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Diagnostics of non-tuberculous mycobacteria

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9 Discussion: diagnostics of non-tuberculous mycobacteria.

Molecular identification of species and recognition of subspecies.

Molecular techniques have replaced conventional methods for appropriate species identification and can be considered the new gold standard. As described in detail in chapter 2, many gene targets have been described to be suitable for species differentiation. Of these, the partial genes for 65 kDa heat shock protein (*hsp*65), RNA polymerase beta-subunit (*rpo*B), essential protein *sec*A1, superoxide dismutase (*sod*A), 16S RNA and the 16S–23S ribosomal RNA internal transcribed spacer (ITS) have been compared in chapter 8 for the species *M. avium, M. kansassii, M. gordonae, M. abscessus/chelonae* and *M. malmoense*. These 6 gene targets are currently applied for mycobacterial species identification in the Netherlands. The most obvious differences between the compared sequence targets are the availability of comparable sequences in public databases and the level of differentiation. The 16S gene is the most conserved among mycobacterial species and allows only differentiation to the species-complex level, while the ITS and the *hsp*65 gene are the most variable. The *sod*A and *sec*A1 genes are variable as well, but of these, few sequences are available in the public databases.

Since the taxonomical and clinical status of subspecies or species variants are unclear, it is uncertain if differentiation of NTM species to the sub-levels is clinically relevant. Several studies have been performed to establish further insight into the clinical significance of for instance *M. kansasii* subtypes *(1, 2).* Some *M. kansasii* subtypes appear more prevalent in human disease, but differences in virulence or pathogenesis have not been determined. The only NTM species of which differences in virulence of subtypes have been determined are *M. avium (3), M. ulcerans (4)* and *M. marinum (5, 6)*. *M. marinum* is often used as a model for *M. tuberculosis* research and through this species, many mutations in coding genomic regions related to virulence and pathogenesis have been identified *(7, 8)*.

Chapter 8 of this thesis represents a pilot study to examine the usefulness of gene-targets in the differentiation of mycobacterial species.

While it remains unclear if differentiating NTM species variants provides relevant information for treatment, the identification of species is relevant from an epidemiologic point of view at least. Therefore, we concluded that the ITS or *hsp*65 sequences, which are abundant in the public databases and provide a sufficient level of differentiation, are the best available sequence targets for species identification at this moment.

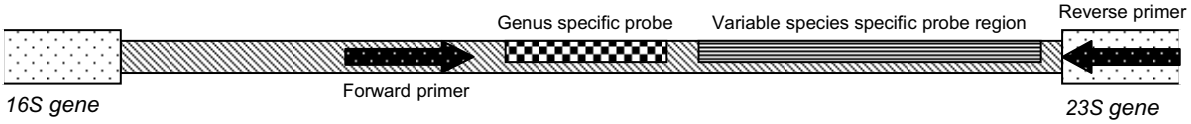
Using these results the next step is to evaluate the targets in their relation to clinical significance of isolates. It might be possible that these or other sequence targets harbour mutations which correlate to virulence or correlate to the same evolutionary lineages.

Real-time PCR for the detection of NTM in clinical materials.

PCR detection in clinical materials enables rapid identification of the mycobacterial pathogen. A conventional PCR with agarose analysis has a low sensitivity due to the amount of amplicon needed for analysis. Moreover, handling PCR products after amplification is a risk for laboratory contamination and sequencing of amplicons (for identification) derived from clinical materials often fails. A possible solution to these problems is the use of real-time PCR. As summarized in the introduction, after a systematic review, most real-time PCR assays are not validated on clinical materials or only capable to detect the *M. tuberculosis*-complex or *M. avium*-complex.

In chapter 3 of this thesis, the development of a real-time PCR assay was described for the direct detection of mycobacterial DNA in clinical specimens. The forward primer and genus specific probe were located in the ITS region and the reverse primer is located in the first part of the conserved 23S gene. The primers yield an amplicon of approximately 175 basepairs (depending on the species) of the ITS region. The primers and genus specific probe for the real-time PCR are located in conserved sequences, but the sequence in between is highly variable and provides enough variation for the design of probes for any other slow-growing species (figure 1). However, because the oligo's were designed on the sequences of slow-growers, mismatches in the forward primer and genus-specific probe have been encountered in several rapid-growing species (i.e. *M. fortuitum* and *M. chelonae*).

Figure 1. Internal Transcribed Spacer region between 16S and 23S genes and location of oligo's.



For the assay in chapters 3, 4 and 5, species-specific probes have been designed for *M. avium, M. tuberculosis* and *M. haemophilum*. In chapter 3, The *M. tuberculosis*-specific probe was applied to exclude the involvement of the species from the *M. tuberculosis*-complex. In chapter 3, 4 and 5, The *M. avium*-specific and *M. haemophilum*-specific probe, to enable the direct recognition of the most common NTM species. Clinical validation revealed that the assay provided a rapid diagnosis of mycobacterial disease in children with mycobacterial lymphadenitis. Addition of the real-time PCR assay to conventional diagnostics (culture and acid-fast staining) performed in 67 children with suspected mycobacterial disease, resulted in the recognition of 13 more children with NTM disease (chapter 3).
While evaluating the application in lymph node specimens, several specimens only yielded a genus-specific signal, while culture remained negative. Sequencing of the amplicons formed in the real-time PCR identified *M. haemophilum*. Therefore, an *M. haemophilum*-specific probe was designed and added to the assay as described in chapter 4. *M. haemophilum*-

specific real-time PCR was superior to culture because only 9 (56%) of the 16 diagnosed *M. haemophilum* infections were positive by culture.
Chapters 3 and 4 reflect only a part of the total study described by Lindeboom *(9).* In total, 135 children with cervicofacial NTM lymphadenitis were included in the study. The addition of the real-time PCR assay to conventional diagnostics revealed *M. avium* to be present in 94 of 135 (70%) of the diagnosed lymphadenitis cases and *M. haemophilum* in 32 of 135 (25%) cases.
No other real-time PCR assay that has been described so far enables genus-specific detection and has been extensively validated for application in clinical materials.
The publications of Shrestha and Garcia are best comparable to the assays we developed and tested as described in chapters 3 and 5 *(10, 11)*. Details of these assays have been described in table 3 in chapter 2. Shrestha and colleagues designed a genus-specific PCR targeting the 16S gene with a 215 bp amplicon *(10)*. An *M. tuberculosis*-specific probe combination (Fluorescence resonance energy transfer principle) was employed for the detection of all species. Because the different mycobacterial species contained variable mismatches in the *M. tuberculosis*-probe region, analysis of the melting points enabled species identification for 9 of the 11 species tested, which included *M. avium* but not *M. haemophilum*. The sensitivity of NTM detection could not be compared to the assay from chapters 3 and 5, because only 3 NTM positive samples were included in the validation.
The method described by Garcia and colleagues comprised a genus-specific probe and targeted the 16S gene as well *(11)*. The clinical validation included 12 NTM positive samples, not including *M. haemophilum*. No culture negative specimens were included from patients with a suspicion of NTM disease. Therefore, the performance of the PCR could only be compared with culture positive specimens. Still, the real-time PCR yielded only 8 samples positive for NTM (66%). This poor sensitivity might be related to the length of the amplicon, which was 475 basepairs. The extraction method might be another reason for the poor sensitivity: alkali washings combined with heating. Mycobacteria are extremely difficult to disrupt and are often intracellular. Therefore, vigorous methods are necessary for successful DNA extraction *(12, 13)*. The assays of Shrestha and Garcia might be as valuable as our own assay in chapters 3 and 5, but a more extensive validation should be performed on NTM containing clinical materials.

In chapter 5, the real-time PCR assay for the detection of mycobacteria was applied to formalin fixed / paraffin embedded (FF/PE) specimens. As far as we are aware, only one publication revealed real-time PCR to be successful in diagnosing NTM disease on FF/PE specimens *(14).* This real-time PCR assay was compared to conventional staining results. However, the assay by Beqaj, detected only *M. tuberculosis*-complex and *M. avium*-complex and not the genus. Based on our experiences described in chapter 4, we consider it very likely that *M. haemophilum* represents an underdiagnosed causative agent of granulomatous inflammations in other clinical manifestations than just lymphadenitis.
Therefore, in chapter 5, the real-time PCR was applied on 30 FF/PE archival skin specimens with granulomatous inflammation collected over the last 25 years. A control group was

assembled of 30 specimens of patients with basal-cellcarcinoma. Surprisingly, DNA of *M. haemophilum* was present in 7 of 13 NTM positive specimens. The oldest *M. haemophilum* positive specimen was from a patient with panniculitis from 1984, initially suspected to be caused by a mycosis. These results clearly prove *M. haemophilum* to be underdiagnosed as a causative agent in granulomatous skin lesions as Sampaio and colleagues suggested previously *(15).* They are not so much "emerging" as suggested by Brown-Elliot *(16),* but appear to have been present years before the real-time PCR detection was introduced.
Unfortunately, one sample of the control group yielded an *M. haemophilum* positive result as well and some of the real-time PCR results could not be confirmed after repeated examinations. The Ct-values (threshold cycles) indicated that the detection limit had been reached. Therefore a higher sensitivity appeared to be necessary in these materials to provide a definite diagnosis of NTM. The true incidence of NTM involvement in granulomatous skin inflammation needs further confirmation in a prospective study using conventional methods for comparison.

Suggestions to improve molecular detection of NTM in clinical materials.

Increasing the sensitivity.
The target for a PCR design can influence the sensitivity in several ways: 1) a small fragment (100-200 bp) is less inclined to form secondary structures than a large fragment (200-1000 bp). The target chosen for the real-time PCR in chapters 3 and 5 is approximately 175 bp; 2) sequence variation should be limited: mismatches in primer sequences decrease effective amplification, mainly in the 3' end of the primers. All known sequences of slow-growing species contain identical primer sequences but the sequences of rapid-growing species contain too much variation; 3) multiple copies of the target sequence increases sensitive detection. Many of the NTM species found in humans are slow-growers. This slow growth rate is related to the presence of only one ribosomal operon in slow growers (and two in some rapid growers) *(17)*. The variety of growth rate in different species is influenced by the amount of promoters at the start of the operon *(17-19)*. Most other bacterial taxa contain several copies of the ribosomal operon and a higher sensitivity is reached when targeting this multi-copy element. Therefore, the ribosomal operon as target in a PCR reaction does not increase the sensitivity of the detection. While repetitive elements applied, for instance, in the detection of *M. tuberculosis* are available for several species, such an element has not yet been identified for all mycobacteria. Some attention might go out to Mycobacterial Intergenic Repetitive Units (MIRUs) *(20)*. These relatively short (approx. 80 bp) repetitive elements are located throughout the mycobacterial genome and have been applied in typing studies of *M. tuberculosis, M. avium-complex, M. marinum* and *M. ulcerans (21-23)*. As mycobacterial MIRU's contain open reading frames, and are therefore fairly stable coding sequences, they might be useful in detecting mycobacteria. When the repeats described in Supply et al *(20)*, are compared to the known genome sequences in the public database, homology is encountered in the genomes of the species *M. paratuberculosis, M. tuberculosis, M. leprae,*

M. bovis and *M. smegmatis,* which are all species of which total genomes have been sequenced and are publicly available. This is in accordance with the homology information given in the article of Supply, where southern Blot analysis yielded homology between *M. tuberculosis, M. bovis* and *M. leprae*. However, other genomes have not been fully sequenced and no knowledge about the presence of sufficient homologous sequences is available. Furthermore, because of the variable amount of repeats of most of the MIRU's, the sensitivity of an assay based on MIRUs can vary in different strains. The identification of a suitable multi-copy DNA target for a diagnostic PCR assay is worth considering, but a more sensitive target than the ITS region we employed for the real-time PCR in chapters 3, 4 and 5 is yet to be discovered.

Optimization for molecular detection in pathological formalin fixed-paraffin embedded materials.

Molecular detection methods applied on pathological formalin fixed-paraffin embedded (FF/PE) materials again often lack sufficient sensitivity levels for diagnostic purposes. Low bacterial loads are a problem in these materials as well, but this is amplified in combination with small paraffin sections. A second problem is encountered in histopathological materials due to DNA degradation by the fixation protocol. This fixation protocol is also responsible for low sensitivity of acid-fast staining procedures. Molecular detection of mycobacteria can yield significantly more positive diagnoses than acid-fast staining, as observed in the comparison of real-time PCR to staining by Fukunaga and collegues *(24)* and also observed for fresh specimens in chapter 3 and 4.

Denaturating properties of formalin fixation used in tissue specimens are known to damage DNA. It has been suggested that the fixation method is the reason that PCR techniques are less successful in materials for histological examination *(25)*. Hepes-glutamic acid buffer mediated Organic solvent Protection Effect (HOPE)-fixation technique provides FF/PE-like morphology together with good preservation of nucleic acids and antigenic structures *(26)*. This fixation technique has been proven to create a higher sensitivity in normal PCR, real-time PCR and hybridization methodology *(25-27)*. A third problem, often encountered in the molecular laboratory, is that DNA from FF/PE materials is difficult to separate completely from the paraffin. Therefore, inhibition of PCR is a common problem in molecular diagnostics of these materials. The protocol used during our study, proved sufficient to eliminate this inhibition.

As described in chapter 5, application of the genus-specific real-time PCR on normal FF/PE materials, was successful in detecting specific mycobacterial sequences, but the sensitivity was limited. A duplex genus specific PCR was only positive in both reactions for 5 specimens and only one of two reactions was positive for 8 specimens. Also, the high threshold-cycles indicate that the detection limit of this assay was reached in these specimens. Application of HOPE fixation in these samples could possibly have led to a higher positivity rate.

Prevention of contamination.

The necessity to detect a low copy number of the bacilli, might compromise the specificity of the assays because contaminating mycobacteria are also detected. This emphasizes the importance of establishing clinical significance in the detection of NTM. However, there is another type of contamination that poses a problem in NTM detection: the components used in molecular assays are all dissolved in water. Experiences in our laboratory and several published articles demonstrate that the PCR mixes are often contaminated with bacterial DNA, presumably present in the water used during preparation of the mixes *(28-30)*. Sensitivity of (real-time) PCR assays is increased by short amplicons and a maximum amount of cycles, as applied in the assay described in chapters 3 and 5. When highly sensitive assays are applied to clinical materials, single copies of contaminated DNA fragments are detected as well. We have encountered this contaminating DNA in PCR mixes through the use of negative controls in diagnostic runs of the assay from chapters 3 and 5. Contaminated batches were observed in Hotstar mastermix (Qiagen) and Platinum taq mastermix (Invitrogen), while batches of IQ mastermix (Biorad) and Universal mastermix (Applied Biosystems) appeared clean. Laboratories should be aware of this: each new batch of PCR mix can potentially contain mycobacterial DNA and should be tested.

Few approaches towards overcoming this problem have been described earlier by cleaning strategies including treatment by 8-methoxypsoralen and different UV wavelengths *(31, 32)*. However, these attempts were demonstrated to be useful in normal PCR programs with 30-35 cycles and detection on agarose, which is less sensitive than the modern real-time approaches. The study of Corless et al, evaluated possible elimination strategies for contaminating DNA. The most effective appeared a combination of *Ava*I and *DNase*I enzymes. UV radiation appeared to reduce the polymerase activity *(30)*. However, the application of nucleases requires inactivation prior to the PCR cycling. Only hotstart polymerase, requiring 10-15 min incubation at a high temperature, will endure this treatment. A control PCR of each batch of mastermix and other contaminating components (i.e. TE buffer and water) will reduce the risk of contamination further. The last component requiring elimination of contaminating DNA is the clinical specimen itself. In other words, the specimens need to be transported in a dry state and all handling should be performed with DNA-free fluids: a very laborious process and difficult to control.

A promising substitution for a DNA target could be an RNA target. While the RNA operon is only present as single copy in most mycobacterial species, the transcripts are available in thousands of copies in intact organisms *(33, 34)*. The Gen-Probe Amplified Mycobacterium Tuberculosis Direct Test (AMTDT, Gen-probe, US California) is a commercially available test which employs the 16S RNA copies from *M. tuberculosis* in direct detection. Several RNA amplification techniques, like Transcription Mediated Amplification (TMA) and Nucleic Acid Sequence Based Amplification (NASBA), might be useful in the detection of NTM.

Extraction of RNA is more difficult than extracting the stable DNA, but this approach might overcome the sensitivity problems as well as the detection of contaminating DNA.

Susceptibility.

Susceptibility testing of NTM for some specific antimycobacterial agents may be valuable for an optimal therapy. However, culture is not always successful and a mycobacterial isolate is required to determine its sensitivity to antimycobacterial agents. Susceptibility tests for mycobacteria require a growth period of several weeks. Also, cut-off values of MIC values for resistancy vary between slow-growing and rapid-growing species *(35)*. For instance, for clarithromycin slow-growing species are considered resistant with a MIC ->32, while rapid-growing species are resistant with a MIC ->8 *(36, 37)*. So far, no molecular tests have been evaluated for antimicrobial resistance in NTM species. The existing assays have been developed for *M. tuberculosis* and for several antibiotics the genetic trait loci have been identified in *M. tuberculosis*. However, for the NTM that have been examined, the loci appeared the same, which creates possibilities for molecular detection approaches.
In general, in vitro susceptibility to clarithromycin is considered to correlate with a clinical response of patients treated with chlarithomycin. Therefore, we evaluated the presence of the 23S mutation in clarithromycin-resistant *M. avium* isolates. Clarithromycin resistance is primarily related to the secondary structure of the ribosomes. Methylation of position 2058/2059 in the structural domain V results in conformational change of the ribosomal binding-site for macrolides, either by mutations in the 23S gene or by direct methylation of the RNA. Of the 6 highly resistant isolates (MIC value >32), all contained a mutation at positions 2058 or 2059 in the 23S gene. However, the intermediate resistant isolates (MIC-value 8 or 16) did not contain the mutation and might be influenced by other resistance-mechanisms, e.g. other known mutations (i.e. in domain II of the 23S gene), direct methylation of the RNA or so far unknown mechanisms *(38)*. The relevance of this intermediate resistance is questionable since clinical failure has never been investigated in correlation to different resistance levels. The rate at which clarithromycin resistance is obtained by an *M. avium* isolate was investigated in our laboratory in a pilot experiment. Three initially susceptible isolates of *M. avium*, were grown for four weeks in Lowenstein media with low concentration of clarithromycin (4 mg/ml). When a colony had grown sufficiently, this colony was passed onto a culture medium with a slightly higher concentration of clarithromycin (8 mg/ml). Subsequently, a colony was transferred to new medium with 32 mg/ml clarithromycin. 23S sequences of the colonies from the highest concentration of clarithromycin were compared to the sequences from the initial strain. The initially susceptible strain yielded wild-type sequences, while the strains obtained in the presence of 32 mg/ml clarithromycin yielded mutations in the 23S sequences at positions 2058 or 2059. These mutations were the same as described previously *(39)* and our results were in concordance with the readily acquired resistance in vivo as described by Thiermann and colleagues: Their research showed the same 23S mutations in clinical *M. avium* strains obtained from AIDS patients after clarithromycin monotherapy, while the strains obtained prior to the therapy showed wild-type sequences *(40)*. Similar experiences were obtained in a retrospective study including isolates from 51 *M. avium* patients who failed clarithromycin

treatment. Many developed resistance during treatment, and 96% of the isolates showed the 23S mutation at position 2058 or 2059 *(39).*

Molecular identification of drug susceptibility patterns, preferably directly in clinical specimens, would enable a rapid initiation of the appropriate antimycobacterial therapy. The macrolide resistance mutations are especially easy to target in an assay, but for other important anti-NTM agents, ethambutol and rifabutin, enough knowledge is also available to develop molecular assays for resistance detection as described in chapter 2. These assays would be fairly easy to develop and could prove a valuable asset to mycobacterial diagnostics.

Typing of NTM for taxonomic purpose and as an epidemiological tool.

In chapters 6 and 7, genotyping was performed on mycobacterial species *M. avium* and *M. haemophilum. M. avium* was typed to investigate animals as a possible source for human infections and *M. haemophilum* was typed to elucidate the possibility of a common source.

In chapter 6, *M. haemophilum* strains from lymphadenitis patients from the Netherlands were compared with strains from other continents and with strains from various clinical manifestations. An Amplified Fragment Length Polymorphism (AFLP) methodololgy was applied which enabled a genome-wide comparison without any sequence information. In total, only 3 published genotyping studies have been performed with *M. haemophilum (41, 42),* including chapter 6. One article describes a Restriction Fragment Length Polymorhism (RFLP) method in which an *M. haemophilum* specific multi-copy sequence was analysed *(41)*. The variable genomic distribution of this “un-named sequence” in *M. haemophilum* was visualized by the RFLP patterns. The method applied by Yakrus and colleagues was a Pulse Field Gel Electroforese (PFGE) *(42)*. This method enables the separation of band patterns created with random priming. In both the studies of Kikuchi and Yakrus, the investigated *M. haemophilum* strains (resp. n=28 and n=19) showed clustered type distribution which might indicate common infection sources among the patients. The strains in these studies were mainly isolated in hospitals in New York. The same strains have been subjected to the AFLP method in chapter 6 to compare the level of discrimination of the methods. The AFLP method was highly discriminatory, and it showed the same clustering as the other two methods. The most striking finding in chapter 6 was a large cluster of 28 strains from the Amsterdam region with one identical pattern. During the CHIMED* study, conducted from 2001 through 2005, a total of 32 children were diagnosed with *M. haemophilum* lymphadenitis *(9)*. Because of this finding, one single niche might be expected for the *M. haemophilum* strain. Another supporting fact was that some of the children included in the study appeared to be living in the same neighbourhood. The only environmental source for *M. haemophilum* described so far is biofilms *(43)*. Water as a reservoir, combined with the finding of clustered cases, might indicate the household water system to be the environmental source of infection. However, we attempted to isolate *M. haemophilum* from one of the patient’s homes, but were only able to detect *Mycobacterium kansasii*. While this patient was the most recent case, there were

several months between the onset of disease and the tapping of household water samples. Therefore, the natural reservoir of *M. haemophilum* remains unknown.

M. avium strains isolated from children with lymphadenitis were genotyped in chapter 7. The applied *IS*1245 RFLP method has previously been described to have a high discriminatory capacity *(44)*. Many papers have been written about genotyping in *M. avium* complex in which a broad spectrum of methods have been applied, ranging from RFLP *(44, 45)*, AFLP *(46)*, Multilocus Variable-number Tandem Repeat Analysis (MLVA) *(45)* and Microarray technology *(47)*. The *IS*1245 method was considered the preferred typing method for *M. avium* and was chosen in chapter 7. The two main objectives in chapter 7 were 1) to establish a correlation of human isolates with isolates from pet birds; and 2) the range of type distribution among human strains. In contrast to *M. haemophilum*, *M. avium* is ubiquitous and has been isolated from soil, animals and water sources *(48)*. While there have been incidences with clustered cases of *M. avium* infection with identical genotypes *(49)* and some cases have been linked to environmental reservoirs *(48, 50)*, we found a large variety of patterns in our typing study and no relation to types isolated from pet birds. Another suggested source of the infecting *M. avium* strains is pig farms. Porcine isolates have been described to be similar to human isolates *(51, 52),* but no clinical cases have been linked to pigs so far. It should be noted, that while so many studies concentrate on the genetic variability and epidemiological behaviour of opportunistic pathogenic mycobacteria, no clear environmental sources have been determined and little research is being performed to find the modes of transmission and risk factors.

Another important finding in chapter 6 is the geographical distribution of *M. haemophilum* genotypes. This phenomenon is recognised in *M. ulcerans* as well *(53-55)*, while type distribution in the closely related species *M. marinum* is not related to geographical origin *(55)*. In chapter 7, also no geographical clustering could be detected for *M. avium* genotypes. The recent article by Johansen and colleagues, support our findings of chapter 7, by determining highly variable RFLP patterns for the human isolates and no geographical relation *(52)*.

These evolutionary or epidemiological differences between mycobacterial species have not been elucidated yet, but should be a highly interesting topic for future research.

* CHIMED: multicenter study to determine the optimal treatment for children with mycobacterial lymphadenitis: medical versus surgical treatment.

<u>A new typing method: Protein profiling.</u>

Previously applied geno(sub)typing methods all have one or more drawbacks: 1) They require sequence information; 2) they have poor reproducibility; 3) they are laborious, as are the methods applied in chapters 6 and 7; and/or 4) they represent only an unknown fraction of the total genomic variation.

New protein profiling methods, the MALDI-TOF (matrix-assisted laser desorption / ionization-time-of-flight mass spectrometry) and SELDI-TOF (surface enhanced laser desorption / ionization time-of-flight mass spectrometry) *(56)*, might overcome all of these drawbacks.

These technologies are designed to provide a characteristic mass spectral fingerprint based on desorbed ions from the cell surface *(57)* but the MALDI-TOF can also be applied on other protein groups, for instance the cord-factors, of which different patterns already have been identified for 12 mycobacterial species *(58, 59).* Therefore, a total protein profile can be obtained from isolates. The methods are applicable on cultured cells without extraction, can be fully automated, and do not need prior information of sequences or proteins. Mass spectrometry has recently been described for the differentiation of 37 mycobacterial species and strains *(57, 58, 60, 61)*. The technology is suitable for both intact and disrupted cells and recent studies have suggested it to be suitable for sub-typing in other bacterial species like *Aeromonas* and *Staphylococcus (62, 63)*. While perhaps already very useful for typing purposes, the methods require an extensive database with protein profiles of species and strains, before it will be fully operational in mycobacterial species identification and of course … it is only applicable on culture isolates.

General conclusion.

Application of the ITS real-time PCR assay on patient materials enables rapid diagnosis of NTM disease. The technique was extremely useful for the rapid detection of the slow-growing *Mycobacterium* species and was successfully applied as a rapid test for the inclusion of patients in a clinical trial. However, sensitivity is limited to 1100 cfu/ml purulent lymph node biopsy material and contamination with saprophytic mycobacterial DNA is problematic for the current genus-specific detection in clinical materials. Therefore, special attention should be given to appropriate controls. Mycobacterial RNA detection might overcome these bottlenecks of PCR techniques.

Addition of species-specific probes to the ITS assay, identified *M. haemophilum* to be present in previously undiagnosed skin inflammation and resulted in the recognition of *M. haemophilum* as the second most common mycobacterial species causing lymphadenitis. Subsequent Amplified Fragment Length Polymorphism (AFLP) analysis of *M. haemophilum* isolates showed this species to posses an extremely low mutation rate. Also, *M. haemophilum* lymphadenitis cases are suspected to have a common source, most likely piped water, in contrast to *M. avium* infections, which appear to originate from variable environmental sources.

Future efforts to shorten and structure mycobacterial diagnostics might be directed towards sensitive molecular detection directly in clinical materials and application of, for instance, the MALDI-TOF methodology, which enables the combined species identification and subtyping of a positive mycobacterial culture within one hour.

References: 9 discussion: diagnostics of non-tuberculous mycobacteria.

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