Diagnostics of non-tuberculous mycobacteria

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2 Diagnosis of NTM disease.

Diagnosing mycobacterial infections quite often is a challenge, in which several medical disciplines are involved. Clinical suspicion will often be the starting point of subsequent specific diagnostic procedures including careful pathological, immunological and microbiological testing, based on appropriate specimens en techniques. The path leading to a definitive diagnosis used to be long and complicated and therefore new approaches are highly relevant to improve the diagnostic process for this purpose.

Clinical diagnosis.

Mycobacterial disease is often aspecific in clinical appearance and the disease usually progresses slowly. Clinical diagnosis starts with the exclusion of other possible diseases. In cutaneous infection, these include viral involvement, fungal involvement, auto-immune disease, tumors or other bacterial pathogens. Clinical appearance differs from subcutaneous nodules (in immunosuppressed patients lesions tend to be less superficial) to superficial plaques. Sporotrichoid lesions are usually caused by *M. marinum* but other species have been reported *(1)*. In lymphadenitis, the differential diagnosis is often extensive: It includes acute pyogenic infections, tuberculosis, fungal infections, toxoplasmosis, cat-scratch disease, infectious mononucleosis, brucellosis, tularemia, foreign body reaction, sarcoidosis, and malignancy. In addition, congenital lesions such as cystic hygroma, thyroglossal cyst, dermoid cyst and branchial cyst should be considered *(2)*. Up to 12 years of age, a positive reaction to skin-tests, few to no complaints, unilateral presence of the involved lymph nodes and several weeks/months duration of swelling are all clinical clues to NTM lymphadenitis.
In pulmonary disease, symptoms other than coughing (60-100%) are often mild or completely absent *(3)*. Differential diagnosis often includes fungal infection and tuberculosis, especially in the classical form of NTM infection which mimics *M. tuberculosis* infection. Infections that present in the classical form are often secondary to underlying lung disease (COPD, prior TB infection, or interstitial lung disease). Caucasian males predominate in the sixth or seventh decade of life. Other risk factors include smoking, alcoholism, cardiovascular disease, chronic liver disease, previous mycobacterial disease and previous gastrectomy *(4)*. In a radiograph, the classical form of NTM (mimics *M. tuberculosis* infection) is characterized by nodular opacities in the apices, cavitation, apical pleural thickening, and bronchogenic spread. Pleural effusion and adenopathy are uncommon *(5)*. Bronchiectasis is the primary finding in the middle lobe on high resolution computed tomography. The second form (atypical) is characterized by a single pulmonary nodule or multiple bilateral nodular and irregular interstitial opacities (<1 cm) *(6)*. It is not necessarily related to any underlying chronic lung disease and is found most often in women.

Immunological tests.

For over a century, purified protein derivatives (PPD) of *M. avium*, *M. kansasii*, M. *scrofulaceum and M. marinum, M intracellulare, M gordonae* and *M fortuitum* have been used for diagnosing NTM infections in humans *(7- 9)*. The most commonly known PPD test is the tuberculin test for the diagnosis of *M. tuberculosis* infection. A few years ago, the PPD of NTM were no longer commercially available. No substitution for this antigenic skin-test exists. The PPD tuberculin test itself, however, does appear useful in the diagnosis of NTM infection in children. Because of the cross-reactivity between immune reactions to different species, the tuberculin test often shows false-positive reactions due to previous encounters with NTM *(10)*. Therefore, the diameter of the skin-reactions is considered for a definite diagnosis of *M. tuberculosis*. Previous NTM encounters are not expected in young children and can therefore be indicative for NTM disease in this patient group *(10).* The tuberculin tests can be applied in initial diagnosis of NTM lymphadenitis with an optimal cut-off value of 5 mm for a positive skin-induration. However, the sensitivity of *M. avium* specific PPD was 93% compared to 70% for the *M. tuberculosis* specific PPD and, therefore, species-specific sensitins would be preferable *(10)*.

The QuantiFERON-TB test, which is significantly more specific in *M. tuberculosis* infection than the tuberculin PPD *(11)*, is built on a principle applicable to all mycobacterial species. This assay is based on a specific elevation in the interferon-gamma concentration that occurs as T cells respond to early-secreted antigenic target 6-kDa protein (ESAT-6) and culture filtrate protein 10 (CFP-10) and is, therefore, directed at a more recent contact phenomenon than tuberculin-PPD, which detects a memory respons *(12)*. It would also be able to overcome the problems of a prior NTM encounter in adults. ESAT-6 and CFP-10 are specifically expressed by *M. tuberculosis* but not by most other species, including *M. bovis* BCG (cross-reactivity is for instance known for *M. kansasii: 13*). The specificity of this test has been evaluated for *M. tuberculosis* compared to NTM disease in children, and showed a 95% specificity for *M. tuberculosis (14)*. ESAT-6 and CFP-10 are also the two proteins to which an enzyme-linked immunospot assay (ELISPOT) is directed *(15)*.

Detection of IgG and IgM directed against mycobacterial A60 is another principle with the same advantages -and therefore great possibilities- in NTM diagnostics *(16, 17)*. So far, the principle has shown promising results in diagnosing *M. leprae*, *M. abscessus* and *M. tuberculosis (16, 17).* Various other antigens of considerable serologic value for *M. tuberculosis*, are antigen 85A, 38-kDa protein, alpha-crystallin (16 kDa), MTB48, and PGL-Tb1 *(18)*. Some of these antigens are secreted or are present in the cell wall of the bacillus. A starting point in the identification of suitable antigens for NTM serology would be protein profiling *(19)*. An especially interesting protein group for the antigen tests would be the cord factors (Trehalose dimycolate). The cord factors are glycolipids which are associated with hydrophobicity and virulence. Because of the variation in the cord factors of different NTM species and their direct involvement in antibody respons, they could be suitable antigens in

NTM serology *(20)*. It should be noted that, in patients suffering from AIDS, in whom the number of T cells is low, determining the humoral response can be an invaluable tool *(18).*

A point of discussion is whether these immunological tests (serological or interferon assays) are cost-effective. After all, the NTM-PPD skin-tests were no longer produced because they were unprofitable. However, as stated that molecular assays encounter considerable problems for the diagnosis of NTM infections, these immunological approaches might be valuable for mycobacterial diagnostics in the future.

Histopathological diagnosis.

The gross pathology of NTM-infected material frequently show necrotizing granulomas, caseating necrosis and masses containing purulent material *(21)*. The pathologist investigates the biopsies further using thin (4-10 μm) sections from the material imbedded in paraffin and fixated with formalin. Microscopic investigation includes direct light microscopy and staining procedures.
Acid-fast staining is applied on imbedded sections with Kinyoun, auramine or Ziehl-Neelsen stains (see further in text). Auramine is considered the most sensitive, but this is correlated to the easy microscopic recognition and not related to the staining procedure. It is also much faster than other staining methods due to the use of a lower microscopic magnification while a high visual contrast is present because of the fluorescent colouring of the mycobacteria. The drawback of auramine staining is that the rapid-growing species occasionally stain poorly and some other bacteria belonging to the family *Mycobacteriaceae* (*Corynebacterium* and *Nocardia*) can be positive in auramine staining as well. The overall sensitivity of staining of histopathological sections is poor: cutting of the sections includes partial destruction of many cells which causes the loss of bacteria and the staining procedure is more difficult to apply on tissue specimens than on “free” bacteria *(22)*. Pathology caused by mycobacteria is often abundant while bacteria are present in low copies. However, the histological manifestation is highly variable. Therefore, the histopathological diagnosis of NTM infection is as difficult as the clinical diagnosis.

Microscopic examination without staining shows necrotizing granulomas of various forms with or without caseating properties in NTM-affected tissue. Only few exceptions exist: the granulomatous reactions were only found in a minority of NTM infected patients with Cystic fibrosis, and histological manifestations of infection with specific species sometimes exhibit no granulomatous reaction at all *(23, 24)*. The granulomas include suppurative granulomas, tuberculoid granulomas, mixed-cell granulomas, sarcoid-like granulomas, ill-defined granulomas and granulomas with a perifollicular distribution *(25, 26)*. Differences between *M. tuberculosis* and NTM infection are significant for a few histological features. Microabscesses, ill-defined granulomas and noncaseating granulomas are more frequently found in *M. tuberculosis* infection, while mixed-cell granulomas, caseation and multinuclear

giant cells are more common in NTM infection *(27)*. Also, a relationship between the chronic evolution of the disease and granuloma formation has been demonstrated *(26)*.

In pulmonary NTM infections the usual manifestations obtained after lobectomy or transbronchial biopsy are bronchiolar inflammation, infiltration of lymphocytes, epithelioid granulomas and giant cells. The wall of cavitary lesions consists of epithelioid cells with multinucleated giant cells, granulomas and caseation and necrosis *(28)*. In the hot-tub lung, moderate to severe granulomatous inflammation is encountered, as well as numerous granulomas with focal caseation and necrosis, interstitial chronic inflammation, and mild and interstitial immature fibrosis with focal pattern *(29)*.

In biopsies from lymph nodes, a fairly stable histopathological picture is seen, with increasing presence of caseation as the infection proceeds. Histologically, the majority of cases show eosinophilic necrosis with nuclear debris and Langhans type giant cells. Different forms of lymphocyte infiltrates and granulomas are possible *(30)*. The most variable histological manifestations are observed in NTM skin infections. Mixed-cell and suppurative granulomas are the most common in NTM skin infections *(31)*. Other histological features are: a diffuse infiltrate of histiocytes with occasionally foamy appearance, acute and chronic panniculitis, acute suppurative folliculitis, diffuse infiltrate of histiocytic foamy cells, non-specific chronic inflammation, cutaneous abscesses and necrotizing folliculitis *(26)*. According to Tang et al, when biopsy specimens show aspecific histological features (fibrotic or hyalinized granulomas, nonspecific chronic inflammation, nonspecific reactive or reparative changes, no significant histologic abnormality or malignancy), additional microbiological culture will not be successful. Only necrotizing granulomas, nonnecrotizing granulomas, poorly-formed granulomas or acute inflammation will contain culturable mycobacteria *(32)*.

Sometimes, the skin abnormalities have different causes at the same time: fungal, mycobacterial, and viral pathogens have concurrently been observed in skin lesions of basal cell carcinomas, Kaposi's sarcoma, melanoma, mycosis fungoides, and squamous cell carcinoma *(33)*. Together with the poor sensitivity of staining procedures and the variable histopathological manifestation of NTM infection, this greatly affects the value of pathological diagnosis of NTM infection. Therefore, additional microbiological investigations are a necessity.

Conventional microbiological diagnosis.

Conventionally, the detection of NTM is performed by acid-fast staining and culture. The acid-fast staining is usually an auramine stain and/or a Ziehl Neelsen stain and is indicative for the presence of mycobacteria. Culture, with or without decontaminating pre-treatment, is followed by the identification of the species.

Acid-fast staining.

Staining procedures for mycobacteria are based on the acid-fast properties of the mycobacterial cell-wall, which is a bacterial cell wall composed of a thin, inner layer of peptidoglycan and large amount of glycolipids such as mycolic acid, arabinogalactan-lipid complex, and lipoarabinomannan. During the acid-fast staining procedure, the acid-fast cell wall enables the bacterium to resist decolorization with acid alcohol and retain the original stain. Mycobacteria are 1-10 µm in length and 0.2-0.6 µm in diameter and visible in different colors depending on the dye *(22)*. In the modified Kinyoun and the Ziehl-Neelsen (ZN) stain this dye is carbol fuchsin and is directly visible with light microscopy. The Auramine stain uses auramine and rhodamine which both fluoresce at short wavelength as green or orange. The different staining methods can be performed either on direct material or on decontaminated material, which can therefore be more concentrated. Sensitivity rates of the ZN and auramine stain are higher than those of the Kinyoun stain *(34, 35)* and are estimated between 60 and 90% compared to culture *(34, 36)*. Low sensitivity of fluorescence microscopy and ZN staining is also negatively influenced by formalin fixation as applied in histopathological examinations *(37)*. The acid-fast staining procedures are not fully specific for mycobacteria, since Nocardia species, some Legionella species and some corynebacteria are also (partially) stained acid fast *(38)*. The positive predictive value is 96% for *M. tuberculosis (36)*. However, no speciation is possible and it is considered a pre-screening of clinical materials.

Decontamination.

Before culture, most clinical specimens are decontaminated because of the possible contaminating commensals present in the non-sterile clinical materials. Several methods are known for this purpose: The Petroff method, N-acetyl-L-cysteine-sodium hydroxide, NaOH-N-acetyl-L-cysteine, Sulphuric acid, Zephiran, Papain-Zephiran, Papain-Pentane-Zephiran, sulphuric acid, chlorhexidine and oxalic acid and the newer method: hypertonic saline with sodium hydroxide (HS-SH) *(39-41)*. While the NaOH-N-acetyl-L-cysteine method is currently recommended for the recovery of NTM, other methods appear to be more efficient like the sulphuric acid, oxalic acid and the chlorhexidine methods *(41- 43).* They yield a higher recovery rate and less contamination and should be considered as the method of choice. A secondary effect of decontaminating pre-treatment is the property to dissolve clinical materials which clears bacilli from intracellular containment. Therefore, decontamination of specimens is also useful in sterile specimens and should be applied to all clinical specimens except sterile fluids.

Culture.

Culturing of mycobacteria is most frequently applied with a radiometric system measuring the assimilation of mycobacteria in Broth medium. Previously, mycobacteria were mainly cultured on solid medium slants (less susceptible to drying during long growth periods). Agar with egg yolk was, and still is, a popular culturing medium. Various commercially available media are possible, of which the Middlebrook media are the most widely used. Guidelines for the culturing of NTM include that samples should be inoculated onto at least one solid medium (Lowenstein-Jensen or Middlebrook variant) and into a liquid medium culture system (BACTEC (Beckton-Dickenson Diagnostics), MB Redox (Heipha Diagnostika), MB/BacT (Organon-Teknika), MGIT (BD Diagnostics) and Septi-check (BD Diagnostics)) *(44, 45)*. Normal growth temperature for mycobacteria is 35-37 ˚C. Most media require additives like Mycobactin and OADC to increase the growth rate (containing oleic acid, albumin, dextrose, catalase and NaCl). Antibiotics are often added to inhibit the growth of contaminants: Panta, containing polymyxin, amphotericin B, nalidixic acid, trimethoprim and azlocillin, and/or PACT, containing polymyxin B, amphotericin B, carbenicillin, and trimethoprim *(46, 47)*. Additives can also inhibit growth of several species, like *M. kansasii* and *M. haemophilum (47, 48)*. Media and additives have been extensively evaluated for *M. tuberculosis* but other (less evaluated) species might require different media for optimal growth. A good example is *M. haemophilum.* Liquid media are preferred for this species and it requires iron supplements such as hemin or ferric ammonium citrate for growth as well as a lower incubation temperature of 30-32˚C *(49)*. This lower temperature is also preferred by *M. marinum* and *M. genavense*. Recommendations of the American Thoracic Society (ATS) include culture in liquid medium as well as on solid medium. Lowenstein-Jensen is an excellent medium for the recovery of *M. tuberculosis,* but is generally inferior to Middlebrook agar as an all-purpose medium for both *M. tuberculosis* and NTM. Incubation periods for liquid media stay within 6 weeks, but cultures on lower temperatures and on solid media might require incubations up to 10 weeks *(3)*. It is recommended by the ATS that skin samples should be incubated at normal and low temperatures. This should be applied to lymphadenitis and soft-tissue specimens as well!

Species identification.

Species cannot be differentiated by colony morphology and further analysis subsequent to culture is necessary. The conventional identification of species consisted of biochemical tests, colony characteristics and growth temperature. Biochemical species differentiation includes niacin secretion, reduction of nitrate, reduction of tellurite, Tween 80 hydrolysis, growth on MacConkey agar without crystal violet, urease activity, caratogenesis, catalase activity at 68˚C, semi-quantitative catalase activity, growth activity with arylsulfatase, acid fosfatase, pyrazinamidase and ß-glucocidase.

The addition of specific growth inhibitors as nitrobenzoic acid (PNB), and nitro-alpha-acetyl-amino-beta-hydroxypropiophenone (NAP), inhibit only species belonging to the *M. tuberculosis-complex*. They can therefore be useful for the differentiation of NTM as a group.

Molecular identification of NTM species.

DNA-DNA hybridization as a tool for the identification of mycobacterial species was first described in 1978 by Baess et al *(50)*. DNA of reference strains is denaturated and sheared to radiolabeled single strand fragments of approximately 500 bp (probe-DNA). Reannealing is allowed in the presence of genomic DNA of the test strain. All single stranded DNA left after hybridization is cut into mononucleotides by S1 nuclease. The proportion of hybridization is detected by the level of radioactivity which represents the double stranded DNA *(51)*. Variations on this method are based on the same hybridization technique but measurements are performed by fluorescence instead of radioactivity or heat stability measurements of the hybrids *(52)*. Nowadays, DNA-DNA hybridization is considered very laborious and many easier techniques have become available for the identification of NTM.

High performance liquid chromatography (HPLC), completely different from all other methods, is a biochemical method and identifies mycobacteria by analysis of mycolic acids. A suspension of acid-fast bacteria is saponified to cleave the mycolic acids bound to the cell wall. Mycolic acids are then converted to esters and separated from all other cell compounds using a microprocessor-controlled pump and are subsequently detected with a UV spectrophotometer. Reproducible chromatographic patterns containing combinations of different diagnostic peaks are formed. Pattern recognition is performed by visual comparison of sample results with mycolic acid patterns from reference species of known mycobacteria *(53- 55)*. This method, as well as DNA-DNA hybridization, is still applied in the identification and the proposal of a new species. HPLC is even still gaining ground in mycobacteriology and new applications for *M. tuberculosis* have recently been described *(56)*. Quantitation of total mycolid acids peaks appear to correlate to susceptibility for ethambutol, isoniazid, pyrazinamide and rifampin *(56)*.

Essentially, all other techniques are based on *Mycobacterium*-specific amplification with subsequent detection and identification of the species-specific amplicons. This analysis can be performed by direct sequencing of amplified fragments, digestion of amplicons with restriction enzymes (PRA) or probe hybridisation methods: micro-array technology *(57, 58)*, blotting (i.e. the commercial hybridization assays) or real-time PCR *(59-61).*

PCR-based sequencing has become the gold standard for the identification of mycobacterial species. The method consists of PCR amplification of mycobacterial DNA with genus-specific primers and sequencing of the amplicons. The organism is identified by comparison of the nucleotide sequence with publicly available reference sequences. An additional advantage of this method is the recognition of potentially new species. The target most commonly used is the last 500 basepairs of the gene coding for the 16S ribosomal RNA, present in all bacterial species and containing both conserved and variable regions. It is thus a valuable target for taxonomic purposes *(59)*. The sequence of the 16S regions of many species is known at this time. Several other target genes have been found suitable for the differentiation of mycobacterial species: the genes coding for the 32 kDa protein, the 65 kDa heat shock protein, the *rpoB* (RNA polymerase beta-subunit), *secA1* (essential protein), *dnaJ* (heat shock induced protein), *sodA* (superoxide dismutase), *recA* (recombination

protein), *gyrA, gyrB* (DNA gyrase) and the 16S–23S ribosomal RNA internal transcribed spacer (ITS) contain sufficient sequence diversity to distinguish most species *(53, chapter 3)*. For the *dnaJ*, *sodA*, *secA1*, *recA, gyrA* and *gyrB* targets, however, few sequences are publicly available. The panel of possible targets for the differentiation of mycobacterial species is still expanding; the elongation factor Tu encoding gene (*tuf*) and transfer mRNA (*ssrA*) gene region have recently been described for the same purpose *(62)*. Both targets have been evaluated with 127 reference strains and the *tuf* gene yielded comparable levels of differentiation with the *hsp*65 gene while the *ssrA* gene yields comparable discriminatory power to that of the 16S gene. For both targets, it must be noted that only the sequences obtained in this study are available for comparison.
For a single pan-mycobacterial PCR, conserved regions flanking the variable target sequence are necessary. For a real-time PCR, the amplified fragment should not exceed 200 bp. In the *rpoB* and *hsp*65 genes, conserved regions are hard to find for at least short fragments. The 16S does not provide differential sequences within species groups. This leaves -as yet- the ITS as the target with the most potential for a genus-specific (real-time PCR) assay.

The ITS region has also been applied in an assay described by Xiong and colleagues *(63)*. These authors describe a genus specific PCR followed by a reverse line blot (as applied in a commercial assay i.e. InnoLipa, which is further described in this text). The target of choice is able to differentiate 34 species, commonly encountered in de microbiological laboratory. The method is based on the hybridization of a biotin-labelled PCR-product to a membrane with species-specific probes. Binding of the product is visualised by chemiluminescence. A variation on this principle is the assay recently described by Wu and colleagues *(64)*. This assay uses dot blots for the identification of 22 species and targets the 16S gene. Positive dots are visualised using colorimetric hybridization.
The microarray technique uses species-specific probes attached to a glass slide on which the PCR product can hybridize *(59, 64, 65)*. Different sequence targets can be used for this application. Tobler et al, described a real-time PCR assay for the identification of the genus mycobacterium and targeted the hsp65 gene. Two primer-pairs were used for the amplification of sequences from the 37 species (and subspecies) tested. NTM species identification was performed separately with an extra amplification on the hsp65 gene and subsequent analysis with microarray technology *(58)*. The hybridised PCR product is visualised by a computer via fluorophore measurements. The main advantage of the method is the possibility to use extensive probe panels, which may also include probes for different targets like resistant markers *(57)*, and are readily equipped with extra probes.
PCR following restriction pattern analysis (PRA) has been described several times for the differentiation of mycobacteria. As a PRA assay, ribotyping was described by Tsitko. The method (PCR of a single unknown ribosomal segment and subsequent cutting with restriction enzymes) could differentiate 32 species, all with individual restriction patterns with the enzymes *pfu*II and *eco*RI *(66)*. Roth and colleagues, described an PRA assay targeting the ITS region *(67)*. This assay has been extended recently with a database of restriction

patterns for the automated analysis of 31 species with 10 different restriction enzymes *(68)*. Most assays have chosen the hsp65 gene as a target with a combination of two different restriction enzymes *(69-75)*. The PRA technique enables identification with limited and inexpensive laboratory equipment. However, while the assays are all evaluated with an extended panel of species, all new or un-evaluated species create undetermined patterns.
A recently described assay from Lim and colleagues used a fluorescent probe real-time PCR approach: a real-time PCR with 21 probe combinations located in the *rpo*B gene which were able to differentiate 18 species and species-complexes from cultured strains *(76)*.

Based on a reverse line probe hybridisation principle, one of the four commercially available tests for the identification of clinically relevant NTM has been developed within the Genotype series (Hain). The test allows identification of 15 species in the main test and an additional 17 in the AS (additional species) test based on 23S sequence differences *(77)*. The second commercial test for the identification of mycobacterial species is the InnoLipa V2 (Innogenetics), also a reverse line probe hybridisation assay based on the ITS region. This test identifies 16 species *(78)*. The third test consists of separate ribosomal DNA probes (AccuProbe, Gen-Probe inc) specific to the species *M. avium, M. intracellulare, M. kansasii* and *M. gordonae (79)*. Comparison of the InnoLipa test with the Accuprobe test shows the InnoLipa assay to be better internally controlled and more accurate in species identification than the Accuprobe assay, since Accuprobe misidentified 4 *M. tuberculosis* and *M. avium* strains *(79)*. The last commercially available test contains a 16S sequencing kit (MicroSeq 500 system, Applied Biosystems) which includes pan-bacterial primers for the amplification and sequencing reactions and software with sequence database for the recognition of obtained sequences *(80)*. All four commercial assays are only applicable on cultured isolates, which is also the case for most in-house assays.
Moreover, almost all of the methodologies described above lack the ability to identify new or unevaluated species. Direct sequencing and HPLC (to a lesser extent) are the only methods available to recognise unknown patterns.

Molecular detection of NTM in clinical specimens.

The main advantage of molecular detection of mycobacteria directly from clinical materials is that the time otherwise required for culture is greatly reduced. Rapid differentiation between *M. tuberculosis* and NTM decrease the time that patients spend in isolation and many of the antibiotic regimens for *M. tuberculosis* differ from those suitable for NTM *(73)*. In several publications, the simultaneous amplification of *M. tuberculosis*-specific and pan-mycobacterial fragments has been applied directly to clinical specimens *(81-84)*. These methods all used the presence of multiple bands as a confirmation of *M. tuberculosis*, while a single band represented NTM. The *hsp*65 gene was the target used in all assays for the pan-mycobacterial amplification. However, the visualisation of pan-mycobacterial amplicons in clinical materials was performed by agarose gel electroforese, which has a poor specificity and sensitivity. PRA technique includes the differentiation of the amplified PCR product. Three published PRA assays, all targeting the *hsp*65 gene, have been evaluated on paraffin-embedded materials and thus offer greater potential in various clinical specimens *(74, 84, 85)*. Staining methods have poor sensitivity in paraffin embedded materials and these assays yield either similar or higher sensitivities for the detection of mycobacteria compared to acid fast staining.

In total, however, only a few molecular detection methods allow detection from clinical materials. A high sensitivity is necessary and often not reached. This is emphasised by the commercially available assays which are almost always exclusively for the identification of NTM from cultured isolates. (Almost) no commercial assays other than for the detection of *M. tuberculosis* are sensitive enough for application on clinical materials, while for these assays the performances are often poor as well. The overall sensitivity of the DR. MTBC Screen assay (DR. Chip Corporation) is 56.6% and the BD ProbeTec ET Mycobacterium tuberculosis Complex Direct Detection (DTB) assay (Becton Dickinson) has a sensitivity of 57-63%. Both assays produced considerable amounts of false-positive results: 13 of 494 patients in the DTB assay and 11 of 494 patients in the DR. MTBC assay *(86, 87)*. The Mycobacterium tuberculosis Amplified Direct Test (AMTDII) (Gen-probe) yields sensitivity rates of 74-92% but has problems with inhibition due to the lack of internal control *(88-90)*. The LCx Mycobacterium tuberculosis assay (Abbott) yields sensitivity rates of 54-88% *(89, 91)*. The COBAS AMPLICOR Mycobacterium tuberculosis Assay (Roche) reaches published sensitivities of 59-94% *(90, 91)*. The overall sensitivity of the RAPID BAP-MTB assay (AsiaGen) was 57-66% and produced significant amounts of false-positive results *(86)*.
Recently, a test has been developed for the detection of *M. tuberculosis* and four clinically relevant NTM (*M. avium, M. intracellulare, M. kansasii* and *M. malmoense*) directly in clinical specimens. The GenoType Mycobacteria Direct (GTMD) (Hain) yielded 77% negative predictive value and a sensitivity of 90% in the only evaluation published so far *(92)*. This is the only commercially available assay for the detection of NTM in clinical materials.

For the direct detection of pathogens in clinical materials, real-time PCR has claimed a prominent position. It yields a higher sensitivity and specificity than other detection methods, mainly because of the addition of specific probes to the primers. The technique allows rapid detection and in most assays is a closed single tube test which decreases the risk of contaminating DNA.
Several detection systems are available for this purpose, of which the ABI Taqman (Applied Biosystems) and the Light-Cycler (Roche Diagnostics) are the most commonly used in the Netherlands. The ICycler IQ4 system (Bio-rad) was one of the first systems to enable multiplex detection by employing halogen light absorption and multiple filters for simultaneous detection of different fluorescent labels.

For the direct detection of *M. avium*-complex or *M. paratuberculosis* many real-time PCR assays have been developed in veterinary medicine, but these are restricted to the detection of *M. avium* only *(93-98)*. Another real-time PCR assay for application on FF/PE materials has been recently published by Beqai and colleagues *(99)*. The assay, however, only detects *M. tuberculosis* and *M. avium* complex. More useful in clinical application are PCR assays that enable NTM detection as a group.
In table 3, four published methods, designed for the real-time detection of the genus *Mycobacterium*, have been summarised. For pan-mycobacterial detection without culture, only a few assays have previously been described *(100-103)*. Of these few, only two *(102, 103)* have been evaluated on clinical materials, the assays of Khan and Kawai were developed for fluidic samples, but might be applicable on clinical materials.
The assay described by Khan and colleagues has been evaluated for metal-working fluids *(100)*. This assay targets the *hsp*65 gene. However, while the assay was designed to be “genus-specific”, no panel of species was tested. Moreover, the detection was performed using sybr-green, which essentially means a normal PCR without the extra specificity of additional probes. Theoretically, the amplicons could be analysed by melting-curve analysis, but this requires an extensive analysis of a species panel.
The assay developed by Kawai and colleagues, a real-time PCR targeting the 16S gene, was only evaluated on water samples for the quantitative detection of bacterial water flora. The assay successfully detected mycobacterial DNA in three samples and enabled quantification *(101)*.
Shrestha and colleagues described a real-time PCR assay for the detection of mycobacteria with the light-cycler system (Roche) *(102)*. The assay uses melting point analysis to identify NTM species by means of mismatches in the *M. tuberculosis* specific probe. Only the species *M. gordonae* was not detected because of too many mismatches and the species *M. kansasii* and *M. marinum* were not differentiated from one another due to overlapping melting points. The assay proved to have the same sensitivity as the Cobas amplicor for *M. tuberculosis* and yielded 96% of positive clinical samples positive for *M. tuberculosis*. Only three clinical samples were positive for NTM which were all detected.
Garcia and colleagues described an assay with simultaneous detection of *M. tuberculosis* and the genus *(103)*. Using three primers for a heminested PCR, amplicons of 475 basepairs

of the 16S gene were formed. This assay was validated on 167 clinical materials, 127 of which were culture positive and 40 were control samples. 12 samples yielded NTM in culture, 8 (66%) of which were detected by real-time PCR. The sensitivity of this assay was probably slightly compromised by the length of the amplicon and no NTM identification was performed.

While a pubmed search for the combined terms “real-time PCR” and “mycobacteria”, yields 210 publications (until November 2007), only four real-time PCR assays are genus-specific and allow detection in direct materials as summarised in table 3 (our own assay described in chapters 3, 4 and 5 is not included). The most extensive clinical validation included 12 NTM positive samples, 8 of which were detected by real-time PCR *(103)*.
This emphasises the need for the development of a sensitive real-time PCR as described further in this thesis, specifically for the genus *Mycobacterium* and validated on clinical materials.

Table 3: Real-time PCR assays for the detection of the genus Mycobacterium directly in materials (PPV = positive predictive value, NPV = negative predictive value).

publication of assay	**design and analytical performance**		**validation and clinical performance**	
Kawai et al, JAP, 2004 *(101)*	Direct recognition in real-time:	genus	Panel of samples used in validation:	48 water samples (3 positive for mycobacteria by PCR, culture results unknown)
	Subsequent identification NTM species:	denaturating gel + sequencing		
	Target:	16S rRNA gene	Clinical sensitivity/specificity/PPV/NPV:	unknown
	Species used for analytical specificity:	unknown		
	Analytical sensitivity:	1,6 copies / reaction		
Khan et al, MCP, 2004 *(100)*	Direct recognition in real-time:	genus	Panel of samples used in validation:	20 water samples (2 positive for mycobacteria by PCR, culture results unknown)
	Subsequent identification NTM species:	no		
	Target:	*hsp65*	Clinical sensitivity/specificity/PPV/NPV:	unknown
	Species used for analytical specificity:	1 species		
	Analytical sensitivity:	130 copies / ml		
Shrestha et al, JCM, 2003 *(102)*	Direct recognition in real-time:	genus and *M. tuberculosis*	Panel of samples used in validation:	50 clinical samples culture positive for *M. tuberculosis*, and 3 culture positive for NTM
	Subsequent identification NTM species:	melting point analysis		
	Target:	16S rRNA gene	Clinical sensitivity/specificity/PPV/NPV:	96% sensitivity for tuberculosis, 100% sensitivity for NTM
	Species used for analytical specificity:	11 species		
	Analytical sensitivity:	unknown		
Garcia et al, JCM, 2002 *(103)*	Direct recognition in real-time:	genus and *M. tuberculosis*	Panel of samples used in validation:	167 clinical samples (127 culture positive of which 12 NTM, 62 smear positive, 40 negative)
	Subsequent identification NTM species:	no		
	Target:	16S rRNA gene	Clinical sensitivity/specificity/PPV/NPV:	75,6% sensitivity (66% sensitivity for NTM) , 100% specificity, 100% PPV
	Species used for analytical specificity:	6 species		
	Analytical sensitivity:	1000 copies / ml		

Susceptibility testing.

Conventional methods.

Due to the variable susceptibility patterns between and within species the susceptibility testing is an important part of mycobacterial diagnostics.

The conventional methods of susceptibility testing in mycobacteria consist of culture in the presence of a panel of antimycobacterial agents. Resistance is measured in the Minimal inhibitory concentration (MIC). Three methods are used for this purpose: the E-test, the broth or agar microdillution and the disc diffusion methods.

The agar and broth microdillution method are based on a two-fold dilution series of agents on solid medium or liquid medium and the MIC is recorded as the lowest concentration of a drug that inhibited “reasonable” growth (either 10% or 50% growth efficiency) *(104)*. The E-test is a method for MIC determinations for antimicrobial agents that is based on a predefined antibiotic gradient on a plastic strip calibrated with a continuous logarithmic MIC scale covering 15 two-fold dilutions *(105)*. Growth occurs only around parts of the strip with concentrations lower than the resistance of the isolate. The disk diffusion method is based on an agar plate with a tablet containing an agent which releases this into the agar. The concentration of the antibiotic then exists in a decreasing gradient from the tablet. The method allows bacterial growth in a radius around the tablet of a concentration within the resistance range of the isolate *(106)*. For all methods, specific growth rates for mycobacteria apply. In rapid-growing species the result is read after 3 days depending on the inoculums used. Slow-growing species need several weeks for trustworthy readings. To help standardize the methods used in the clinical microbiology laboratory for testing the susceptibility of mycobacteria, the Clinical and Laboratory Standards Institute (CLSI, formerly NCCLS) published an updated NCCLS document M24-A: the tentative standard for antimycobacterial susceptibility testing *of Mycobacterium tuberculosis* and NTM *(107, 108)*.

The major drawbacks are similar for all these conventional methods: 1) due to the slow growth of mycobacteria, a high risk of contamination of the plates with other micro-organisms exists; 2) readings are subjective to the observing, but hardly standardised, eye of the technician; 3) for difficult-to-grow or non-culturable mycobacteria, these methods are not applicable or need alternative media/methods; and 4) the accordance of treatment failure with MIC values is unsure. For these reasons, mycobacterial diagnostics will benefit from new methods. However, genetic background of resistance appears to be complicated. For several different antimycobacterial agents, more than one responsible genetic markers have been identified.

Molecular approaches for susceptibility testing.

Due to the emerging of multidrug- and extremedrug-resistant *M. tuberculosis* strains, there is a growing need for molecular identification of drug resistance in *M. tuberculosis* isolates.

Resistance in mycobacteria is linked to the same genetic mechanisms in different species (Table 5). Therefore, research into *M. tuberculosis* give clues to the resistance mechanisms existing in NTM for at least pyraminazine, isoniazin, rifamycins, macrolides, quinolones and

streptamycin *(109-112)*. For rifamycin resistance (rifampin, rifabutin, rifapentine, and KRM-1648), 23 mutations have been identified in the rpoB gene of *M. tuberculosis* of which the 4 most common ones coding for approximately 96% of the resistance *M. tuberculosis* cases are located in the 81 bp long rifamycin hotspot: the "rifampin resistance-determining region" *(113)*. Cross-reactivity for resistance to different rifamycins is recorded in subsets of the known mutations *(114)*. Mutations in codons 513, 526, and 531 of the *rpoB* gene, have been identified in rifampin resistant strains of *M. kansasii (115).* Several molecular tests have been developed for the detection of rifamycin resistance in *M. tuberculosis*, either in cultured or clinical materials *(111, 116-120)*. Two of these are commercially available: the InnoLipa Rif TB and the MTBDR *(111)*. The usefulness of these tests for NTM is variable. The biggest drawback of the use of the assays developed for *M. tuberculosis* is the high sequence variability within the *rpoB* gene. Development of primers specific for all NTM in one assay will be very difficult. In a micro-array assay, developed by Vernet and colleagues, the mutations –accounting for rifamycin resistance- in the *rpoB* gene are probed in the same slide with panmycobacterial 16S sequences. However, while suitable for the mutations identified for both rifampin and rifabutin resistance and possibly applicable to NTM, the authors were only interested in identifying and characterising *M. tuberculosis (57)*.

Genetic alterations in the *inhA* and katG genes account for 70-85% of the Isoniazid (INH) resistant *M. tuberculosis* strains *(111, 121).* Loss of the catalase-peroxidase KatG activity has been shown to be the major resistance mechanism for intrinsic INH resistant *M. avium* strains as well *(122)*. Several assays have been developed for the detection of INH resistance in *M. tuberculosis (109, 111, 116, 119, 121)* and might be useful in NTM that are not intrinsically resistant to INH. However, so far, no sequence information has been obtained for aquired INH resistance in NTM.

Ethambutol (EMB) is widely used in the treatment of NTM infections. Mutations in the *embB* gene have been associated to EMB resistance in NTM. In the “EMB resistance determining region”, several mutations appear to be responsible for resistance in NTM, but seem variable in different species as research of Alcaide and colleagues show *(123)*. Gene transfer of different alleles to *M. smegmatis* results in other MIC values than in the original species (Table 4). Therefore, more research has to be performed to elucidate the EMB resistance mechanisms in NTM.

Fluoroquinolone resistance is related to mutations in de gyrase A and B genes *(124)*. While different resistant mutations occur in different species, species-specific sequences seem conserved *(125)*. These regions might therefore be useful in a molecular assay where a species can be identified with simultaneous detection of quinolone resistance. However, the variability in this gene is too extensive to cover all possible variations in a single PCR unless a hybridisation principle with a large panel of probes is used.

Intrinsic macrolide (clarithromycin, azithromycin) resistance is correlated with the presence of *erm* genes, and is, for instance, found in *M. smegmatis, M. tuberculosis* and isolates of *M. fortuitum (126, 127)*. However, acquired resistance is related to a mutation in the 23S gene. *Erm* genes are responsible for the methylation of 23S-RNA, but direct mutation in the 23S –RNA gene results in the same secondary structure alteration of the ribosomes *(128)*.

Development of molecular tests for the detection of these 23S mutations, might be fairly simple for all NTM, because to date, no other mutations have been identified except in bp 2057-2058 and the sequence variation in this region between mycobacterial species is not extensive.

Overall, since not all genetic markers responsible for antimycobacterial agents are known, and several mechanisms appear complicated, the molecular approach for the detection of resistance is incomplete and conventional culture methods will, for now, provide more information.

Table 4: from Alcaide et al 1997 *(123)*: TABLE 2 Correlation between EMB phenotype and genotype at the ERDR fragment in EmbB[a] . Alignment homology of the EmbB region is represented by stripes. Displayed in the last column are MICs for *M. smegmatis* mc2155 transformed with various *emb* alleles.

[b] A, alanine; R, arginine; N, asparagine; D, aspartic acid; E, glutamic acid; Q, glutamine; G, glycine; H, histidine; I, isoleucine; L, leucine; M, methionine; F, phenylalanine; S, serine; T, threonine; Y, tyrosine; V, valine.

species (phenotype)	ERDR[b]	MIC (mg/ml) or phenotype	MIC (mg/ml) after gene transfer to *M. smegmatis*
M. tuberculosis	SDDGY ILGM ARVADHAGYMSN	2.5	
M. gordonae	----- ---- ----------	2.5	
M. nonchromogenicum	----- ---- ----------	2.5	
M. marinum	----- ---- ----------	4	
M. malmoense	----- ---- ----------	4–8	
M. genavense	----- ---- ----------	S?	
M. kansasii	----- ---- -----R------	8	
M. avium	----- ---- -----R------	8	
M. smegmatis	----- --Q- -T-E-----A--	0.5	10
M. peregrinum	----- --Q ----------	8	
M. fortuitum	----- --Q ----------	8–16	64
M. chelonae	----- QM-- --T-E-----A-	64	
M. abscessus	----- QM-- --T-E-----A-	64	254
M. leprae	----- QMQ- --T---S---A-	R	
M. smegmatis (R)	----- F-Q- --T-E-----A-	100	254
M. tuberculosis (R)	----- ---I ----------	20	
M. tuberculosis (R)	----- ---L ----------	40	
M. tuberculosis (R)	----- ---V ----------	40	

Table 5: Antimycobacterial agents with known resistance inducing mutations.
(all included in susceptibility panel in the Netherlands)

antimycobacterial agent	group	genes with identified mutations	mutations identified in *M. tuberculosis*	mutations identified in NTM	year	reference
Isoniazid		Enoyl acp reductase (inhA)	yes	no	2004	*129*
		Catalase-peroxidase (*katG*)	yes	no	2004	*129*
		Alkyl hydroperoxide reductase (*ahpC*)	yes	no	2004	*129*
		Oxidative stress regulator (*oxyR*)	yes	no	2004	*129*
Rifampicin	rifamycins	RNA polymerase subunit B (*rpoB*)	yes	*M. kansasii, M. avium, M. leprae, M. africanum*	1994	*115, 130*
Rifabutin	rifamycins	RNA polymerase subunit B (*rpoB*)	yes	*M. kansasii*	2001	*115*
Ethambutol		Arabinosyl transferase (*emb A,B* and *C*)	yes	See table 4	1997	*131*
Clarithromycin	macrolides	23S ribosomal RNA	yes	*M. smegmatis, M. intracellulare, M. kansasii, M. avium, M. chelonae, M. abscessus*	2004, 2005	*132*
Streptomycin	aminoglycosides	Ribosomal protein subunit 12 (*rpsL*)	yes	no	2004	*129*
		16s ribosomal RNA (*rrs*)	yes	*M. avium, M. smegmatis*	2004, 1997	*129, 134*
		Aminoglycoside phosphotransferase (strA)	yes	no	2004	*129*
Kanamycin	aminoglycosides	16s ribosomal RNA (*rrs*)	yes	*M. smegmatis*	2005, 1997	*133, 134*
Pyrazinamid		Pyrazinamidase (*pncA*)	yes	no	2004	*129*
Cycloserine	beta-lactam	penicillin binding proteins (*pbp2x*)	no	no	2006	*135*
		histidine protein kinase (*ciaH*)	no	no	2006	*135*
		(related to overproduction of D-alanine racemase (Alr) and D-alanine ligase (Ddl))	*(yes)*	*(M. smegmatis)*	*2003*	*(136)*
Ciprofloxacin	fluoroquinolones	DNA gyrase (*gyr A* and *B*)	yes	*M. avium, M. leprae, M. fortuitum, M. aurum, M. kansasii, M. chelonae, M. smegmatis*	2003, 1995	*124, 125*
		topoisomerase subunits (*parC* and *parE)*	no	no	2006	*137*

Typing of NTM.

Molecular genotyping studies on mycobacterial species provide insights into epidemiological behaviour, evolution and transmission of the bacteria. Evolutionary speed was elucidated by typing studies *(138, 139)* and taxonomical relations have been established between species and subspecies *(140, 141)*. Geno-(sub)typing can follow one of two principles: genome-wide comparisons and comparison of small genome segments (Table 6).

Methods targeting the whole genome have often been applied in mycobacteriology. The methods described most often are Pulse Field Gel Electroforese (PFGE) and Random Amplified Polymorphic DNA (RAPD). These two and Amplified Fragment Length Polymorphism (AFLP), are based on the restriction and/or hybridisation of standard primers over the whole genome in which variation in band patterns can be created either by one single nucleotide polymorphism or the insertion/deletion of larger fragments (*142)*. No sequence information is necessary for these whole genome approaches and they are, therefore, easy to use for less prevalent species. Because of the often applied automated analysis of AFLP, while the other two methods generally depend on visual analysis, the AFLP method is more suitable for large collections of isolates. RAPD is easy to perform and is therefore often applied in the investigation of, for instance, pseudo-outbreaks.

One of the earliest methods for typing mycobacteria was plasmid profiling. In this method the sizes of the plasmids present are compared between strains. However, because this method focuses on extrachromosomal DNA, it does not provide conclusive evidence about the relatedness of strains. In fact, isolates with similar plasmid profiles may belong to different biovariant groups and vice versa. The method has other drawbacks: not all isolates contain plasmids and profiles may change over time, whereas completely different plasmids may have an identical size *(59)*. Other genome segments used in genotyping are repetitive elements like insertion sequences (*IS*). The fact that many *IS* elements are limited to a narrow range of species, and their random distribution within the chromosome, make them useful clinical tools for diagnosis and typing. Thus far, more than 37 different *IS* elements have been described for mycobacterial species *(145)*. *IS*6110 has been widely used as a standard epidemiological tool for identifying and typing *M. tuberculosis* complex isolates *(143)*. The use of insertion sequences for genotyping and identification of NTM has also been reported. *IS*900 and *IS*901 have been used for the identification of *M. paratuberculosis* and *M. avium* strains. *IS*1245 has been standardized for the typing of *M. avium* *(144)*. *IS*1407 was applied for the characterization of *M. celatum*, *IS*1395 for *M. xenopi* and both *IS*2404 and *IS*2606 for *M. ulcerans*. However, the recent detection of *IS*1245, defined as a genetic marker for *M. avium*, in a clinical strain of *M. malmoense* and *IS*2404, an *M. ulcerans* defining element in an *M. marinum* subspecies, seems to indicate that insertion elements may have spread through horizontal transfer to environmental NTM *(145)*.

Mycobacterial Intergenic Repetitive Units (MIRUs) are another group of genetic elements used in genotyping *(146)*. These relatively short (approx. 80 bp) repetitive elements are located throughout the mycobacterial genome and because of their variable number and direct repeats they have recently been applied in typing studies of *M. tuberculosis, M. avium*-

complex, M. marinum and *M. ulcerans (147-149)*. In the research of Stragier and collegues, the 15 MIRU's, applied for typing of *M. marinum* and the related species *M. ulcerans*, revealed evolutionary information for these species. The 15 MIRU's found in *M. tuberculosis* were also able to differentiate between phylogenetic lineages *(148)*. In *M. avium,* 22 MIRU's were identified *(149)*.

Other repetitive sequences, not identified as IS elements, which are species-specific can also act as probes in mycobacterial typing studies *(150)*. Variable Number of Tandem Repeats (VNTR) or microsatellite analyses are methods that are only applicable in species with a (largely) known genomic sequence (*M. tuberculosis, M. leprae, M. ulcerans* and *M. avium*). Tandem repeats or microsatellites are short repetitive sequences which are readily incorporated or deleted by DNA polymerase and therefore highly variable in length between strains *(151-154)*. Ribotyping is applied as a sub-typing method in other bacterial taxa like Clostridia and Vibrios *(155, 156)* but is not very useful in mycobacteriology. Most mycobacteria contain only one copy of the ribosomal operon and ribotyping is based on the comparison of length, sequence variation and copy numbers of this target.

Sequencing is also included in typing studies as a possible approach. When sequencing is applied in subtyping, multiple targets are required. Recent guidelines for Multilocus sequence typing (MLST) describe the use of at least 7 housekeeping genes as sufficient for application in molecular epidemiological studies *(157).* The method however, has hardly ever been described for mycobacteria yet.

Table 6: subtyping methodology applied on NTM species for epidemiological purposes.

method: sequence knowlegde necessary	**discriminatory power**	**laborious**	**species applied**	**year of publication**	**reference**
MIRU's	++	some	*M. ulcerans*	2006	*160*
(known sensitive for horizontal			*M. marinum*	2006	*160*
transfer)			*M. avium*-complex	2005	*149*
microsattelites /short tandem	++	some	*M. avium*-complex	2004	*158*
repeats			*M. lepra*	2004/2005	*159 / 153*
			M. ulcerans	2006	*151*
IS elements / multi copy elements	+++	no	*M. avium*-complex	2006	*163*
(known sensitive for horizontal			*M. ulcerans*	1999	*161*
ransfer)			*M. marinum*	2002	*162*
			M. celatum	1997	*164*
			M. xenopi	1996	*165*
			M. haemophilum	1994	*150*
			M. absessus	2002	*166*
			M. kansasii	1993	*167*
			M. smegmatis	1998	*168*
PRA of *hsp*65	+	no	*M. kansasii*	2006	*169*
			M. gordonae	2002	*170*
			M. terrae	2002	*170*
			M. fortuitum	2002	*170*
			M. avium-complex	2004	*171*
MLST	++	yes	*M. marinum*	1999	*161*
			M. ulcerans	1999	*161*

Table 6 part 2.

method: random genomic approach	discriminatory power	laborious	species applied	year of publication	reference
AFLP (suitable for high troughput application)	+++	yes	*M. kansasii*	2003	*172*
			M. marinum	2001	*173*
			M. ulcerans	2001	*173*
			M. avium	2004	*174*
			M. lentiflavum	2005	*175*
PFGE (highly discriminatory (more so than RAPD) but difficult in use and reproducebility)	+++	yes	*M. haemophilum*	1994	*176*
			M. avium-complex	2003	*177*
			M. abscessus	2003	*178*
			M. terrae	2006	*179*
			M. chelonae	2005	*180*
			M. Imunogenum	2005	*180*
			M. kansasii	2004	*181*
			M. goodii	2004	*182*
			M. mageritense	2004	*183*
			M. furunculosis	2002	*184*
			M. marinum	2002	*185*
			M. szulgai	2002	*186*
			M. malmoense	2002	*187*
			M. gordonae	2001	*188*
			M. celatum	1997	*164*
			M. intracellulare	1993	*189*
			M. ulcerans	2000	*190*
			M. jacuzzii	2003	*191*
			M. fortuitum	2006	*192*
			M. cosmeticum	2004	*193*
			M. simiae	2000	*194*
RAPD (mainly applied for small clusters of isolates to check for clonality, easy to apply)	++	no	*M. jacuzzii*	2006	*191*
			M. fortuitum	2006	*192*
			M. cosmeticum	2004	*193*
			M. gordonae	2006	*195*
			M. abscessus	2005	*196*
			M. szulgai	2002	*197*
			M. avium-complex	2000	*198*
			M. malmoense	1999	*199*
			M. chelonae	1998	*200*
			M. simiae	2000	*194*

Conclusions and design of the thesis.

Many new NTM species have been found and characterised, ranging from non-pathogenic to pathogenic. Molecular methods contributed significantly to the identification of these new species, replacing conventional laborious methods. New criteria have been formulated to determine whether a new species can be officially recognized. The application of these criteria requires that genetic information of at least two targets should be included, but the choice of the targets is not fixed. Therefore, we compared 6 sequence targets (chapter 8), all used for NTM identification in the Netherlands, for their ability in species differentiation.

Because of the highly variable appearance of NTM infections and the large spectrum of species involved, a rapid diagnostic method is required to recognize as many mycobacterial species as possible. We therefore focused on the development of a mycobacterial genus-specific PCR (chapter 3) and on species-specific PCRs, enabling the identification the most frequently found species (chapters 3 and 4). Conventional PCRs are not practical for the microbiological laboratories nowadays, compared to the possibilities of real-time PCR. Since only 2 real-time PCRs have previously been described for the detection of the genus *Mycobacterium* in patient materials, we developed a new assay and applied it directly on variable clinical materials. For this real-time PCR, the ITS region was chosen because it enables the differentiation of all slow-growing species using a short amplicon.

Molecular detection also enables biopsies and other histopathological materials to be examined for the presence of mycobacteria when culturing is not possible due to fixation of the tissue. This offers an excellent opportunity to investigate the presence of newly identified mycobacterial species in stored patient materials (chapter 5). However, the widespread occurrence of NTM in the environment may result in contamination of patient samples during processing of the materials and special precautions will be necessary.

When an emerging mycobacterial species is found in association with a “new” clinical syndrome, questions arise on the epidemiology of the disease. Molecular typing methods play an important role in understanding the epidemiology. Based on an extensive literature review, we decided to develop an AFLP (chapter 6) for the genotyping of *M. haemophilum* isolates and applied a standardised RFLP on *M. avium* isolates (chapter 7). The aims of the application of these techniques were different. AFLP was chosen to compare clinical isolates with each other to trace a possible common source, whereas the RFLP was applied to investigate animals as a possible source for human *M. avium* infections.

References: 2 Diagnosis of NTM disease.

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