on fungal medium only (125) and not on mycobacterial media! It is therefore important to employ media which allow the growth of e.g. *M. chelonae* and *M. abscessus* in any control system for the detection of (pseudo)infection.

The incidence of pseudo-epidemics or mini-epidemics can be easily established by methods that enable the recognition of strain-specific markers.

As indicated above, a variety of DNA fingerprinting methods are now available. Restriction Fragment Length Polymorphism (RFLP), Pulsed Field Gel Electrophoresis (PFGE), Random Amplified Polymorphic DNA (RAPD), repeated sequences and insertion sequences have been frequently used to type environmental mycobacteria (63).

A Line Probe Assay for identification of mycobacterial species has been recently commercialized (Inno-LIPA Mycobacteria; Innogenetics, Ghent, Belgium). This test rapidly identifies most of the pathogenic and opportunistic mycobacterial species (135). Moreover, this test differentiates subgroups in *M. kansasii* and in *M. chelonae-abscessus* and *M. avium* complex, and provides rapid information on the clinical relevance of isolates belonging to these species and complexes, since it can differentiate clinically relevant isolates from environmental isolates (176).

2.7.2. Disinfection

Physicians and laboratory workers should always bear in mind that mycobacteria are much more resistant to disinfectants than other bacteria and viruses. Disinfectants that destroy most bacteria and viruses may have in general no effect on mycobacteria.

Compared to other bacteria, mycobacteria are more resistant to products such as chlorine (34), benzalkonium chloride, cethylpyridinium chloride, quaternary ammonium compounds, and phenolic- and glutaraldehyde-based disinfectants (63, 229). Among mycobacterial disinfectants, 70% alcohol and 5% phenol are noteworthy.

The effect of an antiseptic disinfectant depends upon three factors:

- the concentration of the disinfectant
- the duration of disinfection

- the concentration of mycobacteria in the solution or on the surface to be disinfected.

For example, glutaraldehyde is frequently used to disinfect endoscopes. It is important to know that the eradication of *M. bovis* and *M. tuberculosis* requires a 2% solution of glutaraldehyde to be applied for 20 minutes at 20°C (34). Tubercle bacilli are much more sensitive to disinfectants than NTM (82); therefore the eradication of NTM and in particular rapid growing mycobacteria such as *M. chelonae* or *M. fortuitum* requires much more drastic methods than tubercle bacilli.

Effective disinfection of instruments used for analysis or for specimen collection is not easy. Gubler *et al*. (80) have summarized the recommended minimum procedures for cleaning and disinfecting flexible bronchoscopes when ethylene-oxide-sterilization is not feasible. Frazer *et al*. (70) have compared the effectiveness of endoscope disinfection by automated and manual systems.

In short, the following steps are recommended:

- immediate mechanical thorough cleaning
- autoclaving of removable heat-stable parts
- cleaning with sterile detergent solution
- rinsing with sterile de-ionized water
- terminal rinsing with 70% alcohol
- compulsory drying
- high level disinfection in-between patients
- periodical quality control of disinfection by culturing on appropriate media.

Thorough cleaning is a very important step to remove biofilms in medical devices (148) or in water distribution systems, swimming pools, whirlpools, baths and aquaria. Neither disinfection nor sterilization is likely to be effective if biofilms are not removed by thorough cleaning and brushing.

Finally, any solution (drugs, anaesthetics or other products) should be free of any environmental mycobacteria (preferably by autoclave sterilization). If the solution cannot be autoclaved, sterile water must be used to prepare the solution, and the final products must be sterilized by filtration.

Pseudo-epidemics and mini-epidemics are easy to avoid if:

- all solutions employed for the collection of clinical specimens and isolation of mycobacteria are properly sterilized
- all medical and laboratory instruments are thoroughly cleaned and disinfected with adequate disinfectants used at optimal concentrations for the required amount of time.

2.8. Conclusion

Since NTM are present everywhere in the environment and sometimes colonize healthy individuals, physicians and laboratory staff should be extremely careful and take all necessary precautions in order to avoid the emergence of nosocomial infections or pseudoepidemics. False positive cultures may also originate from contamination in the laboratory. It is essential that a high level of cooperation should exist between the clinicians and the laboratory.

The laboratory must have the capability to detect all contaminations at their earliest stage, rapidly and efficiently, by relying on:

- regular statistical analysis of test results
- regular quality control tests
- efficient communication and regular meetings and discussions with the clinicians.

The role of the physician is to interpret the results of the analysis performed by the laboratory, and to make a diagnostic evaluation of the clinical significance of a mycobacterium in a specimen according to strict standard criteria. Regular feedback and discussion with the laboratory staff will permit detection of cross-contamination as well as confirmation of pseudoinfection or disease due to NTM.

Chapter 3 - LABORATORY SAFETY

3.1. Prevention of mycobacterial infection in the laboratory

Most cases of laboratory-acquired infections result from breathing aerosol particles containing viable microorganisms. Aerosols are produced by handling pathological material or cultures: opening tubes, preparing smears, transferring cultures using pipettes or loops, during centrifugation or shaking of tubes or bottles, and when culture bottles are accidentally broken. Some aerosol formation occurs even with the best techniques. Laboratory staff should take appropriate precautions.

Mycobacteriology laboratories are graded according to the type of services performed (100).

- Level I – Laboratories that collect specimens and forward them to a higher-level laboratory. They may also perform direct smears.
- Level II – Laboratories that perform Level I tasks and cultivate specimens on standard media, identify tubercle bacilli and may do susceptibility tests.
- Level III – Laboratories that perform Level II tasks and perform susceptibility tests, identify all mycobacteria, provide training, supervise proficiency tests and undertake research.

All laboratories should have a biological safety cabinet (BSC) in which clinical specimens are to be handled. Level II and III laboratories should have a containment area, for which standards of accommodation and procedures are prescribed:

- Office area for paperwork only
- Laboratory area for reading cultures and other test results.
- Containment laboratory with BSCs, for work on risk III organisms. This room should be at a negative pressure in relation to the anteroom laboratory. There should be at least 6 and preferably 12 changes of air per hour.

3.2. Biological Safety Cabinets (BSC)

BSC suitable for work with mycobacteria is a class II vertical laminar flow biological safety cabinet that blows HEPA-filtered air over the work area. It is strongly recommended that a BSC should not be installed in a mycobacteriology laboratory unless it is vented into a non-recirculating exhaust system or directly to the outside.

The airflow through the BSC is adjusted by the manufacturer to provide at least 75 linear feet per minute, and should be tested and recertified at least yearly by trained personnel. Periodic checks on the airflow may be performed with an anemometer on a quarterly basis.

The following guidelines should be followed:

- The manufacturer's handbook should be carefully observed
- The user should remember that the cabinet protects only against aerosols, not against spillage
- The user should work in the middle or rear of the cabinet, not within 15 cm of the front
- Gas should be avoided by using plastic disposable loops instead of wire loops which need flaming
- Heating racks should not be kept in the BSC as the heat produced will disturb the air flow
- Unnecessary equipment should not be kept in the cabinet
- All materials required for a period of work should be taken into the cabinet before the work is started. The hands should not be frequently moved to the outside to avoid bringing out aerosols.
- At the end of the working day the cabinet surface should be cleaned with a suitable disinfectant.

3.3. Disinfectants

- Discard jars: phenolic compounds (minimal concentration 2%) or alkalin glutardehyde (minimal concentration 2%), followed by autoclaving.
- Cleaning bench surfaces:
  - phenolic compounds (minimal concentration 2%)
  - alkaline glutardehyde (minimal concentration 2%)
  - 70% alcohol
- decontamination of BSC filters: formaldehyde gas
- emergency use:
  - 70% alcohol
  - phenolic compounds (minimal concentration 2%)

3.4. Safety procedures (60)

- Mouth pipetting is forbidden. Pipetting devices must be provided
- Disposable materials should be used wherever possible
- Syringes and needles should be avoided

- Centrifuges should be equipped with sealable (aerosol-free) safety cups.
- Used pipettes and disposable bench equipment should be placed in a suitable disinfectant, such as a phenolic compound, and then autoclaved.
- To discard specimens and cultures use metal or polypropylene containers which do not leak, and then autoclave.
- Suitable protective clothing should be provided. It should be removed before leaving the laboratory.
- Gloves should be worn when handling patients' specimens, because of HIV and hepatitis risk.
- Hands should be washed with hot water and soap after every interruption of the work. Paper towels should be used to dry hands.
- Eating, drinking, smoking and licking labels in the laboratory is forbidden.

3.5. Health control

Individuals who work in mycobacterial laboratories:

- should have an annual large-film chest X-ray and medical examination.
- should preferably have a positive Mantoux test (5TU) or provide evidence of vaccination with BCG.
- should record all laboratory accidents and report them to the Safety Officer.

3.6. Specimen and culture shipments

For posting cultures follow national and international postal regulations. Important points to notice:

- Be sure the sample is labelled correctly.
- Make sure that the request form and culture match.
- Tighten caps. Do not wax.
- Place culture in plastic sealable bag.
- Wrap culture in absorbent material such as tissues paper.
- Pack wrapped culture firmly in the box so that it cannot move and make sure it is entirely surrounded by at least 2 cm of absorbent material.
- Use containers approved by the postal regulations for this type of samples.

- Put address and telephone number of the sender outside the package.
- Include request form.

Chapter 4 - IDENTIFICATION OF *M. tuberculosis* COMPLEX AND DIFFERENTIATION OF SPECIES BELONGING TO THE COMPLEX

4.1. *Mycobacterium tuberculosis* complex

Mycobacterium tuberculosis complex contains four species included in the approved list (208): *Mycobacterium tuberculosis*, *M. bovis*, *M. africanum* and the rarely isolated *M. microti*. It also contains the vaccine strain Bacille Calmette-Guérin (BCG). Although taxonomic studies based on phenetic characteristics, the relatedness of soluble cytoplasmic antigens and DNA homology clearly indicate that they are very closely related, they are considered separate species but belonging to one single complex.

M. tuberculosis primarily infects human and primates; *M. bovis* is predominantly the cause of tuberculosis in cattle but causes disease in a wide variety of other animals including man; *M. africanum* is an heterogeneous group of isolates found mainly in equatorial Africa and having properties intermediate between *M. tuberculosis* and *M. bovis*; *M. microti* is a rare pathogen of voles and other small mammals, but more recently it was identified as the cause of disease in immunocompromised humans; Bacille Calmette-Guérin (BCG vaccine) was derived from a strain presumed to be *M. bovis* but it now shows characteristics that differentiate it from strains of that species. In addition, members of this complex not directly conforming to these species are occasionally isolated. These include strains isolated from water buffalo, cats, goats, seals and other animals.

Recently proposed subspecies include *M. tuberculosis* subsp. *canettii* (*M. canettii*) (169), *M. caprae* (7, 8, 152), and *M. pinnipedii,* the seal bacillus (38).

Identification of the *M.tuberculosis* complex is essential for diagnostic purposes. Identification of species within the *M. tuberculosis* complex is not essential but desirable. For epidemiological purposes, however, it may be important to identify not only the species but also the geographical variants (classical and Asian) of *M. tuberculosis* and the two geographical variants of *M. africanum*. It is sometimes important to identify specific genotypes using molecular methods, as RFLP (Restriction Length Fragment Polymorphism), spoligotyping, and/or MIRU-VNTR.

4.2. Molecular detection of *M. tuberculosis* in clinical samples

Detection of *M. tuberculosis* by nucleic-acid amplification (NAA)-based methods can be performed directly from clinical specimens. Besides the classical polymerase chain reaction (PCR), other methods like ligase chain reaction (LCR), transcription- mediated amplification (TMA), and strand displacement amplification (SDA), targeting either DNA or RNA have been developed and some of them are available as commercial kits:

AMPLICOR® *M. tuberculosis* Assay (Roche Molecular Systems, Branchburg, NJ) is a PCR-amplified test whose target is a 584 bp segment of the 16S-rRNA gene. Results can be obtained after 6 to 7 hs. The process can be automatically performed on the COBAS AMPLICOR equipment, but a manual version is also commercially available. The US Food and Drug Administration (FDA) have approved this method for testing on smear-positive respiratory specimens.

Amplified™ *Mycobacterium tuberculosis* Direct (MTD) Test (Gen-Probe, Inc., San Diego, Calif.) is an isothermal TMA test in which the target is the mycobacterial 16S-rRNA. The entire process is performed at 42°C and can be completed within 2.5 hs. FDA approves this method for testing on smear-positive and smear-negative respiratory samples.

LCx MTB Assay, ABBOTT LCx probe system (ABBOTT Laboratories, Abbott Park, Ill) is a LCR in which the probe is amplified instead of the target, a chromosomal gene encoding protein antigen b. The assay can be completed in 5 to 6 hs, and the manufacturer recommends its use for respiratory specimens. FDA has not approved this method and it has been withdrawn from the European market.

BD ProbeTec Energy Transfer (ET) System (DTB) (Beckton Dickinson Biosciences Microbiology Products, Sparks, Md) is an isothermal SDA process in which target sequences of IS*6110* and 16S-rRNA gene are co-amplified. Simultaneous amplification and detection is performed at a single temperature in the ProbeTec instrument, within 3.5 to 4 hs. The manufacturer recommends its use for respiratory specimens. Currently the FDA has not approved this method.

In-house protocols have also been tested in several laboratories, targeting amplification of different sequences. Most protocols use the repetitive insertion sequence IS*6110* as a target for amplification. This sequence is specific to *M. tuberculosis* complex and is present in many copies (1-25) in the genome of *M. tuberculosis* isolates. Amplification of a fragment of 123 bp (58) or 245 bp (227) from the IS*6110* element has been used for detection

and identification of *M. tuberculosis* complex isolates. However, *M. tuberculosis* clinical isolates lacking IS*6110* sequences have been isolated, mainly in Asia (262).

Protocols for in house IS*6110*-PCR are given in Section II, Chapter 3.

Currently, NAA tests cannot replace conventional methods for diagnosis and management of tuberculosis, but can be of help when done in conjunction with acid-fast bacilli (AFB) smears and culture. Sensitivity and specificity of molecular tests are excellent when testing pulmonary AFB smear-positive specimens (close to 100%). Performance is lower for AFB smear-negative and extrapulmonary specimens. Considering this, NAA tests will have the greatest benefit in situations where disease caused by NTM is a concern, in which case they can have an impact on patient management and infection control practices (257).

A detailed literature review of publications about the performance of molecular tests for tuberculosis diagnosis is out of the scope of this Manual. Recent reviews concerning commercial and in-house molecular tests, both on respiratory and non- respiratory specimens, have been published by Piersimoni and Scarparo (170); Pai *et al*. (161); Palomino *et al*. (162); Suffys *et al*. (221); and Noordhoek *et al*. (153).

4.3. Differentiation of *M. tuberculosis* complex from other mycobacteria

Differentiation of *M. tuberculosis* complex from NTM is very important for laboratories performing cultures, otherwise they have to report culture positive as AFB only. For this type of laboratories few tests could be used to help differentiate the two groups.

The first step is to examine a ZN-stained smear of all suspicious colonies in cultures to confirm the presence of mycobacteria, to check for purity, and to observe cord formation.

The second step is to examine the colony aspect, verify pigment production, and recognize the morphological characteristics of the *M. tuberculosis* complex. At this point, an experienced technician can usually say whether an AFB is likely to be a member of *M. tuberculosis* complex or one of the other species of mycobacteria.

Then, to confirm identification, a screening method should be used to separate *M. tuberculosis* complex from NTM. For this purpose, a bacterial suspension should be inoculated in media containing ρ-nitobenzoic acid

(PNB) and for the nitrate test. Pick up a few colonies from the original culture for freezing. Use the original tube to perform the niacin test. If growth is not enough for the niacin test, make a subculture from the original tube. For separation of *M. tuberculosis* from NTM follow Table 3.

Table 3 - Differentiation between *M. tuberculosis* complex and NTM

Cord-formation acid-fast bacilli	Growth on PNB	Niacin	Nitrate	Pigment	Identification
+	-	+	+*	absent	*M. tuberculosis* complex
-	+	-/+	-/+	present or absent	NTM

** M. bovis and some M. africanum are negative*
-/+ = mostly negative
PNB = susceptibility to ρ-nitobenzoic acid

In the last decade, major advances in the understanding of the genetic structure of mycobacteria have been made. As a consequence, several genetic probes and amplification systems for diagnosis of tuberculosis have been developed.

Identification of *M. tuberculosis* in culture can be performed using molecular probes. These methods are not very sensitive for direct confirmation from clinical specimens, because they need more than 10,000 organisms in the specimen for positivity.

AccuProbe® MYCOBACTERIUM TUBERCULOSIS Complex Culture Identification Test (Gen Probe, Inc. SanDiego, Calif) can be used for culture confirmation of *M. tuberculosis*. The test is based on degradation of acridinium on the probe, which hybridize with the 16S-rRNA. Results are obtained by using the Gen-Probe's luminometer. The test can give results in about 2 hrs from culture-positive specimens.

INNO-LiPA RIF.TB Assay (Innogenetics NV, Gent, Belgium) consists of a reverse- hybridization method in which the probe is represented by a 70 bp amplicon of the gene encoding the β-subunit of the RNA polymerase (*rpoB* gene). The label (biotin) is incorporated in the second round of amplification of a nested PCR. The amplified biotinylated products are hybridized with 10 oligonucleotides immobilized probes on a membrane-based strip. Finally, hybrids are detected through a colorimetric reaction. LiPA can detect the presence of *M. tuberculosis* and its resistance to rifampicin in the same test, which can be completed in 12 hs.

4.4. Differentiation of members of *M. tuberculosis* complex

Differentiation of members of the *M. tuberculosis* complex can be important in specific clinical settings, for the management of individual patients, and for epidemiological purposes. Several phenotypic and molecular strategies can be used for confirmation of the identity of *M. tuberculosis* complex isolates.

4.4.1. Mycobacterium tuberculosis

Mycobacterium tuberculosis is the most frequent agent of mycobacterial disease in the world and is the pathological agent of tuberculosis. Robert Koch first described it in 1882. The great majority of patients with symptoms compatible with the diagnosis of pulmonary tuberculosis, whose respiratory samples (sputum, bronchial washing, etc) show acid-fast bacilli, are indeed suffering from the disease. However, other mycobacterial infections of the lungs are known to occur in significant numbers; it is therefore recommended to confirm the diagnosis by culture.

The colonies of *M. tuberculosis* in solid media have a rough aspect, and resemble breadcrumbs or cauliflowers. There is no preference for glycerol or pyruvate media. The bacilli are 2-4 x 0.3-0.5 µm, but occasionally shorter or longer cells are observed. The smear from a culture usually shows cord formation. Growth is restricted to temperatures between 34 and 38º C. This species is aerobic, nitratase positive and susceptible to pyrazinamide and cycloserine. It is usually resistant to 2-thiophen- carboxylic acid hydrazide (TCH) and sensitive to ρ-nitrobenzoic acid (PNB). It is usually niacin positive though there are a few exceptions. Isoniazid-susceptible strains give a strongly positive catalase reaction but isoniazid-resistant strains often give weak or negative reactions.

4.4.2. Mycobacterium bovis

The bacilli are generally shorter than those of *M. tuberculosis* usually arranged in clumps but there are fewer tendencies of showing cord formation. The colonies on solid media are flatter and smoother than the human variant. Growth on pyruvate media is usually much better than that on glycerol media. Growth is restricted to temperatures between 34 and 38º C. This species is microaerophilic, susceptible to TCH and cycloserine but resistant to pyrazinamide. The niacin test is negative.

4.4.3. Mycobacterium africanum

There are two major variants of *M. africanum*. The African I variant, mainly of West African origin, is nitratase negative, the colonies in solid media resemble those of *M. bovis*, but may not show preference for pyruvate. The African II variant, of East African origin, is nitratase positive and colonies resemble those of *M. tuberculosis*. Both are microaerophilic and are susceptible to TCH, pyrazinamide and cycloserine. The niacin test for both is variable. Growth is restricted to temperatures between 34 and 38° C.

4.4.4. Bacille Calmette-Guérin (BCG)

This vaccine strain is derived from a bovine strain. However, it shows cultural and morphological characteristics similar to those of *M. tuberculosis*. There is no preference for pyruvate. It is nitratase negative (or weakly positive), aerobic, susceptible to TCH but resistant to pyrazinamide and cycloserine. Niacin reaction is negative.

4.4.5. Mycobacterium microti

This species is very rarely isolated from clinical specimens. According to Yates, 1984, the few strains existing in culture collections have the characteristics of the African I variant of *M. africanum*. Human isolates have been recently characterized using novel genetic markers (235). AFB are sometimes curved.

4.4.6. Mycobacterium canettii

M. canettii was added to the list of *M. tuberculosis* complex organisms in 1997. It shows a smooth-colony, and there are few reports of its isolation until now. It gives negative niacin results and positive nitratase reduction. It is resistant to TCH, pyrazinamide and streptomycin.

4.4.7. Mycobacterium caprae

This is a strain that occurs primarily in Spanish goats. It is an *M. tuberculosis* complex organism, showing preference to grow in medium with sodium pyruvate instead of glycerol.

4.4.8. Mycobacterium pinnipedii

It was originally isolated from cases of tuberculosis in sea lions, seals and Brazilian tapir. It is an *M. tuberculosis* complex organism, showing preference to grow in medium containing sodium pyruvate instead of glycerol.

4.4.9. Phenotypic and genotypic approaches for differentiation of members of the M. tuberculosis complex

Differentiation of members of the *M. tuberculosis* complex can be achieved by analysis of phenotypic and genotypic special tests. Useful phenotypic characteristics are shown in Table 4.

Table 4 - Characteristics of species belonging to *M.tuberculosis* complex

Tests	*M. tuberculosis*	*M. bovis*	BCG	*M. africanum*	*M. microti*	*M. canettii*
Colony morphology	eug rough	dys rough	eug rough	dys rough	dys rough	eug smooth
SP > glycerol	-	+	+	-	-	-
PZAse	+	-	-	+	+	+
Niacin	+	-	-	+/-	+	-
Nitrate	+	-	-	+/-	-	+
Urease	+	-	+	+/-	+/-	+
TCH	R	S	S	S	S	R
SM	S	S	S	S	S	R
O_2 preference	aerobic	micro	aerobic	micro	micro	ND

+/- = variable result, R = drug resistant, S = drug susceptible, ND = no data, eug = eugonic, dys = dysgonic, micro = microaerophilic, SP>glycerol = sodium pyruvate growth preference, PZAse = pyrazinamidase, TCH = susceptibility to thiophen-2-carboxylic acid hydrazide, SM = susceptibility to streptomycin.

Protocols for these tests are shown in Section II, chapter 2.

Genotypic methods that have been used for the discrimination of the members of this complex are described below.

Spoligotyping is a PCR-based method, developed for fingerprinting of *M. tuberculosis* strains (96). It is based on polymorphisms in the direct repeat (DR) region that depend on the presence or absence of specific spacer regions sequences between two DR regions. Specific spacers for differentiation between members of *M. tuberculosis* complex have been identified. *M. tuberculosis* does not hybridize to spacers 33 to 36, *M. bovis* and *M. bovis* BCG do not hybridize to spacers 39 to 43 (96). Many strains of *M. microti* hybridize only to spacers 37 and 38 (235), while *M. africanum* does not hybridize to spacers 8, 9, and 39 (240).

***GyrB*-RFLP** differentiates *M. tuberculosis*/*M. africanum* II from *M. africanum* I/*M. pinnipedii, M. microti,* and *M. bovis*/*M. bovis* BCG. After PCR-amplification, amplicons are subjected to restriction using *Taq*I and *Rsa*I enzymes. The band patterns are analyzed after agarose gel electrophoresis (99, 151).

Deletions - Comparative genomics studies employing different methods revealed the existence of regions of difference (RD) representing the loss of genetic material in *M. bovis* BCG compared to *M. tuberculosis* H37Rv (16, 77). Some RD loci were shown to be restricted to a single *M. tuberculosis* complex member, while others appear to be differentially distributed among them (24). PCR-amplification of RDs has been used for differentiation of members of *M. tuberculosis* complex (92, 166).

Multiplex PCR includes primers for amplification of genus specific *hsp65* gene, *M. tuberculosis* complex specific IS*6110*, *M. bovis*/*M. africanum* I specific spacer 33/34 (99 bp) and *M. bovis* BCG specific spacer 33/34 (172 bp). The analysis of band patterns generated after PCR-amplification allows differentiation between *M. tuberculosis*/*M. africanum* II, *M. bovis*/*M. africanum* I and *M. bovis* BCG (260).

***mtp40*-PCR** - The *mtp40* sequence is present in most but not all isolates of *M. tuberculosis*, *M. africanum*, and *M. microti* and is consistently absent in *M. bovis* and *M. bovis* BCG isolates. Therefore, it has a potential use for differentiation between *M. tuberculosis* and *M. bovis*/*M. bovis* BCG (51). However the recent finding of *M. tuberculosis* clinical isolates lacking the *mtp40* sequence must be considered when interpreting the results (239). The product of *mtp40* was detected in mycobacteria isolates from seals (119) and in *M. tuberculosis* subsp. *canettii* (24), but not in *M. bovis* subsp. *caprae* (152).

Detailed protocols for *gyrB*-RFLP and *mtp40*-PCR are shown in Section II, chapter 3.

Chapter 5 – MOST FREQUENTLY ISOLATED NTM

5.1. *Mycobacterium abscessus*

- **General characteristics**: the previously named *M. chelonae* subsp. *abscessus* was recognized as new species in 1992 by Kusunoki *et al*. (108). *M. abscessus* is a non-chromogenic rapidly growing mycobacteria characterized by its inability to grow at 42°C. It grows on McConkey agar and 5% NaCl, does not produce nitrate reductase and is unable to utilize citrate as the sole carbon source. Colony morphology is undistinguishable from *M. chelonae* (Table 6). This species has two PRA patterns (53).
- **Epidemiology**: *M. abscessus* is found in the environment. The most common source is drinking water, but it can also be found in soil. In humans it causes skin and soft tissue infections. *M. abscessus* is the second non-tuberculous mycobacterium more commonly isolated from respiratory infections, after *M. avium* in patients with cystic fibrosis, and is second in frequency after *M. fortuitum* associated to extra pulmonary sites of infection. It doubles the frequency of *M. chelonae* as a cause of extra pulmonary infections. Most of the post-injection cases that have been described are associated to *M. abscessus* (265). It was implicated in outbreaks of post-injection abscesses in Colombia, one of them involving a total of 297 persons (30, 59, 241) and in post-surgical infections after liposuction and liposculpture in Venezuela (147).

5.2. *Mycobacterium asiaticum*

- **General characteristics**: *M. asiaticum* was first isolated from lymph nodes and viscera of healthy monkeys imported from India and kept at the research institute of Hungary (98). The species description was published in 1971 by Weiszfeiler *et al* (250). It is a photochromogen slow grower and can be differentiated by its positivity in the Tween hydrolysis test, and its negative result for niacin and nitrate reduction. Table 10. shows some characteristics of *M. asiaticum* among the other photochromogenic slow growers. This species shows one PRA pattern (26).
- **Epidemiology:** the first indication that *M. asiaticum* was pathogen for humans was described in 1983 from five Australian patients with pulmonary disease (18).

5.3. *Mycobacteriun avium* complex

- **General Characteristics:** *M. avium* complex (MAC) consists of two closely related slow growing species *M. avium* and *M. intracellulare* that are non-pigmented and that do not hydrolyze Tween 80. Distinction between these species can be ultimately achieved by using DNA probes based on rRNA gene sequences, rather than biochemical tests (AccuProbe, Gen Probe). However, some of the phenotypic characteristics such as arylsulphatase activity and growth at 45°C may allow initial differentiation. Three different subspecies have been ascribed to *M. avium* species, namely *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis* and *M. avium* subsp. *silvaticum*. These three subspecies have phenotypic, genotypic and epidemiologic differences (179, 228, 248). *M. avium* isolates from humans and other mammals were recently designated *M. avium* subsp. *hominissuis* and separated from *M. avium* bird-type isolates named *M. avium* subsp. *avium* (134). As a complex they can be readily identified using biochemical characteristics (Table 5). Other tools that have been used for identification are analysis of mycolic acids using high-pressure liquid chromatography (HPLC), thin layer chromatography and analysis of cell wall fatty acids by gas liquid chromatography. *M. avium* and *M. intracellulare* can be distinguished by PRA, and different PRA patterns have been described for both species (110, 157, 222). Smole *et al*. recently published a detailed review about 15 different PRA types of MAC. They identified 3 PRA types of *M. avium* and 2 of *M. intracellulare,* which represent the clinically significant isolates, and 10 PRA types related to bacteria named "MAC other", found mostly in the environment (209). Colonies are mostly smooth, flat and transparent; however, opaque colonies are also seen, especially when grown on Löwenstein-Jensen medium with prolonged incubation periods. Opaque colonies tend to develope yellow pigmentation when cultures are kept at room temperature.
- **Epidemiology:** *M. avium* is isolated from man-made environments as potable water, which is believed to be a common source of infection for AIDS patients. Several studies have demonstrated the presence of the same strain in tap water and in patients infected with *M. avium* in diverse settings (140, 242). This species is also a common etiological agent of infections in pigs and poultry and similarities have been found between animal and human strains.

However, transmission from animals to humans does not appear to be important (129). Most infections caused by MAC in AIDS patients are caused by *M. avium.* In contrast, *M. intracellulare* has been found more frequently causing diseases in non-AIDS patients. For this reason distinction between both species is recommended, especially in the context of AIDS (174). In immunocompetent patients pulmonary diseases are more frequent in patients with previous pulmonary damage (cystic fibrosis, reduced pulmonary function), and cervical lymphadenitis, which is a sporadic disease in children. In immunodeficient patients, particularly in AIDS patients, the most common manifestation of disease is mycobacteremia associated with low CD4 cell counts and higher mortality. MAC is the predominant NTM species causing mycobacteremia in HIV positive patients (1, 67, 159) and is the most frequently mycobacteria isolated from other specimens in AIDS patients in Latin America (15, 41, 177). MAC is also the most frequent NTM isolated from HIV negative patients In Latin America, either as a contaminant or implicated in disease (12, 13, 76, 83, 116, 202). Implementation of highly active antiretroviral therapy (HAART) had a protective effect against infection by MAC but did not protect against MAC colonization (73).

Table 5 - Differential biochemical tests for *M. avium* and *M. intracellulare*

Tests	***M. avium***	***M. intracellulare***
Growth at 25°C	+/-	+
Growth at 45°C	+/-	+/-
INH	+	+/-
Niacin	-	-
Catalase 68°C	+/-	+
Arylsulfatase	-	+/-

+/- = mostly positive
INH = isoniazid

5.4. *Mycobacterium celatum*

- **General characteristics**: *M. celatum* was first described in 1993 (29) and the strains were from clinical origin, specially from the respiratory tract and also from blood. Biochemical characteristics of this microorganism are similar to those of *M. xenopi*. Colonies have been described as non-pigmented but the majority of strains are pale yellow. The most important distinctive biochemical test seems to be positive arylsulfatase activity. Growth at 45°C is negative,

which differentiates it from *M. xenopi.* Two PRA patterns of *M. celatum* are included in the PRASITE (http://app.chuv.ch/prasite)

- **Epidemiology**: *M. celatum* has been reported to cause infection in AIDS patients. Recently Piersimoni *et al.* (171) reported a case of serious pulmonary infection in an apparently immunocompetent patient.

5.5. *Mycobacterium chelonae*

- **General characteristics**: *M. chelonae* was first described in the Bergey's manual in 1923. In 1953, Moore and Frerichs recovered mycobacteria from a knee abscess (141). The authors identified it as a new species *M. abcessus*. Stanford (219) and Pattyn (167) first reported studies on clinical isolates and among them were isolates from post-injection abscess outbreaks in Holland and England. This study resulted in the official name *M. chelonae*. *M. chelonae* is a nonchromogen rapidly growing mycobacteria. Colonies are mucoid rather than rough and appear in the first week of incubation using conventional culture media. Biochemical characteristics are positive arylsulfatase test in 3 days, growth on McConkey without crystal violet, negative iron uptake and nitrate reduction tests (Table No. 6). This species should be distinguished from other potential non-pigmented rapidly growing pathogenic species such as *M. fortuitum* and *M. abscessus*, since treatment may vary according to the species. *M. chelonae* has a unique PRA pattern described by Telenti *et al*. (226) which is different from the pattern described by Steingrube *et al*. (220). Devallois *et al*. (53) reported both PRA patterns of *M. chelonae*.
- **Epidemiology**: This species is abundant in man-made environments like drinking water but has also been recovered from lakes, freshwater rivers, seawater, and wastewater systems. Its widespread presence in the environment makes this species a common contaminant in the laboratory. For the same reason it can contaminate solutions, medical equipment and surgical wounds. In humans it is associated to pulmonary, soft skin, or disseminated infections, mainly in patients with risk factors such as kidney transplantation, surgery, corticosteroid therapy etc. (31, 95, 256). It has been recently implicated in several outbreaks after ophthalmologic surgery (LASIK, cataract, trauma) (3, 71) and aesthetic procedures (inoculations, plastic surgery, mesotherapy) (133).

5.6. *Mycobacterium flavescens*

- **General characteristics**: *M. flavescens* has been described by Bojalil *et al.* (22). *M. flavescens* is a schotochromogen mycobacterium with intermediate growth rate (7-10 days). Colonies are smooth and yellow-orange pigmented and growth can be observed between 25 and 42°C. Biochemical characteristics are positive nitrate reductase, hydrolysis of Tween, and growth in the presence of 5% NaCl, negative iron uptake, and variable tellurite reduction test. Four different PRA patterns were described for this species: *M. flavescens* I and II in Telenti *et al.* (226) and Devallois *et al.* (53), *M. flavescens* III in the PRASITE (http://app.chuv.ch/prasite), and a new pattern described by da Silva Rocha *et al.* (46).
- **Epidemiology**: *M. flavescens* is an environmental mycobacterium. It can be a common isolate in clinical specimens, although most of the time it is considered as non-pathogenic. Nevertheless, several reports have associated this species to human respiratory, skin and disseminated infections.

5.7. *Mycobacterium fortuitum*

- **General characteristics**: *M. fortuitum* has been described by da Costa Cruz in 1938 (43). *M. fortuitum* is an environmental nonchromogen rapidly growing mycobacteria. Main biochemical characteristics are positive arylsulphatase test at 3 days, growth on McConkey without crystal violet, growth at 42°C and in the presence of 5% NaCl, nitrate reductase activity positive and they are unable to utilize inositol and mannitol as carbon sources. Colonies are non-pigmented with rough aspect rather than smooth. Tests with carbohydrates and sodium citrate are useful for separation of bacteria classified as belonging to *M. fortuitum* complex by traditional identification (20) (Table No. 6). Two PRA patterns are present in the PRASITE (http://app.chuv.ch/prasite). Steingrube *et al.* (220) and da Silva Rocha *et al.* (46) analyzed different isolates of *M. fortuitum*, and described additional PRA patterns.
- **Epidemiology**: This species has been recovered from drinking water, water distribution systems and from a variety of soil worldwide (14, 113, 160). In humans, it is frequently associated to cutaneous infections (156, 182, 183) and rarely to pulmonary or disseminated disease (37). It causes infection after mammoplasty and other plas-

tic surgery procedures (261) and is a common etiological agent in sternal wound infections after cardiac surgery. In some outbreaks of *M. fortuitum* infection, an environmental source has been identified.

Table 6 - Differential biochemical tests for rapid growers

Tests	*M. fortuitum*	*M. peregrinum*	*M. chelonae*	*M. abscessus*
Arylsulphatase	+	+	+	+
NaCl	+	+	-	+
Nitrate	+	+	-	-
Iron uptake	+	+	-	-
β-galactosidase	-	-	+	-
Citrate	-	-	+	-
Mannitol	-	+	-	-

5.8. *Mycobacterium gastri*

- **General characteristics**: *M. gastri* was isolated from human gastric lavage specimen, or sputum specimen as a casual resident, not considered an etiologic agent of the disease (246). *M. gastri* is a nonpigmented, slowly growing mycobacterium. Phenotypic characteristics are similar to *M. kansasii*, although differentiation between both species can be achieved based on pigmentation, nitrate reduction, Tween hydrolysis and catalase tests (Table 7. Sequencing of the 16S rRNA confirms that both species are closely related. Colonies are smooth to rough and growth is observed in a range of 25°C to 40°C. A unique PRA pattern has been described for this species (PRASITE - http://app.chuv.ch/prasite)
- **Epidemiology**: *M. gastri* has been isolated from gastric lavage specimens, occasionally from sputum and from soil, but it is not a common isolate in the clinical laboratory. In general it is considered nonpathogenic for humans, although it has been occasionally implicated in infections as peritonitis, arthritis and seminal vesiculitis.

Table 7 - Differential characteristics between *M. gastri*, *M. gordonae* and *M. kansasii*

Tests	*M. kansasii*	*M. gordonae*	*M. gastri*
Tween	+	+	+
Nitrate	+	-	-
Catalase SQ	+	+	-
Urease	+	-	+
Pigmentation	photochromogen	scotochromogen	non-chromogen

SQ = semiquantitative

5.9. *Mycobacterium genavense*

- **General characteristics**: *M. genavense* was first isolated in 1992 (23) from the blood of an AIDS patient in Geneva, Switzerland (the name is derived from the city Geneva). Because of its failure to grow in Löwenstein Jensen medium, 7H11 agar or other classical solid media routinely used for identification of mycobacteria, many laboratories miss its identification. *M. genavense* was first isolated in BACTEC 13A medium. Middlebrook 7H11 agar supplemented with mycobactin supports the growth of *M. genavense* (40) and Middlebrook 7H10 medium requires human blood (124). For primary isolation, microaerophilic conditions promote growth of *M. genavense* (2,5% or 5 % oxygen) in liquid medium as Middlebrook 7H12 at pH 6.0 (180). Mycolic acids are identical to those of *M. simiae* and *M. malmoense* (α α' k mycolates). One PRA pattern was described by Telenti *et al.* (226).
- **Epidemiology**: the source of *M. genavense* in humans is unknown, although an intestinal source has been proposed (40). *M. genavense*, most commonly, causes disease in AIDS patients. Infection by *M. genavense* may be undetected because of the slow growth and fastidious requirements of the organism. It may occur in combination with other mycobacteria, like *M. avium* complex, in infections in AIDS patients. A case of *M. genavense* disseminated infection was reported by Hadad *et al.* (84).

5.10. *Mycobacterium gordonae*

- **General characteristics**: *M. gordonae* is extremely common in the environment and the 16S rRNA sequence comparison has demonstrated a separation between *M. gordonae* and *M. asiaticum* (186,

218). *M. gordonae* is a slowly growing schotochromogenic mycobacterium. This species hydrolyses Tween 80, is not able to reduce nitrate, is urease negative and catalase positive (Table 7). Colonies are smooth and yellow or orange pigmented, optimal growth is observed at 37°C but it is able to grow in a range from 25 to 37°C. *M. gordonae* is a highly polymorphic species. Several PRA patterns have been described. Telenti *et al*. (226) identified five patterns, and in the PRASITE there are now nine different patterns. Additional patterns have recently been described by da Silva Rocha *et al*. (46) and Häfner *et al*. (85). *M. gordonae* I and III are the most frequent alleles in most studies.

- **Epidemiology**: *M. gordonae* is an environmental mycobacterium found commonly in water (150). This species is the most common contaminant in clinical mycobacteriology laboratories. It is considered nonpathogenic. However, several reports have implicated this species in human infections, and few of them have enough information to confirm *M. gordonae* as the etiologic agent (189).

5.11. *Mycobacterium haemophilum*

- **General characteristics**: *M. haemophilum* was first described in 1978 in Israel by Sompolinsky *et al*. (212) as the cause of cutaneous infections in patients with Hodgkin's disease. *M. haemophilum* is a slowly growing mycobacterium that has special requirements for growth, as iron supplementation (hemin or ferric ammonium citrate). Most commonly, Löwenstein-Jensen medium with 2% ferric ammonium citrate or Middlebrook 7H10 or 7H11 agar supplemented with 39 µg/ml of hemin is used. The optimal temperature for growth is between 30°C and 32°C, like *M. marinum, M. ulcerans*, also cutaneous pathogens. Table 8 summarizes useful phenotypic/biochemical tests for identification of this species. This table is a compilation from different references (62, 102, 175, 196). One PRA pattern was described for this species (226).
- **Epidemiology**: *M. haemophilum* is a pathogen of immunocompromised patients, particularly those with AIDS and organ transplants (renal transplantation). Sampaio *et al.* (193) described the isolation of *M. haemophilum* from three patients in Brazil.

Table 8 - Differential biochemical tests for *M. haemophilum*

Tests	*M. haemophilum*
Growth at 25°C	+
Growth at 37°C	+/-
Growth at 45°C	-
Pigment	-
Niacin	-
NaCl	-
Hemin, iron supplement	+
INH	+
PZAse	+
Acid phosphatase	-
Nitrate	-
Urease	-
β-galactosidase	-
Catalase (semi quantitative and 68°C)	-
Tween	-
Tellurite	-
Arylsulfatase	-
Fatty acid pattern	α, m, k mycolates

INH = isoniazid, PZAse = pyrazinamidase

5.12. *Mycobacterium kansasii*

- **General characteristics**: Since the description of *M. kansasii* in 1955 (89) a number of reports documented the presence of *M. kansasii* in water samples. *M. kansasii* is a slow growing photochromogen mycobacterium. It grows at temperatures between 32°C and 42°C, better at 37°C but not at 45°C. The main biochemical characteristics are production of catalase, urease, pyrazinamidase, nitrate reductase and the ability to hydrolyze Tween 80 (Table No. 10). Colonies are rough or smooth with irregular edges. Genetic diversity has been demonstrated among isolates by using specific DNA probes, rRNA sequence and RFLP with a DNA probe made from a repetitive sequence (2). Alcaide defined five PRA types of *M. kansasii* after a comprehensive study of a collection of 276 isolates. *M. kansasii* I represents the most common isolate from humans.
- **Epidemiology**: *M. kansasii* has been recovered mainly from potable water, rarely from soil. In humans, it causes disseminated or pulmonary disease in immunodeficient patients (213). However, pulmonary disease is the characteristic form of the disease in im-

munocompetent patients (206). Disease caused by this species has been described worldwide, but predominantly in patients from urban areas (66).

5.13. *Mycobacterium lentiflavum*

- **General characteristics**: *M. lentiflavum* was described in 1996 (217) on the basis of 22 isolates. Out of 11, 4 were from gastric fluid, 4 from sputum, 2 from urine and 1 from a biopsy specimen. The others were associated with a contaminated bronchoscope. *M. lentiflavum* is a slow grower schotochromogen, which grows better at temperatures between 22 to 37°C. Most biochemical characteristics are negative and, because of this, biochemical identification is not possible. TLC features are not distinguishable from other species as *M. simiae, M. genavense*, *M. intermedium* and *M. malmoense*, while HPLC patterns are slightly different from those of *M. simiae*. Four PRA patterns have been described in the PRASITE (http://app.chuv.ch/prasite).
- **Epidemiology**: This species has not been isolated from the environment. However, many isolates from humans do not have a clear clinical significance. Nevertheless, several cases of human infections have been described recently such as cervical lymphadenitis, cavitary pulmonary disease and disseminated infections. There is a report of isolation of *M. lentiflavum* I in Brazil, without confirmation of involvement in disease (45).

5.14. *Mycobacterium leprae*

- **General characteristics**: *Mycobacterium leprae*, which causes leprosy, now denominated Hansen disease, was discovered by G.A. Hansen in 1873, being the first bacterium to be identified as causing disease in humans. *M. leprae* differs from all other mycobacteria in that it has never been cultivated in vitro. *M. leprae* is extremely slow to grow in the mouse model, it takes 20-30 days to divide and its optimum temperature for growth is around 30°C. It is possible to detect acid-fast bacilli in the skin, nodules, plaques, and in the nasal mucosa of infected patients by microscopic examination. The polymerase chain reaction (PCR) has also been applied for detection of *M. leprae* in clinical specimens (94). Inoculation into the

mouse footpad has also been used for confirmation of the presence of *M. leprae* in clinical samples. It presents a unique PRA pattern (172, 178).

- **Epidemiology**: It is the only mycobacterial species infecting peripheral nerves. Humans seem to be the main natural host of *M. lepra*e, although the nine-banded armadillo and nonhuman primates can also naturally develop the disease. Experimental infection in these animals has been used to provide a source of bacterial material for research purposes. Disease caused by *M. leprae* remains highly endemic in some areas of the world. Brazil, India, Madagascar, Mozambique, Myanmar, and Nepal account for 90% of the prevalence of the disease in the world in early 2002 (259).

5.15. *Mycobacterium malmoense*

- **General characteristics**: *M. malmoense* was first described in 1977 (197) in Sweden, in the city of Malmö, on the basis of the unique set of biochemical and culture characteristics shared by seven isolates. Laboratory identification of *M. malmoense* is sometimes difficult because it can be confused with other slowly growing nonpigmented mycobacteria like *M. gastri, M. avium* complex, or *M. shimoidei*. The Tween 80 hydrolysis test is positive and allows rapid differentiation from MAC. However, low-pH and pyruvate containing media are recommended. Analyses of fatty acids and mycolic acids are very useful for confirmation of identification of *M. malmoense,* with a characteristic pattern of α-mycolates, α'-mycolates and keto-mycolates. Table 9 shows two characteristic tests of *M. malmoense* that differentiate it from other non- pigmented slowly growing mycobacteria. There are two distict *M. malmoense* PRA types in the PRASITE (http://app.chuv.ch/prasite). An additional pattern was described (46).
- **Epidemiology**: *M. malmoense* has only sporadically been recovered from the environment. The organisms have been isolated from natural waters in Finland and soil in Japan (93,192). *M. malmoense* is a pathogen causing pulmonary disease; infection is restricted mainly to the lungs. In immunodeficient patients *M. malmoense* is often disseminated (264). It is possible that *M. malmoense* is a human commensal.

Table 9 - Differential biochemical tests for non-chromogen slow growers

Tests	*M. malmoense*	*M. gastri*	*M. avium* complex	*M. shimoidei*
Tween	+	+	-	+
Acid phosphatase	-	+	-	+
Urease	+	+	-	-
Catalase	+	-	-	-

5.16. *Mycobacterium marinum*

- **General characteristics**: *M. marinum* was first described in 1926 by Aronson *et al*. and was isolated from diseased fish and aquaria. *M. marinum* is a slow grower photochromogen, that shows better growth between 25-32ºC. Colonies are smooth and flat and the pigmentation can be observed either at 25ºC or at 32ºC (it can be maintained at 37ºC after several subcultures). The main biochemical characteristics are: negative nitrate reduction, negative semi-quantitative catalase, positive hydrolysis of Tween 80 (most but not all strains), positive arylsulphatase and urease tests (Table No. 10, 14). Only one PRA pattern was described (226).
- **Epidemiology**: *M. marinum* is an environmental mycobacterium, mostly associated to infections in fish. In humans, these infections are related to exposure to contaminated water or animals (114). Most of the infections are in extremities in the form of skin lesions (33, 54, 66, 79, 142, 195, 233), but disseminated infections have been sporadically described in immunodeficient patients.

Table 10 - Differential biochemical tests for photochromogens

Test	*M. kansasii*	*M. marinum*	*M. simiae*	*M. asiaticum*
Tween	+	+/-	-	+
Nitrate	+	-	-	-
Niacin	-	-/+	+	-
PZAse	-	+	-/+	ND
Urease	+	+	-/+	-

PZAse = pyrazinamidase, +/- = mostly positive, -/+ = mostly negative, ND = no data

5.17. *Mycobacterium mucogenicum*

- **General characteristics**: In 1982, *M. mucogenicum* was first called *M. chelonae- like organism* (MCLO) (11) because it is biochemically

similar to *M. chelonae*. In 1995, a genetic investigation, based on 16S rRNA revealed its diversity from other rapid growers and the status of a new species, *M. mucogenicum,* was proposed (216). *M. mucogenicum* is a non-chromogenic rapid grower. It has typical smooth and slighty non-pigmented mucoid colonies, but it is very difficult or impossible to identify correctly by using only conventional biochemical tests. The mycolic acid patterns are identical to that of some slow growing mycobacteria *(M. terrae, M. xenopi)* but it has a unique pattern among the rapid growers (146). For definitive identification of *M. mucogenicum*, analysis by TLC, HPLC or molecular biology may be necessary. Two different PRA patterns were identified by Steingrube *et al.* (220). An additional pattern, named V, was described by Häfner *et al.* (85).

- **Epidemiology:** *M. mucogenicum* is commonly recovered from tap water and most strains are mucoid (giving origin to its name). It is rarely seen in human infections, may cause nosocomial infections and, most commonly, post- traumatic wound infections (243).

5.18. *Mycobacterium nonchromogenicum*

- **General characteristics**: *M. nonchromogenicum* was described by Tsukamura in 1965. *M. nonchromogenicum* is a nonchromogenic, slow grower. This species belongs to the *M. terrae* complex together with *M. triviale*. The main biochemical characteristics are hydrolysis of Tween 80, growth at 42°C and positivity for pyrazinamidase production, but it is nitratase negative (Table 13). Fatty acid analysis production, HPLC and 16S rRNA sequencing allows further and more precise identification to the species level. Two PRA patterns have been described (53), PRASITE - http://app.chuv.ch/prasite)
- **Epidemiology**: *M. nonchromogenicum* is an environmental mycobacterium. It has been recovered from soil and water sources. This species is considered non-pathogenic, but it is the member of *M. terrae* complex most frequently implicated in human infections, including chronic tenosynovitis after trauma and rare reports related to pulmonary and disseminated infections (131, 253).

5.19. *Mycobacterium paratuberculosis*

- **General characteristics**: *Mycobacterium avium subsp. paratuberculosis* is the etiological agent of paratuberculosis or Johne's disease, affecting mainly ruminants; it was first isolated by Johne and Frothingham in 1895 and causes chronic progressive enteritis in ruminants. The organism has also been isolated from primates, including humans. *Mycobacterium avium subsp. paratuberculosis* is difficult to cultivate. It is a slowly growing mycobacteria requiring up to 20 weeks to produce colonies on solid medium and can be cultured only on media containing mycobactin (253). *Mycobacterium avium subsp. paratuberculosis* can be identified by PCR amplification of insertion sequence *IS900* (78) with DNA isolated from tissue samples. The radiometric BACTEC culture system combined with *IS900* PCR analysis give relatively rapid confirmation of *Mycobacterium avium subsp. paratuberculosis* (39, 181). *M. paratuberculosis* and *M. avium* show undistinguishable PRA paterns.
- **Epidemiology**: The disease is economically important in cattle industry. This species has been implicated as one of the etiologic agents of Crohn's disease, a chronic inflammatory disease of the gastrointestinal tract in humans (143).

5.20. *Mycobacterium peregrinum*

- **General characteristics**: *M. peregrinum* was first proposed in 1962 (22) based on physiological characteristics and the name *M. peregrinum* was proposed because they were the only non-pigmented strains in a group that included *M. smegmatis* and *M. phlei*. (21). *M. peregrinum* is a non-chromogen rapid grower, which was formerly a subspecies belonging to the *M. fortuitum* complex. It can be distinguished from *M. fortuitum* by not growing at 42°C, being able to grow in 5% NaCl and by using mannitol as carbon source (Table 6). It does not have special characteristics besides rapid growth and the ability to grow on McConkey without crystal violet. Three PRA patterns were reported by Devallois *et al*. (53) and are included in the PRASITE (http://app.chuv.ch/prasite). A new pattern, similar to type one, was described by da Silva Rocha *et al*. (46). Blanco *et al*. used additional tests with carbohydrates and sodium citrate and PRA for analysis of isolates previously identified as *M. fortuitum*

complex, and detected 2 isolates of *M. peregrinum*, stressing the complexity of identification of this species (20).

- **Epidemiology**: *M. peregrinum* is an environmental mycobacteria. In humans, it has been associated to localized but not disseminated infections. Among the former are sternal wound infections, chronic lung disease and cutaneous diseases. Due to its presence in the environment this species has been involved in pseudo-outbreaks of respiratory infections and outbreaks of sternal wound infections. Reports on *M. fortuitum* infections can refer to *M. peregrinum*, especially before 1992, when the taxonomic validity of this species was demonstrated (108).

5.21. *Mycobacterium phlei*

- **General characteristics**: *M. phlei* was described by Lehmann *et al.* in 1899 and is widely distributed in nature. *M. phlei* is a scotochromogen rapid grower. Colonies are rough and yellow. Optimal temperature for growth is between 22°C and 52°C. Differential biochemical characteristics are negativity for arylsulphatase (3 days), positivity for acid phosphatase and nitrate reductase and utilization of citrate as a single carbon source (Table 11). One PRA pattern was described for *M. phlei* (PRASITE - http://app.chuv.ch/prasite)
- **Epidemiology**: *M. phlei* is an environmental mycobacterium isolated from soil and plants. It is considered nonpathogenic for humans. Sporadic cases associated to human infections have been reported (204).

Table 11 - Differential biochemical tests for pigmented rapid growers

Tests	*M. phlei*	*M. vaccae*	*M. thermoresistibile*
Growth 45°C	+	-	+
Pigment	scoto	photo	scoto
Citrate	+	+	-
Acid phosphatase	+	-	-

scoto = scotochromogen, photo = photochromogen

5.22. *Mycobacterium scrofulaceum*

- **General characteristics**: *M. scrofulaceum* infection has been associated with cervical lymphadenitis in children (130). *M.scrofulaceum* has been isolated from natural lakes and rivers and aerosols (64,

251). *M. scrofulaceum* is schotochromogen slowly growing mycobacteria. It grows better at 37°C, does not hydrolyze Tween 80 and is negative in the nitrate reduction test. It is usually positive for urease and semiquantitative catalase (Table 12). Colonies are smooth yellow to orange. This species has been associated in the past to *M. avium* since it shares several genetic, pathogenic, ecological and phenotypic characteristics. However, it is considered a distinct species. One PRA pattern of *M. scrofulaceum* was reported by Telenti *et al.* (226) and Devallois *et al.* (53) and it is included in the PRASITE (http://app.chuv.ch/prasite). Khosravi *et al.* (101) reported seven different PRA patterns for this species. Da Silva Rocha *et al.* (46) reported one additional PRA pattern.

- **Epidemiology**: *M. scrofulaceum* has been isolated from the environment mostly from lakes and rivers in warm climates. However, in last years, a decrease in the number of isolates has been noted. This can be explained by the description of new species isolated from lymphadenitis that in the past could have been mistaken as *M. scrofulaceum*. In humans, this species has been associated to lymphadenitis in children, limited to cervical and mandibular lymph nodes. The association to other clinical presentations is rare but has been described causing disseminated infections, chronic ulcerative skin nodules and lung diseases in AIDS patients (5, 66, 67, 155).

5.23. *Mycobacterium shimoidei*

- **General characteristics**: *M. shimoidei* was first described in 1975 (232) from a respiratory infection in a Japanese patient. It shares some biochemical characteristics with other slow growing nonchomogenic species, mainly *M. malmoense* and *M. terrae* complex. Key tests are growth at 45°C but not at 25°C and positive test for Tween 80 hydrolysis (10 days). The typical mycolic acid pattern with a α α' k mycolates and w carboxymycolate is very useful for its identification. (Table 9). One PRA pattern was described by Telenti et al. (226) and Devallois et al. (53) and it is also present in the PRASITE (http://app.chuv.ch/prasite).
- **Epidemiology**: It is a potential pathogen in humans and is difficult to identifiy by routine methods because only few cases have been reported.

5.24. *Mycobacterium simiae*

- **General characteristics**: *M. simiae* was first isolated from monkeys in 1965 (98). *M. simiae* is a photochromogen mycobacterium (Table 10). It grows optimally at 37°C, slowly at 25°C and fails to grow at 45°C. In some clinical isolates *M. simiae* may resemble MAC. Niacin test can differentiate these species. It is not always easy to differentiate *M. simiae* from *M. scrofulaceum* based on traditional tests like pigment and niacin production, so methods of analysis of mycolic acid patterns or other molecular methods must be used to confirm *M. simiae.* Two PRA patterns, types I and II, were described by Devallois *et al*. (53). Four PRA patterns, types I, III, IV, and V, are present in the PRASITE (http://app.chuv.ch/prasite).
- **Epidemiology**: *M. simiae* is usually an environmental contaminant rarely associated with human disease. It has been isolated from tap water and soil samples in some areas (109). Clinical disease is similar to that caused by MAC including chronic pulmonary disease. Cases of *M. simiae* infection in AIDS patients have been reported (115, 194, 236).

5.25. *Mycobacterium smegmatis*

- **General characteristics**: *M. smegmatis* was first isolated by Lustgarten *et al*. in 1885 (121) and was named for the genital a secretion (smegma) from which it was recovered in a patient with a penile ulcer. *M. smegmatis* is a nonpigmented rapid grower. Main biochemical characteristics include growth in the presence of 5% NaCl, negative arylsulphatase test (3 days), positive nitrate reductase test, and growth at 45°C. Some authors mention the group *M. smegmatis* including the recently described species *M. wolinskyi* and *M. goodie* (25). One PRA pattern was described (226) and is present in the PRASITE (http://app.chuv.ch/prasite).
- **Epidemiology**: *M. smegmatis* is an environmental mycobacterium isolated from soil and water. This species has been commonly implicated in human infections such as soft tissue lesions associated with trauma and infections in AIDS patients, rarely pulmonary infections.

5.26. *Mycobacterium szulgai*

- **General characteristics**: *M. szulgai* is an unusual pathogen in humans and was first described in 1972 by Marks *et al.*(126). It is important to be able to differentiate *M. szulgai* from the other scotochromogens because of its pathogenicity. *M. szulgai* is a scotochromogen at 37°C but photochromogen at 25°C. Its key characteristics are the positivity for nitrate reduction and urease. Table 12 shows some distinguishing characteristics of slowly growing schotochromogen mycobacteria. One PRA pattern was described (226), and is present in the PRASITE (http://app.chuv.ch/prasite).
- **Epidemiology**: Pulmonary disease indistinguishable from that caused by *M. tuberculosis* is the most common type of infection caused by *M. szulgai*. Disseminated *M. szulgai* infection has been reported in AIDS patients and immunocompromised patients (65, 66, 207, 238, 263).

Table 12 - Differential biochemical tests for scotochromogen slow growers

Tests	*M. scrofulaceum*	*M. xenopi*	*M. gordonae*	*M. flavescens*	*M. szulgai*
Nitrate	-	-	-	+	+
Urease	+	-	-	+	+
Arylsulfatase	-	-/+	-	-	+/-
NaCl	-	-/+	-	+	-

+/- = mostly positive, -/+ = mostly negative

5.27. *Mycobacterium terrae*

- **General characteristics**: *M. terrae* described by Wayne *et al.* in 1966 (246) was isolated in 1968 from sputum and gastric lavage specimens from humans. It has been considered casual residents rather than pathogens. *M. terrae* is a non- chromogenic slow grower. The main biochemical characteristics are: positive Tween 80 hydrolysis, and nitratase tests, negative arylsulphatase, urease, and pyrazinamidase tests, and no growth at 42°C (Table 13). Colonies are rough and non-pigmented. Other species as *M. nonchromogenicum* and *M. triviale* have been considered as belonging to the *M. terrae* complex. However, on the basis of biochemical characteristics, mycolic acid analysis and 16S-rRNA sequencing they can be readily identified. One PRA pattern has been described for this species (226) and is also present at the PRASITE (http://app.chuv.ch/prasite). Da Silva Rocha (46) reported three additional PRA patterns.

- **Epidemiology**: *M. terrae* is an environmental mycobacterium that has been isolated from water, soil and vegetables. Its recovery from clinical samples should be regarded as contamination, unless when repeatedly isolated from the same source. Nevertheless, this species has been documented as cause of human infections such as joint, synovial, pulmonary, and rarely disseminated infections in AIDS patients.

Table 13 - Differential biochemical characteristics of the *M. terrae* complex

Tests	*M. triviale*	*M. terrae*	*M. nonchromogenicum*
NaCl	+	-	-
Nitrate	+	+	-
β-galactosidase	-	+	+
Tween	+	+	+

5.28. *Mycobacterium thermoresistibile*

- **General characteristics**: The original strain was recovered from moist soil and from house dust (231). *M. thermoresistibile* is a pigmented scotochromogen rapid grower. Colonies are yellow rough or smooth. Optimal growth temperatures are between 37°C and 52°C. Main biochemical characteristics are positive nitrate reductase and Tween 80 hydrolysis tests, negative acid phosphatase and citrate tests and ability to grow in 5% NaCl (Table 11). One PRA pattern is present in the PRASITE (http://app.chuv.ch/prasite).
- **Epidemiology**: *M. thermoresistibile* is an environmental mycobacterium that has been isolated from house dust. It is considered nonpathogenic for humans, although sporadic reports have associated this species to infections after mammoplasty, pulmonary abscesses, and disseminated infections in inmunodeficient patients.

5.29. *Mycobacterium triviale*

- **General characteristics**: *M. triviale* described by Kubica *et al*. in 1970 is nonchromogenic and slow grower. It has been traditionally included in the *M. terrae* complex because of similar biochemical characteristics; however, some distinctive characteristics are present: positive nitrate reduction and arylsulfatase (3 days) tests. It is able to grow in the presence of 5% NaCl, hydrolyze Tween 80 and it does not grow at 42°C (table 13). One PRA pattern has been described (226) and it is also present in the PRASITE (http://app.chuv.ch/prasite).

- **Epidemiology**: *M. triviale* has been recovered from the environment. This species, as other members of this complex, is regarded as a contaminant when isolated in the laboratory. However, there are rare reports in the literature that implicate this species in human infections.

5.30. *Mycobacterium ulcerans*

- **General characteristics**: *M. ulcerans* was first described in 1948 in Australia in a biopsy from an ulcer in a young child (123). *M. ulcerans* is a slowly growing environmental mycobacteria that may be slightly pigmented. It grows on common mycobacteriological Löwenstein-Jensen medium and in liquid BACTEC medium, or Middlebrook 7H9 under microaerophilic conditions (2,5 - 5% oxygen). It grows best at low temperature of 30°C. (Table 14) shows phenotypic characterisitcs of *M. ulcerans* compared to the closely related *M. marinum.* PRA can differentiate *M. ulcerans* and *M. marinum*. One PRA pattern was described by Devallois *et al*. (53), but here we show that *M. ulcerans* has two PRA patterns.
- **Epidemiology**: *M. ulcerans* causes a necrotizing human skin disease called Buruli ulcer or Bairnsdale ulcer. In 1998, WHO recognized *M.ulcerans* as an emerging pathogen.

Table 14 - Differential biochemical tests for *M. ulcerans* and *M. marinum*

Tests	***M. ulcerans***	***M. marinum***
Pigment	nc*	photo
Growth at 37°C	-	-
peptone agar	-	+
INH	+/-	+/-
TCH	+	+
HA	-/+	+
NaCl	-	-
Catalase SQ	-	-
Tween	-	+
Urease	-/+	+
Niacin	-/+	-
Nitrate	-	-
Acid phosphatase	-/+	+

* = some strains are scotochromogen
INH = isoniazid, TCH = thiophen-2-carboxylic acid hydrazide, HA = hydroxylamine, SQ = semiquantitative, nc = non-chromogen, photo = photochromogen, +/- = mostly positive, -/+ = mostly negative

5.31. *Mycobacterium vaccae*

- **General characteristics:** *M. vaccae* was described by Bonike *et al.* in 1964. *M. vaccae* is a pigmented photochromogen rapid grower. Colonies are smooth yellow to orange. It can be non-pigmented if growing in complete darkness but acquire pigmentation rapidly after exposure to light. Growth is observed at a temperature range of 22 to 40°C. The main biochemical characteristics include positive arylsulphatase, Tween hydrolysis, iron uptake and tellurite reduction tests, and growth in 5% NaCl (Table 11). One PRA pattern has been described (53, 226) but no pattern for *M. vaccae* is present in the PRASITE (http://app.chuv.ch/prasite).
- **Epidemiology**: *M. vaccae* is an environmental mycobacterium. It has been isolated from soil, ponds, and wells, from cattle udder and skin lesions. This species is considered non-pathogenic. However sporadic cases that implicate it with human infections such as skin and pulmonary infections have been reported (144).

5.32. *Mycobacterium xenopi*

- **General characteristics**: *M. xenopi* was first described in 1959 and isolated from skin lesions in an African toad (201). The main characteristic of *M. xenopi* is its ability to grow at 45°C. It can grow slowly at 37°C, but not at 25°C.It is arylsulphatase positive in 3 days and does not hydrolyse Tween 80 after 14 days. These characteristics are important because *M. xenopi* can be confused with *M.avium and M. intracellulare* . Table 15 shows how to differentiate *M. xenopi* from the other. One PRA pattern has been described (53, 226) and it is also present in the PRASITE (http://app.chuv.ch/prasite).
- **Epidemiology**: In 1965, lung infection in a human due to *M. xenopi* has been reported in England (127) and then also in other countries. Extrapulmonary and disseminated infections have been described in immunocompromised patients; AIDS is a risk factor for *M. xenopi* infections (203). Häfner *et al.* (85) reported *M. xenopi* as the most common species identified in routine laboratory, but in Latin America it has been rarely reported (1). It has been recovered from aquatic environments in a Brazilian study (150).

Table 15 - Differential biochemical tests for *M. xenopi* and MAC

Tests	***M. xenopi***	***M. avium***	***M. intracellulare***
Growth at 25°C	-	+	+
Growth at 45°C	+	+/-	-/+
Arylsulfatase	+	-	+/-
Tellurite	-	+	+

+/- = mostly positive, -/+ = mostly negative

Chapter 6 - MOLECULAR IDENTIFICATION OF NTM

In the last years, alternative rapid methods for identification of mycobacteria were developed and evaluated for clinical use, as DNA probe hybridization, mycolic acid analysis (Chapter 7), DNA sequencing, microarrays, and tests based on PCR. Improved culture methods as the BACTEC system and the Mycobacterial Growth Indicator Tube (MGIT) used in conjunction with DNA probes for identification of species are currently employed in many laboratories throughout the world, but are expensive for developing countries. Mycolic acid analysis and DNA sequencing require technical expertise and special equipment.

6.1. DNA probes

AccuProbe® Culture Identification Tests (Gen Probe, Inc. SanDiego, Calif) includes probes for culture confirmation of *M. tuberculosis*, *M. kansasii*, MAC (individual probes for *M. avium* and *M. intracellulare* are also available), and *M. gordonae*. A single-stranded DNA probe with a chemiluminescent label is complementary to the ribosomal RNA of the target organism. The labeled DNA/RNA hybrids are measured in a Gen-Probe luminometer.

INNO-LiPA Mycobacteria v. 2 (Innogenetics NV, Gent, Belgium) consists of a reverse-hybridization method in which the probe is represented by an amplicon of 400-550 bp from the 16S-23S rRNA spacer region. Primers are biotinylated. The amplified biotinylated product is hybridized with 14 probe lines immobilized on a typing strip. Finally, hybrids are detected through a colorimetric reaction. Twelve line probes react specifically with *M. tuberculosis* complex (MTB probe line), *M. kansasii* (MKA-1, MKA-2, MKA-3), *M. xenopi* (MXE), *M. gordonae* (MGO), *M. genavense* (MGV), *M. simiae* (MSI), *M. marinum-M. ulcerans* (MMU), *M. celatum* (MCE), *M. avium* (MAV), *M. intracellulare* (MIN-1, MIN-2), *M. scrofulaceum* (MSC), *M. malmoense* (MML), *M. haemophilum* (MHP), *M. chelonae* (MCH-1, MCH-2, MCH-3), *M. fortuiutm* (MFO), and *M. smegmatis* (MSM). One probe line is specific for the genus *Mycobacterium* (MYC) and one for the MAIS complex (MAIS - which reacts with *M. avium, M. intracellulare, M. scrofulaceum* and MAC other).

6.2. DNA sequencing

The reference molecular method for identification of mycobacteria is the determination of sequences of **16S ribosomal DNA (16S-rDNA)**. The

16S-rRNA molecule is highly conserved, with phylogenetically meaning sequence changes in certain positions, which are specific for microorganisms at the species level. Hypervariable regions within the molecule can be useful for identification purposes because they contain relatively high differences between species but relatively low variability within a species. Rogall *et al*. (186) described a method for determination of 16S-rRNA sequences by direct sequencing of a 1 kb DNA fragment containing the 5' part of the gene. Two hypervariable regions were identified – region A (positions 123 to 273) and region B (positions 430 to 500). Most species show unique sequences in the hypervariable region A, but *M. gordonae* isolates show intraspecific gene variability (104). The closely related species *M. kansasii* and *M. gastri* have identical 16S-rRNA gene sequences. *M. ulcerans* and *M. marinum* have nearly homologous 16S-rRNA gene sequences and cannot be distinguished by hypervariable regions A and B sequence analysis. The same occurs with *M. chelonae* and *M. abscessus*.

A database containing 123 5' 16S-rDNA mycobacterial sequences (from position 54 to 510) obtained from 199 culture colection isolates is publicly accessible at http:// www.ridom.de (88).

Other DNA sequences were described in the last years for differentiation of mycobacteria, but still need interlaboratory validation for their use in routine sets.

16S-23S internal transcribed spacer (ITS) ITS sequencing represents a supplement to 16S rRNA gene sequencing for the differentiation of closely related species. Sequences of *M. avium*, *M. conspicuum*, *M. gastri*, *M. genavense*, *M. kansasii*, *M. malmoense*, *M. marinum*, *M. shimoidei*, *M. simiae*, *M. szulgai*, *M. triplex*, *M. ulcerans*, *M. xenopi, M. intracellulare*, *M. phlei*, *M. smegmatis*, and *M. tuberculosis* showed low divergence (0 to 2%) when different isolates belonging to the same species were tested. *M. marinum* and *M. ulcerans* possess identical ITS sequences (187).

gyrB - The *gyrB* sequences have been shown to be useful phylogenetic markers for the identification of species. Analysis the *gyrB* sequences of 43 isolates belonging to 15 speceis (*M. africanum, M. asiaticum, M. avium, M. bovis, M. gastri, M. gordonae, M. intracellulare, M. kansasii, M. malmoense, M. marinum, M. microti, M. scrofulaceum, M. simiae, M. szulgai, M. tuberculosis*) showed that the frequencies of base substitutions were comparable to those in the ITS sequences (99). The ITS sequences of four species belonging to the *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. africanum*, and *M. microti*) were 100% identical, while four synonymous substitutions were found in the *gyrB* sequences of these strains (99). Based on these findings, a *gyrB*-RFLP test was developed for differentiation of members of the *M. tuber-*

culosis complex (151) (see Chapter 3 and ANNEX 3). A *gyrB* database is available at http://www.mbio.co.jp/icb for taxonomic studies using *gyrB* sequences.

rpoB - Comparative sequence analysis of a 342 bp fragment of the *rpoB* gene, encoding the β subunit of RNA polymerase, was investigated in 44 reference strains of mycobacteria and 107 clinical isolates by Kim *et al.* (103). Slowly and rapidly growing groups of mycobacteria were clearly separated. Low-level sequence divergence was found among isolates of the same species. Pathogenic *Mycobacterium kansasii* was easily differentiated from nonpathogenic *M. gastri*. Sequence analysis of amplified *rpoB* DNAs can be used in parallel with traditional culture methods and as a supplement to 16S rDNA gene analysis. Furthermore, in the case of *M. tuberculosis*, rifampin resistance can be simultaneously determined (103). Based on these findings, a PCR- Restriction Analysis test was proposed. Another group studied a different fragment (360 bp – nucleotides 902 to 1261) from the *rpoB* gene and developed tests for differentiation of species based on PCR-Restriction Fragment Length Polymorphism analysis (112) and dot-blot hybridization (111).

hsp65 - Partial sequencing of a 441-bp fragment of the *hsp65* gene was used for the identification of rapidly growing mycobacteria (RGM) by Ringet *et al.* (185). Sequences from *M. abscessus, M. brumae, M. chelonae, M. chitae, M. confluentis, M. fortuitum, M. mucogenicum, M. peregrinum, M. senegalense* and *M. smegmatis* were unique, and one species could be clearly distinguished from the others. There were few sequence differences within each species (<2% of base differences), and they had no effect on species assignment. The *hsp65* sequencing differentiated *M. chelonae* and *M. abscessus*, two species difficult to identify by classical methods and 16S rRNA gene sequencing. Smole *et al.* (209) obtained sequences of the *hsp65* 441-bp fragment from 278 MAC isolates cultured from human specimens and environmental sources and defined 18 different alleles, based on 54 polymorphic sites.

Other groups have also analysed DNA sequences of the *dnaJ* gene (224), the gene encoding the **32 kDa** protein (211), the superoxide dismutase (*sod*) gene (266) and the *recA* gene (19) for differentiation of species.

6.3. Microarrays

Hybridization patterns produced on a high-density oligonucleotide array (DNA chip) with probes complementary to 705 bases of *M. tuberculo-*

sis rpoB gene sequence were used for the differentiation of 121 isolates comprising *M. avium, M. chelonae, M. fortuitum, M. gordonae, M. intracellulare, M. kansasii, M. scrofulaceum, M. smegmatis, M. tuberculosis,* and *M. xenopi*. Identification using arrays was similar to species assignments derived from an analysis of 180 bp of 16S sequences (74).

Fukushima *et al*. (72) recently described a microarray assay based on *gyrB* gene sequences that can be used for speciation of mycobacteria. A set of oligonucleotide probes for specific *gyrB* gene regions was spotted onto sialylated glass slides that were used for hybridization with fluorescently labeled RNA derived from amplified sample DNA. This microarray produced unique hybridization patterns for *M. tuberculosis, M. bovis, M. avium, M. intracellulare, M. kansasii*; *M. gordonae, M. gastri, M. africanum, M. asiaticum, M. malmoense, M. marinum, M. scrofulaceum, M. simiae,* and *M. szulgai* and could differentiate closely related bacterial species. Moreover, results corresponded well with those obtained by conventional identification methods.

Results obtained using microarrays demonstrate the general capability of such systems to provide important information about genes of related mycobacterial organisms that have not been sequenced previously. It can be expected that, in the near future, algorithms will be used to analyze results of genome-wide surveys to identify these organisms.

6.4. Tests based on PCR

Several authors have proposed tests based on PCR for identification of mycobacteria from cultured and non-cultured clinical isolates. These tests can be less expensive and training of personnel is easily performed. PCR is technically less demanding than direct sequencing and cheaper than identification with species-specific commercial probes.

Amplification of the *dnaJ* gene and subsequent hybridization with species-specific probes was used by Takewaki *et al*. (224) and allowed differentiation of *M. tuberculosis, M. avium, M. intracellulare* and *M. kansasii.*

Fiss *et al*. (69) incorporated digoxigenin labeled UTP to the *hsp65* gene amplified by PCR and detected *M. tuberculosis* and *M. avium* by chemiluminescence using reverse dot-blots.

De Beenhouwer *et al* (49) used the products of amplification of the 16S rRNA gene in a capture assay hybridizing with 7 species-specific probes for *M. tuberculosis, M. avium, M. intracellulare, M. scrofulaceum, M. xenopi, M. genavense* and *M. chelonae*.

Kulski *et al* (107) developed a multiplex PCR with 6 primers to amplify the 16S rRNA gene and the MPB70 gene allowing identification of *M. tuberculosis, M. avium* and *M. intracellulare,* according to the sizes of the amplified fragments.

Vaneechoutte *et al*. (237) combined PCR amplification of the 16S rRNA gene with enzymatic digestion with *Cfo*I and *Mbo*I. This strategy allowed differentiation between *M. avium, M. chelonae, M. flavescens, M. fortuitum, M. gordonae, M. intracellulare, M. marinum, M. nonchromogenicum, M. simiae, M. terrae* and the species belonging to *M. tuberculosis* complex.

Telenti *et al*. (226) described a method of mycobacterial differential diagnosis, named PRA (PCR-Restriction Enzyme Analysis), based on the amplification of 441 bp fragment of the *hsp65* gene by PCR, and followed by digestion of the amplified product with the restriction enzymes *Bst*E II and *Hae* III. In the original identification table proposed by Telenti, 29 species and subspecies were identified by analysis of restriction patterns obtained with both enzymes. Several groups have used this technique for mycobacterial identification and have described new PRA patterns that differentiate other mycobacterial species, sub-species, and allelic variants (2, 26, 44-46, 53, 85, 110, 157, 209, 225), among others). Brunello *et al*. (26) studied 121 reference and clinical strains of both slowly and rapidly growing mycobacteria belonging to 54 species using DNA sequencing and PRA. The PRA restriction digests were separated by 10% polyacrylamide gel electrophoresis (PAGE). By including a size standard in each sample, the restriction fragment profile was calculated using a computer-aided comparison program. An algorithm describing these 54 species (including 22 species not previously described) was proposed.

A Database on the Internet (PRASITE) (http://app.chuv.ch/prasite) was developed to help interpretation of PRA results. Seventy-four different PRA patterns have been included to date in the PRASITE database.

A detailed flowchart for molecular identification is shown in Annex 1.

Chapter 7 - IDENTIFICATION BY MYCOLIC ACID ANALYSIS

7.1. Thin Layer Chromatography

Thin-layer chromatography (TLC) has been used by several laboratories for fast confirmation of mycobacterial identification according to their mycolic acid profiles. TLC is a useful technique due to its simplicity, length of the procedure and requirement for small quantities of material.

TLC consists of a stationary phase immobilized on a glass or plastic plate and a solvent. The sample, either liquid or dissolved in a volatile solvent, is deposited as a spot on the stationary phase. The constituents of a sample can be identified by simultaneously running standards with the unknown sample. One edge of the plate is then placed in a solvent reservoir and the solvent migrates up the plate by capillarity. When the solvent front reaches the other edge of the stationary phase, the plate is removed from the solvent reservoir. The different components in the mixture migrate up the plate at different rates and the separated spots are visualized.

In Table 16 the mycolic acid profiles are shown for the most commonly isolated species of mycobacteria.

Table 16 – Mycolic acid profiles. This table is a compilation from (29, 40, 47, 87, 117, 136-139, 146, 165, 175)

Species	Mycolates profile
I. Slow grower scotochromogens	
M. gordonae	α m k
M. szulgai	α m k
M. flavescens	α k w
M. scrofulaceum	α k w
II. Slow grower non-chromogens	
M. xenopi	α k w
M. marinum	α m k
M. microti	α m k
Mulcerans	α m k
M. simiae	α α′ k
M. malmoense	α α′ k
M. avium-intracellulare	α k v
M. terrae	α k w
M. nonchromogenicum	α k w
M. tuberculosis	α m k
M. bovis	α m k
M. bovis BCG	α k
M. kansasii	α m k
M. gastri	α m k
M. triviale	α
M. shimoidei	α α′ k w
M. farcinogenes	α e
M. celatum	α k w
M. avium	α k w
M. asiaticum	α m k
III. Rapid growers	
M. phlei	α k w
M. chelonae	α α′
M. fortuitum	α α′ e
M. smegmatis	α α′ e
M. peregrinum	α α′ e
M. thermoresistibile	α α′ m k
M. vaccae	α α′ k w
M. mucogenicum	α w
IV. Non cultivable and difficult to grow mycobacteria	
M. leprae	α k
M. paratuberculosis	α k w
M. genavense	α α′ k
M. haemophilum	α m k

The mycolate designations are those previously used by Daffé *et al.* (47)
α = α mycolate (type I), long nonoxygenated mycolate
α′ = α′ mycolate (type II), short nonoxygenated mycolate
m = methoxymycolate (type III)
k = ketomycolate (type IV)
e = epoxymycolate (type V)
w = w-carboxylic mycolate (type IV)or dicarboxylic mycolate

7.2. High Performance Liquid Chomatography

The method is based on detection of mycolic acids by High Performance Liquid Chomatography (HPLC), in which every mycobacteria species has a particular mycolic acid pattern.

The method consist of several steps, briefly: harvesting of cells from LJ culture, extraction of fatty acids, evaporation of the extract, derivatization of fatty acids (with bromophenacyl bromide), clarification of samples, UV-HPLC analysis of mycolic acids, interpretation of mycolic acid pattern and identification of the species (28).

The advantages of this method are that it can be completed in few hours, it is not expensive (the cost of each identification is calculated in USD 3.00), and it can identify a wide range of species as well as reveal unknown species. Disadvantages are the initial cost of the equipment, the need for heavy growth to obtain a mycolic acid pattern, dependability on standardized growth conditions and good laboratory expertise and proficiency for the interpretation of patterns (145).

Mycolic acid patterns are characterized by having single, double, triple or multiple peak clusters. Peaks are identified by their heights and according to RRT (relative retention time) as compared to an internal standard (75). Using this method, more than 45 species of mycobacteria can be identified. A support web site for mycobacteria identification by HPLC is maintained by HPLC users group at http://hplc.cjb.net.

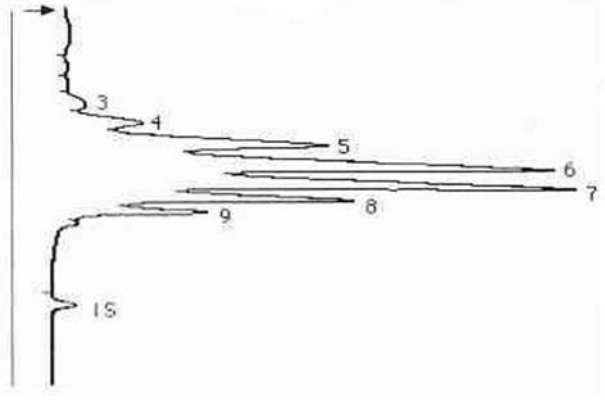

Figure 1 - Single cluster peaks of *M.tuberculosis* by HPLC (145)