Nontuberculous Mycobacteria

From Gene Sequences to Clinical Relevance

Jakko van Ingen

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from gene sequences to clinical relevance

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Sweet to ride forth at evening from the wells
When shadows pass gigantic on the sand,
And softly through the silence beat the bells
Along the Golden Road to Samarkand.

We travel not for trafficking alone:
By hotter winds our fiery hearts are fanned:
For lust of knowing what should not be known
We make the Golden Journey to Samarkand.

James Elroy Flecker, Golden Journey to Samarkand (1913)

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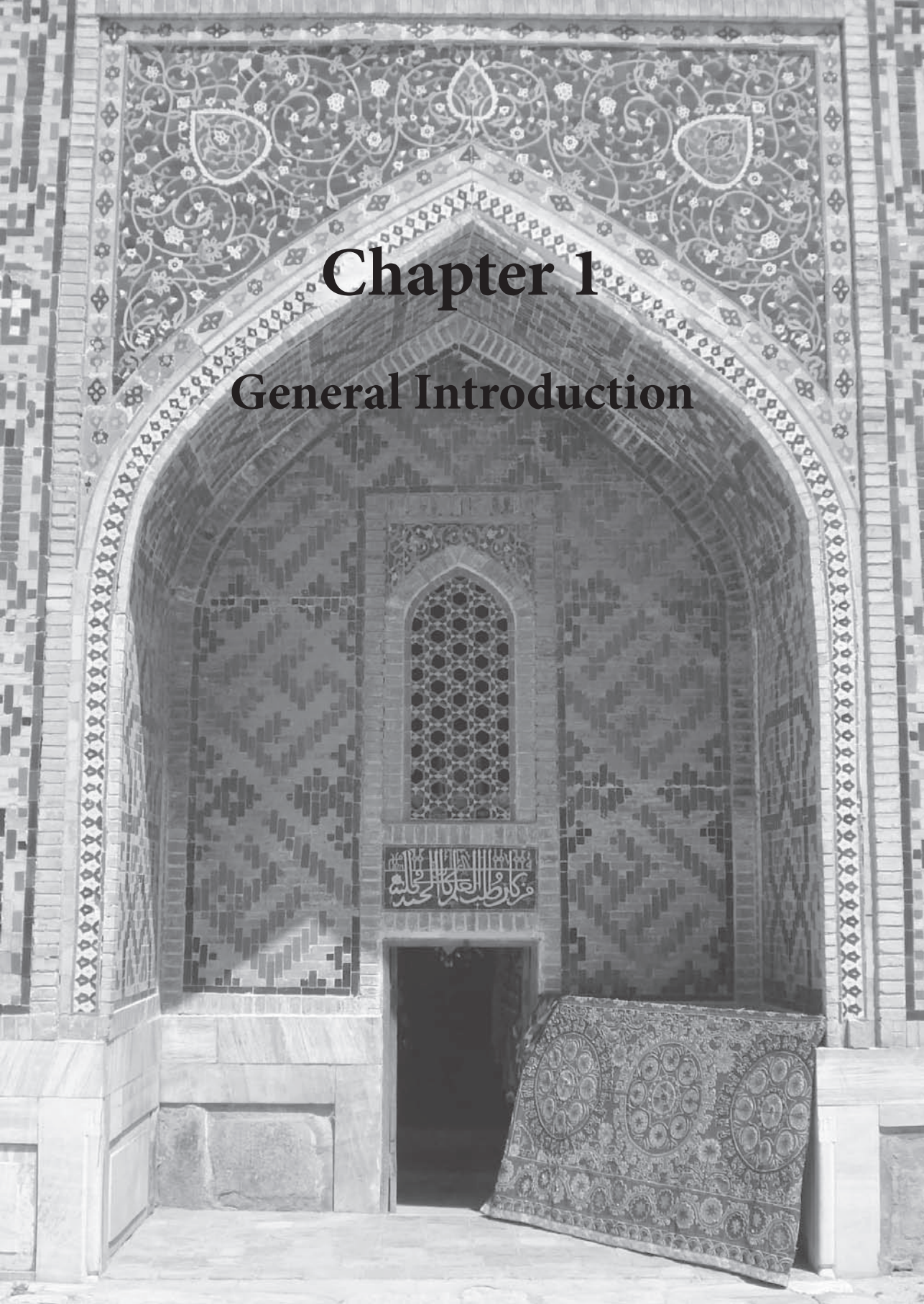


Chapter 1

General Introduction

Chapter 1: General Introduction

A historical perspective

In the years following the ground-breaking discovery of *Mycobacterium tuberculosis* by Robert Koch in 1882, similar mycobacteria were isolated from a wide variety of animal and environmental sources. At the time, these were all considered "atypical *M. tuberculosis*".[1] In the following decades, it became evident that the genus *Mycobacterium* comprises three separate parts: I) the *Mycobacterium tuberculosis* complex, causative agents of tuberculosis in humans and animals, II) *M. leprae*, the causative agent of leprosy, and III) the "atypical" mycobacteria. From the 1950's on, the pathogenicity of these "atypical" mycobacteria to humans was increasingly recognized.[1] This recognition followed a key publication by Buhler and Pollack,[2] in which they described two cases of pulmonary disease similar to tuberculosis, but caused by what they called the "yellow bacillus". The "yellow bacillus" was named after its production of a bright yellow pigment after exposure to light; this species is today known as *M. kansasii*.

In addition to "atypical pulmonary tuberculosis", specific diseases were attributed to the "atypical" mycobacteria, including pediatric cervical lymphadenitis and skin disease by *M. marinum* (swimming pool granuloma) and *M. ulcerans* (Buruli ulcer).[1] At the time, it was often suggested that these "atypical" mycobacteria had arisen in some way as a result of the introduction of chemotherapy for tuberculosis, with streptomycin, isoniazid and para-aminosalicylic acid.[3] Various attempts were even made to create "atypical" mycobacteria, by repeated *in vitro* exposure of *M. tuberculosis* to various drugs; although drug resistance, a hallmark feature of "atypical" mycobacteria, could be induced, the resulting strains were still *M. tuberculosis*.[3]

Just four years after Buhler and Pollack, the first cases of disease caused by "atypical" mycobacteria were recorded in the Netherlands. Manten reported three patients who had been admitted to a sanatorium on a clinical and radiological suspicion of tuberculosis. All their sputum samples grew the "yellow bacillus" on culture, in large quantities.[4] The increasing recognition of their ability to cause human disease led to a long discussion on the correct nomenclature of the "atypical" mycobacteria. Different authors in different periods have proposed the names anonymous mycobacteria, environmental mycobacteria, atypical mycobacteria, opportunist mycobacteria, mycobacteria other than tubercle bacilli (MOTT) and nontuberculous mycobacteria.[1] As these mycobacteria make up the majority of species within the genus *Mycobacterium*, the term "atypical" is debatable. Moreover, many of these mycobacteria are no longer anonymous and there is no evidence that they are environmental or opportunistic pathogens by definition. Hence, we prefer the term nontuberculous mycobacteria (NTM) and it will be used throughout this thesis.

To facilitate structured clinical and taxonomical study, Runyon proposed a classification of NTM into four major groups, based on growth rate and colony pigmentation, in his seminal 1959 review.[3] His bacteriological classification, which remained in use until the wide-scale application of molecular techniques, is summarized in Table 1.

Table 1: Classification of nontuberculous mycobacteria according to Runyon[3]

Group	Characteristics	Important species
I	Slow growth; Pigmentation after exposure to light (Photochromogens)	*M. kansasii, M. szulgai, M. simiae*
II	Slow growth; Pigmentation without light exposure (Scotochromogens)	*M. scrofulaceum, M. xenopi, M. gordonae*
III	Slow growth; No pigmentation (Non-chromogens)	*M. avium, M. intracellulare, M. malmoense*
IV	Rapid growers	*M. fortuitum, M. abscessus, M. chelonae*

Much of the early research in the Netherlands also focused on the classification of mycobacteria based on bacteriological features. Coster made an important contribution with his animal inoculation experiments. These experiments revealed that NTM could be distinguished from *M. tuberculosis* complex bacteria by inoculation of the bacteria in the anterior chamber of the eye of rabbits.[5] In the rabbit eye, NTM caused only local nonspecific inflammation, while *M. tuberculosis* caused destructive granulomatous disease.[5]

The bacteriological focus was strongly criticized by Manten in his 1965 landmark review. He stated that knowledge on clinical NTM disease in the Netherlands lagged behind on that of physicians and researchers in other countries.[6] This review first coined the issue of clinical relevance of NTM isolation from non-sterile sites, for which Manten proposed a scale based on culture results (Table 2).

Applying these criteria to 240 patients with NTM isolates in the 1956-1964 period, he identified 135 cases of definite NTM disease. The majority were

Table 2: Manten's scale of clinical relevance of nontuberculous mycobacteria

Culture results	Interpretation
Repeated positive cultures yielding high numbers of bacterial colonies	Definite infection
Low numbers of bacteria isolated on a few occasions, or a single positive culture with high numbers of colonies	Probable infection
A single positive culture with a low number of colonies	Possible infection

adult males with pulmonary *M. kansasii* disease, followed by children with *M. scrofulaceum* cervical lymphadenitis. Both *M. avium* and the rapidly growing mycobacteria were infrequently isolated and rarely caused disease.[6]

With the limited array of antimycobacterial drugs available at the time and extensive *in vitro* resistance of NTM to those drugs, Manten painted a grim picture of the therapeutic options. He stated that "[effective] chemotherapy for these infections is an illusion". Surgical resection was considered the only option for NTM disease.[6]

In the 1960s, a growing number of cases of pulmonary NTM disease were reported. The demographics of the patients described were strikingly similar: predominantly males, with an average age of 50 to 55 years and pre-existent pulmonary diseases including chronic obstructive pulmonary disease and prior tuberculosis.[7] The predominant species isolated differed by region. Based on the publications by Manten and colleagues, Selkon reported that in the Netherlands and neighbouring countries, *M. kansasii* was most frequent; in most parts of the USA, isolates of Runyon class III predominated.[7]

Two interesting clinical reports were published in this period. First, the first case of *M. marinum* skin infection ("swimming pool granuloma") was recorded in the Netherlands in 1968.[8] Second, there was the observation that some coal miners suffered disease due to photochromogenic mycobacteria, mainly *M. kansasii*, rather than the presumed silico-tuberculosis.[9] Wolinsky had already recorded that induction of silicosis in guinea pigs, by quartz powder inhalation, increased their susceptibility to pulmonary *M. kansasii* disease.[10]

Despite the growing recognition of the capacity of NTM to cause disease in humans, the focus of research in the Netherlands remained on bacteriology. Extending the work on classification and identification initiated by Coster,[5] Schuitemaker introduced phage typing of mycobacteria in the Netherlands in 1963.[11] Phage typing is a technique based on exposing bacteria to a set of previously selected bacteriophages, viruses that can infect and kill bacteria. Bacteria will be able to survive some phages and will be killed by others. This pattern is generally species-specific and can thus serve as a method of identification (Figure 1).[11]

Schuitemaker's phage typing technique enabled the later work on the etiology of NTM disease. The first important contribution on this topic in the Netherlands was a study performed in the city of Rotterdam, where sampling of tap water resulted in almost 50% of the samples growing *M. kansasii*. Some of the *M. kansasii* phage types encountered in water samples were also noted in clinical specimens in the same region, suggesting tap water as a source of human infection.[12] This interesting finding led to a nationwide study of swimming pool, whirlpool and tap water samples. From these samples, the potentially pathogenic *M. kansasii*, *M. avium*, *M. szulgai* and *M. fortuitum* were isolated.[13]

Studies on environmental presence of NTM were conducted in many parts of the world in this period; NTM were commonly found in soil, natural water

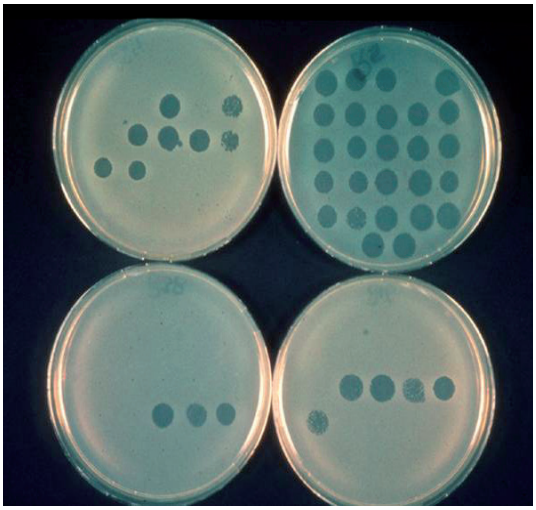


Figure 1: Phagetyping results of four different *Mycobacterium* isolates. The plates are enriched with series of phages, which kill susceptible bacteria. The dark spots in the plates are zones where the locally present phage has killed the bacterium. The pattern resulting from the susceptibility of the bacterium to the different phages can be used for identification and typing purposes.

and on plants. Nontuberculous mycobacteria can be isolated from such a wide variety of environmental samples because they can grow at wide ranges of temperature, pH, salinity, and oxygen tension. Their common resistance to disinfectants and capacity for biofilm formation enables their survival in man-made environments including water systems.[14]

Clinical NTM disease again came to attention during the early stages of the human immunodeficiency virus (HIV) epidemic. In patients with very low CD4 counts, typically below 50 cells/μL, disseminated NTM disease was among the most frequent and most severe infections.[15] Over 95% of these infections are caused by *M. avium*, although over the course of time many species have been recorded as causative agents of HIV-related disseminated NTM disease.[15] The advent of prophylactic therapy, first with rifabutin, later with macrolide antibiotics, and later of highly active antiretroviral therapy (HAART) significantly reduced the incidence, morbidity and mortality of HIV-associated disseminated NTM disease,[16] at least in the USA and Europe where prophylaxis and HAART are available.

Clinical features of disease caused by nontuberculous mycobacteria

Now, 50 years after the seminal review by Runyon and the first diagnoses of pulmonary NTM disease in the Netherlands by Manten, it is clear that NTM are environmental bacteria capable of causing a wide range of infections in humans, all in very specific groups of patients. Pulmonary NTM disease has three distinct presentations. Cavitary disease resembles conventional tuberculosis both clinically and radiologically. This disease mostly affects the upper lung lobes and patients often have a productive cough, weight loss and fatigue. Although the symptoms are very much like pulmonary tuberculosis, the clinical course is generally more prolonged. This type of disease is most common in patients

with pre-existing pulmonary disease, including chronic obstructive pulmonary disease (COPD) and prior pulmonary tuberculosis. A wide variety of NTM species have been recorded as causative agents of cavitary disease. Patients are mostly males, their average age is 50-60 years.[17] The second type of pulmonary NTM disease is nodular-bronchiectatic disease. Radiologically, the disease is characterized by bronchiectasis and multiple small nodular lesions commonly affecting the right middle lobe and the lingula. This disease type is mostly caused by *M. avium* complex or *M. abscessus* bacteria and commonly affects an older, female population without a significant history of pulmonary disease. These patients tend to have a distinct body habitus with scoliosis, pectus excavatum and mitral valve prolaps. Pulmonary NTM disease in these women has been labeled the "Lady Windermere syndrome", after the main character of Oscar Wilde's famous 1893 play "Lady Windermere's Fan".[18] The name was chosen to convey the fastidious behavior that Reich and Johnson believed was the reason that this disease primarily affected women: habitual voluntary suppression of cough, as it is considered socially unacceptable behavior.[18] The third type of pulmonary disease caused by NTM is a disease very similar to hypersensitivity pneumonitis. This rarest form of pulmonary NTM disease has a very distinct etiology. Patients are exposed to NTM, mainly *M. avium* complex bacteria in aerosols produced by "hot tubs"; small steam baths for personal use. Hence, this disease is often referred to as "hot tub lung". Clinically, patients present with cough, dyspnea and fever. Radiological findings include diffuse infiltrates with nodular lesions throughout all lung fields. Ground glass opacities and a mosaic pattern are often present on high-resolution computed tomography (HRCT) scans. Although the exact pathogenesis remains controversial, it is thought to be a combination of infection and inflammation; the latter may be provoked by NTM antigens.[19] The histopathology is that of granulomas, usually non-necrotizing; organizing pneumonia or interstitial pneumonia is rare.[20] Patients are usually nonsmokers, similar to patients with other forms of hypersensitivity pneumonitis.

Extrapulmonary disease due to NTM is generally rare. An important exception is pediatric lymphadenitis caused by NTM. This relatively benign disease affects immunocompetent children below six years of age, although this age range is different in different NTM species. In the Netherlands the annual incidence has been estimated at 77 cases per 100.000 children. Children usually present with a painless swollen submandibular or cervical lymphnode in the absence of generalized symptoms.[21] The second most important extrapulmonary disease due to NTM is disseminated disease, which generally affects severely immunocompromised patients, including those infected by HIV and patients using immunosuppressive drugs. In HIV-infected patients, disseminated NTM disease is mainly caused by *M. avium* and presents insidiously with fever, weight loss and night sweats.[22] Disseminated disease caused by *M. chelonae*, *M. abscessus* and *M. kansasii* tends to affect patients with an impaired immunity due to factors other than HIV. These diseases present with multiple

subcutaneous nodules or abscesses.[14,17] Localized skin disease also occurs. *Mycobacterium marinum* is its most common causative agent. These infections are contracted from contaminated water sources, such as fish tanks. Infections often emerge in wounds acquired during cleaning of the fish tanks. Hence, this localized disease due to *M. marinum* is often referred to as the "fish tank granuloma"; it has been called "swimming pool granuloma" as well, after another frequent source of infection.[1,8,14]

Most ear and eye infections are caused by rapidly growing mycobacteria. *Mycobacterium chelonae* and *M. abscessus* infections have been noted after eye surgery, especially after laser in situ keratomileusis (LASIK).[23] *Mycobacterium abscessus* is also the most frequent causative agent of ear infections in children, often after tympanostomy tube placement.[24] Both are severe and difficult to treat infections, which may result in permanent visual disability and hearing loss.

The wide range of infections caused by NTM is reflected by the wide array of sources from which NTM are isolated in the Netherlands. Table 3 provides an overview of the sources of the isolates submitted to the Dutch National Mycobacteria Reference Laboratory (part of the Laboratories for Infectious Diseases and Perinatal Screening, National Institute for Public Health and the Environment; RIVM) in the 2000-2006 period. Pulmonary isolates make up 83% of all NTM isolates referred to the RIVM. It is obvious from Table 3 that all other types of extrapulmonary NTM disease are clinical rarities in the Netherlands. Nevertheless, their obscurity should not preclude their thorough study.

The impact of nontuberculous mycobacteria on other disease processes

The NTM are not just causative agents of human infections. A role for *Mycobacterium avium paratuberculosis* in the pathogenesis of Crohn's disease (a chronic inflammatory bowel disease) is still debated.[25,26] *Mycobacterium avium paratuberculosis* causes Johne's disease in cattle, which shares some clinical features with Crohn's disease in humans. The bacteria are present in milk of infected cows; it has been estimated that 30-70% of Dutch cow herds are infected by *M. avium paratuberculosis*.[27]

A second aspect of NTM has received a lot of attention is the influence of NTM exposure to the efficacy of Bacille Calmette Guérin (BCG) vaccination. Previous researchers have suggested that strong environmental exposure to NTM reduces the protection of BCG against pulmonary tuberculosis.[28] This exposure differs by region, due to different environmental niches. Hence the protective effect of BCG also differs by region.[28] On the other hand, many countries have reported rising incidences of pediatric lymphadenitis caused by NTM after cessation of mass BCG vaccination.[29,30] Apparently, BCG vaccination does offer some protective immunity to pediatric NTM disease. Prior tuberculosis also has such a protective effect against NTM disease, at least in HIV-infected persons.[31,32]

Table 3: Sources of NTM isolates submitted to the RIVM in the 2000-2006 period

Source	2000	2001	2002	2003	2004	2005	2006	Total
Pulmonary	401	416	382	403	491	532	631	3256
Lymph node	24	18	12	33	79	59	42	267
Skin	10	10	11	7	20	9	15	82
Feces	5	12	5	2	11	11	3	49
Urine	9	7	5	3	6	8	4	42
Blood	2	11	4	3	7	3	11	41
Gastric juice	8	1	2	4	4	4	4	27
Joint	3	2	2	1	2	7	3	20
Ear	1	2	1	1	0	3	5	13
Bone marrow	1	2	2	3	1	2	1	12
Eye	1	0	0	0	0	5	1	7
Other	15	12	12	11	13	25	27	115
Total	480	493	438	471	634	668	747	3931

Advances in laboratory diagnosis

Until the late 1990's, the laboratory diagnosis of NTM disease in the Netherlands relied on microscopy and culture on solid media; the RIVM identified NTM isolates by their colony morphology, growth rate and a delicate series of biochemical tests. These tests were notoriously irreproducible and identification results could take weeks or months to obtain. For clinicians, the long turnaround time severely limited the utility of these results and few NTM isolates were submitted. It was only in the late 1990's that the fast and robust molecular tools for NTM identification became widely available to clinical and reference laboratories.[33] For laboratories without access to DNA sequencers, the line-probe assays were developed, which hybridize an amplified DNA fragment with a series of short DNA stretches, called oligonucleotides, from various NTM species, which are attached to a paper strip. The hybridization with a specific oligonucleotide leads to a color reaction at the binding site of the oligonucleotide on the paper strip. The localization of the resulting colored band reveals which species is present. The major drawback of these methods is the fact that an identification result is based on hybridization with a small part of a single gene or spacer. As a result, divergence within the rest of the target gene, spacer, or in fact in the entire mycobacterial genome is disregarded. This creates a situation comparable to identification of a person by the color of the eyes only. Although these systems offer identification results within 24 hours after DNA isolation, the result should probably be considered as an approximate.

As automated liquid culture systems were introduced, *Mycobacterium* cultures and identification finally became a matter of weeks instead of months.

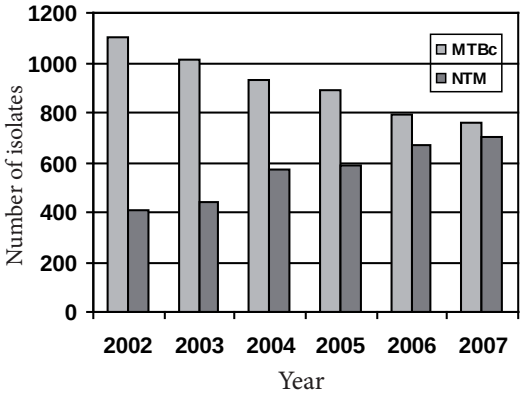
Moreover, the liquid media proved far more sensitive and saw the number of cultures yielding NTM rise in many laboratories.[34,35]
Drug susceptibility testing (DST) of nontuberculous mycobacteria has always been a difficult issue in the laboratory diagnosis of NTM disease. For most drugs, the *in vitro* susceptibility does not correlate with *in vivo* outcome of treatment; susceptibility breakpoints have not been confirmed to be clinically meaningful There is ongoing debate on the preferential testing method. Currently, the Clinical and Laboratory Standards Institute (CLSI; formerly National Committee for Clinical Laboratory Standards, NCCLS) propagates the broth microdilution method for most NTM species.[36] The RIVM currently uses an agar dilution method, since this same platform is also in use for *M. tuberculosis* complex bacteria.[37] The fact that, in the Netherlands, only the RIVM routinely performs DST for NTM is testimony to the fact that this technique is difficult both to perform and to interpret.

The changing isolation frequency of nontuberculous mycobacteria

The advent and spread of the improved laboratory diagnostic tools facilitated robust studies of the isolation frequency of NTM. Consequently, many studies reported rising isolation frequencies.[38-41] This observation however, had already been made prior to the advent of these improved detection and identification techniques.[38,39] In the Netherlands, the number of clinically isolated NTM submitted to the RIVM has also risen since the late 1990's. In the same period, the number of submitted *M. tuberculosis* complex isolates has decreased (Figure 2).
Although it is tempting to conclude that this increasing isolation frequency is essentially a result of the technical improvements, there are various reasons for the increase in NTM isolation frequency. Of course, the recent technical developments such as liquid culture systems have had a major impact,[34,35] but there may also be a growing number of patients who get infected by NTM and develop true NTM disease. Repetitive skin testing surveys using a purified protein derivative of *M. scrofulaceum* in elementary school children in the Netherlands recorded sensitization rates rising from 4 to 13% over the 1965 to 1985 period.[42] Recently, Marras and co-workers demonstrated that the sensitization to *M. intracellulare* in the United States, also assessed by skin testing, rose from 11% in 1971-72 to 17% in 1999-2000.[43]
Several medical and social factors could lead to an increase in the prevalence of NTM disease. The population in the Netherlands is ageing and with growing age there is an increase in the prevalence of chronic disease (the "epidemiological transition"). Chronic pulmonary disease is also common due to the (past) smoking habits and work-related exposure to harmful compounds. Chronic pulmonary diseases, especially COPD, are well known risk factors for pulmonary NTM disease.[1,14,17] Hence, the number of patients at risk has increased. The aspects of smoking and work-related exposure to

Figure 2:
Annual number of *Mycobacterium* isolates submitted to the Dutch National Mycobacteria Reference Laboratory (RIVM)

MTBc: *Mycobacterium tuberculosis* complex; NTM: nontuberculous mycobacteria



harmful compounds also explain the predominance of males among patients with pulmonary NTM disease.[1,14] A recent chronic pulmonary disease prevalence survey (among 358741 persons) in the Netherlands found that 1.3% of the Dutch population is formally diagnosed with COPD.[44] In a country of 16.2 million inhabitants, that makes for 210600 COPD patients. The life expectancy of patients with cystic fibrosis has also risen. Patients with cystic fibrosis are also highly susceptible to NTM disease, frequently caused by *M. abscessus*.[45,46] The HIV epidemic is another cause of the increasing numbers of patients with NTM disease, although this situation has now stabilized; highly active antiretroviral therapy (HAART) significantly reduced the number of patients with HIV-related NTM disease[13,14] and the prevalence of HIV in the Netherlands is relatively low.[47] More recently, a new category of patients susceptible to mycobacterial disease was recognized. Patients using the new immunosuppressive agents for rheumatic disease, mainly the tumor necrosis factor alpha-neutralizing (anti-TNF) agents, are at increased risk to develop mycobacterial disease.[48]

Mass BCG vaccination was never applied in the Netherlands, so an increasing NTM disease incidence can not be related to cessation of BCG vaccination.

The increasing isolation frequency may also result from non-medical factors. The use of showers for personal hygiene means that many people are exposed to aerosols on a daily basis. Many NTM have been demonstrated to be present in tap water and aerosol formation favors their inhalation.[12,13,49] Recently, DNA fingerprinting techniques clearly demonstrated that *M. avium* disease in a patient could be linked to presence of the same *M. avium* strain in his shower.[50] Due to their presence in natural and man-made environments, it has been estimated that humans are exposed to 50 to 500 NTM bacilli per day.[51] In the light of the large number of patients with NTM disease in whom no predisposing conditions are found, it has also been suggested that the NTM may be becoming more virulent.[52]

A growing interest in nontuberculous mycobacteria in the Netherlands

Alongside the increase in sample volume (Figure 2 & Table 3), the RIVM received a growing number of questions on the clinical relevance of these isolates and diagnosis and treatment of NTM disease. A similar trend of increasing interest in NTM was noted at the University Lung Centre Dekkerswald, one of two reference hospitals for mycobacterial disease in the Netherlands. The number of patients referred to Dekkerswald for treatment of their NTM infection started to rise, alongside a growing number of questions on clinical management of NTM disease from pulmonary physicians and infectious diseases specialists throughout the Netherlands. The apparent difficulties in the diagnosis and treatment of NTM disease led to a collaboration between Dekkerswald and the RIVM, focusing on the clinical relevance of NTM isolation. The first action of the research group, referred to as the Research Atypical Mycobacteria (RAM)-team, was to explore the current standards of bacteriological handling of NTM. These were explored during interviews with bacteriologists of all clinical laboratories with facilities to culture mycobacteria. Then, as a pilot project, 2 separate studies started. The first was a retrospective analysis of the clinical relevance of NTM isolated in four associated hospital within a single region, in the 1999-2004 period. This regional study confirmed the rising isolation frequencies of NTM **(Chapter 2.1)**. The second was an exploration of the clinical relevance of a new NTM species, which had previously been considered a variant of *M. xenopi* **(Chapter 5.2)**. Both studies unambiguously demonstrated serious flaws in the diagnosis and treatment for NTM disease in the Netherlands. Very few cultures were performed, even in patients with prior positive cultures and some centers only identified cultured NTM upon request of the acting physician. During a meeting in which we presented the findings of our initial clinical studies, a microbiologist from a sizeable institute in the Netherlands stated: "whenever we have a negative PCR result [for *Mycobacterium tuberculosis* complex] on an isolated *Mycobacterium*, we discard the culture outright".

During both pilot studies, we recorded that some patients received treatment in absence of evidence of NTM disease; other did not receive treatment despite such evidence. Apparently, not all clinicians were able to define true NTM disease. This definition is troublesome, due to various reasons. Firstly, the NTM are environmental pathogens, present in natural water, soil and tap water and resistant to common disinfectants such as glutaraldehyde.[14,53-55] Therefore, a positive culture from a non-sterile source, such as the respiratory and digestive tract, alone does not prove NTM disease. In fact it may result from recent environmental exposure, thus coincidental presence in the respiratory tract, or even contamination of the sample during collection or processing. Even rinsing of the mouth with water, prior to sputum collection, may lead to contamination of the sputum sample with the NTM present in the tap water. Contamination may also take place in the microbiological laboratory.[56]

Box 1: Summary of the American Thoracic Society diagnostic criteria for pulmonary nontuberculous mycobacterial infection[17]

Clinical.
1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules.
and
2. Appropriate exclusion of other diagnoses.

Microbiological.
1. Positive culture results from at least two separate expectorated sputum samples. (If the results from the initial sputum samples are nondiagnostic, consider repeat sputum AFB smears and cultures.)
or
2. Positive culture results from at least one bronchial wash or lavage.
or
3. Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.
4. Expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination.
5. Patients who are suspected of having NTM lung disease but who do not meet the diagnostic criteria should be followed until the diagnosis is firmly established or excluded.
6. Making the diagnosis of NTM lung disease does not, *per se*, necessitate the institution of therapy, which is a decision based on potential risks and benefits of therapy for individual patients.

Patients who meet these criteria are likely to have true pulmonary NTM disease; their NTM isolates can be considered clinically relevant.

The most notorious sources of NTM (pseudo-)outbreaks are contaminated medical instruments. The common resistance to disinfectants allows NTM to survive in washers for bronchoscopes and other medical instruments.[57,58] Pseudo-outbreaks of NTM have also been noted in the Netherlands.[54] Using contaminated instruments may lead to false-positive cultures or even inoculate NTM into tissues and thus cause true NTM disease, for instance during surgery with contaminated materials.[59]
Thus, the clinical relevance of a positive culture yielding NTM should always be assessed by compiling clinical and microbiological data. To aid in the differentiation between coincidental culture positivity of a respiratory sample and true pulmonary NTM disease, both the British Thoracic Society (BTS) and American Thoracic Society (ATS) have published diagnostic criteria.[17,60,61] The clinical relevance of an isolated NTM can be assessed using these criteria, which all combine clinical features, mainly symptoms and radiological features, with microbiological data regarding the number of positive cultures and results of microscopy for acid-fast bacilli of the original clinical samples. The most recent ATS diagnostic criteria are summarized in Box 1.

Treatment of disease caused by nontuberculous mycobacteria

After the difficult diagnosis of NTM disease is made, treatment may be required. There has been significant progress in this field, and Manten's 1965 statement that "[effective] chemotherapy for these infections is an illusion"[6] can be partially refuted. Initially, pulmonary NTM disease was treated as conventional tuberculosis, with isoniazid and para-aminosalicylic acid, though this proved ineffective.[1,6] Surgical resection was the therapy of choice until newer drugs including cycloserine, viomycin and ethionamide became available. The efficacy of regimens including these drugs was reviewed by Selkon, who noted a 56% cure rate for *M. kansasii* disease; cure rates for *M. avium* complex disease were disappointing, these ranged between 17 and 43%.[7] The original studies were observational in nature, rather than comparative or even randomized and controlled. In 1981 Hunter reviewed the results of treatment for pulmonary disease caused by *M. avium* complex bacteria. Various treatment regimens were used, though only combinations of isoniazid, rifampicin, and either ethambutol or streptomycin of 24 months duration were successful. Eighty-four percent of all patients treated with these combinations converted to negative cultures, although 14% relapsed within a year after treatment. Treatment regimens using multiple second line drugs were less effective and highly toxic.[62]

The use of treatment regimens including rifampicin and ethambutol became more widespread after the *in vitro* studies by Banks, who clearly demonstrated synergy between these two drugs.[63] The first prospective trial was done for pulmonary *M. kansasii* disease and used a rifampicin and ethambutol regimen of nine months duration; of 175 patients, all but one converted to negative cultures, although 10% suffered a relapse within 4 years of follow-up after treatment.[64] In the same period, the first studies of macrolide-based multi-drug therapy for *M. avium* complex disease were published. In 50 patients with pulmonary *M. avium* complex disease, 92% achieved prolonged culture conversion.[65] The macrolide-based regimens also proved highly effective in HIV-related disseminated *M. avium* disease.[66] The BTS published another important randomized controlled trial in 2001, which compared rifampicin and ethambutol regimens with and without isoniazid of 24 months duration in pulmonary disease caused by the NTM species important in North-Western Europe: *M. avium*, *M. xenopi* and *M. malmoense*. Generally, addition of isoniazid added little benefit, but resulted in more reported adverse events.[67] This trial was followed by a comparative trial of the rifampicin and ethambutol regimen with adjunctive ciprofloxacin or clarithromycin. Interestingly, this recently published trial failed to demonstrate superiority of a macrolide or fluoroquinolones based regimen over rifampicin and ethambutol only.[68] Combined, the BTS trials observed cure rates of 28% after five years of follow-up, with 14% relapses.[68] These results frankly conflict with previous North American studies of macrolide-based regimens, the causes of which remain

to be investigated. The added benefit of clarithromycin for treatment of pulmonary NTM disease remains controversial. In HIV-related disseminated disease, the macrolide based regimens are the recommended treatment.[17,66]
For a select group of patients, surgical excision of the affected part of the lung, combined with multi-drug treatment before and after surgery can prove successful.[17]
For extrapulmonary NTM disease, there is even less evidence on efficacy of treatment regimens. Pediatric NTM lymphadenitis usually responds well to excisional surgery alone;[69] this is the recommended treatment.[17,69] Other extrapulmonary disease types are generally treated by excisional surgery (or surgical debridement) combined with multi-drug treatment.[17]
A special reference should be made to disease caused by *M. abscessus*. Bacteria of this species are naturally resistant to virtually all classes of antibiotics, except the macrolides. Disease caused by *M. abscessus* is an enormous challenge for clinicians. Pulmonary *M. abscessus* disease is a virtually incurable disease for most patients and suppressive, instead of curative, treatment may be the only option,[17] especially in CF patients. In pulmonary *M. abscessus* disease, too, surgery may be applied in selected cases.[17]

Nontuberculous mycobacteria in areas with a high prevalence of tuberculosis

Traditionally, the NTM have been studied mainly in Europe, Australia and North America, where they were noted during the decline in the prevalence of tuberculosis. In Asia, Africa and South America the prevalence of tuberculosis has remained high. Moreover, many countries in these regions lack the financial and laboratory resources for *Mycobacterium* cultures at all. Diagnosing tuberculosis in these regions often depends on clinical assessment, including chest radiographs where available, and acid-fast bacilli microscopy. As a result, distinguishing TB from NTM disease is virtually impossible. The few historical studies that have looked at NTM isolation from patients suspected of pulmonary tuberculosis found very few; a survey of 7580 cultures from patients in various African countries identified only 86 (1.1%) as NTM.[70] Similarly, a prospective study early in the HIV-epidemic revealed that the prevalence of disseminated *M. avium* complex disease in Kenya was ten times lower than in the USA and Northern Europe;[71] HIV-associated tuberculosis was more frequent in Kenya. Later studies in West Africa found prevalence rates very similar to those in the USA.[72] Most recently, several studies were conducted in Zambia, which noted clinically relevant NTM disease in both HIV-positive and negative patients.[73] Though the clinical relevance of NTM in areas with a high prevalence of tuberculosis remains unsettled, it may exceed previous expectations.

Aims and outline of this thesis

Diagnosis and treatment of disease due to nontuberculous mycobacteria remains complicated. For most species, it is not clear whether their isolation is likely to be a sign of true NTM disease or whether they can be regarded as probable contaminants. Therefore, a single positive culture from a non-sterile source demands careful follow-up.
Within this thesis, we have aimed to quantify the clinical relevance of the most frequently isolated NTM species in the Netherlands. To quantify clinical relevance, we assessed the percentage of patients that meet the ATS diagnostic criteria for pulmonary NTM disease, as well as the frequency and clinical features of extrapulmonary disease, per species (Chapter 2). Within Chapter 3, we focus on a new category of patients at risk for NTM disease, those with rheumatic or other inflammatory disease treated with the new immunosuppressive agents including the anti-TNF agents. The poor outcome of treatment for NTM disease is discussed in Chapter 4, where we study the role of drug susceptibility testing, adjunctive surgery and a potential novel drug. Hereafter, we shift the focus towards the pathogens. Chapter 5 details on the issue of unidentifiable mycobacteria. We have studied the prevalence of such unidentifiable mycobacteria in our reference laboratory. Selected groups of strains were studied in-depth and described as new species, with an assessment of their clinical relevance. In Chapter 6 we focus further, as we studied the genetic divergence within NTM species. This divergence could provide important insights in the bacterial factors in the issue of clinical relevance. We discuss our findings with an emphasis on the issue of clinical relevance. Finally, in Chapter 7, we have looked across our borders, to assess the clinical relevance of NTM isolation in very different geographical regions, with a different prevalence of tuberculosis and HIV.

Ultimately, our efforts should provide a basis for improved diagnosis and treatment of NTM disease. This is a clinical issue that can no longer be ignored. The period of considering NTM as possible but improbable causative agents of "atypical tuberculosis" needs to make way for a vision of NTM disease as a completely separate clinical entity, affecting different patients in different settings, to be treated in a way different from conventional tuberculosis.
Our data should assist in the creation of guidelines specifically for the clinical and laboratory diagnosis and treatment of NTM disease, in the Netherlands as well on an international level. Our pilot studies have clearly laid bare the necessity of such guidelines.

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Chapter 2

Clinical relevance of isolation of nontuberculous mycobacteria in the Netherlands

2.1 The clinical relevance of nontuberculous mycobacteria isolated in the Nijmegen-Arnhem region, the Netherlands.
Thorax 2009; 64(6): 502-6.

2.2 *Mycobacterium xenopi* clinical relevance and determinants, the Netherlands.
Emerg Infect Dis 2008; 14(3): 385-9.

2.3 Clinical relevance of *Mycobacterium simiae* in pulmonary samples.
Eur Respir J 2008; 31(1): 106-9.

2.4 Clinical relevance of *Mycobacterium szulgai* in the Netherlands.
Clin Infect Dis 2008; 46(8): 1200-5.

2.5 Clinical *Mycobacterium conspicuum* isolation from two immunocompetent patients in the Netherlands.
J Clin Microbiol 2007; 45(12): 4075-6.

2.6 Clinical relevance of *Mycobacterium malmoense* isolation in the Netherlands.
Eur Respir J: Epub April 22nd, 2009.

2.7 Relevance of *Mycobacterium chelonae* and *Mycobacterium abscessus* isolation in 95 patients.
In Revision

2.8 Impact of new American Thoracic Society diagnostic criteria on management of nontuberculous mycobacterial infection.
Am J Respir Crit Care Med 2007; 176(4): 418.

2.9 The changing pattern of clinical *Mycobacterium avium* isolation in the Netherlands.
Submitted

2.10 Otomastoiditis due to nontuberculous mycobacteria in the Netherlands.
In Revision

The clinical relevance of non-tuberculous mycobacteria isolated in the Nijmegen-Arnhem region, the Netherlands

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Abstract

Background: The frequency of clinical isolation of nontuberculous mycobacteria (NTM) in the Netherlands is increasing, but its clinical relevance is often uncertain.

Objectives: To assess the frequency and clinical relevance of isolation of NTM in four associated hospitals in a single region in the Netherlands.

Methods: Medical files of all patients from whom NTM were isolated between January 1999 and January 2005 were reviewed retrospectively. Diagnostic criteria for nontuberculous mycobacterial disease published by the American Thoracic Society (ATS) were used to determine clinical relevance.

Results: 232 patients were found, from whom NTM were isolated from the respiratory tract in 91% of cases. Patients were mostly white men, with an average age of 60 years and pre-existing pulmonary disease. Fifty-three of 212 patients (25%) with pulmonary isolates met the ATS diagnostic criteria for pulmonary NTM disease; this percentage differed by species. Most patients were treated with rifampicin, ethambutol and clarithromycin. Treatment outcome for pulmonary NTM disease was suboptimal but differed by species: overall improvement was seen in 67% of treated patients, but in only 50% of those with pulmonary *M. avium* disease. Lymphadenitis was the most common extrapulmonary disease type.

Conclusions: Twenty-five percent of all patients with pulmonary NTM isolates met the ATS criteria. Clinical relevance differs by species. NTM isolation increases over time. Species distribution differs from that of neighboring countries and the *M. avium* complex isolates have traits different from those reported in the USA. Adherence to diagnostic and treatment guidelines can be improved.

Introduction

The clinical isolation of nontuberculous mycobacteria (NTM) increases in many countries where the incidence of tuberculosis is decreasing, with marked geographic differences in the species encountered.[1-3] The NTM are often opportunistic pathogens, capable of causing disease in patients with impaired immunity, either local due to pre-existing pulmonary disease or systemic – for example, hematological malignancy, immunosuppressive drug treatment or HIV/AIDS.[1] The clinical relevance of isolated NTM is often unclear. The NTM are common in the environment and can survive in flowing water systems.[4] Moreover, NTM resist common disinfectants.[5] Thus, pseudo-infection due to occasional presence of NTM in clinical samples as a consequence of contamination of medical tools should always be considered.[1,6,7]

To differentiate true infection from pseudo-infection, and establish the clinical relevance of an NTM isolate, is of paramount importance since treatment of NTM disease is time consuming and often complicated. To assist in this differentiation, the American Thoracic Society (ATS) established general criteria for the diagnosis of pulmonary nontuberculous mycobacterial infection (Box 1).[1]

We performed a retrospective case study to assess the prevalence and clinical relevance of NTM isolated in the four associated training hospitals of the Nijmegen-Arnhem region, the Netherlands, using the ATS diagnostic criteria to differentiate NTM disease from pseudo-infection or contamination.

Methods

To determine clinical relevance, we examined medical records of all in- and outpatients in four collaborating hospitals in the Nijmegen-Arnhem region of the Netherlands from whom NTM were isolated between January 1999 and January 2005. We recorded demographic, clinical and microbiological data and status according to the diagnostic criteria for pulmonary NTM disease by the ATS.[1] We considered pulmonary isolates clinically relevant if the patient met the ATS diagnostic criteria in absence of evidence of other pulmonary infections.

For patients treated for NTM disease, we defined cure as symptomatic improvement and reversion to negative cultures sustained throughout our follow-up period. We focused on pulmonary isolates.

The Dutch National Institute for Public Health and the Environment (RIVM) subjected isolates of all patients to laboratory diagnosis. The RIVM is the national reference laboratory that provides identification, drug susceptibility testing and genotyping of mycobacterial isolates for all hospitals in the Netherlands. To identify NTM, the INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Ghent, Belgium) reverse line blot assay was used, after ruling out membership of the *M. tuberculosis* complex using a GenoType MTBC line probe assay (Hain Lifescience, Nehren, Germany). If no species-specific result was obtained, 16S rDNA gene sequencing (151bp hypervariable region A)

Box 1: Summary of the American Thoracic Society diagnostic criteria for pulmonary nontuberculous mycobacterial infection.[1]

Clinical.
1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules.
and
2. Appropriate exclusion of other diagnoses.

Microbiological.
1. Positive culture results from at least two separate expectorated sputum samples. (If the results from the initial sputum samples are nondiagnostic, consider repeat sputum AFB smears and cultures.)
or
2. Positive culture results from at least one bronchial wash or lavage.
or
3. Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.
4. Expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination.
5. Patients who are suspected of having NTM lung disease but who do not meet the diagnostic criteria should be followed until the diagnosis is firmly established or excluded.
6. Making the diagnosis of NTM lung disease does not, *per se*, necessitate the institution of therapy, which is a decision based on potential risks and benefits of therapy for individual patients.

was performed. Prior to 2004, 16S rDNA gene sequencing was performed, after ruling out membership of the *M. tuberculosis* complex or *M. avium* complex using the AccuProbe MTB and *M. avium* complex DNA probe kits (GenProbe, San Diego, USA). Sequencing results were compared with the GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) database. A Pearson χ^2-test was used for statistical correlations. The regional ethics committee approved the study.

Results

We found 232 patients with NTM isolates, mostly from the respiratory tract (n=212, 91%). Primary isolates were cultured from sputum (n=158; 75%), broncho-alveolar lavages (n=47; 22%) and lung biopsy specimens (n=7; 3%). The annual number of patients from whom NTM were isolated rose during our research period, mainly owing to an increase in pulmonary isolates, especially *M. avium* complex (MAC) isolates (Figure 1). The annual percentage of patients meeting the ATS criteria did not change.
Table 1 presents the baseline characteristics of the 212 patients with pulmonary NTM isolates.

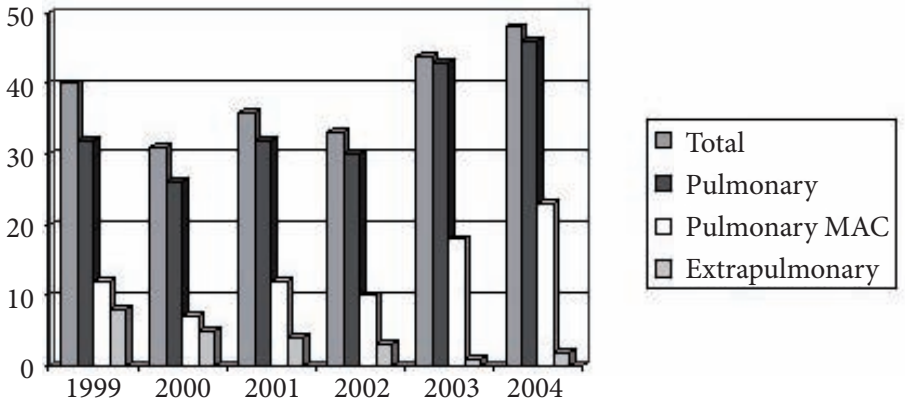

Figure 1: Total number of patients with NTM isolates, per year and isolation site
MAC: M. *avium* complex

Fifty-three patients (25%) met the ATS diagnostic criteria and were thus likely to have pulmonary NTM disease. Of the 159 patients who did not meet the ATS criteria, 146 (92%) failed to meet microbiological criteria and 149 (94%) failed to meet radiological criteria. For these patients, follow-up for a mean duration of 47 months (range 29-98 months) did not yield additional evidence of NTM disease.

Figure 2 visualizes the clinical relevance of the various NTM species, defined by the percentage of patients who met the ATS diagnostic criteria. Four cases of pulmonary NTM disease were actually relapses of disease episodes prior to our study period (two *M. avium*, one *M. xenopi*, one *M. kansasii*).

Eighty-five patients (40%) had MAC isolates (59 *M. avium*, 16 *M. intracellulare*, 10 other MAC) of whom 26 (24 *M. avium*, 2 *M. intracellulare*; 31%) met the ATS diagnostic criteria. *M. avium* isolation was clinically more relevant than *M. intracellulare* (24/59=41% vs. 2/16=13% of patients met the ATS diagnostic criteria). A radiographic presentation with fibrosis and cavities was more prevalent than nodular bronchiectatic disease (12 vs. 3 cases for *M. avium* and 1 case each for *M. intracellulare*).

Whereas MAC was most frequently seen, the degree of clinical relevance was higher for isolation of *M. malmoense* (2/3), *M. kansasii* (12/17, 71%), *M. xenopi* (3/5), *M. szulgai* (4/4), *M. celatum* (2/2) and *M. genavense* (1/1; Figure 2).

Symptoms recorded at presentation varied widely; only fever (p=0.014, OR 2.422, 95%CI 1.179-4.978) and fatigue (p<0.001, OR 3.477, 95%CI 1.721-7.024) at presentation were associated with meeting the ATS diagnostic criteria (Table 1). Chest radiographs were performed for 206 of the 212 patients and abnormalities were noted in 161 patients (78%; Table 1). Abnormalities did not differ significantly by NTM species. Three patients with pulmonary NTM disease presented with a pulmonary mass, mimicking malignancy. Computed tomography (CT) scans were performed in 107 patients (50%); among patients

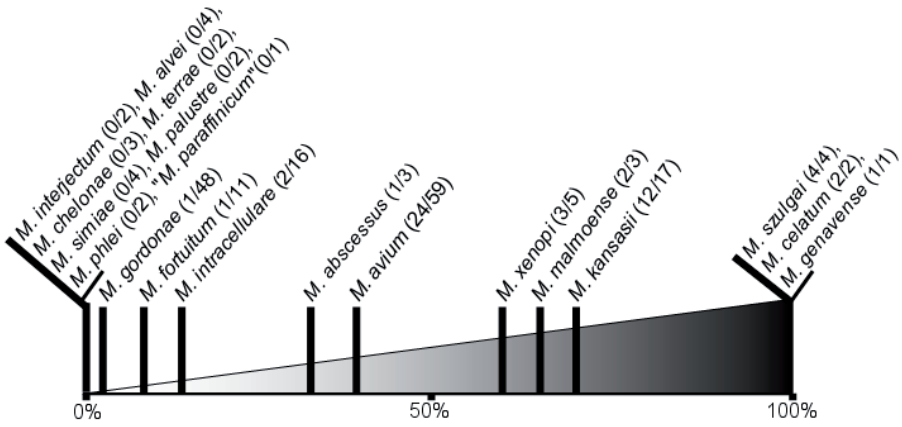

Figure 2: Clinical relevance of pulmonary NTM isolates in this study, per species (x/y): number of patients that meet the ATS diagnostic criteria / total number of patients per species

meeting the ATS diagnostic criteria, five cases of fibrocavitary disease and six cases of nodular-bronchiectatic disease were noted in patients with inconclusive chest radiographs. On chest radiographs, these cases of fibrocavitary disease presented with dense airspace opacities; the nodular-bronchiectatic disease appeared as coarse linear scarring.

Drug treatment for mycobacterial infection was started in 44 patients; 31 started treatment for tuberculosis (TB) and switched to an NTM treatment regimen, 13 patients received standard TB treatment only. Of these 44 patients, 30 met the 1997 ATS diagnostic criteria available during the study period.[8] Among those 30, 20 (67%) were cured, defined by symptomatic improvement and reversion to negative cultures, five died during treatment (three with *M. avium*, one with *M. intracellulare*, one with *M. xenopi* disease; 17%) and five had a relapse after treatment (four with *M. avium*, one with *M. celatum* disease; 17%). The mean interval between the end of treatment and culture-proven relapse was 31 months (range: 23-44). Of 14 patients treated for pulmonary *M. avium* disease, only seven improved. The regimens used mostly consisted of rifampicin and ethambutol, despite frequent *in vitro* resistance (15/25 primary isolates were resistant to rifampicin, 18/25 to ethambutol; 5 were not tested); isoniazid was added in 11 patients (despite *in vitro* resistance in all cases), clarithromycin in 19, ciprofloxacin in four. Clarithromycin and ciprofloxacin were only used in patients with susceptible isolates. Three patients received 6 months of standardized TB treatment only. The mean treatment duration was 13 months (range 1-37). Three patients received additional surgical treatment that consisted of resection of a solitary pulmonary mass in two and lobectomy for persisting fibrocavitary disease in one; all three were cured. The mean duration of follow-up after treatment was 34 months (range 17-91).

Table 1: Baseline characteristics of patients with pulmonary NTM isolates

		ATS+ (n=53)	ATS- (n=159)	Total (n=212)
Demography	Males	37 (70%)	95 (60%)	132 (62%)
	Mean age (yr)	57	61	60
	Dutch origin	48 (91%)	145 (91%)	193 (91%)
Predisposing conditions	Pre-existing pulmonary disease	37 (70%)	125 (79%)	162 (76%)
	COPD	23 (43%)	80 (50%)	103 (49%)
	Lung cancer	4 (8%)	17 (11%)	21 (10%)
	Healed Tuberculosis	6 (11%)	22 (14%)	28 (13%)
	Previous pulmonary NTM disease	4 (8%)	0	4 (2%)
	Asthma	4 (8%)	21 (13%)	25 (12%)
	Bronchiectasis	8 (15%)	8 (5%)	16 (8%)
	Current or past smoker	35 (66%)	94 (59%)	129 (61%)
	Alcohol abuse	8 (15%)	16 (10%)	24 (11%)
	High dose steroid use*	2 (4%)	6 (4%)	8 (4%)
	HIV infection	6 (11%)	7 (4%)	13 (6%)
	Hematologic malignancy	1 (2%)	4 (3%)	5 (2%)
	Anti TNF treatment	1 (2%)	1 (1%)	2 (1%)
	Otherwise impaired immunity	3[#]	3 **	6
Symptoms	Productive cough	40 (75%)	126 (79%)	166 (78%)
	Hemoptysis	10 (19%)	28 (18%)	38 (18%)
	Dyspnea	29 (55%)	85 (54%)	114 (54%)
	Fever	19 (36%)[†]	30 (19%)	49 (23%)
	Night sweats	7 (13%)	16 (10%)	23 (11%)
	Weight loss	14 (26%)	29 (18%)	43 (20%)
	Fatigue	28 (53%)[§]	37 (23%)	65 (31%)
Chest radiograph features	Airspace opacities	22 (42%)	42 (26%)	64 (30%)
	Cavity	15 (28%)	2 (1%)	17 (8%)
	Nodules	4 (8%)	2 (1%)	6 (3%)
	Bronchiectasis	4 (8%)	5 (3%)	9 (4%)
	Emphysema	14 (26%)	23 (15%)	37 (17%)
Microbiology	Mean no of cultures performed	4.9	3.5	3.8
	Mean no of positive cultures	2.9	1.1	1.5
	AFB smear positive	26 (49%)[¶]	6 (4%)	32 (15%)

* >15mg prednisone/day for >3 months before primary NTM culture
[#] anorexia nervosa, lymphopenia and complement C4 deficiency, diabetes mellitus (all n=1)
** intravenous drug abuse (n=2), azathioprine use (n=1)
[†] p=0.014, OR 2.422, 95%CI 1.179-4.978
[§] p<0.001, OR 3.477, 95%CI 1.721-7.024
[¶] p<0.001, OR 23.583, 95%CI 8.777-63.125

Treatment was not given in 168 patients, though eight of them met the 1997 ATS diagnostic criteria.[8] Four patients were considered to have too few symptoms or radiographic deterioration, one refused treatment and one patient spontaneously reversed to negative cultures. Slow radiographic deterioration of pulmonary disease was apparent in all seven persistently symptomatic and culture-positive, untreated patients.

During the study period, NTM were isolated from extrapulmonary samples only of 20 patients. Lymphadenitis was the most common extrapulmonary NTM disease type; six children and one elderly female had cervical lymphadenitis (three *M. avium*, two *M. malmoense*, one *M. intracellulare*, one *M. haemophilum*) and one patient with AIDS (CD4 count 1) had axillar and mediastinal lymphadenitis with extensive abscess formation, caused by *M. gordonae*. We recorded three cases of skin infection: two caused by *M. marinum* in fish tank owners and one by *M. malmoense* in a patient with hematological malignancy. We also noted single cases of *M. kansasii* tenosynovitis in a gardener, *M. abscessus* otomastoiditis after tympanostomy tube placement in a child and disseminated *M. avium* disease in an AIDS patient (CD4 count 40). The remaining six patients had MAC isolated from the digestive tract, in the absence of further evidence of NTM disease.

Discussion

Twenty-five percent of all patients in this study had pulmonary NTM disease according to the ATS diagnostic criteria, and the clinical relevance differed significantly by species (Figure 2). Isolation of *M. malmoense*, *M. xenopi*, *M. szulgai*, *M. kansasii*, *M. celatum* and *M. genavense* warrants special attention, as this usually reflects true NTM disease; an observation confirmed in previous studies.[1] Although in the current study the number of isolates of many species is too low to permit firm conclusions, similar rates of clinical relevance have already emerged for *M. xenopi* (21/45 patients; 47%), *M. chelonae* (7/35; 20%), *M. abscessus* (13/39; 33%), *M. simiae* (3/28; 11%) and *M. szulgai* (11/15; 73%) in nationwide studies of pulmonary isolates in the Netherlands.[9-12] These data and the additional data in our current study strengthen a presentation such as that shown in Figure 2. The 25% of patients that met the ATS criteria is similar to the 33% recently found in a Canadian referral center study, 25% recorded among patients in Korea, and the results of earlier studies reviewed by Marras and Daley.[2,13,14]

A difference in clinical relevance by species has also been noted in the recently published Korean study with results similar to ours, except for a very limited relevance of *M. szulgai* (2/32 patients; 6%) and *M. celatum* (1/11; 9%).[13] These differences in clinical relevance between NTM species emphasize the major role of the bacteriological laboratory in the management of these emerging infections; correct identification of NTM isolates is a prerequisite for correct patient handling. The apparent regional differences in clinical relevance of NTM species require further study.

The ATS diagnostic criteria are based on experience with *M. avium*, *M. kansasii* and *M. abscessus*;[1] their applicability to less studied species may be limited.[1,9] The clinical applicability of the ATS criteria is supported by those patients who did not meet the ATS criteria and which had no clinical event or new radiological events during follow-up. Very few follow-up cultures were performed (see Table 1), although this was advocated in the ATS statement available during the study period.[8]

Although for some species the low number of isolates decreases the weight of the conclusions of the analysis, the difference in clinical relevance of the various NTM species may mean that for the species to the left of Figure 1 the ATS criteria should be very strictly applied or even be made more stringent, whereas among the upper half (50-"100"% clinical relevance) a diagnosis of NTM disease may be justified after the first positive culture.

The increase in the annual number of patients from whom NTM were isolated (Figure 1) is a cause of concern and has also been noted in previous studies.[1,2,13,15] This increase was often thought to be related to improvements in laboratory techniques; in the Netherlands, the use of (automated) liquid culture systems increased during the study period.[1,16] A recent study, however, demonstrated that skin sensitization to *M. intracellulare* has also increased significantly over recent decades in the USA, consistent with observed increases in the rate of pulmonary NTM infections.[17] Several aspects may influence this increase in NTM isolation. First, the Dutch population, as in many developed countries, is ageing and an increasing prevalence of chronic obstructive pulmonary disease is observed.[18-20] The baseline characteristics of our study group, predominantly men at an average age of 60 years, similar to previous NTM studies in societies with low HIV prevalence,[1,13,15] reflect these changes. Second, for chronic inflammatory diseases, immunosuppressive drugs, including tumor necrosis factor α neutralizing agents, are increasingly used. Both aspects may have an impact on the future prevalence of NTM disease. In addition, the exposure to aerosols of tap water may have increased over time, as frequency of showering has risen dramatically over recent decades. The minor role of HIV infection probably reflects the low prevalence of HIV infection in the Netherlands, compared to chronic pulmonary disease, and the advent of highly active antiretroviral treatment (HAART). This limits the number of cases of severe HIV immunosuppression and its co-infections,[21] which may be reflected in the decreasing NTM isolation from extrapulmonary samples (Figure 2). Besides, the hospitals participating in the study are situated in the eastern part of the Netherlands, where HIV incidence is lower than in the more urbanized western part.[22]

The limited associations between symptoms, chest radiograph results and NTM disease according to the ATS diagnostic criteria underline the fact that diagnosis of NTM disease remains difficult. Hence, the NTM merit special attention by doctors and microbiologists.

Despite inherent limitations, the species found in this study and their frequencies probably reflect the current epidemiological situation for NTM in the Netherlands. This situation is remarkably different from nearby southeast England, where *M. xenopi* is most frequent and Scotland and Scandinavia, where *M. malmoense* is especially prevalent.[1,14,23] The species distribution and clinical relevance among MAC isolates seen in this study differs from recent reports from the USA where *M. intracellulare* is the more common respiratory pathogen,[1] clinically more relevant than *M. avium* in non-HIV patients[24] and nodular-bronchiectatic pulmonary MAC disease, associated with postmenopausal immunocompetent women, is as prevalent as cavitary disease.[24] Possibly, the predominance of *M. avium* and cavitary disease in our study population are interrelated; different MAC subtypes are known to cause different disease types in humans.[25] Cases of nodular-bronchiectatic disease may have also been missed owing to the infrequent use of CT scanning and low number of follow-up cultures performed; diagnosing this paucibacillary disease type generally demands prolonged, intense follow-up.[1] Alternatively, since we used hospital in- and outpatient file review, cases of nodular-bronchiectatic disease may have been missed as they are less frequently referred to hospitals for diagnosis, owing to a more indolent clinical course compared with cavitary MAC disease.[1]

Previous studies have recorded high degrees of clinical relevance of *M. kansasii*, comparable to the 71% in the present study.[14] Whereas the latest ATS statement states that for *M. kansasii* the treatment decision may be based on a single positive culture in select cases,[1] we recorded single positive sputum cultures in five patients with non-suspect radiographic changes and repeatedly negative follow-up cultures. This would have meant an unnecessary treatment for these patients when applying the new ATS criteria.

We observed possible instances of both under-treatment (not treating those who meet the diagnostic criteria) and over-treatment (treatment of those who do not meet diagnostic criteria), as well as the use of treatment regimens that have never proved to be effective. This may harm patients. This, as well as the fact that few (high-resolution) CT scans and follow-up cultures, both strongly advocated in the ATS criteria,[1,8] were performed in patients with NTM isolates in non-cavitary pulmonary disease, could reflect a lack of knowledge of and experience with the diagnosis and management of NTM disease in doctors, or clinical circumstances not captured in our file review. All these aspects emphasize the need for centralization of knowledge, experience and care for patients with (suspected) NTM disease or increased expert consultation.

Treatment duration recorded in our study was not in accordance with the British Thoracic Society (BTS) and ATS guidelines.[1,26] A favorable outcome for 67% of patients with pulmonary NTM disease is higher than the 36% observed in the recently published BTS clinical trial comparing regimens with clarithromycin or ciprofloxacin as adjuncts to rifampicin and ethambutol.[27] In that trial,

patients with MAC and *M. xenopi* had worse outcomes than those with *M. malmoense*.[27] The differences in treatment outcome between our study and the BTS trial may result, first, from our more lenient definition of "cure", second, our limited follow-up period and finally, the NTM species causing pulmonary disease in our patients. Whereas the first trials of macrolide-based regimens in pulmonary MAC disease in HIV-negative patients in the US reported cure rates of 59-92%, with comparable definitions of cure,[1] the outcome of treatment for pulmonary MAC disease observed in our study and the BTS trial was disappointingly poorer. The treatment results, overrepresentation of men and cavitary disease, MAC species distribution and clinical relevance all suggest differences in MAC organisms encountered in the USA and Europe.
Although generally, outcome of NTM treatment is suboptimal,[1] various factors may have influenced our results. First, the conditions predisposing to NTM disease influence the outcome of the patient. Second, the 1997 ATS diagnostic criteria[8] available to doctors in the study period, might not have selected the group of patients who would have benefited from treatment. More patients meet the recently published criteria,[12] though herein it is stated that meeting the criteria does not of itself necessitate treatment.[1] Still, this may lead to improved selection of patients to receive treatment and thus improve outcome rates. In this perspective, the term under-treatment will become invalid. Third, previous trials for pulmonary MAC disease have recorded superior treatment outcome using similar definitions, but in a different MAC population. In our group more patients had cavitary disease, which in general is associated with a worse treatment outcome.[1] Finally, one of the centers incorporated in this study is a referral center for NTM disease treatment, which may create a selection bias.
Extrapulmonary disease was rare, though its overall frequency (9%) and distribution of disease types are in line with previous studies, summarized in the recent ATS statement.[1]

In conclusion, clinical isolation of NTM was relevant in 25% of the 212 patients with pulmonary isolates. Clinical relevance differs markedly by species and there are important regional differences in species distribution and relevance. This, as well as the rising isolation rates of NTM, warrants special attention by doctors and microbiologists. Treatment outcome of pulmonary NTM disease was disappointing. Both over-treatment and insufficient treatment were noted. Extrapulmonary NTM disease is rare. Actions to increase awareness of the ATS diagnostic criteria and management guidelines and centralization of NTM disease management are strongly recommended.

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Mycobacterium xenopi clinical relevance and determinants, the Netherlands

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Abstract

In the Netherlands, isolation of *Mycobacterium xenopi* is infrequent, and its clinical relevance is often uncertain. To determine clinical relevance and determinants, we retrospectively reviewed medical files of all patients in the Netherlands in whom *M. xenopi* was isolated from January 1999 through March 2005, by using diagnostic criteria for nontuberculous mycobacterial infection published by the American Thoracic Society. We found 49 patients, mostly white men, with an average age of 60 years and pre-existing pulmonary disease; of these patients, 25 (51%) met the diagnostic criteria. Mycobacterial genotype, based on 16S rRNA gene sequencing, was associated with true infection. Most infections were pulmonary, but pleural and spinal infections (spinal in HIV-infected patients) were also noted. Treatment regimens varied in content and duration; some patients were overtreated and some undertreated.

Introduction

Mycobacterium xenopi was first described by Schwabacher in 1959; it was isolated from skin lesions in a clawed frog and named after the official species designation of the frog, *Xenopus laevis*.[1] Thereafter, these slow-growing mycobacteria have been recovered from heated water systems in many countries and more recently from natural waters in Finland.[2] Transmission to humans is believed to originate from the environment, through aerosol inhalation or ingestion. Human-to-human transmission and transmission from animal reservoirs remain controversial because these routes have not been proven by molecular typing.[3,4] Pulmonary *M. xenopi* infections are most common, but extrapulmonary and disseminated infections have also been recorded.[5,6] A predisposing factor is impaired immunity, either local (e.g., pre-existing pulmonary disease) or systemic (e.g., hematologic malignancy, immunosuppressive medication, or HIV/AIDS).[5,7]

Its survival in flowing water systems and resistance to common disinfectants enables *M. xenopi* to contaminate laboratory samples and medical devices such as bronchoscopes, thus causing healthcare-acquired (pseudo)infections and laboratory cross-contaminations.[3,6,8,9] Differentiating true infection from pseudoinfection is of paramount importance because treatment of *M. xenopi* infections is time-consuming and often complicated. The British Thoracic Society (BTS) trial in 2001 established that treatment for pulmonary infections should consist of a 2-year course of rifampicin and ethambutol; regimens including macrolides or fluoroquinolones are still being investigated.[10] The American Thoracic Society (ATS) established general criteria for the diagnosis and treatment of nontuberculous mycobacterial, not specifically *M. xenopi*, infections. The treatment guidelines are similar to those by the BTS, although the ATS guidelines advocate macrolide-containing regimens.[5] To assess frequency and clinical relevance of *M. xenopi* isolation and its determinants in the Netherlands, we performed a retrospective case study. We used the ATS diagnostic criteria available during the study period to differentiate true infection from pseudoinfection.

Methods

To determine clinical relevance, we examined medical records of all patients in the Netherlands from whom *M. xenopi* had been isolated from January 1999 through March 2005. The following variables were extracted from the records: sex, age, predisposing factors, symptoms, chest imaging results, treatment and outcome, and drug susceptibility and status according to the ATS diagnostic criteria.[5]

Laboratory diagnosis of the isolates was made by the Dutch National Institute of Public Health and the Environment (RIVM) or by a local hospital laboratory. RIVM acts as the national reference laboratory that provides identification, drug-susceptibility testing, and genotyping of mycobacterial isolates for all

hospitals and other healthcare institutions in the Netherlands. To identify a mycobacterial isolate, a GenoType MTBC reverse line-blot (Hain Lifescience, Nehren, Germany) was used after PCR-based amplification to determine whether an isolate was a member of the *M. tuberculosis* complex. If the reaction was negative, an INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Gent, Belgium) reverse hybridization multiple DNA probe assay was used to differentiate between the more common species of nontuberculous mycobacteria, including *M. xenopi*.[11] Before 2004, 16S rDNA gene sequence analysis was performed, after ruling out membership in the *M. tuberculosis* complex, by using the AccuProbe MTB DNA probe kit (GenProbe, San Diego, CA, USA). The result was compared with the RIVM and GenBank (National Center for Biotechnology Information, www.ncbi.nlm.nih.gov) 16S rDNA gene sequence databases.[12]

On the basis of the results at position 90 in the 151bp hypervariable region of the 16S gene, two *M. xenopi* genotypes were discerned; a C at position 90 distinguished *M. xenopi* I and a T distinguished *M. xenopi* II. Retrospectively, the 16S rRNA genes of all *M. xenopi* isolates at RIVM were sequenced and assigned to their respective genotypes.

Susceptibility testing was performed by using an agar dilution method.[13] Drugs in the susceptibility testing panel were isoniazid, rifampicin, ethambutol, streptomycin, cycloserine, prothionamide, amikacin, ciprofloxacin, clofazimine, clarithromycin, and rifabutin. The Pearson χ^2 test was used for statistical correlations. The local ethics committee approved the study.

Results

We found 49 patients with *M. xenopi* isolates (Table 1); of these, 25 (51%) met the ATS diagnostic criteria. Isolates from 46 patients were identified to the genotype level, I or II. Sequencing failed for two, and one was unavailable at RIVM. *Mycobacterium xenopi* I was found for 28 patients, *M. xenopi* II for 13, and mixed (types I and II) for five. Isolation of type II was significantly associated with fulfillment of the ATS criteria compared with isolation of type I only (77% vs 39%; odds ratio [OR] 5.1, 95% confidence interval [CI] 1.2–23.0, p = 0.025). When we defined *M. xenopi* II cultures as "involving *M. xenopi* II," and thus included the mixed cultures, the correlation increased in significance (OR 5.4, 95% CI 1.4–20.8, p = 0.011).

Clinical signs and symptoms varied widely and were not associated with fulfillment of the ATS diagnostic criteria (Table 1). Chest radiographs were taken for all patients, except for two who had spinal infection (Table 1). Cavitation was the only radiographic finding significantly associated with fulfillment of the ATS diagnostic criteria (OR 14.3, 95% CI 2.7–75.6, p = 0.001). Results of additional computed tomography scanning, performed for 27 patients, were not associated with fulfillment of the ATS diagnostic criteria (data not shown).

Table 1: Baseline population characteristics of 49 patients with *M. xenopi* isolates, the Netherlands, January 1999 through March 2005*

Characteristic	ATS+ (n = 25)	ATS– (n = 24)	Total (%)
Demographics			
Male sex	19	18	37 (76)
Mean age, y	60	60	60
Dutch origin	24	20	44 (90)
Concurrent and predisposing conditions			
Pre-existing pulmonary disease	21	18	39 (80)
Chronic obstructive pulmonary disease	17	14	31 (63)
Lung cancer	1	3	4 (8)
Prior tuberculosis	0	2	2 (4)
Recurrent pulmonary infection†	5	2	7 (14)
Bronchiectasis	2	4	6 (12)
Smoker, current/ past	15/ 6	11/ 3	35 (71)
Alcohol abuse	2	3	5 (10)
High-dose steroid use‡	3	5	8 (16)
HIV infection	2	5	7 (14)
Mean CD4 count in HIV-infected patients, cells/mL	226	126	159
Hematologic malignancy	0	1	1 (2)
Otherwise impaired immunity§	2	1	3 (6)
Signs and symptoms			
Productive cough	21	20	41 (84)
Hemoptysis	5	4	9 (18)
Dyspnea	14	9	23 (47)
Fever	11	6	17 (35)
Weight loss	12	7	19 (39)
Malaise	16	10	26 (53)
Chest radiographic abnormalities			
Infiltrate	15	12	27 (55)
Cavity	12¶	3	15 (31)
Pleural thickening	3	4	7 (14)
Emphysema	9	9	18 (37)
Space-occupying lesion	1	3	4 (8)

*ATS+, American Thoracic Society diagnostic criteria for nontuberculous mycobacterial infection met; ATS–, ATS diagnostic criteria for nontuberculous mycobacterial infection not met.
†>3 requiring treatment in 6 months before primary *M. xenopi* culture.
‡>15 mg prednisone/day for >3 months before primary *M. xenopi* culture.
§Diabetes mellitus, cisplatinum chemotherapy, anorexia nervosa (all n = 1).
¶Significant association (odds ratio 14.3, 95% confidence interval 2.7–75.6, p = 0.001).

We found four cases of extrapulmonary disease, two cases of pleural *M. xenopi* infection, and two cases of spondylodiscitis (the latter in HIV–co-infected patients). The pleural infections were diagnosed by biopsy of pleural tissue for one patient and repeated pleural fluid culture for the other, after chest radiograph demonstrated pleural thickening and fluid collection. The spinal infections were diagnosed by bone biopsy. In the pleural and bone biopsy specimens, granulomatous lesions with central necrosis were observed.

For most patients, *M. xenopi* was first isolated from sputum (51%), bronchoalveolar lavage fluid (35%), or lung biopsy sample (4%). Remaining isolates were from bone biopsy samples (4%), pleural fluid (2%), pleural biopsy samples (2%), and stool samples (2%). Acid-fast bacilli were detected with direct microscopy of primary samples for 39% of patients. An acid-fast bacilli–positive primary sample, regardless of its nature, was significantly associated with fulfillment of the ATS diagnostic criteria (OR 8.2, 95% CI 2.1–31.6, $p<0.001$).

Treatment was started for 25 of 49 patients, of whom 19 met the ATS diagnostic criteria. Therapy consisted of medication for 21 patients, surgery for two, or both for two. Surgery consisted of lobectomy, pulmonary wedge resection, Clagett pleurostomy, and vertebral surgery with psoas muscle abscess drainage. Medication regimens varied widely but generally included rifampicin, isoniazid, ethambutol, clarithromycin, ciprofloxacin, and pyrazinamide in various 3- to 4-drug combinations. Duration of therapy varied between five days and 2.5 years, with a mean duration of nine months. Macrolides were included in regimens for 58% and quinolones for 37% of the patients who met the ATS diagnostic criteria and received drug treatment.

Antimycobacterial treatment cured 11 out of the 19 (58%) patients who met the ATS diagnostic criteria: seven with *M. xenopi* II, two with *M. xenopi* I, and two with *M. xenopi* I and II. We defined cure as resolution of symptoms and negative cultures after finishing treatment, until the end of our study period (range 0–60 months, median 25 months). Treatment failure, defined as protracted culture positivity for *M. xenopi* during and after adequate treatment, was noted for four (21%). Four other patients died. Treatment failure or death was not associated with genotype, susceptibility pattern, predisposing conditions, or radiographic imaging results.

Although they fulfilled the ATS diagnostic criteria, four patients did not receive treatment. Of these, one recovered spontaneously; two remained positive for acid-fast bacilli, culture, or both throughout the study period and one died. These outcomes were not associated with specific genotypes or patient factors.

Susceptibility testing was performed for 47 isolates from 42 patients. For five patients, cultures failed to grow for testing; for two others, cultures were not available. Results for isoniazid, rifampicin, and ethambutol are shown in Table 2. Isolates were susceptible to all other compounds tested. Susceptibility testing of follow-up cultures was performed for five patients. *Mycobacterium xenopi*

Table 2: Baseline *in vitro* susceptibility of primary isolates from 42 patients with *M. xenopi* infection, the Netherlands, January 1999 through March 2005

Susceptibility	No. (%) susceptible to		
	Isoniazid	Rifampicin	Ethambutol
Susceptible	9 (21) MIC 0.2 mg/L	29 (69) MIC <1mg/L	5 (12) MIC 5 mg/L
Intermediate	32 (76) MIC 0.5–1.0 mg/L		11 (26) MIC 10 mg/L
Resistant	1 (2) MIC >1 mg/L	13 (31) MIC >1 mg/L	26 (62) MIC >10 mg/L

bacteria in two patients treated with rifampicin became resistant to rifampicin and to ethambutol in one patient. For nine patients, susceptibility testing results influenced the treatment regimens, mostly by inclusion or exclusion of rifampicin and ethambutol or by adding a quinolone or macrolide agent.

For six patients with cavitation visible on chest radiograph, of whom four met the ATS diagnostic criteria, fungi were cultured simultaneously (*Aspergillus fumigatus* from four, *A. flavus* from one, and *Scedosporium apiospermum* from one). Antifungal treatment was initiated for four patients, which meant true *M. xenopi* infection was left untreated for two.

Four patients who had received antimycobacterial treatment for *M. xenopi* infection before (mean duration eight months) had relapses. The mean interval between discontinuation of drug treatment and relapse was 28 months (range 12–39). We found no evidence of geographic clustering, which suggests nosocomial transmission or a pseudo-outbreak. The number of new isolates per year remained steady at ~8 per year. At least five patients were treated in preventive isolation for 2–15 days until *M. tuberculosis* complex infection was excluded by PCR.

Discussion

Clinical relevance of *M. xenopi* isolation, defined by fulfillment of the ATS diagnostic criteria, was likely in 51% of patients; mycobacterial genotype II was a major determinant. To our knowledge, this phenomenon and its causal mechanisms have not been described. If further evidence emerges, 16S rDNA gene sequencing may become a relevant addition to the diagnostic algorithm of *M. xenopi* infection.

The ATS diagnostic criteria are designed for *M. avium*, *M. kansasii,* and *M. abscessus* infections, although the authors state "there is no reason to believe these criteria would not be applicable to other species".[5] Because the BTS statement focuses on management rather than specific diagnostic criteria,[14] the ATS diagnostic criteria are recommended for the clinical setting. Of the main ATS diagnostic components, two were each significantly associated with true infection in our study (cavitary lesions on chest radiograph and acid-fast bacilli on primary samples), thereby supporting the ATS criteria.

The ATS diagnostic criteria, however, have one limitation. Patients with pre-existing cavitary lesions are likely to have respiratory symptoms; they meet the radiologic criteria and are more likely to harbor mycobacteria in the cavity, which are not necessarily responsible for their symptoms and cavity formation. Cavity characteristics cannot reliably predict the cavity's origin or pathogenesis.[15] The uncertainty is compounded when fungi are cultured simultaneously, which suggests matching requirements for *in vivo* success of these microorganisms or selective impaired immunity. Determining which organism causes disease in the patient is difficult.

The undertreatment and overtreatment that we noted indicate a relative lack of knowledge in physicians, mainly those specializing in pulmonary diseases, concerning nontuberculous mycobacteria infections. Unnecessary drug treatment could harm the patient in terms of adverse effects and costs,[16] and undertreatment of patients who fulfill the diagnostic criteria is potentially harmful to the patients' health.

The baseline characteristics of our study group are similar to those in studies of nontuberculous mycobacteria patients in societies with low HIV prevalence.[5,10] The North American series included more HIV-infected patients, which lowered the mean age of patients in these studies.[17,18] Pre-existing pulmonary diseases are major predisposing factors and may be causally associated with isolation of *M. xenopi*. However, *M. xenopi* may have been isolated more often because physicians were more focused on mycobacterial cultures for this category of patients.

Minor predisposing factors were HIV infection or other causes of impaired immunity, which, in addition to the causal relationship, probably reflects the low prevalence of HIV infection compared with chronic pulmonary disease in the Netherlands. Also, because most HIV-infected patients receive highly active antiretroviral therapy, fewer cases of severe HIV immunosuppression and its co-infections are seen. HIV infection predisposes patients to extrapulmonary *M. xenopi* infection, especially spinal infection.[6,19] Those with HIV-associated spinal *M. xenopi* infection had rising CD4 counts after starting or changing highly active antiretroviral therapy regimens (Table 1). Possibly, their *M. xenopi* infection was an expression of immune reconstitution inflammatory syndrome.[20] We found no previous reports of pleural infections.

Although the treatment regimens recorded in our study were not in accordance with the current standards of the BTS[14] and ATS,[5] cure rates were high. Although partly the result of the restricted definition of "cure" resulting from our research methods, this finding does bring the validity of the ATS diagnostic criteria into question. Despite meeting these criteria, some patients might have cleared *M. xenopi* infection without treatment. This possibility is also endorsed by the spontaneous recovery of a minority of patients who met the ATS criteria but were not treated. Alternatively, the regimen of 24 months of rifampicin and ethambutol advised by the BTS may not be better

than similar regimens of shorter duration. The addition of macrolides and quinolones to therapy regimens might also account for the high cure rates after relatively short treatment durations.

Susceptibility testing results were similar to those published previously,[21] but their value in clinical practice is uncertain. Interpretation of the laboratory results is difficult because of discrepancies between *in vitro* susceptibility and *in vivo* response to treatment.[5,21] In our study, results rarely influenced treatment regimens; when they did, choice of regimen was controversial and not in accordance with ATS and BTS guidelines.[5,14] Increasing use of PCR to rule out *M. tuberculosis* infection can be valuable for preventing or shortening patient isolation.

In conclusion, clinical isolation of *M. xenopi* was relevant for 51% of the patients; mycobacterial genotype was a major determinant. Currently, the ATS diagnostic criteria are the best tool for determining clinical relevance. We strongly recommend increased awareness of these diagnostic criteria and management guidelines by ATS and BTS.

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Clinical relevance of *Mycobacterium simiae* in pulmonary samples in the Netherlands

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Abstract

The aim of the present study was to determine the clinical relevance of *Mycobacterium simiae* isolation from clinical samples.

The medical files of patients in the Netherlands from whom *M. simiae* was isolated between 1999 and 2006 were reviewed in order to assess frequency and clinical relevance. Clinical relevance was defined as fulfilment of the diagnostic criteria of the American Thoracic Society.

From the files, twenty-eight patients were identified, of whom six (21%) met the American Thoracic Society diagnostic criteria. A slight female predominance (54%) was observed, which is uncommon for NTM isolation. Fulfilment of the diagnostic criteria and initiation of treatment were not in agreement; treatment results were poor.

Only a minority of clinical *M. simiae* isolates are clinically relevant and, applying the ATS diagnostic criteria, the number of true infections is overestimated. Physicians in the Netherlands do not always use these criteria in daily practice, resulting in both over- and underdiagnosis of *M. simiae* infection. Further studies are needed to improve diagnostic criteria and treatment regimens.

Introduction

In 1965, Karassova *et al.* reported the isolation of a new species of nontuberculous *Mycobacterium* (NTM), which they named *Mycobacterium simiae* as it was isolated from *Macacus* rhesus monkeys (Latin; *simiae*: of monkeys).[1] In 1975 Weiszfeiler and Karczag found that the previously described species *M. habana* was in reality also *M. simiae*.[2]

Clinical *M. simiae* isolation at first seemed restricted to the south of the USA, Israel and Cuba.[3] However, in due course, *M. simiae* isolation has been reported from many places in the world.[4-6]

Mycobacterium simiae is present in the environment and can contaminate medical equipment and laboratory samples.[3,7] Therefore, *M. simiae* isolation is not clinically relevant *per se*. Reports on *M. simiae* in the literature mainly describe single cases of infection; few authors have reported clinical relevance. The American Thoracic Society (ATS) has published guidelines for the diagnosis and treatment of NTM infections. Their diagnostic criteria are designed to distinguish between true and pseudo-infection, and thus to assess clinical relevance.[8]

In the current study, the frequency and clinical relevance of *M. simiae* isolation in the Netherlands were determined using the ATS diagnostic criteria available during the study period.[8]

Methods

The medical records of all patients in the Netherlands from whom *M. simiae* was isolated between January 1999 and January 2006 were examined. We recorded demographic data, clinical data, drug susceptibility and whether the ATS diagnostic criteria available at the time were met.[8] The impact of the renewed ATS diagnostic criteria published in 2007 was analyzed separately.[9]

The isolates of the patients were subjected to laboratory diagnosis by the Dutch National Institute of Public Health and the Environment (RIVM, Bilthoven, the Netherlands). This is the national reference laboratory, providing identification, drug susceptibility testing and genotyping of *Mycobacterium* isolates for all health-sector institutions in the Netherlands.

After ruling out membership of the *M. tuberculosis* complex, using the GenoType MTBC (Hain Lifescience, Nehren, Germany) reverse line blot, an INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Gent, Belgium) reverse line blot was used to identify common species of NTM, including *M. simiae*. Prior to 2004, 16S rDNA gene sequence analysis (151bp) was performed after ruling out membership of the *M. tuberculosis* complex using the AccuProbe MTB DNA probe kit (GenProbe, San Diego, USA).

Drug susceptibility testing was performed using the agar dilution method.[10] The drugs tested were isoniazid, rifampicin, ethambutol, streptomycin, cycloserine, prothionamide, amikacin, ciprofloxacin, clofazimine, clarithromycin and rifabutin. Pearson χ^2 and Fisher exact tests were used for correlations. The study was approved by the regional ethics committee.

Table 1: Baseline population characteristics

	ATS+ (n=6)	ATS- (n=22)	Total (n=28)
Male	2	11	13 (46%)
Female	4	11	15 (54%)
Mean age (yr)	73*	63	65
Dutch origin	6	20	26 (93%)
Pre-existing pulmonary disease	6	20	26 (93%)
COPD	5	16	21 (75%)
Lung cancer	0	2	2 (7%)
Prior tuberculosis	2	1	3 (11%)
Bronchiectasis	2	3	5 (18%)
Current smoker	0	3	3 (11%)
History of smoking	3	11	14 (50%)
Alcohol abuse	0	1	1 (4%)
HIV infection	0	1	1 (4%)
Hematologic malignancy	0	1	1 (4%)
Otherwise impaired immunity#	0	2	2 (7%)

* significant difference, 95%CI: 3.0-18.0; p=0.008
# methotrexate-associated pancytopenia, cystic fibrosis (both n=1)

Results

Mycobacterium simiae was isolated from 28 patients during the study period. No extrapulmonary isolates or disease were noted during the study period. The baseline patient characteristics are shown in Table 1. Six patients (21 %) met the ATS diagnostic criteria, based on features detailed in Table 2.

Patients who met the ATS diagnostic criteria for true NTM infection were significantly older than those who did not (73 vs. 63 years; 95%CI: 3.0-18.0; p=0.008; Table 1).

Productive cough (96%), dyspnea (54%) and malaise (32%) were the most commonly reported symptoms, although only patients who reported weight loss were significantly more likely to meet the ATS diagnostic criteria (p=0.038 OR 9.0; 95%CI 1.3-10.5).

Chest radiographs at the time of primary sampling mostly showed infiltrates (54%), pre-existent emphysema (25%) or fibrosis (25%) and suspected malignant lesions (11%). Of the radiographic abnormalities, both nodular (p=0.040) and cavitary lesions (p=0.040) on chest X-ray were significantly associated with meeting the ATS diagnostic criteria.

The *M. simiae* isolates were cultured from sputum (44%), bronchoalveolar lavage (BAL) fluid (52%) and lung biopsy specimens (4%). Of all primary samples, 22% were positive for acid fast bacilli (AFB) on direct microscopy. An AFB positive primary sample was significantly associated with meeting the ATS diagnostic criteria (p=0.010; OR 20.0; 95%CI 1.7-30.9).

Table 2: Characteristics of patients with true *M. simiae* infections

Patient	1	2	3	4	5	6
Sex, Age	F, 79	F, 68	M, 75	F, 72	M, 76	F, 70
Predisposing conditions	COPD	TB	COPD	COPD, Bronchiectasis	COPD, TB, Bronchiectasis	
Symptoms	C,H,W,M	C,H	C,D,F,M	C,W	C,H,F,W,M	C,H,W
Cultures/ positive	2/ 2 (Smear+)	4/ 4 (Smear+)	5/ 3 (Smear-)	9/ 6 (Smear+)	6/ 5 (Smear+)	3/3 (Smear-)
Chest X-ray	Infiltrate	Cavities	Infiltrate	Nodular	Cavities, Nodular	Infiltrate
Histiology	-	GI	-	NSI	GI	NSI
Treatment	2ECipCla	2HRZE6ECipCla	None	None	1HRZ3RbECla	None
Outcome	Improved	Relapse	Stable	Deteriorated	Relapse; died	Stable

COPD=chronic obstructive pulmonary disease; TB=tuberculosis; C=productive cough; H=hemoptysis; W=weight loss; M: malaise/asthenia; F=fever; GI=granulomatous inflammation; NSI=non-specific inflammation; E=ethambutol; R=rifampicin; Rb=rifabutin; H=isoniazid; Z=pyrazinamide; Cip=ciprofloxacin; Cla=clarithromycin

Six patients started drug treatment; three of them had met the ATS diagnostic criteria. One of these three was cured (defined by improvement of symptoms and negative cultures until the end of the study period), one relapsed and one died due to progression of *M. simiae* disease (Table 2). Three received treatment without having met the ATS diagnostic criteria, for a mean duration of 30 days (range 14-56 days), until an alternative diagnosis was made (disseminated *Histoplasma capsulatum* infection and malignancy (each n=1)) or identification of the NTM as *M. simiae* was communicated (n=1).
Most commonly used drugs were rifampicin, ethambutol, ciprofloxacin and clarithromycin. Three patients met the ATS diagnostic criteria but did not receive treatment. In two, this decision was based on the estimated chance of successful treatment and the burden of treatment on the patients involved; in one the stable clinical picture was the main reason. Two of the patients remained clinically stable and one showed radiological deterioration (Table 2). Drug susceptibility testing was performed for a single isolate of 25 patients, and invariably demonstrated *in vitro* resistance to rifampicin, ethambutol, isoniazid, streptomycin, amikacin and rifabutin. Resistance to ciprofloxacin (72% of isolates) and clarithromycin (84%) were frequent. Isolates were all susceptible to clofazimine and cycloserine and most (76%) were susceptible to prothionamide.
The ATS has recently published new diagnostic criteria for NTM infections with stricter radiological but less strict microbiological criteria for true infections. In addition, the criteria now state "making the diagnosis of NTM lung disease does not, *per se*, necessitate the institution of therapy".[9] Eight patients in our study (29%) met the new criteria, due to the less strict microbiological criteria.

Discussion

In 21% of the 28 patients, *M. simiae* isolation represented clinically relevant disease, as defined by the ATS diagnostic criteria. This low degree of clinical relevance demonstrates the probably limited pathogenicity of *M. simiae* in humans.

In contrast to other NTM species, a small majority of *M. simiae* isolates were cultured from samples in women. The low number of patients in the current study, however, prevents firm conclusions on gender distribution. Predisposing conditions, mainly pre-existent lung disease, were similar to those observed for other NTM species.[8,11] During a nationwide survey in the USA, O'Brien *et al.* recorded a similar degree of clinical relevance (21%) and a comparable gender distribution (48% females) in 67 patients.[11] The causes of this possibly unusual gender distribution remain unknown. Extrapulmonary infections are seldomly reported and restricted to immunocompromised hosts.[8,11] Cases have occurred in the Netherlands before the present study period.[12]

In those patients who met the ATS diagnostic criteria, treatment results were poor, with frequent relapses (Table 2). Those who met the ATS diagnostic criteria but were left untreated remained clinically stable. Therefore, benefit of current drug treatment regimens may be questioned, although our study group is too small to draw firm conclusions. Alternatively, the ATS diagnostic criteria may not select the patients for whom treatment would be beneficial. Although designed for *M. avium*, *M. kansasii* and *M. abscessus*, the authors state that "there is no reason to believe these criteria would not be applicable to other species".[8] The present data, however, suggest that the ATS criteria are not applicable for *M. simiae*, as they over-diagnose true pulmonary *M. simiae* infections. Applying the latest ATS criteria,[9] with more lenient microbiological criteria, further increased the number of true infections, since meeting the clinical and radiological criteria was common in our study group; the microbiological criteria were the major hurdle.

Increasing the number of positive cultures required for diagnosis to more than three in one year, or reservation of the diagnosis for histologically proven infections alone, may lead to improved selection of patients to benefit from treatment. In the present study, this would result in three cases (11% of all patients) of true infection, requiring treatment (Table 2; patients 2,4,5). In line with this observation, Rynkiewicz *et al.*, on the basis of stricter clinical and histological criteria, found 9% of 23 patients having true *M. simiae* infections.[13] Limited treatment results in the present study group demonstrate that the content and duration of treatment regimens require further study.

Although not suffering true infections, three patients in the present study were treated for NTM infection. This overtreatment may harm patients in terms of adverse effects and costs.[14] Moreover, it indicates the difficulty in diagnosing true infections. The limited exposure of physicians in the Netherlands to NTM disease leads to limited experience with the currently available, albeit

imperfect, diagnostic criteria. Probably this is one of the main reasons of the observed over- and undertreatment.
When considering treating a patient, age and concomitant diseases might be a factor in the decision; the significantly higher age of patients who met the ATS criteria complicates this situation. The contribution of drug susceptibility testing results to the drug treatment decision remains limited, as the association between *in vitro* susceptibility and the *in vivo* response has not been established for most drugs.[8] Application of the new ATS criteria not only leads to diagnosis of more *M. simiae* infections, but also leaves the treatment decision more to the physician. For the infrequently encountered NTM, centralization of expertise and easily accessible expert consultation is therefore important, as acknowledged in the new statement.[9]
In conclusion, clinical *M. simiae* isolation occurs infrequently in the Netherlands, with a slight female predominance uncommon to other NTM. A minority of isolates represents true infection, which suggests limited pathogenicity in humans. The available diagnostic criteria are inadequate for the selection of patients for whom drug treatment for true *M. simiae* infection would be beneficial. Critical evaluation of more patients from whom NTM have been isolated will help to fine tune the criteria for improved diagnosis and treatment of NTM infection.

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Clinical relevance of *Mycobacterium szulgai* in the Netherlands

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Abstract

Background: The clinical relevance of *Mycobacterium szulgai* isolates is unknown, and available literature focuses on case reports of *M. szulgai* disease. We assessed the clinical relevance of *M. szulgai* isolated from patients in the Netherlands.

Methods: We reviewed medical files for all 21 patients in the Netherlands from whom *M. szulgai* was isolated during 1999-2006, applying the diagnostic criteria of the American Thoracic Society for nontuberculous mycobacterial infection. Random amplified polymorphic DNA genotyping was performed using IS*986*, OPA-2 and OPA-18 as primers.

Results: Of the 21 patients, 16 (76%) met the American Thoracic Society diagnostic criteria and were thus likely to have *M. szulgai* disease. Pulmonary *M. szulgai* disease was the most common presentation, with extrapulmonary disease restricted to patients with an impaired systemic immunity. Although treatment regimens varied in content and duration, the outcomes were mostly favorable. Both overtreatment and undertreatment were noticed.

Random amplified polymorphic DNA genotyping revealed a higher degree of interpatient variability, with limited intrapatient variability, suggesting persisting monoclonal infection and good reproducibility. No genotype was associated with clinical relevance.

Conclusions: Clinical isolation of *M. szulgai* generally represents true disease and demands careful follow-up. Extrapulmonary disease occurs in patients with impaired immunity. Adherence to diagnostic guidelines can be improved.

Introduction

Mycobacterium szulgai is a slow-growing nontuberculous mycobacterium (NTM) that was first described in 1972 by Marks et al. and that was named after Dr. T. Szulga, who developed the lipid analysis method that identified this NTM as a new species.[1] Nontuberculous mycobacteria are opportunistic pathogens, with local events (e.g. chronic obstructive pulmonary disease [COPD] or healed tuberculosis) or systemic impaired immunity (e.g. receipt of immunosuppressive medication, HIV infection or hematological malignancy) as the predisposing condition.[2] Pulmonary *M. szulgai* disease, which resembles tuberculosis, is the most common presentation, although extrapulmonary and disseminated disease has been recorded.[1-6]

Mycobacterium szulgai has been recovered from environmental sources, including a snail, aquarium water, swimming pool water, and tropical fish.[3-6] The environment is the suspected source of human NTM infection.[4,7] Therefore *M. szulgai* disease has to be distinguished from pseudoinfection because of the occasional presence of *M. szulgai* in clinical samples or contamination of samples in the laboratory.[2,7]

The American Thoracic Society (ATS) published guidelines for diagnosis and treatment of NTM infections, which consist of clinical, radiological and bacteriological criteria and are summarized in Box 1.[2]

We reviewed the medical files of all patients in the Netherlands from whom *M. szulgai* was isolated during the period from January 1999 through January 2006. We assessed the frequency and clinical relevance of *M. szulgai* isolation using the current ATS diagnostic criteria[2] and we evaluated drug susceptibility, treatment regimens and outcome. In addition, we analyzed the genotypes of all clinical isolates that were evaluated, to study the molecular epidemiology of *M. szulgai* in The Netherlands.

Methods

To determine clinical relevance, we examined the medical records of all patients in the Netherlands from whom *M. szulgai* was isolated between January 1999 and January 2006. We recorded sex, age, predisposing factors, symptoms, chest imaging results, treatment and outcome, drug susceptibility and status in accordance with the diagnostic criteria of the ATS.[2] We defined cure as clinical and radiographic improvement during treatment and absence of positive culture results after completion of treatment.

In all patients, the isolates were subjected to laboratory diagnosis by the Dutch National Institute for Public Health and the Environment (RIVM), or a self-sufficient hospital laboratory. All self-sufficient hospitals used 16S rDNA gene sequence analysis for identification and granted access to their databases, to ensure full national coverage. The RIVM is the national reference laboratory that provides identification, drug susceptibility testing and epidemiological typing of mycobacterial isolates for all hospitals in the Netherlands. At the

Box 1: Summary of the 2007 American Thoracic Society diagnostic criteria

American Thoracic Society Diagnostic Criteria of Nontuberculous Mycobacterial Lung Disease

Clinical criteria

1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules.
and
2. Appropriate exclusion of other diagnoses.

Microbiological criteria

1. Positive culture results from at least two separate expectorated sputum samples. (If the results from the initial sputum samples are nondiagnostic, consider repeat sputum AFB smears and cultures.)
or
2. Positive culture results from at least one bronchial wash or lavage.
or
3. Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.

RIVM we used 16S rDNA gene sequence analysis to identify mycobacteria, after ruling out membership of the *M. tuberculosis* complex, using a GenoType MTBC strip (Hain Lifescience, Nehren, Germany), or the more common species of NTM, with an INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Gent, Belgium) reverse line blot. *Mycobacterium szulgai* is not incorporated in this line blot.

We used the agar dilution method for drug susceptibility testing. Drugs tested were isoniazid, rifampicin, ethambutol, streptomycin, cycloserine, prothionamide, amikacin, ciprofloxacin, clofazimine, clarithromycin and rifabutin.[8]

Genotypes were determined using Random Amplified Polymorphic DNA (RAPD) fingerprinting.[9] We selected OPA2, OPA18 and IS*986* as primers. The study was approved by the regional ethics committee.

Results

Files were reviewed for 21 patients. The patients' clinical characteristics are detailed in table 1. Sixteen patients (76%) met the current ATS diagnostic criteria and therefore we considered them to have *M. szulgai* disease. Eighteen patients (86%) were male, and 20 (95%) were of Dutch origin; the mean age was 56 years. Most primary *M. szulgai* isolates were obtained from pulmonary samples (sputum samples, 7 isolates [33%]; bronchoalveolar lavage fluid samples, 8 isolates [38%]). Remaining primary isolates were obtained from skin specimens (two isolates [10%]) and from feces, joint aspirate, lymph node biopsy, and pleural fluid samples (one isolate each [5%]). Primary samples tested positive for acid-fast bacilli (AFB) by direct microscopy in

Pt.	Sex, Age	Cult/Pos (smear)	Sample	Risk factor	Symptoms	Chest Radiograph	ATS criteria	Treatment (months)	Outcome
1	M, 62	7/5 (-)	Skin	Steroids[a]	Nodular skin lesions	No abnormalities	Met	12EClaCip	Cured
2	M, 19	1/1 (-)	Lymph node	IFN-γ RD	Painless swollen neck	No abnormalities	Met	9EClaCip	Cured
3	F, 90	1/1 (-)	Pleural fluid	None	PC, D, M	Infiltrate, pleural fluid collection	Not met	None	n.a.
4	M, 37	4/1 (-)	Feces	HIV (CD4: 20)	PC, D, WL, M	Nodules	Met	26RbECla	Cured
5	M, 41	4/4 (+)	Skin	HIV (CD4: 40),B2G	Skin ulcers	No abnormalities	Met	6RbEClaOfl	Death
6	M, 17	1/1 (+)	Joint	IFN-γ RD	Ankle joint swelling, F, NS, WL, M	Nodules, coarse linear scarring	Met	2MerOflDox 18REClaOfl	Cured
7	M, 71	7/3 (-)	BAL	COPD, TB	PC	Infiltrate, coarse linear scarring	Met	12HRECip	Death
8	M, 55	3/1 (-)	Sputum	COPD	PC, D, F	Infiltrate, bulla, air-fluid level, emphysema	Not met	12RECla	n.a.
9	M, 87	5/2 (-)	BAL	B2G	PC, WL, M	Infiltrate, coarse linear scarring	Met	None	Death
10	F, 54	1/1 (-)	BAL	COPD, TB	H	Emphysema	Not met	None	n.a.
11	M, 44	9/6 (-)	Sputum	Diabetes	PC, F, WL	Cavities, infiltrate	Met	12RECla	Cured
12	M, 53	12/6 (-)	BAL	Anti-TNF, COPD	PC, D, F	Infiltrate, cavities, emphysema	Met	12RECla	Cured
13	M, 49	7/1 (-)	BAL	COPD	PC, D	Pulmonary mass	Not met	none	n.a.
14	M, 67	4/2 (+)	Sputum	COPD, BR	H, WL, M	Cavities, pleural thickening, BR, emphysema, coarse linear scarring	Met	1HRZE 12RECla	Cured
15	M, 70	6/6 (-)	BAL	COPD, TB	PC, D	Fibrosis, coarse linear scarring	Met	Refused	Deteriorated
16	M, 44	4/4 (+)	Sputum	Metalworking fluid exposure, smoking	PC, D, WL	Cavities	Met	5HRZE 7RECla	Cured
17	M, 68	3/2 (-)	BAL	COPD, BR, B2G	PC, D, F, WL, M	Infiltrate, emphysema, BR, nodules, pleural thickening, coarse linear scarring	Met	1HRZE 6RECla	Cured
18	M, 77	2/1 (-)	BAL	COPD	PC, NS, WL, M	Infiltrate, cavities, pleural thickening, coarse linear scarring	Met	None	Death
19	M, 38	3/1 (-)	Sputum	HIV (CD4: 280)	PC, D, F, WL, M	No abnormalities	Not met	None	n.a.
20	M, 59	4/2 (-)	Sputum	Asthma, recurrent RTIs	H	Emphysema, BR, coarse linear scarring	Met	None	Cured
21	F, 70	6/4 (-)	Sputum	COPD, TB	H, D, F, WL, M	Cavities, emphysema, pleural thickening, coarse linear scarring	Met	None	Death

Table 1: Clinical characteristics of all 21 patients with *Mycobacterium szulgai* isolates

Note: M, male; F, female; Cult, cultures performed; Pos, total number of positive cultures; BAL, broncho-alveolar lavage; HIV, human immunodeficiency virus; IFN-γ RD, interferon γ receptor deficiency; B2G, Billroth II gastrojejunostomy; RTI, respiratory tract infection; COPD, chronic obstructive pulmonary disease; BR, bronchiectasis; TB, prior tuberculosis; anti-TNF, tumor necrosis factor alpha neutralizing treatment; PC, productive cough; H, hemoptysis; D, dyspnea; F, fever; WL, weight loss; M, malaise/fatigue; NS, night sweats; R, rifampicin, H, isoniazide, Z, pyrazinamide, E, ethambutol; Rb, rifabutin; Cla; clarithromycine; Cip, ciprofloxacin; Ofl, ofloxacin; Mer, meropenem; Dox, doxycyclin; n.a., not applicable.

[a] >15mg prednisone/day for >3 months

just four patients (19%). There were no relapses of prior *M. szulgai* disease. Remarkably, three patients had a history of Billroth II gastrojejunostomy; all three had *M. szulgai* disease. Impaired systemic immunity was associated with extrapulmonary disease (Odds ratio 4.0; 95% CI 1.71-9.35; p=0.006), but not with true disease overall (p=0.647).

Of the 15 patients with pulmonary isolates (table 1; patients 7-21), 11 (73%) had true disease (table 1). Thirteen patients (87%) were male, and all were of Dutch origin; the mean age was 60 years. Twelve patients (80%) had pre-existing pulmonary disease, mostly COPD (ten patients [67%]), and 11 patients (73%) were current or past smokers. Impaired immunity was noted in two patients (diabetes for one patient and use of tumor necrosis factor-α-neutralizing agents for the other).

Reported symptoms included productive cough (14 patients [93%]), hemoptysis (four patients [27%]), dyspnea (eight patients [53%]), weight loss (eight patients [53%]), and fever and malaise (six patients for each [40%]). All patients underwent chest radiography, and abnormalities were noted in 14 patients. Infiltrates (seven patients [47%]), cavities (six patients [40%]), bronchiectasis (three patients [20%]), nodular opacities (one patient [7%]), pleural thickening (four patients [27%]), and scars of previous lung disease (seven patients [47%]) were observed. In ten patients, additional computed tomography (CT) scans of the thorax were performed, revealing one additional case of cavitary disease and one additional case of nodular bronchiectatic disease (in patients 7 and 20, respectively) (table 1). All other CT findings were concomitant with the findings on the chest radiograph.

Six patients (patients 1-6) had *M. szulgai* isolates from extrapulmonary sites (table 1). Five out of six (83%) patients met the ATS diagnostic criteria, having skin (two patients), joint, intestinal and lymph node infections (one patient each). The skin, joint and intestinal diseases represented disseminated disease. Five patients (83%) were male, and five (83%) were of Dutch origin; the mean age was 44 years. Predisposing conditions were HIV infection (two patients [33%]), interferon-γ receptor deficiency (two patients [33%]), and receipt of high-dose steroid treatment (one patient [17%]). Aside from local signs and symptoms, that are detailed in Table 1, patients reported a productive cough

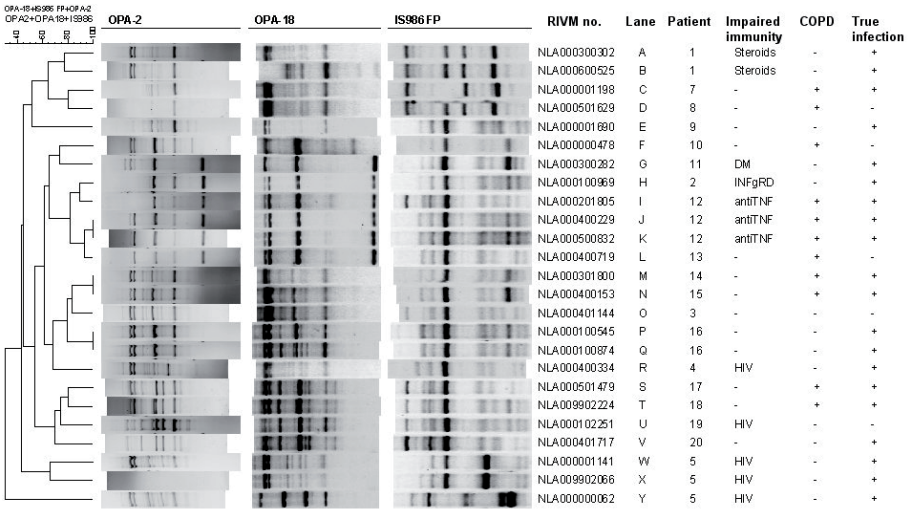
(three patients [50%]), dyspnea (four patients [67%]), malaise (three patients [50%]), and weight loss (two patients [33%]). Three patients had abnormalities noted by chest radiography that consisted of nodular opacities in two patients (patients 4 and 6) and an infiltrate and pleural fluid collection in one patient (patient 3). Similar findings were noted in additional CTs of the thorax.

Reported symptoms and radiographic abnormalities were not significantly associated with fulfillment of the ATS diagnostic criteria, neither overall or specifically among patients with pulmonary or extrapulmonary isolates.

A total of 12 patients received antimycobacterial treatment, of whom 11 met the former ATS diagnostic criteria, which were available during the studied period.[10] On average, treatment lasted for 12 months (range, 6-26 months) and consisted of rifampicin or rifabutin with ethambutol and clarithromycin and/or a quinolone antibiotic. Two patients (patients 2 and 6) received concurrent treatment with interferon-γ, due to proven underlying interferon-γ receptor deficiency. Follow-up sputum microscopy or culture during treatment was not performed at regular intervals; five patients with pulmonary *M. szulgai* disease were known to have negative cultures after six months of treatment. Treatment outcomes are detailed in table 1; we recorded no failures or relapses. Two patients died: one during treatment, and the other died just after therapy (due to HIV-related cryptococcal meningitis in one and lung cancer in the other). The average duration of follow-up after treatment was 42 months (range, 9-84 months). Three patients met the former ATS diagnostic criteria but were not treated; one died, one refused treatment, and one patient experienced conversion of culture results to negative. No signs of active NTM disease were noted during the follow-up for patients who did not meet the ATS criteria. Isolation measures were taken for four patients until PCR results for *M. tuberculosis* complex were proven to be negative.

In vitro, intermediate susceptibility to isoniazid (MIC 0.5-1 μg/ml) and susceptibility to all other compounds in the test panel was noted for all isolates. The MICs for rifampicin, ethambutol, and clarithromycin were 0.5-1, 2-5, and <2 μg/ml, respectively.

Genotyping of the isolates from our study period revealed little intrapatient and good interpatient variability (figure 1). Four major groups of strains are apparent. No associations between genotype and predisposing factors, patient origin, or clinical relevance were observed. Clustering that potentially indicates (pseudo-)epidemics or laboratory cross-contamination was not recorded. Two patients (patients 14 and 15, represented by lanes M and N in figure 1) had similar genotypes of *M. szulgai*, which was cultured in different laboratories and during different periods. They were not epidemiologically linked. In one patient (patient 5, represented by lanes W/X and Y in figure 1) two different *M. szulgai* strains were isolated; one sample (shown in lane Y) was cultured from feces, whereas the others (shown in lanes W and X) were from skin lesions.



RIVM no.	Lane	Patient	Impaired immunity	COPD	True infection
NLA000300302	A	1	Steroids	-	+
NLA000600525	B	1	Steroids	-	+
NLA000001198	C	7	-	+	+
NLA000501629	D	8	-	+	-
NLA000001690	E	9	-	-	+
NLA000000478	F	10	-	+	-
NLA000300282	G	11	DM	-	+
NLA000100969	H	2	INFgRD	-	+
NLA000201805	I	12	antiTNF	+	+
NLA000400229	J	12	antiTNF	+	+
NLA000500832	K	12	antiTNF	+	+
NLA000400719	L	13	-	+	-
NLA000301800	M	14	-	+	+
NLA000400153	N	15	-	+	+
NLA000401144	O	3	-	-	-
NLA000100545	P	16	-	-	+
NLA000100874	Q	16	-	-	+
NLA000400334	R	4	HIV	-	+
NLA000501479	S	17	-	+	+
NLA009902224	T	18	-	+	+
NLA000102251	U	19	HIV	-	-
NLA000401717	V	20	-	-	+
NLA000001141	W	5	HIV	-	+
NLA009902066	X	5	HIV	-	+
NLA000000062	Y	5	HIV	-	+

Figure 1: RAPD genotyping results of 25 strains isolated from 19 patients

Four major strain types are discerned, type 1 in lanes A-E, type 2 in lanes F-R, type 3 in lanes S-V and type 4 in lanes W-Y; no association with geographical region, clinical relevance or specific predisposing conditions exists. Limited intra-patient variability is noted in patients with >2 year intervals between follow-up cultures (lanes A&B, I&J). Patient 5 has 2 distinct types from different body sites (lanes W/X&Y).
Note: DM, diabetes mellitus type 1; INFgRD, Interferon-γ receptor deficiency; Anti-TNF, tumor necrosis factor α neutralizing treatment; HIV, Human immunodeficiency virus.

Discussion

Mycobacterium szulgai isolation was found to be clinically relevant in 16 (76%) of the 21 patients included in this study. O'Brien *et al.* also reported a high rate of true *M. szulgai* infection (8 [57%] of 14 patients) in the first national survey from the United States.[11] The current ATS criteria state "cultures yielding *M. szulgai* almost always have a pathological significance";[2] our findings are in accordance with this. No specific determinants significantly associated with *M. szulgai* disease were observed; the limited number of patients and the observed co-morbidities do not allow greater statistical certainty. Previous literature on *M. szulgai* disease focuses mainly on case reports.[1,3-6,12,13] To our knowledge, we report the largest group of isolates examined thus far for their clinical relevance. Still, we may have missed *M. szulgai* isolates, because 16S rDNA gene sequencing is not a free service at the national reference laboratory, whereas identification by the INNO-LiPA MYCOBACTERIA v2 reverse line blot method - which does not recognize *M. szulgai* - is. However, few strains that are unidentifiable to the species level by this method are not sequenced, on the basis of an appraisal of the costs by the referring hospital. If cases of *M. szulgai* isolation have been missed, they will thus be very few. In addition, strains submitted for drug susceptibility testing are always identified to the species level, with 16S rDNA sequencing applied as appropriate.

The rate of extrapulmonary disease (31% of all cases of *M. szulgai* disease) was similar to that reported in prior publications by Marks *et al.* (43%),[1] Benator *et al.* (29%)[3] and Maloney *et al.* (33%).[12] This may reflect the degree of impairment of immunity seen in this patient category, although it might also be a species-specific phenomenon. We considered one positive pleural fluid culture for a patient with *Streptococcus pneumoniae* pneumonia with pleural effusion to have been contaminated on the basis of the patient's clinical and radiographic improvement after receipt of treatment with penicillin, with complete resolution of the pleural fluid collection. The stool sample from one patient with HIV/AIDS was considered to be representative of a disseminated infection on the basis of a low CD4 count, symptoms, associated chest radiograph and CT abnormalities, which improved - as did the patient's symptoms - after the initiation of antimycobacterial therapy; both interpretations remain debatable, because the pathway to diagnosis was not optimal.

The clinical and demographic features of our study group are comparable to those in previous reports.[1-5,12,13] The number of patients with previous Billroth II gastrojejunostomy was an unexpected observation; gastric surgery has been recognized as a risk factor for the development of tuberculosis.[14] Maloney *et al.* reported one case of *M. szulgai* disease with partial gastrectomy, smoking and alcohol abuse as predisposing factors.[12] The three patients in our study also had multiple predisposing factors: they were smokers with AIDS, COPD and old age as individual risk factors. It remains controversial whether gastric surgery is a predisposing condition or a result of other predisposing conditions (e.g. heavy smoking and alcohol abuse) that cause gastric ulcers that require surgery. Contrary to reports of *M. avium* complex or *M. abscessus* pulmonary disease in the United States,[2] we noted pulmonary *M. szulgai* disease only in patients with established predisposing conditions. This finding may reflect a difference in pathophysiology of infections with different NTMs.

In our cohort, we observed a favorable treatment outcome for all but two patients, and none of the cases in our study constituted relapses of prior *M. szulgai* disease. Previous studies recorded similar results, with the few relapses attributable to noncompliance with drug treatment or receipt of an inadequate drug regimen.[1,3,12,13] Our findings imply that the observed average treatment regimen - 12 months of rifampicin, ethambutol and clarithromycin - leads to favorable outcomes without bacteriological relapse. *In vitro* drug susceptibility testing results supported this assumption. Randomized, controlled trials are needed to investigate optimal drug treatment and its duration. We realize, however, that these are difficult to perform, because they require a large, multiple-center approach to collect a large enough sample size. In our cohort, the treatment regimens were based on data from the available literature or expert consultation;[10] the susceptibility of the isolates to the most frequently used drugs did not necessitate changes in therapy regimens.

We observed possible instances of both undertreatment (i.e., patients who met the diagnostic criteria were not treated) and overtreatment (i.e., treatment of persons who did not meet diagnostic criteria). This, as well as the lack of bacteriological follow-up during treatment and handling of the uncertain cases of extrapulmonary disease discussed above, could reflect a lack of knowledge of and experience with the diagnosis and management of NTM disease in physicians or clinical circumstances that were not captured in our file review. Both over- and undertreatment can be harmful for patients.

The mostly unique genotypes imply acquisition of *M. szulgai* from the local environment rather than human-to-human transmission or laboratory contamination, as was found in previous studies.[4,7,9] The uncertain pathogenesis and environmental reservoirs, as well as the low frequency of isolation are important drawbacks to these molecular epidemiological studies and to the interpretation of their results. The absence of an association between RAPD genotype and fulfillment of the ATS diagnostic criteria may reflect the importance of patient - rather than mycobacterial - factors in the etiology of *M. szulgai* disease. The limited intra-patient variability suggests good reproducibility and persisting monoclonal infections. In one patient (patient 5), two infecting strains were found in specimens from separate body sites (as denoted in lanes W/X and Y in figure 1). The minor changes observed in serial isolates may hint at either limited reproducibility of RAPD genotyping or genetic drift of the infecting strain during chronic infection.

In conclusion, the vast majority (76%) of 21 patients with *M. szulgai* isolates in our study experienced true *M. szulgai* disease. Therefore, clinical *M. szulgai* isolation demands careful follow-up. Extrapulmonary disease occurs in patients with impaired immunity. Stricter adherence to diagnostic guidelines seems desirable. Treatment with rifampicin, ethambutol and a macrolide antibiotic leads to favorable outcomes; its optimal duration, as well as the efficacy of alternative regimens, requires additional study. *Mycobacterium szulgai* is most likely contracted from the environment, although this subject also requires further study.

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Clinical *Mycobacterium conspicuum* isolation from two immunocompetent patients in the Netherlands

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Case Reports

The first patient, a 63-year-old Dutch male living in the south of the Netherlands, presented at a regional hospital with dyspnea and productive cough. No fever, malaise, hemoptysis, night sweats or weight loss was reported. His medical history included chronic obstructive pulmonary disease. Since a chest radiograph revealed a single opacity in the right upper lobe, computed tomography scanning (CT) was performed, demonstrating calcification in a mediastinal lymph node and a solid mass with spiculae in the right upper lobe, suggestive of malignancy. In the diagnostic work-up, a bronchoalveolar lavage was performed. No malignant cells were found in the cytology sample of the lavage fluid. Although no acid-fast bacilli (AFB) were seen on direct microscopy, the routine mycobacterial culture of the lavage fluid yielded *Mycobacterium conspicuum*. Serological examination lowered the suspicion of an infectious process, with an erythrocyte sedimentation rate (ESR) of 5mm/h and white blood cell (WBC) count of 7.8 per nl. Although no malignancy was cytologically proven, a mediastinoscopy was performed for staging of the suspected malignancy. No evidence of either malignancy or mycobacterial infection was found in the lymph node samples. Based on the radiographic image and mediastinoscopy result, the patient underwent a lobectomy of the right upper lobe and a squamous cell carcinoma was diagnosed histologically. No histological evidence of mycobacterial infection was found. Based on the single positive culture, nonsuspect radiographic image, and alternative diagnosis, the *M. conspicuum* isolate was considered a contaminant; hence, no antimycobacterial treatment was started.

The second patient was a 65-year-old Dutch male from the eastern part of the Netherlands whose medical history consisted of radiographic stage IV sarcoidosis. His sarcoidosis was not treated with immunosuppressive agents. The patient presented at another regional hospital with dyspnea and a productive cough, without a fever, night sweats, weight loss, or malaise. A chest radiograph and CT revealed further progression of pre-existent interstitial nodular opacities, thick septa, bronchiectasis, fibrotic scarring, bullae, emphysema, and a ground glass aspect. During the next two years, 13 mycobacterial sputum cultures were performed, 10 of which yielded *M. conspicuum*; three cultures remained negative, although two samples were initially PCR positive for a nontuberculous mycobacterium (NTM), identified as *M. conspicuum* by sequencing of the PCR product. All AFB smears were negative. Serological parameters of infection were slightly raised; the ESR ranged between 21 and 32 mm/h, the C-reactive protein concentration ranged between 13 and 44 mg/liter and the WBC counts were 12.2 per nl. The disease progressed in these two years, with increasingly frequent *Pseudomonas aeruginosa* infections and chronic hypoxia, making the patient oxygen-dependent. It was decided that the sarcoidosis, rather than the *M. conspicuum* infection, would be treated, and the patient underwent bilateral lung transplantation 2 years and 8 months after the first *M. conspicuum* culture. The last sputum culture yielding *M. conspicuum* was performed 8 weeks prior to transplantation. The patient died of respiratory failure 17 days after transplantation; the donor lungs were colonized by *P. aeruginosa* and an *Acinetobacter* species. Autopsy revealed an acute necrotizing pneumonia in both lungs, most likely caused by *P. aeruginosa*. The native lungs were not examined.
Based on the progression of symptoms and radiographic abnormalities as well as multiple positive cultures, this patient met the American Thoracic Society (ATS) diagnostic criteria,[1] and thus was likely to have *M. conspicuum* pulmonary disease. However, fulfillment of these criteria does not necessitate treatment *per se*, this is a decision based on potential risks and benefits of therapy for the individual patient.[1] In addition, the progression of symptoms and radiographic abnormalities may have been due to sarcoidosis alone. For these reasons, no antimycobacterial treatment was initiated. Histological examination of the native lungs could have provided additional proof of *M. conspicuum* infection. Previously, the condition of persistent culture positivity with little or no clinical and radiographic deterioration has been referred to as colonization. However, airway colonization without infection is an unproven condition for NTM and recent studies with high-resolution CT, summarized in the ATS statement, have revealed progressive nodular-bronchiectatic disease, as in our patient, considered to be due to the NTM.[1]

Since its isolation and subsequent description as a novel *Mycobacterium* species in 1995 by Springer *et al.*, no reports on clinical *M. conspicuum* isolation have

Table 1: *In vitro* drug susceptibility results for four *Mycobacterium conspicuum* strains

Drug	MIC (µg/ml) for indicated isolate				Overall status
	1 (patient 1)	2 (patient 2)	3 (patient 2)	4 (patient 2)	
Isoniazid	10	2	5	5	Resistant
Rifampicin	>5	5	>5	5	Resistant
Rifabutin	0.5	0.5	0.5	0.5	Susceptible
Ethambutol	5	=<1	2	2	Susceptible
Streptomycin	10	5	5-10	5	Variable
Amikacin	10	10	20	10	Resistant
Clarithromycin	=<2	=<2	=<2	=<2	Susceptible
Ciprofloxacin	=<1	=<1	=<1	=<1	Susceptible
Cycloserine	10	10	10	10	Susceptible
Prothionamide	5	5	5	2	Susceptible
Clofazimine	2	=<0.5	1	1	Susceptible

been recorded in the literature.[2] *Mycobacterium conspicuum* is a rare clinical isolate phylogenetically most closely related to *M. malmoense* and *M. szulgai* on the basis of 16S rDNA gene sequences.[2]

In our country, over an eight year period, bacteria of this species have been isolated only from the pulmonary samples of the two patients presented. The mycobacteria were cultured in the MB/BacT system (Biomerieux, Boxtel, the Netherlands) as well as on Middlebrook 7H10, Ogawa and Coletsos-T (BioMerieux) solid media at 35° C. Growth was observed after 13 days in the MB/BacT liquid medium and after 28-35 days on the solid media. No growth was observed after 35 days on Löwenstein-Jensen media with and without pyruvate incubated at 35° C. On Middlebrook 7H10 media, colonies were small, smooth and white.

Identification was performed at the Dutch national mycobacteria reference laboratory (RIVM) by sequencing of the 151bp hypervariable region in the 16S rDNA gene, after ruling out membership of the *M. tuberculosis* complex using the GenoType MTBC (Hain Lifesciences GmbH, Nehren, Germany) line probe assay and the more common species of NTM using the INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Ghent, Belgium) reverse line blot assay. The obtained sequences were compared to those in the Ribosomal Differentiation of Medical Microorganisms database (http://rdna.ridom.de). All isolates from patient 2 had also been identified as *M. conspicuum* in the regional hospital laboratory, by 16S rDNA gene sequence analysis.

We performed drug susceptibility testing for four *M. conspicuum* isolates (one from patient 1 and three from patient 2), using a previously published Middlebrook 7H10 agar dilution method.[3] Drugs included in the panel,

with their breakpoint concentrations in parentheses, are isoniazid (1 µg/ml), rifampicin (1 µg/ml), ethambutol (5 µg/ml), streptomycin(5 µg/ml), cycloserine (50 µg/ml), prothionamide (5 µg/ml), amikacin (5 µg/ml), ciprofloxacin (2 µg/ml), clofazimine (2 µg/ml), clarithromycin (16 µg/ml) and rifabutin (2 µg/ml). Growth at the breakpoint concentration is reported as susceptible, and growth at higher concentrations of the drug is considered resistant. The results are detailed in Table 1.

The original species description featured two immunocompromised patients suffering from disseminated infections.[1,2] The patients presented here were not known to suffer from impaired systemic immunity, but their preexisting pulmonary diseases predisposed them to NTM infection. For patient 1, follow-up cultures in the three years since his *M. conspicuum* isolation have yielded other NTM (one *M. avium* and one *M. kansasii* isolate), indicative of increased susceptibility to mycobacterial infections in general. The demographics, with the patients being male, white and older than the average tuberculosis patient, are common for NTM infections and probably reflect the characteristics of the predisposing lung disease.

To assess the clinical relevance of the *M. conspicuum* isolation from these patients, we used the diagnostic criteria set in the recent ATS statement.[1] Although these criteria fit best with the *M. avium* complex, *M. kansasii* and *M. abscessus*, the authors state that "it is assumed, but not proven, that the concepts outlined in these guidelines are pertinent for other less common NTM respiratory pathogens".[1]

Our findings indicate that *M. conspicuum* is not only a causative agent of disseminated infections in immunocompromised patients. This species may occasionally cause pulmonary infections in immunocompetent persons with pre-existing pulmonary diseases or be a mere contaminant. This is in line with most other NTM species.[1]

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Clinical relevance of *Mycobacterium malmoense* isolation in the Netherlands

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Abstract

Uncertainty exists about the clinical relevance of *Mycobacterium malmoense* isolation, especially in pulmonary samples. We therefore determined clinical relevance, treatment and outcome of *M. malmoense* isolation in the Netherlands.

A retrospective medical file study was conducted for all patients in the Netherlands from whom *Mycobacterium malmoense* had been isolated between January 2002 and January 2006. Diagnostic criteria for nontuberculous mycobacterial (NTM) disease published by the American Thoracic Society (ATS) were used to determine clinical relevance. Treatment was compared with guidelines published by the British Thoracic Society.

We found 51 patients from whom *M. malmoense* was isolated. Forty patients (78%) had pulmonary isolates, 32 of them met the ATS diagnostic criteria (80%). Cavitary disease was most common (n=28; 88%). Patients with pulmonary disease were mostly males, with an average age of 56 years and pre-existing chronic obstructive pulmonary disease. Cervical lymphadenitis was the most common extrapulmonary disease type. Adherence to treatment guidelines was poor. A good clinical response to treatment was observed in 70% and 73% of patients treated for pulmonary and extrapulmonary disease, respectively.

In conclusion, *M. malmoense* is a clinically highly relevant NTM in the Netherlands causing serious pulmonary morbidity. Adherence to treatment guidelines is not satisfactory.

Introduction

First described as a respiratory tract pathogen in 1977 by Schröder and Juhlin,[1] *Mycobacterium malmoense* is among the most frequently isolated and clinically relevant nontuberculous mycobacteria (NTM) in northern Europe.[2,3,4] The environment is the suspected source of transmission of NTM to humans through aerosols and ingestion. Person-to-person transmission or transmission from animal sources has not been proven.[3,5,6] The presence of NTM in the environment implies that a NTM cultured from a non-sterile body site, such as the respiratory tract, may result from contamination or occasional presence of the NTM in a sample. Hence, it is important to distinguish contamination from true NTM disease. The American Thoracic Society (ATS) has published guidelines to assist in this distinction.[3] The clinical relevance of a NTM species can be quantified by assessing the percentage of patients with positive cultures of the respective NTM who meet the ATS diagnostic criteria. In this study, we quantified the clinical relevance of *M. malmoense* isolation in the Netherlands between 2002 and 2006 by applying the ATS diagnostic criteria, and evaluated treatment and outcome.

Methods

We retrospectively reviewed medical records of all patients from whom *Mycobacterium malmoense* was cultured in the January 2002 to January 2006 period. We recorded demographic data, clinical data, drug susceptibility, treatment and outcome, and status according to the 2007 ATS diagnostic criteria (summarized in Box 1).[3]

Treatment was compared with guidelines published by the British Thoracic Society: a NTM based treatment regimen was defined as consisting of rifampicin or rifabutin and ethambutol.[7] An adequate response to treatment was defined as symptomatic improvement and reversion to at least three subsequent negative cultures.

The National Institute for Public Health and the Environment (RIVM) subjected isolates of most patients to laboratory diagnosis. The RIVM is the national mycobacteria reference laboratory that provides identification and drug susceptibility testing for all hospitals in the Netherlands. All isolates were subcultured in both liquid and solid media and identified using the INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Gent, Belgium) reverse line blot assay which has specific probes for *M. malmoense*. Prior to 2004, 16S rDNA gene sequencing (151bp hypervariable region A) was performed, after ruling out membership of the *M. tuberculosis* or *M. avium* complex using the AccuProbe assays (GenProbe, San Diego, USA). Remaining isolates were identified at local hospitals, by 16S rDNA gene sequencing.

Drug susceptibility was tested using the agar dilution method.[8] Drugs included in the test panel were isoniazid, rifampicin, ethambutol, streptomycin, cycloserine, prothionamide, amikacin, ciprofloxacin, clofazimine, clarithromycin, and rifabutin.

Box 1: Summary of the 2007 American Thoracic Society diagnostic criteria

American Thoracic Society Diagnostic Criteria of Nontuberculous Mycobacterial Lung Disease

Clinical criteria

1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules.

and

2. Appropriate exclusion of other diagnoses.

Microbiological criteria

1. Positive culture results from at least two separate expectorated sputum samples. (If the results from the initial sputum samples are nondiagnostic, consider repeat sputum AFB smears and cultures.)

or

2. Positive culture results from at least one bronchial wash or lavage.

or

3. Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.

HRCT, High resolution computed tomography; NTM, nontuberculous mycobacteria;
Note: Three or more pulmonary samples should be analyzed to apply these criteria.

Pearson χ^2 and Fisher exact tests were used for statistical correlations. The regional ethics committee approved the study.

Results

Mycobacterium malmoense was isolated from 51 patients in the study period. Forty patients (78%) had pulmonary isolates, in 11 cases (22%) these were of extra-pulmonary origin. No patients in the study group were HIV-infected. During the study period, no increase in the annual notification of *M. malmoense* isolation was observed.

Pulmonary isolates

Of all 40 patients with pulmonary *M. malmoense* isolates, 32 (80%) met the ATS diagnostic criteria and were likely to suffer *M. malmoense* lung disease. The baseline patient characteristics are detailed in Table 1.

The predominant patient profile is a male with pre-existing pulmonary disease, mainly chronic obstructive pulmonary disease (COPD). The seven patients without a previous diagnosis of pre-existing pulmonary disease were mostly smokers, with radiographic features suggestive of pulmonary disease. Most patients reported productive cough (n=37; 93%), weight loss (n=24; 60%), and fatigue (23; 58%). Night sweats (n=10; 25%), hemoptysis (n=7; 8%) or fever (n=11; 28%) were infrequently reported. Only patients who reported weight loss were more likely to meet the ATS diagnostic criteria (p=0.048; OR

Table 1: Baseline characteristics of patients with pulmonary *M. malmoense* isolates

	ATS criteria met	ATS criteria not met	Total	P value
N	32	8	40	
Males	21 (66)	7 (88)	28	0.67
Mean age (range)	56 (28-81yr)	57 (33-83yr)	56 (28-83yr)	0.84
Pre-existing pulmonary disease	26 (81)	7 (88)	33	0.57
COPD	21 (66)	5 (63)	26	0.59
Prior TB	2 (6)	2 (25)	4	0.18
AFB smear positive	27 (84)	2 (25)	29	0.03
Cavitary lesion	28 (88)	1 (13)	29	<0.001
Nodular lesion(s)	4 (13)	1 (13)	5	0.74

Data are presented as n or n (%). ATS, American Thoracic Society; COPD, Chronic obstructive pulmonary disease; TB, tuberculosis; AFB, acid-fast bacilli

7.333; 95%CI 1.072-50.145). In the group of the patients that did not meet the ATS diagnostic criteria, four failed to meet the bacteriological criteria (three because only one sputum sample was collected) and four failed to meet the bacteriological and radiological criteria. Seventy-five percent (n=24) of the 32 patients that met the ATS criteria for pulmonary NTM disease presented with cavitary lesions visible on chest radiographs. Additional computed tomography scanning revealed four extra cases of cavitary disease (total n=28; 88%), not identified as such using plain chest radiographs. Two patients presented with multiple nodular opacities on chest radiograph; two had a single pulmonary mass.

Thirty patients who met the ATS diagnostic criteria for pulmonary NTM disease started treatment. Figure 1 summarizes treatment and outcome in the study group. The mean duration of antimycobacterial treatment was 12 months (range 1–26 months). Macrolides were added for 22 patients (clarithromycin for 18 and azithromycin for four patients; 92%), fluoroquinolones in six (ciprofloxacin for four and moxifloxacin for two patients; 25%). Nine patients received therapy for presumed tuberculosis, prior to the diagnosis of NTM disease, for a mean duration of 48 days (range 2-123 days), and completed a NTM based regimen afterwards. Six patients with *M. malmoense* pulmonary disease only received a complete first-line tuberculosis treatment.

Of 30 patients treated, 21 patients (70%) showed an adequate response, five suffered a failure or relapse (17%; mean time to relapse 13 months, range 5-24 months) and four died (13%) (Figure 1). Although the percentage of patients with an adequate response was lower in those receiving macrolide containing regimens (43 vs. 63%), this difference was not statistically significant (p=0.344). The mean duration of treatment among patients who later relapsed was shorter than for patients with an adequate response, though not significantly (320 vs.

358 days; p=0.709). The frequency of adequate response was not significantly different between patients treated with a TB based regimen and those treated with a NTM regimen (p=0.260). Two patients who met the ATS criteria refused treatment, one patient died, the other showed progressive disease. Follow-up of patients not meeting ATS diagnostic criteria was uneventful; no more positive cultures have been recorded. Symptoms regressed in absence of antimycobacterial treatment.

Six (20%) patients received the 24 months of rifampicin and ethambutol regimen based on the British Thoracic Society (BTS) trials. This did not affect the percentage of patients with an adequate response (BTS regimen vs. other: 83% vs. 71%; p=0.426).

Contact-tracing studies were initiated for two patients with pulmonary *M. malmoense* isolates; both were presumed to have pulmonary tuberculosis. Some contacts received six months of isoniazid, based on a tuberculin skin test conversion.

In this study, one case of disseminated *M. malmoense* disease was noted in a patient who received immunosuppressive treatment after kidney transplantation. He presented with pulmonary *M. malmoense* disease, which extended to

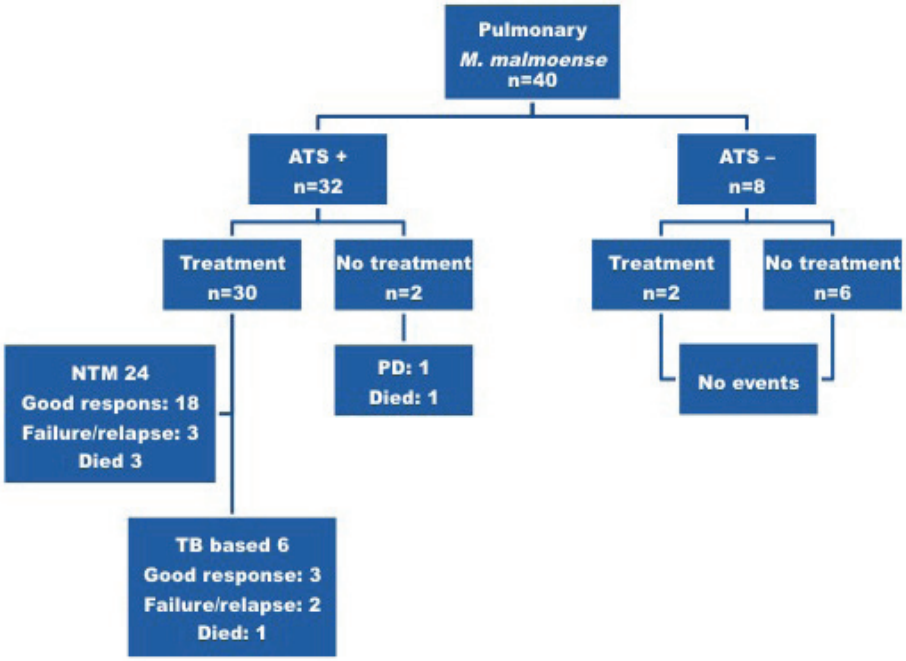

Figure 1: Frequency, treatment and outcome of pulmonary *M. malmoense* disease in the Netherlands between 2002 and 2006

NTM based, treatment given to treat nontuberculous mycobacteria; TB based, group of patients treated only for a presumed tuberculosis infection; ATS+, fulfillment of the criteria of the American Thoracic Society; PD, progressive disease; Abbreviations: AFB, Acid-fast bacilli

histologically and bacteriologically proven lymphadenitis and mediastino-esophageal fistula, with blood cultures yielding *M. malmoense*. Interestingly, this patient had strong epidemiological links to a patient diagnosed with smear positive pulmonary *M. malmoense* disease one year before.

Extra-pulmonary isolates

Eleven patients had extra-pulmonary *M. malmoense* isolates; we noted 10 cases of cervicofacial lymphadenitis, including two in elderly patients. One case of tenosynovitis of the 2nd and 3rd digit of the right hand was observed in a plant handler with a history of multiple wounds to the right wrist. He had an adequate response after surgical debridement followed by a macrolide based regimen of 13 months duration. The eight pediatric cases of lymphadenitis were three boys and five girls without predisposing conditions, with a mean age of 36 months (range 22-46 months). All presented with painless cervical or submandibular swelling, without fever or other symptoms. Surgical excision was the most frequent treatment and resulted in an adequate response in all patients. One of the elderly patients had a relapse after surgery, the other had an adequate response. Overall, eight patients (73%) with extra-pulmonary isolates had an adequate response after the initial therapy.

In vitro drug susceptibility testing was performed on the primary isolates from 46 patients. Isolates were resistant to isoniazid (all), streptomycin (70%), amikacin (70%), ciprofloxacin (61%); intermediately susceptible (39%) or resistant (46%) to ethambutol; and susceptible to rifampicin (72%), rifabutin (96%), clarithromycin (all), cycloserine (98%), prothionamide (96%) and clofazimine (all).
Relapse or treatment failure among patients with pulmonary *M. malmoense* disease was not associated with *in vitro* rifampicin or ethambutol resistance (p=0.327 and p=0.405 respectively).

Discussion

Mycobacterium malmoense is one of the most clinically relevant NTM in the Netherlands. Eighty percent of patients with pulmonary isolates met the ATS diagnostic criteria, compared to 21 (47%) relevant infections among 45 patients with pulmonary *M. xenopi* isolates, and 11 (73%) among 15 patients with pulmonary *M. szulgai* isolates.[9,10] We observed *M. malmoense* disease exclusively in HIV-negative patients, which is in contrast to *M. avium*, *M. kansasii*, *M. xenopi* and *M. szulgai*.[3,9,10] Patients were mainly males with pre-existing pulmonary disease. The ATS diagnostic criteria were designed for infections with *M. avium*, *M. kansasii* and *M. abscessus*; they may be less applicable to *M. malmoense.* Because of the high degree of true pulmonary *M. malmoense* infections observed, judgment on the clinical relevance of pulmonary *M. malmoense* isolates could probably be based on less strict

criteria, as is advocated for *M. kansasii*,[3] to prevent a prolonged period of inadequate treatment. The high degree of clinical relevance is in accordance with previous studies in northern Europe that reported 70% to 84% clinical relevance using either ATS criteria or a modification of these criteria.[11-14]
Interestingly, a dramatically lower clinical relevance of 10% was found in a retrospective case study of 73 patients in the USA.[15] There is no explanation for this difference; however, it suggests strains of *M. malmoense* in northern America are less pathogenic than those in northern Europe. There are no known bacterial virulence factors for *M. malmoense.* Phylogenetically related *M. szulgai* and *M. kansasii*, both of which are suggested to be among the most pathogenic NTM,[3,9] are known to harbor a region of difference 1-like genetic element (including *esat-6* and *cfp-10* genes) which is a well-known virulence factor for *M. tuberculosis*.[16] *Mycobacterium malmoense*, however, lacks this element.[17] To date, immunological studies have focused on *M. avium* and *M. abscessus*,[18,19] rather than *M. malmoense*. Studies of *M. malmoense* pathogenesis and virulence in murine models are warranted, as are studies on the role of host genetic factors in *M. malmoense* disease.
In a recently published retrospective study of the prevalence of all NTM in Ontario, Canada, in the 1997 to 2003 period, *M. malmoense* was not isolated.[20] This observation is in contrast with the increase in *M. malmoense* notification in Europe since 1980, including increasing numbers of countries reporting isolation of *M. malmoense*.[21] This contrast suggests environmental niches favoring transmission to humans in Europe. Whether this observation can be linked with the lower clinical relevance observed in the United Sates needs to be studied.
Human transmission has never been proven, even in a setting of geographic clustering of cases. In a study published by Doig and coworkers, small differences observed using pulsed-field gel electrophoresis were sufficient to show a lack of correlation between strain type and epidemiological or patient characteristics, making person-to-person spread unlikely.[22]
Treatment regimens in the current study deviated from the BTS treatment guidelines for pulmonary *M. malmoense* disease, both in content and duration of treatment. Treatment of *M. malmoense* disease was often preceded by, or consisted only of, TB treatment. This observation reflects the similar clinical presentation of pulmonary *M. malmoense* and *M. tuberculosis* complex infection and is a cause of concern. Increasing the use of PCR to rule out *M. tuberculosis*, providing a quick and definite NTM diagnosis, will probably decrease morbidity and mortality and prevent initiation of unnecessary contact tracing studies. Although hampered by our limited study group size and duration of follow-up, successful clinical response in this study in the optimally treated group (83%) is comparable with a 75% successful outcome reported by Henry *et al.*[23] The failure and relapse rates found in the recently published BTS trial (12% in the group treated with R and E, 5% in the group

treated with R and E combined with clarithromycin or ciprofloxacin) are lower compared to the rate found in our study (17%).[24] Probably, the shorter mean duration of antimycobacterial therapy negatively influenced treatment outcome in our population.
The mortality rate found among adequately treated patients in this study (13%) is comparable to that found by Banks *et al* (15%)[12] and Henry *et al* (11%).[23] The NTM disease related mortality after 5 years of follow-up is found to be low (3.6%).[24] Mortality has been related to the length of delay between diagnosis and start of the treatment, while the occurrence of relapse has previously been associated with total time span of treatment.[11] Other factors suggested to independently affect mortality are *in vitro* resistance to ethambutol and the involvement of more than one lung zone.[15] In our study population, there was no significant association between ethambutol resistance and treatment failure. The recently published BTS trial showed no additional benefit of adjunctive clarithromycin or ciprofloxacin over the 24RE regimen for pulmonary *M. malmoense* disease. Addition of clarithromycin even led to more side-effects.[24] These data are clinically important considering the extent of adjunctive macrolide and/or fluoroquinolone use in our study group.
The frequency and types of extrapulmonary disease in our study are similar to those found in a survey in Sweden in which 21% of 221 patients had extra-pulmonary isolates, mainly lymphadenitis.[14] Pediatric cases of cervicofacial lymphadenitis are most frequent and tend to affect children in a limited age range, which may be related to environmental exposures specific to this age category,[25] or the state of development of the immune system in children. Contrary to pulmonary disease, *M. malmoense* lymphadenitis is a relatively benign condition. Surgery is considered to be the optimal treatment and yields good results.[3,26]
Tenosynovitis due to *M. malmoense* is rare, though case reports are available in the international literature.[27] Extra-pulmonary infection with *M. malmoense* is rare and dissemination is only observed in patients with severely impaired immunity, although rarely in HIV/AIDS.

In conclusion, pulmonary *Mycobacterium malmoense* isolation is clinically relevant in 80% of all patients in the Netherlands, reflecting a level of virulence unmatched by other NTM species. Pulmonary disease resembling tuberculosis and pediatric lymphadenitis are the most common types of *M. malmoense* disease. Some patients are incorrectly treated for tuberculosis for a lengthy period. We recommend the use of molecular diagnostic tools for every sample positive for mycobacteria to enable quick initiation of adequate therapy. Treatment outcome is relatively favorable when compared to other NTM infections. Future studies are necessary to optimize treatment regimens and to discern host and pathogen factors determining virulence and transmission to humans.

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Clinical relevance of *Mycobacterium chelonae* and *Mycobacterium abscessus* isolation in 95 patients

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Abstract

Objectives: To determine the clinical relevance of *Mycobacterium. abscessus* and *M. chelonae* isolation from clinical samples.

Methods: We retrospectively reviewed medical files of all patients from whom these mycobacteria were isolated between January 1999 and January 2005. We applied the American Thoracic Society (ATS) diagnostic criteria to establish clinical relevance.

Results: Ninety-five patients were traced; 49 had *M. abscessus* and 46 *M. chelonae* isolates. Most isolates were cultured from pulmonary samples in patients with pre-existing pulmonary disease. Thirty-three percent (13/39) of patients with pulmonary *M. abscessus* isolates met the ATS criteria, opposed to twenty percent (7/35) for patients with *M. chelonae* isolates. Extrapulmonary disease usually presented as disseminated skin disease, with eye disease specific for *M. chelonae* and otomastoiditis for *M. abscessus*. Treatment, especially for pulmonary *M. abscessus* disease, yielded limited results.

Conclusions: One third of the patients with pulmonary *M. abscessus* isolates meet the ATS criteria, versus one fifth for *M. chelonae*. Pre-existing pulmonary disease is the main predisposing condition, different from previous studies. *Mycobacterium abscessus* isolation in cystic fibrosis patients warrants special attention. The ATS criteria may be too lenient to diagnose *M. chelonae* or *M. abscessus* disease. Increased adherence to treatment guidelines may improve outcome.

Introduction

For a long time, *Mycobacterium chelonae* and *Mycobacterium abscessus* have been thought to represent subgroups of a single species, due to overlap in biochemical and genetic properties; only in 1992 *M. abscessus* was granted a separate species status, supported by DNA-DNA hybridization of less than 70%.[1] In the Netherlands, these two species are the most frequently encountered rapidly growing nontuberculous mycobacteria (NTM), making up 55% of all referred rapid growers. (Source: National Mycobacteria Reference Laboratory). In general, NTM are opportunistic pathogens and pulmonary infections mostly affect patients with pre-existing pulmonary disease. Extrapulmonary disease generally occurs after trauma or in patients with systemic impaired immunity, i.e. immunosuppressive medication, HIV infection or hematological malignancy.[2] Improvements in laboratory facilities for culture and species identification, increasing notification, and growing awareness of their pathogenic potential have led to increased interest in the NTM in general.[2] *Mycobacterium abscessus* infections in cystic fibrosis (CF) patients, and the problematic resistance of these bacteria to antimycobacterial drugs, have received special attention.[1-5]

The environment is the suspected source of infections by NTM, as man-to-man transmission has not been proven.[2,3] Bacteria of both species have been recovered from water and soil.[1,2] Their presence in water and resistance to common disinfectants can result in pseudo-outbreaks due to contamination of laboratory materials[6] or medical equipment such as bronchoscopes.[7] Hence, clinical *M. chelonae* or *M. abscessus* isolation, especially from the respiratory tract, does not represent disease *per se*. To aid in the differentiation between NTM disease and pseudo-infection or contamination, the American Thoracic Society (ATS) provides diagnostic criteria, summarized in Box 1, with a specific emphasis on *M. abscessus*.[2]

In the current study we establish the clinical relevance of *M. chelonae* and *M. abscessus* isolation by studying the clinical and demographical data of patients from whom *M. chelonae* or *M. abscessus* was isolated and determining the percentage of patients that meet the ATS criteria.

Methods

The medical records of all patients in the Netherlands from whom *M. chelonae* or *M. abscessus* was isolated between January 1999 and January 2005 were retrieved. We recorded demographical, clinical and microbiological data and status according to the diagnostic criteria by the ATS.[2]

All patient isolates were subjected to laboratory diagnosis at the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands). The RIVM is the national reference laboratory that provides identification and drug susceptibility testing of mycobacteria for all hospitals in the Netherlands. For identification, the GenoType MTBC (Hain Lifescience, Nehren, Germany) is used to determine whether an isolate is a member of the

M. tuberculosis complex. If this test is negative, an INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Ghent, Belgium) reverse line-blot is used to differentiate between the more common species of NTM, including *M. chelonae* and *M. abscessus*. Prior to 2004, 16S rRNA gene sequence analysis was performed for all isolates, after ruling out membership of the *M. tuberculosis* complex using the AccuProbe MTB DNA probe kit (GenProbe, San Diego, USA). The DNA sequence results were compared to the GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov) sequence database. Distinction between *M. chelonae* and *M. abscessus* was subsequently performed using the picric acid test.[8]

Susceptibility to isoniazid, rifampicin, ethambutol, streptomycin, cycloserine, prothionamide, amikacin, ciprofloxacin, clofazimine, clarithromycin and rifabutin is tested using an agar dilution method.[9]

Pearson χ^2, Fisher exact and t-tests were used for statistical correlations. The regional ethics committee approved the study.

Results

We found 95 patients, of whom 49 had *M. abscessus* isolates and 46 *M. chelonae* isolates. The baseline characteristics of all 95 patients are detailed in Table 1. Though more patients with *M. abscessus* than with *M. chelonae* isolates met the ATS diagnostic criteria (n=21; 43% vs. n=14; 30%) this was not statistically significant (p=0.210).

Most isolates were from pulmonary specimens. Thirty-three primary *M. abscessus* isolates were from sputum, 6 were from broncho-alveolar lavages; for *M. chelonae* this was 31 and 4, respectively. Thirty-three percent (n=13) of all patients with pulmonary *M. abscessus* isolates met the ATS diagnostic criteria. This percentage was lower for *M. chelonae* (20%; n=7), though not statistically significant (p=0.197). Primary samples were AFB smear positive in eight patients with pulmonary *M. abscessus* isolates (21%), opposed to 6 (17%) in those with pulmonary *M. chelonae* isolates (Table 2).

The clinical characteristics are detailed in Table 1; symptoms did not predict the results of microbiologic and radiographic diagnostic criteria, for both pulmonary and extrapulmonary patients. In patients with pulmonary *M. abscessus* isolates, bronchiectasis visible on chest radiograph (p=0.022; OR 8.57; 95%CI 1.36-54.15) and smear positivity of the primary sample (p=0.001; OR 29.17; 95%CI 2.99-284.26) were significantly associated with meeting the ATS diagnostic criteria. No such associations were found for *M. chelonae*.

Pre-existing pulmonary disease was the most common predisposing condition for pulmonary *M. abscessus* and *M. chelonae* disease (Table 1). Pulmonary function was relatively preserved in the patients with pulmonary isolates, 43% had an FEV1>70% predicted and another 28% scored within the 50-70% predicted range, compatible with GOLD I/II COPD.[10] None of the patients were co-infected with HIV.

Table 1: Baseline patient characteristics, by species

	M. chelonae			*M. abscessus*			Total
	Pulm.	EP	Total	Pulm.	EP	Total	
Demography							
N (%)	35 (76%)	11	46	39 (80%)	10	49	95
% males	60	36	54	62	50	59	57
Mean age (range)	57 (12-89)	51 (1-82)	56 (1-89)	51 (7-87)	52 (4-84)	51 (4-87)	53 (1-89)
AFB positive	6 (17%)	2 (18%)	8 (17%)	8 (21%)	6 (60%)	14 (29%)	24 (25%)
Predisposing conditions							
Smoking	15 (43%)	1 (9%)	16 (35%)	19 (49%)	1 (10%)	20 (41%)	36 (38%)
Alcohol abuse	1 (3%)	1 (9%)	2 (4%)	1 (3%)	0	1 (2%)	3 (3%)
COPD	14 (40%)	1 (9%)	15 (33%)	11 (28%)	1 (10%)	12 (24%)	27 (28%)
Prior tuberculosis	1 (3%)	1 (9%)	2 (4%)	6 (15%)	1 (10%)	7 (14%)	9 (9%)
Cystic Fibrosis	2 (6%)	0	2 (4%)	5 (13%)	0	5 (10%)	7 (7%)
Non-CF bronchiectasis	3 (9%)	0	3 (7%)	6 (15%)	0	6 (12%)	9 (9%)
Hematol malign	1 (3%)	0	1 (2%)	2 (5%)	0	2 (4%)	3 (3%)
Steroids*	2 (6%)	3 (27%)	5 (11%)	1 (3%)	2 (20%)	3 (6%)	8 (8%)
Symptoms							
Productive cough	33 (94%)	1 (9%)	34 (74%)	33 (85%)	1 (10%)	34 (69%)	68(72%)
Hemoptysis	6 (17%)	0	6 (13%)	7 (18%)	0	7 (14%)	13(14%)
Dyspnea	17 (49%)	0	17 (37%)	22 (56%)	1 (10%)	23 (47%)	40 (42%)
Weight loss	5 (14%)	1 (9%)	6 (13%)	9 (23%)	0	9 (18%)	15 (16%)
Night sweats	2 (6%)	0	2 (4%)	4 (10%)	0	4 (8%)	6 (6%)
Fever	3 (9%)	1 (9%)	4 (9%)	12 (31%)	2 (20%)	14 (29%)	18 (19%)
Malaise/ Fatigue	9 (26%)	1 (9%)	10 (22%)	13 (33%)	4 (40%)	17 (35%)	27 (28%)
Chest imaging							
Infiltrate	10 (29%)	0	10(22%)	20 (51%)	0	20 (41%)	30 (32%)
Cavities	3 (9%)	0	3 (7%)	2 (5%)	0	2 (4%)	5 (5%)
Nodules	7 (20%)	0	7 (15%)	5 (13%)	1 (10%)	6 (12%)	13 (14%)
Bronchiectasis	3 (9%)	0	3 (7%)	7 (18%)	0	7 (14%)	10 (11%)
2007 ATS criteria met	7 (20%)	7 (64%)	14 (30%)	13 (33%)	8 (80%)	21 (43%)	35 (37%)

Pulm, Pulmonary; EP, extrapulmonary; AFB, acid-fast bacilli smear; COPD, chronic obstructive pulmonary disease; CF, cystic fibrosis; Hematol malign, hematological malignancy; ATS, American Thoracic Society

* >=15mg prednisone per day (or equivalent) for > 3months

Among cystic fibrosis patients, of *M. abscessus* was more commonly isolated than *M. chelonae*; four out of five CF patients (80%) with pulmonary *M. abscessus* isolates met the ATS diagnostic criteria; the two CF patients with *M. chelonae* isolates did not. Pulmonary *M. abscessus* isolates more often represented true infections in CF than in non-CF patients (p=0.035; OR 11.1; 95%CI 1.09-113.06). All cases of CF were diagnosed clinically and confirmed by CFTR genotyping.
Six (17%) patients with pulmonary *M. chelonae* and two (6%) patients with pulmonary *M. abscessus* isolates had esophageal disorders.
Ten patients started treatment for *M. abscessus* lung disease; regimens, duration and outcome are detailed in Table 2. The average duration of treatment in patients who met the ATS criteria was nine months. For four patients, these regimens were followed by long-term monotherapy with clarithromycin. Four patients were cured, which was defined by symptomatic improvement and conversion to negative cultures, three experienced treatment failure, i.e. persistent symptoms and positive cultures, and four patients died during treatment (Table 2); no autopsies were performed. Two patients were treated for *M. chelonae* lung disease. One failed, remaining symptomatic and culture positive, the other improved and converted to negative cultures (Table 2). Twelve patients (6 *M. abscessus*, 6 *M. chelonae*) started TB treatment, based on a clinical suspicion of tuberculosis. The average duration of TB treatment was 1 month. One patient received nine months of standard TB treatment only and improved (Table 2, patient 2).
Extrapulmonary *M. abscessus* isolates represented disease in eight out of ten patients (80%), as opposed to 64% (n=7/11) for *M. chelonae*. The few extrapulmonary isolates that did not represent disease were cultured from the digestive tract without other signs of disease or were suspected contaminants that could not be repeatedly cultured from the original clinical sample. Extrapulmonary disease mostly followed trauma (n= 8) or immunosuppressive drug treatment (chronic high-dose steroid use, or post-transplantation; n=4). None of the patients were co-infected with HIV. Extrapulmonary infections with both species are detailed in Table 3. Among patients with extrapulmonary isolates, smear positivity was noted in 6 (60%) in the *M. abscessus* and 2 (18%) in the *M. chelonae* culture positive materials (Table 3). Disseminated skin infections by both species presented with multiple nodules, with various degrees of ulceration. Specific extrapulmonary disease types were seen for *M. chelonae* (eye disease; n=2) and *M. abscessus* (chronic otitis media; n=2; Table 3).
In vitro drug susceptibility testing was performed for 26 *M. chelonae* isolates from 20 patients and 46 *M. abscessus* isolates from 33 patients. Primary *M. abscessus* isolates were mostly susceptible to clarithromycin (67%) and occasionally to ciprofloxacin (18%), prothionamide (9%), amikacin (3%) and rifabutin (6%), though resistant to all other compounds tested. Acquired resistance to clarithromycin was noted in one patient after two months of clarithromycin treatment (Table 3, pt. 10). Primary *M. chelonae* isolates

were mostly susceptible to clarithromycin (60%), occasionally susceptible to ciprofloxacin (16%), prothionamide (10%) and amikacin (5%) and resistant to all other compounds in the test panel. No acquired resistance for bacteria of this species was noted.
Three patients with pulmonary *M. abscessus* isolates had simultaneously obtained cultures yielding *M. avium*, *M. interjectum* and *M. gordonae*. Among those with pulmonary *M. chelonae* isolates, four patients had simultaneous positive pulmonary cultures yielding *M. avium*, *M. kansasii*, *M. mucogenicum* and an unidentifiable species, respectively. All were single isolates, none represented NTM disease.
We recorded no temporal or geographical clustering of isolates, suggesting (pseudo)outbreaks or contamination.

Discussion

Mycobacterium chelonae and *M. abscessus* isolation and disease occur in almost equal frequency in the Netherlands. Pulmonary NTM disease as defined by the ATS criteria was likely in 20% of patients with *M. chelonae* isolates and 33% of those with *M. abscessus* isolates. Vigilance is warranted in specific patient categories such as cystic fibrosis patients; isolation of *M. abscessus* generally represents disease in this group.
The ATS criteria presumably fit well with pulmonary *M. abscessus* isolates.[2] However, short regimens, as well as regimens including mainly first-line anti-tuberculosis drugs considered inactive against *M. chelonae* and *M. abscessus*, lead to favorable outcome in several pulmonary patients (Table 2; patients 1, 2, 9 & 12). Although meeting the ATS criteria, it is doubtful whether these patients had true NTM disease. Despite the obvious drawbacks of our retrospective study design, this suggests that the ATS criteria are insufficiently stringent to diagnose true pulmonary *M. chelonae* or *M. abscessus* disease. Moreover, this demonstrates that knowledge on the treatment of disease due to these species is to be improved. If we do not consider the four patients with dubious pulmonary disease, true pulmonary disease was likely in only 14% (5/35) of patients with *M. chelonae* and 28% (11/39) of patients with *M. abscessus*. Stricter criteria should be imposed, possibly including more positive cultures or histological evidence where appropriate.

MCH, *Mycobacterium chelonae*; MAB, *M. abscessus*; Cult/pos/(smear), Number of cultures performed/number of positive cultures/(acid-fast bacilli smear result); M, male; F, female; RTI respiratory tract infections (>=3 in 6 months); COPD, chronic obstructive pulmonary disease; ALS, amyotrophic lateral sclerosis; TB, tuberculosis; IVDA, intravenous drug abuse; PC, productive cough; H, hemoptysis; D, dyspnea; NS, night sweats; M, malaise/fatigue; WL, weight loss; F, fever; scar, coarse linear scarring; I; infiltrate; PT, pleural thickening; M/HLE, mediastinal/hilar lymph node enlargement; PF, pleural fluid; H, isoniazid; R, rifampicin; Z, pyrazinamide; E, ethambutol; Cla, clarithromycin; Pro, prothionamide; Ctm, co-trimoxazole; Ami, amikacin; Cef, cefoxitin; Imi, imipenem; Azi, azithromycin; Levo; levofloxacin

Table 2: Clinical data of patients meeting the 2007 ATS criteria for pulmonary NTM disease

Pt.	Species	Sex, Age	Cult/pos/ (smear)	Predisposing conditions	Symptoms	Chest imaging	Treatment	Outcome
1	MCH	M, 76	4/3 (+)	None	PC	Scar	1HRZE	Cured
2	MCH	M, 71	2/1 (+)	Diabetes	PC, H	Nodules, PT	9HRZE	Cured
3	MCH	F, 29	6/4 (+)	Asthma, Recurrent RTI	PC,D,NS,M	I, bronchiectasis	1HRZE	Failure
4	MCH	F, 71	7/2 (-)	Asthma, Recurrent RTI	PC,D,F,M	I, bronchiectasis	24RbClaPro	Cured
5	MCH	M, 57	8/6 (-)	COPD, Recurrent RTI	PC	Solitary nodule, scar	None	Death
6	MCH	M, 78	7/4 (+)	COPD, silicosis	PC	I, scar	8Cla 10Ctm	Failure
7	MCH	M, 48	5/5 (-)	ALS, Recurrent RTI, ventilator	PC,D,F,M	I, nodules	None	Death
8	MAB	M, 87	2/2 (-)	Chest wall trauma	PC, WL	I, cavities, MLE, HLE, PF	None	Death
9	MAB	M, 47	9/2 (-)	COPD, bronchiectasis	PC	Bronchiectasis, scar	None	Cured
10	MAB	M, 21	3/3 (-)	None	PC	I, cavities	None	Lost
11	MAB	F, 42	8/8 (-)	Asthma, bronchiectasis	PC,D,NS,WL,M	I, cavities, bronchiectasis, nodules	Lobectomy, 28ECla	Failure
12	MAB	F, 72	2/2 (+)	Prior TB, bronchiectasis	PC,D,F,M	I, bronchiectasis	1HRZE 1Ctm 1RECla	Cured
13	MAB	M, 80	5/5 (+)	Prior TB, COPD	PC,H,D	I, scar	1HRZE 1RECla	Death
14	MAB	M, 47	9/4 (+)	Prior TB, COPD	PC,H,D	I, cavities, nodules, PT, scar	2HZE	Failure
15	MAB	M, 73	3/2 (+)	Malignancy	PC,H	Cavities	1HRZ	Failure
16	MAB	M, 40	9/6 (-)	IVDA, COPD	PC,F,D, NS	I, nodules, scar	1HRZE 5HRECla	Cured
17	MAB	M, 28	24/18(+)	Cystic Fibrosis	PC,H,D,F,WL,M	I, bronchiectasis, nodules, scar	12Cla	Death
18	MAB	M, 7	6/6 (+)	Cystic Fibrosis	PC,D,WL,M	I, bronchiectasis, scar	16ClaAmiCefImi 9Cla	Death
19	MAB	F, 28	7/4 (-)	Cystic Fibrosis	PC,D,M	Bronchiectasis, nodules, scar	12AziLevo	Cured
20	MAB	M, 12	3/1 (+)	Cystic Fibrosis	PC,WL	I, bronchiectasis, scar	1CtmCla 5Cla	Cured

Patients with bronchiectasis from whom *M. abscessus* was isolated were significantly more likely to meet the ATS diagnostic criteria, probably because bronchiectasis is a part of the criteria. Still, it remains uncertain whether bronchiectasis predisposes to, or results from, *M. abscessus* disease.

Pulmonary *M. abscessus* or *M. chelonae* disease, although infrequent, is a clinical challenge, due to the extensive drug resistance that characterizes bacteria of these species. Pulmonary *M. abscessus* disease is difficult to treat and suppressive, instead of curative, treatment may be the only option,[2] especially in CF patients. More than half of the patients treated for *M. abscessus* pulmonary disease in our study experienced treatment failure, relapsed after treatment or died during therapy. The low number of patients treated for *M. chelonae* pulmonary disease hampers analysis of their treatment outcome. The use of parenteral drugs (amikacin, cefoxitin, imipenem) was very limited in our study, despite their central role in the ATS and British Thoracic Society (BTS) treatment guidelines for disease due to rapidly growing mycobacteria.[2,11] Increased use of multi-drug regimens including parenteral agents and more extensive testing of new drug classes, such as glycylcyclines and oxazolidinones, may provide additional therapeutic options and improve outcome of chemotherapy in the Netherlands. The recent finding that *M. abscessus*, but not *M. chelonae*, has an *erm* gene that induces macrolide resistance[12] emphasizes the urgency of these issues. Surgical resection of the involved lung, combined with chemotherapy, may further improve treatment outcome in selected cases.[2,11,13] However, the only patient in our study group who underwent adjunctive surgery ultimately experienced treatment failure (Table 2; patient 11).

Pre-existing pulmonary disease was the major predisposing condition for pulmonary NTM disease in our study, whereas in North American reports, *M. abscessus* or *M. chelonae* pulmonary disease is often associated with previously healthy women over 60 years of age.[2,13,14] Its indolent course[2,13] may prevent it from being diagnosed in this patient category in the Netherlands. Among our patients with pulmonary *M. abscessus* disease, the percentage of CF patients, whose susceptibility to *M. abscessus* disease has been widely published,[4,5] is higher than the 6% recorded by Griffith *et al.*[13] Moreover, *M. chelonae* and *M. abscessus* were isolated in equal frequency, while in the 154 patients with disease caused by rapid growing NTM studied by Griffith *et al.*, only one had *M. chelonae* disease.[13] The background of these differences demands further research.

The extent of pre-existing pulmonary disease in our patients meant that symptoms and radiographic abnormalities had limited value in diagnosing pulmonary *M. chelonae* or *M. abscessus* disease. The reported associations between esophageal disease and pulmonary disease due to rapidly growing NTM[2,13,15] as well as concurrent recovery of other NTM species,[2,13] were infrequent and statistically insignificant in our study group.

Table 3: Clinical data of patients with extrapulmonary disease

Pt.	Species	Site	Sex	Age	Predisposing conditions	Treatment	Outcome
1	MCH	Skin (single lesion)	M	58	Trauma	Surgery, 1ClaCip	Cured
2	MCH	Skin (diss.)	F	60	Prednison & cyclosporine use	Surgery, 3Cla	Cured
3	MCH	Bone & joint	F	69	None	Surgical debridement (2x), 5ClaOflClz	Lost to follow up
4	MCH	Lymph node	F	6	None	3RbCla	Cured
5	MCH	Tendon	M	67	Steroid injection	Surgical debridement, 6HRZOfl	Cured
6	MCH	Eye (endophthalmitis)	F	82	Cornea transplantation	4CipCla with topical ofloxacin	Cured; blind
7	MCH	Eye (corneal ulcer)	F	47	Trauma	3Cla with topical tobramycin	Cured
8	MAB	Skin	F	35	Prior cosmetic surgery (Caribbean region)	Surgical excision (3x)	Relapse
9	MAB	Skin (single lesion)	M	67	None	6ClaDox	Cured
10	MAB	Skin & bone & pleura (diss.)	M	65	COPD; prior TB; Surgery, sternotomy	4ClaCipImiLin, surgical debridement	Failure, Death
11	MAB	Skin & joint (diss.)	F	44	Prednisone & cyclophosphamide use	4ClaAmiLevo	Cured
12	MAB	Skin (diss.)	M	56	Prednisone, cyclosporine & mycophenolate use	1RECla 1ClaAmiImi 4CipCla 4ECipCla 1LinCipCla 1MinCipCla 14Cla	Relapse
13	MAB	Tendon	F	84	Steroid injection, thioguanine use	6Cla	Cured
14	MAB	Ear & mastoid	F	11	Prior tympanostomy tube placement	Surgical debridement (2x), 2CipCla	Cured
15	MAB	Ear	M	4	PDCD; IgG deficiency	3Cla	Cured

MCH, *Mycobacterium chelonae*; MAB, *M. abscessus*; (diss.), disseminated disease; COPD, chronic obstructive pulmonary disease; TB, tuberculosis; PDCD, pyruvate dehydrogenase complex deficiency; Cla, clarithromycin; Cip, ciprofloxacin; Ofl, ofloxacin; Clz, clofazimine; Rb, rifabutin; H, isoniazid; R, rifampicin; Z, pyrazinamide; Dox, doxycyclin; E, ethambutol; Ami, amikacin; Imi, imipenem; Levo, levofloxacin; Lin, linezolid; Min, minocyclin

Box 1: Summary of the 2007 American Thoracic Society diagnostic criteria

American Thoracic Society Diagnostic Criteria of Nontuberculous Mycobacterial Lung Disease

Clinical criteria

1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules.
and
2. Appropriate exclusion of other diagnoses.

Microbiological criteria

1. Positive culture results from at least two separate expectorated sputum samples. (If the results from the initial sputum samples are nondiagnostic, consider repeat sputum AFB smears and cultures.)
or
2. Positive culture results from at least one bronchial wash or lavage.
or
3. Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.

HRCT, high resolution computed tomography; AFB, acid-fast bacilli; NTM, nontuberculous mycobacteria
Note: Three or more pulmonary samples should be analyzed to apply these criteria

The observed differences in clinical relevance of isolation of the two species may reflect a difference in pathogenicity, as recently indicated in a murine model.[16] Among CF patients, the clinical relevance of *M. abscessus* pulmonary isolates is higher than that of *M. chelonae* and that of *M. abscessus* in non-CF patients. Apparently, infection and growth of *M. abscessus* in the lung requires pre-existing tissue damage, a changed biochemical environment, such as that resulting from CF, or both. This is in line with the observation in the murine model that, after systemic administration, *M. abscessus* fails to maintain itself in the lung.[16]

Extrapulmonary isolates were rare in our cohort, but almost invariably represented disease for both species. Immunosuppressive drug treatment and local trauma were the main predisposing conditions, which is line with the available literature.[1,2] *Mycobacterium abscessus* skin infection after cosmetic surgery in the Caribbean (Table 3; patient 8) has also been recorded in previous studies.[17] *Mycobacterium chelonae* and *M. abscessus* are uncommon isolates in HIV patients. Although in the Netherlands this is probably due to the low HIV prevalence, it could also have a causal relation.

Specific extrapulmonary disease types were seen for *M. chelonae* (eye disease) and *M. abscessus* (chronic otitis media). Both have been previously reported,[18,19] though the mechanism causing this species-specific tissue tropism is unknown. The settling of the taxonomical positions of *M. chelonae* and *M. abscessus* seems short-lived. *rpoB* gene sequencing now allows distinction between *M.*

abscessus and closely related *M. bolletii* and *M. massiliense*.[20] The presence of these two novel species among strains labeled *M. abscessus* by our identification methods has not yet been studied in the Netherlands. These two species may have a different clinical relevance or cause specific disease types.
In conclusion, one third of all patients from whom *M. abscessus* is cultured from a respiratory specimen meets the ATS diagnostic criteria for pulmonary *M. abscessus* disease. For *M. chelonae*, this is the case in one fifth of all patients. Cystic fibrosis patients are highly susceptible to *M. abscessus* disease and warrant special attention. The ATS criteria may be too lenient, overdiagnosing true *M. chelonae* or *M. abscessus* disease. The characteristics of our study group differ from previous studies in the USA, mainly in age, gender distribution, extent of pre-existing pulmonary disease and the equal isolation frequency of *M. chelonae* and *M. abscessus*. Extrapulmonary isolates are rare, but should generally be considered indicative of NTM disease. Bacteria of both species cause specific extrapulmonary diseases. Treatment of these infections, especially pulmonary *M. abscessus* disease, yields limited results. Adherence to treatment guidelines and surgical treatment may improve treatment outcome.

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Impact of new American Thoracic Society diagnostic criteria on management of nontuberculous mycobacterial infection

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The American Thoracic Society has recently published new diagnostic criteria for nontuberculous mycobacterial (NTM) diseases.[1] We retrospectively assessed the impact of the new criteria on clinical practice by reviewing medical files of all patients in whom *Mycobacterium chelonae*, *M. abscessus*, *M. simiae* or *M. szulgai* was isolated between January 1999 and January 2005 in the Netherlands. Isolates were earlier identified at the national mycobacteria reference laboratory (RIVM), by either reverse line blot assay (InnoLipa Mycobacteria v2, Innogenetics, Ghent, Belgium) or 16S gene sequencing, or by a self-sufficient hospital laboratory.

7.7 % (9/117) more pulmonary patients met the 2007 diagnostic criteria,[1] compared to the 1997 criteria,[2] although this differed by NTM species (Table 1). The new diagnostic criteria contain more specific radiological criteria, whereas bacteriological criteria have become more lenient: a single NTM culture from bronchial washing fluid, in a well-defined class of patients, or two positive sputum cultures now suffice to establish the diagnosis.

As more patients are likely to meet the new criteria, more patients might receive antimycobacterial treatment. The authors, however, repeatedly state that meeting these criteria does not, *per se*, necessitate the institution of therapy.[1] This was not emphasized in the former statement. We believe such comments only add to the confusion for the clinician. Which factors should influence this treatment decision in individual patients? Ten years of experience with the former criteria apparently have not provided the answer to that question. More lenient criteria even increase the responsibility of physicians in the treatment decision.

Table 1: Fulfillment of the diagnostic criteria, by species and site

Species	Site	n	Age (yr)	1997 ATS+, n (%)	2007 ATS+, n (%)
Mycobacterium	P	35	57	5 (14%)	7 (20%)
chelonae	EP	11	51	8 (73%)	7 (64%)
M. abscessus	P	39	51	11 (28%)	13 (33%)
	EP	10	52	9 (90%)	8 (80%)
M. szulgai	P	15	60	8 (53%)	11 (73%)
	EP	6	44	6 (100%)	5 (83%)
M. simiae	P	28	65	6 (21%)	8 (29%)

Definition of abbreviations: Age= mean age; ATS+= American Thoracic Society diagnostic criteria met; EP= extrapulmonary isolates; P= pulmonary isolates

Increased exposure to antimycobacterial drugs can harm patients, both in terms of adverse events and cost.[3] The variable cure rates achievable per type of NTM disease and persistence of the conditions predisposing to NTM infection further complicate the assessment of the benefit of treatment. Centralization of expertise and easily accessible expert consultation is important, as acknowledged in the new statement.

For extrapulmonary NTM isolates, no criteria for additional proof of infection are mentioned in the summarized criteria, contrary to the prior statement.[2] This complicates assessment of the significance of NTM isolated from pleural fluid, stool or gastric juice, without a context of disseminated disease, and in the HIV-negative patient, without histological proof of infection (Table 1). Extrapulmonary NTM warrant own diagnostic criteria.

More research is needed to improve diagnosis and treatment of this increasing problem in clinical practice.

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The changing pattern of clinical *Mycobacterium avium* isolation in the Netherlands

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Abstract

Background: The isolation frequency of nontuberculous mycobacteria is increasing in the Netherlands. Laboratory improvements have been considered key factors in this increase. We assessed the role of factors other than laboratory improvements in the increasing isolation frequency of nontuberculous mycobacteria in the Netherlands.

Methods: Retrospective laboratory database analysis. All clinically isolated nontuberculous mycobacteria referred to the national mycobacteria reference laboratory between January 2000 and January 2007 were retrieved from the laboratory database and divided by species, patient age group and sample origin.

Results: Clinical *Mycobacterium avium* isolates accounted for most of the increase in referred nontuberculous mycobacteria. The number of respiratory *M. avium* samples in patients >40 years of age has increased over time. *Mycobacterium avium* isolation from lymph nodes in children remained stable, whereas extrapulmonary *M. avium* isolation in the middle age group, which includes HIV-associated bloodstream isolates, decreased.

Conclusions: The increasing nontuberculous mycobacteria notification in the Netherlands largely results from an increase of clinical *M. avium* isolation, especially from the emergence of *M. avium* in respiratory samples in patients over 40 years old. This specific increase is unlikely to result from laboratory improvements only; the ageing population with an increasing prevalence of chronic obstructive pulmonary disease and decreasing exposure to tuberculosis inferring cross-protection to nontuberculous mycobacterial disease as well as environmental changes may specifically favour *M. avium*.

Introduction

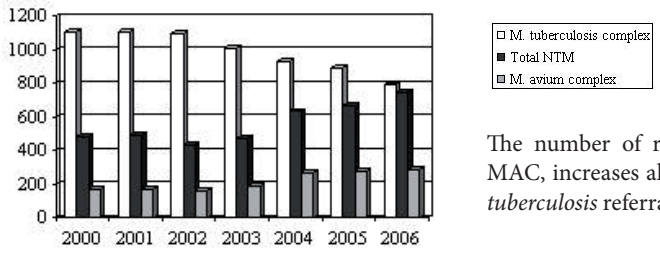
Nontuberculous mycobacteria (NTM) are mostly opportunistic pathogens that are widely present in natural and man-made environments.[1] The isolation frequency of nontuberculous mycobacteria (NTM) is increasing in many countries, mainly those where the incidence of tuberculosis is in decline.[1-3] This increase in isolation frequency has previously been related to laboratory improvements for detection and identification of NTM, including automated liquid culture systems and molecular identification kits.[1,2] The pathogenic potential of these NTM, however, is increasingly recognized, especially in patients at risk due to local or systemic impaired immunity.[1]

At the Dutch national mycobacteria reference laboratory (RIVM), we also noted an increase in the number of referred nontuberculous mycobacteria, most notably isolates belonging to the *Mycobacterium avium* complex (MAC). In the current study, we explored the factors underlying this increase in NTM isolation.

Methods

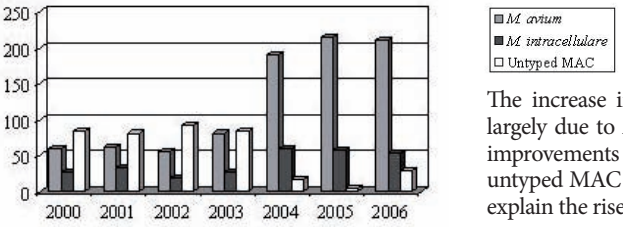
Records of all clinical isolates of nontuberculous mycobacteria referred in between January 2000 and January 2007 were extracted from the reference laboratory database and divided by species, sample origin (respiratory, lymph node or other) and age group. Age was defined as the age at the date of primary sampling. We chose the following categories: 1-12yrs old to include mostly the children with lymphadenitis; 13-39yrs to include mostly immunocompromised patients, including co-infection with the Human Immunodeficiency Virus (HIV) and cystic fibrosis patients; over 40yrs to include mostly patients with pre-existent pulmonary disease, e.g. chronic obstructive pulmonary disease (COPD).[4] The latter group was subdivided in the 40-60 and over 60yrs groups. The isolates of all patients were subjected to laboratory diagnosis by the National Institute for Public Health and the Environment (RIVM). The RIVM is the national reference laboratory that provides identification, drug susceptibility testing and genotyping of *Mycobacterium* isolates for all hospitals in the Netherlands. To identify mycobacteria, a GenoType MTBC reverse line probe assay (Hain Lifesciences, Nehren, Germany) is used after PCR-based amplification to determine whether an isolate is a member of the *M. tuberculosis* complex. If the reaction is negative, an Inno-LiPA Mycobacteria v2 (Innogenetics, Gent, Belgium) reverse line blot assay is used to differentiate between the more common species of NTM. If no species-specific result is obtained, 16S rDNA gene sequencing is performed.

Prior to 2004, 16S rDNA gene sequence analysis was performed on request of referring physicians, if AccuProbe *M. tuberculosis* complex, *M. avium* complex, *M. intracellulare* and *M. avium* DNA probes (GenProbe, San Diego, USA) yielded negative results.



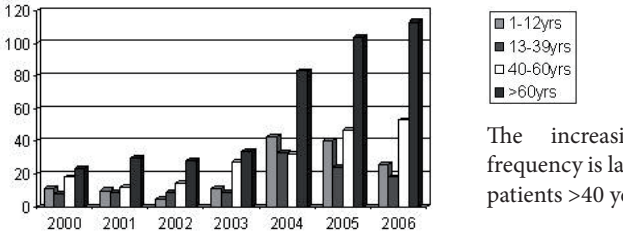
The number of referred NTM, including MAC, increases alongside a decrease in *M. tuberculosis* referral.

Figure 1: Total number of TB, NTM and MAC isolates received per year



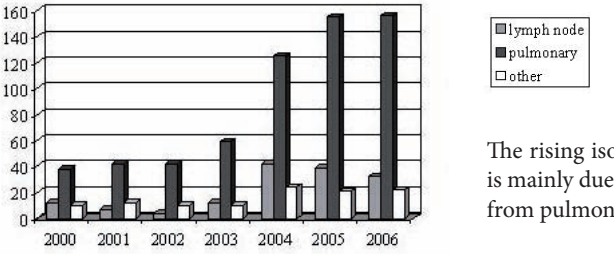
The increase in referral of MAC isolates is largely due to *M. avium* isolates. Laboratory improvements resulted in a decline in untyped MAC isolates, though insufficient to explain the rise in *M. avium* isolates.

Figure 2: MAC isolates referred per year, by species



The increasing *M. avium* isolation frequency is largely due to its isolation from patients >40 years of age.

Figure 3: The number of referred *M. avium* isolates per year, by age group



The rising isolation frequency of *M. avium* is mainly due to a rising isolation frequency from pulmonary samples.

Figure 4: The number of referred *M. avium* isolates per year, by sample site

Results

During the study period, the annual number of referred NTM rose in absolute terms, from 480 to 747 per year, as well as relative to *M. tuberculosis* isolation (Figure 1). Isolates belonging to the *Mycobacterium avium* complex (MAC), made up of *M. avium* and *M. intracellulare*, are the most commonly isolated NTM in the Netherlands and their number has risen sharply during the study period from 170 to 292 per year, making up 35% to 39% of annually referred NTM, respectively (Figure 1).

During our study period, the increase in referred MAC isolates was caused mainly by an increase in *M. avium* isolates (Figure 2) from 60 isolates in the year 2000 to 210 in 2006. The number of *M. intracellulare* isolates was stable (Figure 2).

This rise in *M. avium* isolates has two important features. Firstly, it is most pronounced in patients over 40 years of age (Figure 3). Secondly, it is mainly caused by a rise in *M. avium* isolates from pulmonary samples (Figure 4). These pulmonary isolates are cultured almost exclusively from the patients over 40 years of age; in general, the sample origin recorded was in agreement with the origins expected within the age group (Table 1).

Numbers of extrapulmonary samples, including those from children with cervical lymphadenitis, have remained relatively stable throughout the study period (Figure 4).

The increase in the annual number of referred isolates is not confined to *M. avium*, although for this species it is most pronounced. *Mycobacterium gordonae* isolates are also increasing in number, whereas isolation of other important species including *M. intracellulare*, *M. malmoense* and *M. kansasii* has been mostly stable (Figure 5).

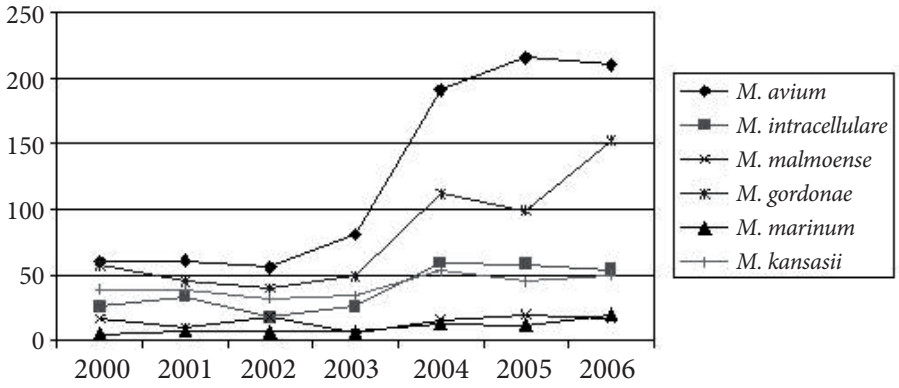


Figure 5: The annual number of referred isolates of the most common NTM species

The increase in the isolation frequency of *M. avium* is not mirrored in other NTM species, including *M. kansasii*, *M. malmoense* and *M. marinum*. Many NTM species were referred in low numbers and not included in the graph to increase clarity.

Table: The number of MAC isolates by sample origin and age group

Age group	Lymph node	Other	Pulmonary	Total
0-12yrs	159	16	10	185
13-39yrs	7	70	112	189
40-60yrs	10	52	326	388
>60yrs	6	24	748	778
Total	182	162	1196	1540

Discussion

The rise in isolation frequency of nontuberculous mycobacteria in the Netherlands is caused mostly by increasing numbers of *Mycobacterium avium* isolates from respiratory samples in patients over 40 years of age. These are probably patients suffering COPD, the most common predisposing condition for nontuberculous mycobacterial infection within this age group in the Netherlands and an argument for frequent culture of sputum samples.[5] A recent study in the Netherlands demonstrated that *M. avium* was more common than *M. intracellulare* in pulmonary samples. Moreover, pulmonary *M. avium* isolation represented true disease in 24 of 59 cases (41%) as opposed to 2 out of 16 cases (13%) for *M. intracellulare*, based on the 2007 ATS criteria.[1,5] This study also demonstrated that among pulmonary MAC isolates, the percentage representing true MAC disease did not change significantly in the 1999-2004 period;[5] this suggests that the increase in isolation frequency is not a mere result of an increased sample volume. Reports from the USA have noted that the nodular-bronchiectatic type of pulmonary MAC disease is most frequent there and *M. intracellulare* its most common causative agent.[6] These differences remain largely unexplained. Possibly, *M. avium* bacteria isolated in the Netherlands differ in virulence from those isolated in the USA; previous studies have identified genetic differences in MAC isolates from different human and environmental sources.[7,8]

The improvements in culture and identification techniques often held responsible for rising NTM isolation frequencies[1,2] are also relevant in the Netherlands. Automated liquid culture systems were introduced in 2003, to spread in the years following. Improvements in the identification methods in 2004 led to a decrease in the number of untyped MAC strains and increase in the number of isolates identified as *M. intracellulare* or *M. avium* (Figure 2). It is evident from Figure 2 that this provides only a partial explanation of the increase in *M. avium* isolates in 2004. Figures 1-4 show that, although boosted by improved identification methods, the increase in *M. avium* isolation, especially among the oldest age groups, set in before the improvements in laboratory techniques. Moreover, the annual number of isolates of other species, including the commonly isolated *M. kansasii*, *M. intracellulare* and

M. malmoense did not benefit from these technical improvements (Figure 5), neither did the number of extrapulmonary *M. avium* isolates (Figure 4). Skin sensitization to *M. intracellulare* has increased over the last decades in the USA, along with the incidence of *M. intracellulare* infection.[9] Combined, this suggests that laboratory techniques have a role in the increase in NTM, thus *M. avium*, isolation, but are not its sole explanation.

Host factors provide a second partial explanation for the rise in NTM isolation frequency. The Dutch population is ageing and the prevalence of COPD is increasing.[10] The COPD prevalence may partially explain the increase in NTM notification in pulmonary samples (Figure 5). Still, this offers no explanation for the rise in isolation specifically of *M. avium* unless COPD predisposes to infection by *M. avium* rather than other NTM species. In the Netherlands, the fibrocavitary disease type is more prevalent, primarily affects COPD patients and is mostly caused by *M. avium*.[5] It is uncertain whether the predominance of cavitary MAC disease results from the strong presence of *M. avium* as opposed to *M. intracellulare*, or from host factors including COPD. Rather than rising COPD prevalence, the simultaneous decline in tuberculosis incidence (Figure 1) may imply that exposure to tuberculosis infers cross-protection to NTM disease; this is supported by the fact that countries that halted Bacille Calmette-Guerin (BCG) vaccination subsequently noticed an increase in the incidence of paediatric cervicofacial lymphadenitis caused by MAC.[11,12]

A third explanation for the increase in *M. avium* isolation in the Netherlands may be changes in the environment, either natural or man-made, that favour *M. avium* and impede or influence other NTM; these could result in increased exposure of humans specifically to *M. avium*. Similarly, in the 1970's *M. scrofulaceum* was replaced by MAC as the leading causative agent of cervical lymphadenitis in children. This phenomenon has been attributed to chlorination of tap water, which should select for the more chlorine resistant *M. avium*.[3] Studies reviewed by Collins and co-workers, however, did not demonstrate important differences in chlorine susceptibility between NTM species and considered tap water chlorine levels too low to significantly reduce mycobacterial loads.[13]

The number of *M. avium* isolates from lymph node samples referred to our reference laboratory has remained relatively stable; the temporary elevation in 2004-2005 results from a clinical trial in that period.[14] The stable low numbers of other extrapulmonary *M. avium* isolates probably result, in part, from the availability of highly active antiretroviral therapy (HAART) and MAC prophylaxis for patients infected with HIV. A decline in the incidence of disseminated *M. avium* disease during and after HAART introduction has been recorded and reviewed before.[15] Nontuberculous mycobacterial infection usually affects patients with very low CD4 counts and their numbers have been reduced after the introduction of HAART.

In summary, the rise in notification of nontuberculous mycobacteria at the

reference laboratory in the Netherlands was mainly caused by increasing isolation of *M. avium* from respiratory samples in patients over 40 years of age. This group most likely has pre-existent pulmonary disease, mainly COPD. Changes in laboratory techniques are unlikely to be the sole explanation of this increase; the ageing population with an increasing prevalence of chronic diseases including COPD and decreasing cross-protection to NTM disease from exposure to tuberculosis and environmental changes may specifically favour *M. avium*.

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Otomastoiditis caused by non-tuberculous mycobacteria, the Netherlands

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Abstract

An increase in incidence of otomastoiditis caused by nontuberculous mycobacteria was noticed in the Netherlands. These infections affect children with tympanostomy tubes, a history of ear infection and ototopical antibiotic treatment and can result in permanent hearing loss. Diagnostic delay and limited adherence to available treatment guidelines warrant increased attention.

Introduction

Nontuberculous mycobacteria (NTM) are common in the environment and increasingly recognized as mostly opportunistic human pathogens.[1] NTM usually cause pulmonary disease. Extrapulmonary and disseminated disease is typically diagnosed in immunocompromised patients.[1] Lymphadenitis and otomastoiditis in children are notable exceptions to this rule.
Otomastoiditis is a rare extrapulmonary NTM disease type first described in 1976 and mostly affecting children.[1-3] In the Netherlands, we noted a sudden rise in NTM isolates derived from otological samples in 2006. This induced the current retrospective analysis of the clinical features, diagnosis and treatment of NTM otomastoiditis in the Netherlands.

The Study

We searched the national reference laboratory database and identified thirteen patients with otological samples yielding NTM over the January 1993 to June 2007 period. We re-subjected their isolates to molecular identification applying the INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Gent, Belgium) reverse line blot assay. *Mycobacterium abscessus* was the most frequent causative agent (10/13; 77%), followed by *M. avium* (n=2) and *M. avium* complex (MAC), though not *M. avium* or *M. intracellulare* (n=1). The latter three will be collectively referred to as MAC. Susceptibility testing was performed using the agar dilution method.[4] All primary isolates were susceptible to clarithromycin and resistant to aminoglycosides and fluoroquinolones; *M. abscessus* bacteria in two patients acquired clarithromycin resistance during treatment.
The peak in the number of patients in 2006 has only partly abated; Figure 1 details the timing of the thirteen cases in this report, as well as two new cases from 2007 and three in the first half of 2008.

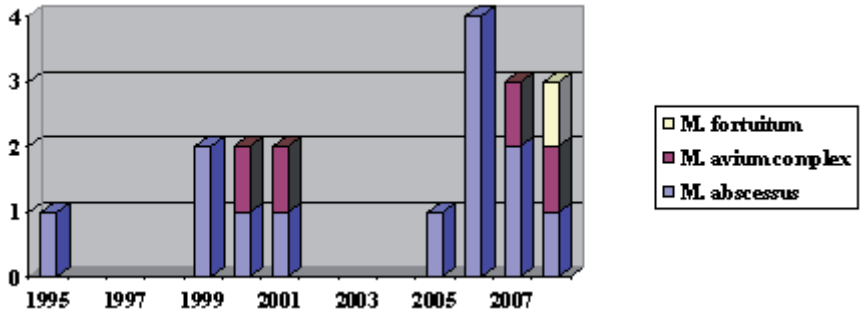

Figure 1: Notification of NTM otomastoiditis over time, by species. After a prior emergence in 1999-2001, increased notification of cases was apparent from 2005 until May 2008

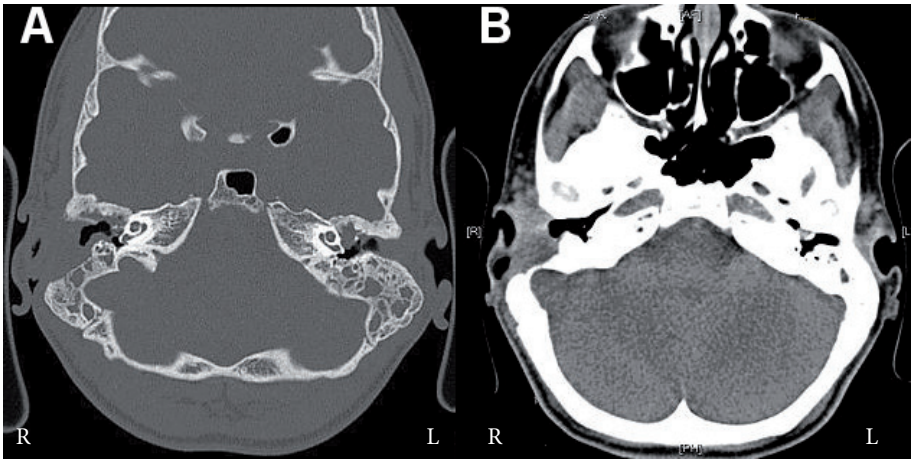

Figure 2 (A+B): Computed tomography images of a patient with *Mycobacterium abscessus* otomastoiditis. There is extensive bone destruction in the right mastoid and associated right-sided mucosal swelling.

We examined medical records of the thirteen patients; the main results are recorded in the Table. Two patients with *M. abscessus* otomastoiditis are siblings (Table, patients 5 & 6). Among immunocompetent patients, those with *M. abscessus* otomastoiditis were older than patients with MAC disease (6 vs. 2 years).

All patients had a history of ear infections and twelve had a history of tympanostomy tube placement. Eleven patients had used ototopical medication, including quinolone antibiotics (n=6), steroids and aminoglycosides (n=3) or both (n=2). The clinical presentation was non-specific, with persistent tympanic membrane perforation, chronic painless otorrhea resistant to antibiotic therapy and hearing loss.

The mean interval between first symptoms and diagnosis was 155 days for *M. abscessus* otomastoiditis (range 14-360) and 39 days for MAC otomastoiditis (range 28-49). In all thirteen patients acid-fast bacilli microscopy was performed; eleven were positive. In six cases the primary isolate was from biopsy material, in seven from otorrhea fluid. Seven patients had a computed tomography (CT)-scan performed which revealed fluid in the mastoid (n=6), bone erosion of the mastoid (n=3) and mucosal swelling (all); figure 2 displays typical findings.

The treatment regimens are detailed in the Table. Patients with *M. abscessus* infections received drug treatment for a mean duration of three months (range 28-150 days) and 1.8 episodes of surgery, versus 14 months of drug therapy and one episode of surgery for those with MAC otomastoiditis. Five patients with *M. abscessus* otomastoiditis received clarithromycin monotherapy, five received multi-drug therapy with fluoroquinolones (n=3), fluoroquinolones

Table: Clinical data of patients with NTM otomastoiditis

Pt	Species	Sex, Age	Predisposing factors	Side	Symptoms	Cultures/ pos/(AFB)	Days to diagnosis	Treatment (n=months)	Outcome
1	*Mycobacterium abscessus*	M, 2	TT, OD	right	O,H,P,F, M	5/2(-)	147	XW-X-X,AD, 1RE3ECipCla	Cured, H
2	*M. abscessus*	F, 10	TT	right	O,H,P,fi,M	5/1(+)	330	XW-X-X,2CipCla	Cured
3	*M. abscessus*	M, 4	TT, OD	left	O,H,P,F,M	2/1(+)	60	X,3Cla	Cured, H
4	*M. abscessus*	M, 4	TT, OD	left	O,P	2/1(+)	14	1Cla-8Cla	Failure
5	*M. abscessus*	M, 5	TT, OD	left	O,H	3/3(+)	100	4Cla	Cured
6	*M. abscessus*	M, 3	TT, OD	right	O,H,P,M	4/3(+)	60	X,5Cla-X	Cured
7	*M. abscessus*	F, 10	TT, OD	right	O,H,P,M, L	1/1(+)	360	2CipCla-X,CR	Cured
8	*M. abscessus*	M, 12	TT, OD	right	O,H,P,A,fi,M, F,L*	12/8(+)	270	X- XW-RD, 1ClaMox-X,LY-CR	Cured, H
9	*M. abscessus*	F, 6	TT, OD	Left	O,H,A,F,M,T,V,L	4/2(+)	90	X-X-XW1Cip-X,5ClaMer,AD	Failure, H
10	*M. abscessus*	F, 5	TT,OD	left	O,H,P,M,V,L	7/3(+)	120	X-1Cla	Cured, H
11	MAC	F, 2	TT, OD	left	O,M,L	4/2(+)	28	X,6RbCla	Cured
12	*M. avium*	F, 1	TT, OD	right	O,H,P,M,L*	6/1(+)	49	XW,6RCla-X,LY	Cured, H
13	*M. avium*	F, 15	Immunodeficiency	right	O	3/3(-)	21	30RbCla	Died

MAC, *M. avium* complex; TT, ventilation tubes; OD, otic drops; O, chronic otorrhea; H, hearing loss; P, tympanic membrane perforation A, otalgia; F, fever; fi, fistula; M, mastoiditis; F, facial nerve palsy; V, tinnitus; V, vertigo; L, lymphadenitis (*culture proven); X, surgery; RD, radical debridement; Cla, clarithromycin; Rb, rifabutin; Cip, ciprofloxin; R, rifampicin; E, ethambutol; Mer, meropenem; Mox, moxifloxacin; AD, retroauricular abscess drainage; LY, cervical lymphnode excision; W, delayed wound healing

and first-line anti-tuberculosis drugs (n=1) or meropenem (n=1; Table). The patients with MAC otomastoiditis received rifabutin and clarithromycin.
Complications of surgery comprised mainly delayed wound healing (n=5) and fistula formation (n=2; Table). For two patients, incus removal and later chain reconstruction surgery was necessary (Table, patient 7 and 8).
Although extrapulmonary NTM infections are considered localized infections, we recorded culture-proven regional spread of the infection to cervical lymph nodes (Table; patients 8 & 12) with a retro-auricular abscess, fistula and partially reversible facial nerve palsy in one patient (patient 8).
Ten patients are now considered to be cured. Two patients still received treatment at the time of the data collection. Cure was defined by symptomatic improvement and in some cases confirmed by negative cultures. One patient died after deliberately discontinuing treatment for multiple opportunistic infections (Table, patient 13). The mean interval between the start of the first treatment and cure was 341 days (range 202-570). Audiograms demonstrated persistent conductive hearing loss after treatment in five patients (42%; range 30-80dB; Table 1).

Conclusions

The number of cases of NTM otomastoiditis cases rose in 2006 and remained elevated, with three new cases in the first 6 months of 2008 (Figure 1). Whether this is a true increase or a result of improved laboratory facilities or awareness in clinicians remains uncertain. The latter seems unlikely as Dutch guidelines advise against performing cultures in children with chronic otorrhea. Laboratory improvements like liquid culture and molecular identification techniques predate the rise in notification.
The predominance of *M. abscessus* is remarkable, as MAC is the overall most frequently isolated NTM and the most common causative agent of pulmonary and disseminated disease in adults as well as cervical lymphadenitis in children.[1] Apparently, *M. abscessus* has a predilection to cause otomastoiditis.
The patient characteristics and NTM species are similar to previous studies.[5-7] Early reports have identified *M. fortuitum* or *M. chelonae* as causative agents, which may be due to the fact that the taxonomy of the rapid growing mycobacteria has long been debated.[8,9]
MAC otomastoiditis is usually diagnosed in patients younger than those with *M. abscessus* disease.[5-7] Lindeboom *et al.* found similar associations between *M. avium* infection and young age, in their comparison of causative agents of pediatric NTM lymphadenitis.[10] This may result from age-specific environmental exposure, e.g. playing in sandpits or swimming.[10]
American Thoracic Society (ATS) guidelines for treatment of skin, soft tissue and bone infections caused by *M. abscessus* advocate 4 to 6 months of triple-drug therapy with a macrolide, an aminoglycoside and cefoxitin or a carbapenem, based on *in vitro* drug susceptibility test results, combined with

surgical debridement when possible.[1] Treatment regimens observed in our study deviated both in duration (3 months) and content; the clarithromycin monotherapy is likely to invoke resistance[1] and simultaneously administered fluoroquinolones dampen clarithromycin activity.[11] Moreover, use of parenteral agents was limited; its reasoning was not generally captured in our file review. Aminoglycosides may have been withheld due to their ototoxicity.[1] The patients with MAC otomastoiditis were treated with a combination of rifabutin and clarithromycin, without the ethambutol recommended by the ATS.[1] The frequent deviation from treatment guidelines, necessity of multiple rounds of surgery to attain cure and acquired clarithromycin resistance may be interrelated, although our study group is too small for such conclusions. This situation may improve if adherence to ATS treatment guidelines increases.

We noted significant diagnostic delays. Otorrhea unresponsive to antibiotic therapy should raise a clinical suspicion of NTM otomastoiditis,[3] especially in patients with bone destruction visible on CT images. Routine *Mycobacterium* cultures in such patients may reduce diagnostic delay, preventing further damage. Tissue biopsy is the most sensitive means of obtaining a specimen for culture.[1]

In summary, otomastoiditis caused by NTM is a rare but serious condition that may be emerging in the Netherlands. *Mycobacterium abscessus* and MAC are the predominant causative agents and affect children with previous ear infections, tympanostomy tubes and ototopical antibiotic or steroid use. The diagnostic delay and treatment regimens should be improved, mainly for *M. abscessus* disease. Treatment with surgery and evidence-based multi-drug regimens may prevent deterioration and prevent or limit permanent hearing loss.

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Chapter 3

Nontuberculous mycobacterial disease in patients treated for rheumatic disease

3.1 Pulmonary *Mycobacterium szulgai* infection and treatment in a patient receiving anti-tumor necrosis factor therapy.
Nat Clin Pract Rheumatol 2007; 3(7): 414-9.

3.2 Mycobacterial disease in patients with rheumatic disease.
Nat Clin Pract Rheumatol 2008; 4(12): 649-56.

Pulmonary *Mycobacterium szulgai* infection and treatment in a patient receiving anti-tumor necrosis factor therapy

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Summary

Background: A 54-year-old man with a 22-year history of rheumatoid arthritis and an 8-year history of chronic obstructive pulmonary disease presented with dyspnea on exertion, nonproductive cough and fatigue of 1 month's duration. His medication at presentation consisted of etanercept, azathioprine, naproxen and inhaled fluticasone and salbutamol.

Investigations: At presentation, the patient underwent physical examination, chest X-ray and high-resolution CT, blood tests, and bronchoalveolar lavage fluid analysis including auramine stains and gene sequence analysis of cultured *Mycobacterium szulgai*. The patient underwent minithoracotomy after 6 months, and bronchoalveolar lavage fluid analysis, culture and chest X-ray after 18 months. Further chest imaging and culture of sputum samples were performed another year later.

Diagnosis: Pulmonary *M. szulgai* infection.

Management: Triple drug therapy with rifampicin, ethambutol hydrochloride and clarithromycin. Anti-tumor necrosis factor treatment was continued.

The Case

A 54-year-old man presented to the respiratory department of a regional hospital with dyspnea on exertion, nonproductive cough and fatigue, which he had experienced for 1 month. The patient had a 22-year history of rheumatoid-factor positive, severe, nodular, erosive rheumatoid arthritis (RA; Steinbrocker class III) and an 8-year history of chronic obstructive pulmonary disease (COPD; Global Initiative for Chronic Obstructive Lung Disease stage I). The patient's RA had been treated with subcutaneous etanercept 25 mg twice weekly for 24 months, azathioprine 50 mg twice daily for 8 months and naproxen 500 mg twice daily for over 10 years. Previous treatment with sub cutaneous methotrexate 30 mg once weekly had not been successful. The patient's COPD had been treated for 3 years with inhaled fluticasone propionate 500 μg twice daily and salbutamol 100 μg 1-6 times daily, as needed.

Physical examination of the chest revealed decreased breath sounds but no wheezes, crackles or prolonged expiration. A chest X-ray (Figure 1A) and high-resolution CT at presentation showed generalized emphysema, an infiltrate in the lingula, apical bullae, cavities with fibrosis in the right upper lobe, and diffuse peribronchial alveolar consolidations. Chest X-rays, which had been performed before initiation of etanercept treatment, had not shown these abnormalities. Bronchoalveolar lavage fluid analysis at presentation revealed acute, nonspecific inflammation with an elevated cell count, lymphocytosis and an elevated CD4/CD8 T-lymphocyte ratio. Blood tests showed that the CD4/CD8 T-lymphocyte ratio was normal in the serum. Three auramine stains of the bronchoalveolar lavage fluid were negative; one of three corresponding cultures yielded *Mycobacterium szulgai*, a nontuberculous mycobacterium. No other micro-organisms were cultured. At this time, the *M. szulgai* was considered to be a contaminant in the culture, and a firm diagnosis of nontuberculous mycobacterial infection could not be made according to the American Thoracic Society 1997 criteria (Box 1).[1] A minithoracotomy was performed 6 months later for histological diagnosis, which revealed panacinar emphysema, bronchiolitis and low-intensity granulomatous pneumonitis with epithelioid cell granuloma. The differential diagnosis now included sarcoidosis, tuberculosis, fungal infection, *M. szulgai* infection, and occupational pulmonary disease such as asbestosis, berylliosis and silicosis. At this stage, the patient's stable pulmonary and rheumatologic condition did not warrant a therapy change.

At a routine follow-up visit 18 months later, bronchoalveolar lavage fluid analysis again yielded one positive culture for *M. szulgai*. Radiological progression of disease was noted, with progression of cavitary and fibrotic lesions, traction-bronchiectasis and resolution of patchy consolidations (Figures 1B and 2A). A tentative diagnosis of stage IV sarcoidosis was made on the basis of results from chest X-rays and histology, and because the diagnostic criteria for nontuberculous mycobacterial infection according to the American Thoracic

Box 1 Summary of the American Thoracic Society diagnostic criteria for nontuberculous mycobacterial infection.[1]

Patients can be diagnosed with nontuberculous mycobacterial infection if they fulfill the following criteria:
- They have pulmonary symptoms

plus
- Infiltrate

or
- Nodular or cavitary disease

or
- A high-resolution CT scan that shows multifocal bronchiectasis, with or without multiple small nodules

plus
- If three sputum or bronchial wash results are available from the previous 12 months: three positive cultures with negative AFB smear results, or two positive cultures and one positive AFB smear

or
- If only one bronchial wash is available, and sputum samples can not be obtained: positive culture with a 2+, 3+, or 4+ AFB smear, or 2+, 3+, or 4+ growth on solid media

or
- If sputum or bronchial wash evaluations are nondiagnostic or another disease cannot be excluded: transbronchial or lung biopsy yielding an NTM, or biopsy showing mycobacterial histopathologic features (granulomatous inflammation, with or without AFB) and one or more sputum samples or bronchial washings that are positive for an NTM even in low numbers

Abbreviations: AFB, acid-fast bacilli; NTM, nontuberculous mycobacteria.

At least three respiratory samples should be evaluated from each patient. Other reasonable causes for the disease should be excluded. Expert consultation should be sought when diagnostic difficulties are encountered.

Society had not been met (Box 1).[1] Etanercept treatment was terminated, and treatment with adalimumab 40 mg every 2 weeks was started with the intention of treating both RA and the potential sarcoidosis.

Another year later, the patient returned with a mild cough, malaise and night sweats. Chest imaging (Figures 1C and 2B) revealed thickening of cavity walls, reappearance of consolidations, a nodular pattern in the right middle lobe, and ground glass appearance. Three sputum samples were positive for acid-fast bacilli on microscopy, and corresponding cultures yielded *M. szulgai.* At this time, the patient met the American Thoracic Society diagnostic criteria for nontuberculous mycobacterial infection (Box 1).[1] Treatment was started with rifampicin 600 mg once daily, ethambutol hydrochloride 1400 mg (20 mg/kg) once daily and clarithromycin 500 mg twice daily for a duration of 18 months. Adalimumab therapy was continued during the antimycobacterial treatment. The patient's cough and dyspnea diminished within the first month after initiation of antimycobacterial treatment, constitutional symptoms resolved

after 4 months, and radiological abnormalities had improved after 1 year of therapy (Figures 1D and 2C).

The patient's erythrocyte sedimentation rate and serum C-reactive protein concentration were repeatedly measured during the course of the patient's disease and after antimycobacterial treatment was initiated (Figure 3). Genotyping of all the patient's *M. szulgai* isolates with random amplified polymorphic DNA analysis as previously described,[2] using OPA18 and IS*986* FP as primers, was performed at the Dutch national tuberculosis reference

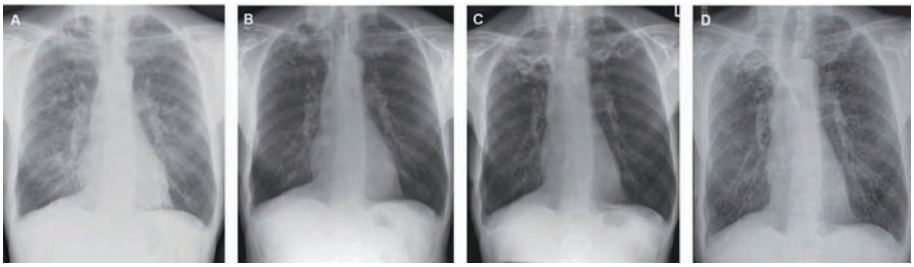

Figure 1: The patient's chest X-rays

(**A**) At initial presentation. The image shows patchy consolidations in both lungs. (**B**) 2 years after initial presentation and 1 year prior to antimycobacterial therapy. The image shows progression of the patchy consolidations to fibrotic, cavitary lesions in the upper lobes. (**C**) 3 years after initial presentation and at the start of antimycobacterial treatment. The image shows progression of the fibrotic and cavitary lesions in both upper lobes. (**D**) After antimycobacterial treatment. The image shows that the fibrotic, cavitary lesions in the upper lobes are slightly decreased in size.

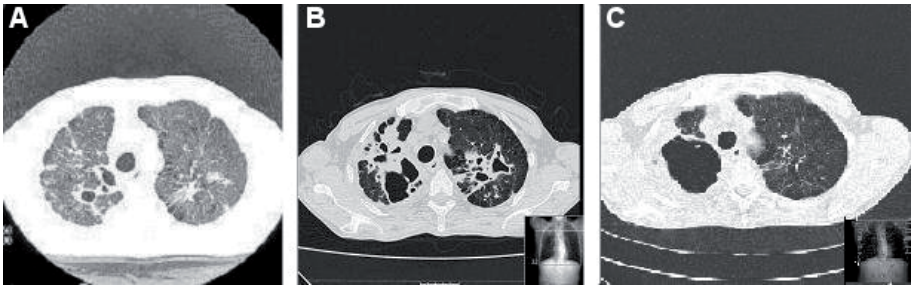

Figure 2: The patient's CT images

(**A**) 2 years after initial presentation and 1 year prior to antimycobacterial therapy. The image shows fibrotic and cavitary lesions. (**B**) 3 years after initial presentation and at the start of antimycobacterial therapy. The image shows that the fibrotic and cavitary lesions have worsened since the previous year. The smaller image in the bottom right-hand corner shows the anatomical position of the section displayed. (**C**) 4 years after initial presentation and 1 year after antimycobacterial treatment. The image shows that the fibrotic and cavitary lesions have partially regressed. The smaller image in the bottom right-hand corner shows the anatomical position of the section displayed

laboratory after the patient's symptoms resolved. The results of the genotyping showed that all the *M. szulgai* isolates were of a similar, distinct genotype (Figure 4).

Mycobacterial smears and cultures were negative after 1 year of antimycobacterial treatment and remained negative on subsequent tests. This patient is still treated with adalimumab and his RA remains stable.

Discussion of Diagnosis

The patient developed a nontuberculous mycobacterial infection while receiving anti-tumor necrosis factor (TNF) therapy for the treatment of RA. The similar mycobacterial genotypes, which were obtained using random amplified polymorphic DNA analysis, indicated that the same strain of *M. szulgai* was isolated on all three occasions (Figure 4). Pulmonary *M. szulgai* isolates from other patients within the same geographical region and the same period, which were mostly cultured in the same hospital laboratory, seemed to be unrelated to those obtained from this patient; therefore, contamination of this patient's cultures, or reinfection, is unlikely to have occurred. Theoretically, repeated exposure to a single environmental source might have caused monoclonal reinfections; however, this possibility is considered improbable. It is assumed, therefore, that the primary *M. szulgai* culture, which was cultured 3 years prior to treatment, already represented true infection. The increases in erythrocyte sedimentation rate and serum C-reactive protein concentration in a period when RA was stable also supported this assumption.

The patient's early symptoms, radiological features and *M. szulgai* isolates probably represented subclinical or latent nontuberculous mycobacterial infection; however, the American Thoracic Society diagnostic criteria for nontuberculous mycobacterial infection (Box 1)[1] were not met, and the patient was not treated for nontuberculous mycobacterial infection at this time. Histological samples were obtained from the patient at the initial presentation, and the results of the samples were interpreted as sarcoidosis. The differential diagnosis also included tuberculosis, *M. szulgai* infection, fungal infection, and occupational pulmonary disease such as asbestosis, berylliosis and silicosis. The absence of asbestos, beryllium or silica exposure, and absence of cultured *M. tuberculosis* complex bacteria or fungi, however, narrowed the differential diagnosis to sarcoidosis and *M. szulgai* infection.

The possibility that the patient's histological findings resulted from the nontuberculous mycobacterial infection, influenced by anti-TNF therapy, is supported by the finding that bronchiolitis and epithelioid cell granuloma, with or without central necrosis, have been recorded in histological studies of patients with nontuberculous mycobacterial infection.[3] Also, the absence of radiological abnormalities 2 years prior to the initial presentation indicated that there was an association between the patient's radiological features and the *M. szulgai* culture. Mycobacteria might have a role in the etiology of

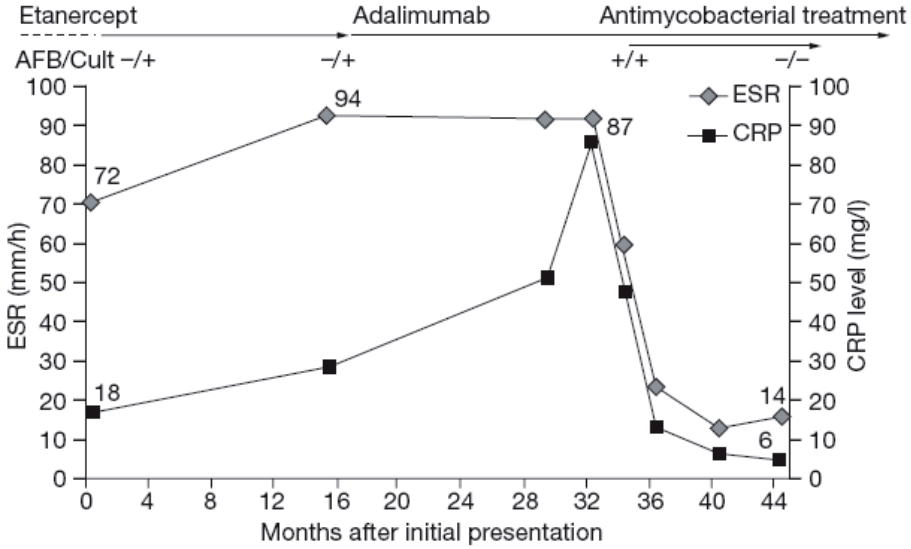

Figure 3: Microbiological and serological parameters during the course of the patient's disease.

This figure shows the culture results and the ESR and CRP levels, which rise after the first *Mycobacterium szulgai* culture, with CRP levels rising more pronouncedly after adalimumab treatment is started. Both ESR and CRP levels regress to their normal range during combined antimycobacterial and anti-TNF treatment. This figure indicates that the patient had chronic infection, which deteriorated during adalimumab therapy, but which was successfully treated without cessation of adalimumab therapy.
Abbreviations: AFB, acid-fast bacilli (visible on direct microscopy); CRP, C-reactive protein; Cult, culture results for *M. szulgai*; ESR, erythrocyte sedimentation rate.

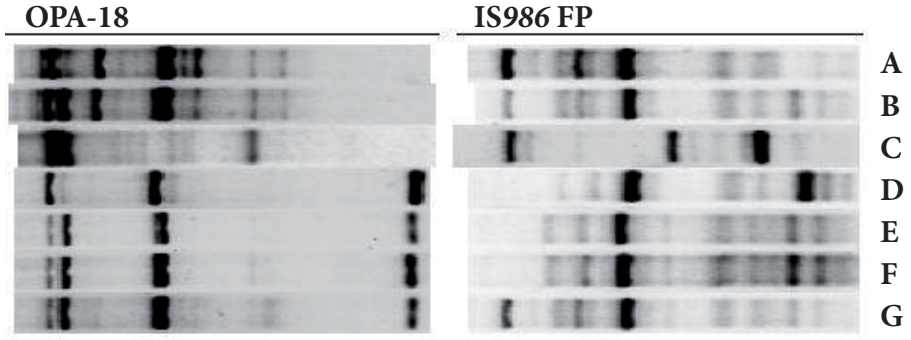

Figure 4: A photograph of genotyping results of the patient's *Mycobacterium szulgai* isolates.

The genotyping was performed using random amplified polymorphic DNA analysis with OPA18 and IS*986* FP as primers. Lanes A–D show the genotyping for pulmonary samples from four patients in the same geographical region as the case patient. Lanes E–G show the genotyping for the case patient's pulmonary samples: 3 years after initial presentation and at the start of antimycobacterial therapy (E); 2 years after initial presentation and 1 year prior to the start of antimycobacterial therapy (F); and at initial presentation (G, extra band in IS986 FP). This figure shows that in the case patient, the same strain of *M. szulgai* was isolated on all three occasions. Pulmonary *M. szulgai* isolates from the four other patients within the same geographical region and the same period were unrelated to those obtained from the case patient.

sarcoidosis;[4] therefore, *M. szulgai* could theoretically have caused sarcoidosis in this patient. This possibility is unlikely, however, because mycobacterial DNA fragments, not viable mycobacteria, have been demonstrated in tissue samples of patients with sarcoidosis, and the bacteriological course of this patient's disease is strongly indicative of mycobacterial infection rather than sarcoidosis.[4]

Treatment with etanercept was discontinued in this patient, and treatment with adalimumab was initiated. Adalimumab is closely related to the anti-TNF agent infliximab, which is effective in the treatment of sarcoidosis.[5] Paradoxically, anti-TNF agents (mainly etanercept) have been described as a possible causative agent of sarcoidosis;[6] this finding strengthened the diagnosis of sarcoidosis in this patient during the early stages, as he was being treated with etanercept at this time. Adalimumab inhibits the action of TNF and the production of interferon-γ (INFγ);[7] these properties increase patients' susceptibility to nontuberculous mycobacterial infection. This patient's infection by *M. szulgai*, which was cultured at the initial presentation, was clinically stable during treatment with etanercept but slowly deteriorated after initiation of adalimumab therapy. This observation might demonstrate the high extent to which TNF and INFγ inhibition is achieved with adalimumab.

TNF has an important role in granuloma formation and maintenance, which is essential in host defense against mycobacteria;[8] therefore, mycobacterial infection or reactivation of latent infection can be expected as a complication of anti-TNF treatment. Reports have documented the emergence of nontuberculous mycobacterial infection in patients who receive anti-TNF treatment. In 2004, Wallis *et al.*[9] reported 29 unspecified nontuberculous mycobacterial infections in patients treated with anti-TNF agents from the FDA adverse events reporting system. These reports are not necessarily 29 true nontuberculous mycobacterial infections, as the reporting procedure might not be sufficient to fulfill the American Thoracic Society diagnostic criteria. Two true nontuberculous mycobacterial infections are described in separate case reports;[10,11] one concerned a psoas muscle abscess caused by *M. avium* induced by etanercept use, and the other a *M. abscessus* skin infection induced by infliximab use. These infections occurred after 12 and 13 months of anti-TNF treatment, respectively, as in this patient, whose symptoms deteriorated after 12 months of adalimumab therapy. These reports highlight that the length of time between the start of anti-TNF treatment and the onset of nontuberculous mycobacterial disease is longer than for the onset of tuberculosis, where a median interval of 12 weeks is observed.[8,9]

Among patients who receive anti-TNF therapy, tuberculosis infection or reactivation is more common than nontuberculous mycobacterial infection or reactivation.[8-11] Several possibilities exist for the cause of this finding, including a higher prevalence of latent tuberculosis compared with latent nontuberculous mycobacterial disease. Another explanation could be the ability of tuberculosis

to infect previously healthy individuals, whereas nontuberculous mycobacteria, being opportunistic pathogens, generally affect patients with local (e.g. COPD, as in this patient) or systemic impaired immunity. The finding could also arise from the possibility that TNF has a less prominent role in the pathogenesis of nontuberculous mycobacterial infection than it has in tuberculosis infection. The concept of 'latent' nontuberculous mycobacterial infection is controversial, and missed diagnoses of active nontuberculous mycobacterial infection might also contribute to a higher recorded prevalence of tuberculosis than nontuberculous mycobacterial infections in patients who receive anti-TNF therapy.

Various authors have produced recommendations for the screening of latent tuberculosis in candidates eligible for anti-TNF therapy, which usually involves tuberculin skin testing and chest X-ray.[8] For nontuberculous mycobacteria, these tests might not suffice: tuberculin skin test results are mostly negative in patients with nontuberculous mycobacterial infections, and chest X-rays show diverse, partly species-specific patterns.[1] In candidates for anti-TNF treatment with known lung disease, specifically COPD and bronchiectasis, their sputum should be repeatedly cultured for mycobacteria.

In this patient, it should be considered that COPD itself predisposed him to nontuberculous mycobacterial infection, independently of anti-TNF therapy. The localized pulmonary presentation in this patient, as opposed to the extrapulmonary or disseminated mycobacterial infections seen in patients who receive anti-TNF treatment, suggests that the host response to infection was relatively intact.[8] It is not possible, therefore, to establish whether or not anti-TNF treatment was the sole, definite cause of his *M. szulgai* infection. As the number of indications for anti-TNF treatment increases, more patients with additional risk factors for nontuberculous mycobacterial infection might receive treatment with this class of agents; therefore, the case patient might represent an emerging issue of nontuberculous mycobacterial infection in anti-TNF recipients.

Discussion of treatment options

Whether or not anti-TNF agents can be safely continued during antimycobacterial treatment remains the subject of debate. Continuation of these agents might be feasible for the following three reasons. First, the disruption of granuloma achieved by anti-TNF agents could increase the exposure of mycobacteria to anti mycobacterial drugs, thereby resulting in improved treatment outcome for the infection.[12] Second, although an increased risk of paradoxical response to antimycobacterial therapy has been reported in infliximab-treated patients with tuberculosis, which is characterized by clinical deterioration during treatment in patients who initially improve, maintenance of low doses of anti-TNF agents might produce immunologic regulation beneficial to this group.[13] Third, rheumatologic complaints remain

well-controlled with anti-TNF therapy. A case reported by Matsumoto *et al*. showed that antimycobacterial therapy followed by anti-TNF therapy can be safe and effective for the treatment of mycobacterial infection; infliximab therapy was safely restarted after 2 months of antituberculosis treatment.[14] Anti-TNF treatment was not discontinued during antimycobacterial therapy in the patient described here, and his *M. szulgai* infection improved (Figures 1-3) while his rheumatologic complaints remained minimal and no paradoxical reaction occurred.

Both the American Thoracic Society and the British Thoracic Society have released management guidelines for nontuberculous mycobacterial infections. Although not designed for *M. szulgai*, both groups specifically mention this species. The American Thoracic Society advises treatment with rifampicin 600 mg, ethambutol hydrochloride 25 mg/kg in the first 2 months, then 15 mg/kg, and streptomycin (dosage depending on weight, age and phase of therapy); the British Thoracic Society advises rifampicin 450 mg for patients who weigh <50 kg and 600 mg for those who weigh >50 kg, ethambutol hydrochloride 15 mg/kg, clarithromycin 500 mg twice daily, and also the option of surgical treatment.[1,15] Previous case series have reported an almost 100% cure rate of *M. szulgai* infection, with the few failures or relapses resulting from inadequate drug regimens or proven non-compliance.[16] This patient was treated with clarithromycin to avoid the use of drugs that require injection. The optimal duration of therapy has not yet been established in clinical trials; however, both the American Thoracic Society and the British Thoracic Society currently advise 18-24 months of treatment if the patient has a satisfactory clinical and bacteriological response to chemotherapy, as this patient had.

Conclusions

The important role that TNF has in granuloma formation and maintenance, which is essential in host defense against mycobacteria, has been demonstrated by the many cases of tuberculosis reactivation described.[7-9] Few cases of nontuberculous mycobacterial infections in patients receiving anti-TNF therapy have been reported.[9-11] Given the low incidence and lack of good diagnostic instruments, specific screening for nontuberculous mycobacterial infections is currently not feasible; however, candidates for anti-TNF treatment with known lung disease, specifically COPD and bronchiectasis, should have their sputum repeatedly cultured for mycobacteria. Furthermore, clinicians involved in anti-TNF treatment should be aware of the potential emergence of nontuberculous mycobacterial infections in their patients. If a nontuberculous mycobacterial infection occurs, strictly supervised continuation of anti-TNF agents during antimycobacterial treatment might be safe and effective.

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Mycobacterial disease in patients with rheumatic disease

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Summary

This Review focuses on the emergence of mycobacterial disease in patients undergoing treatment for rheumatic disease with four new drug classes—tumor necrosis factor (TNF) inhibitors, human interleukin (IL)-1 receptor antagonists, anti-CD20 antibodies and CD4+ T-cell costimulation modulators—collectively referred to as biologic agents. Mycobacterial disease is a major cause of severe infection in patients undergoing anti-TNF therapy. Reports are now emerging of an association between mycobacterial infection and antirheumatic treatment with anti-IL-1 or anti-CD20 antibodies. Although tuberculosis is the most common mycobacterial disease, nontuberculous mycobacterial (NTM) disease is an increasingly recognized problem in this setting. Among the antirheumatic drugs currently in development, agents that target IL-17, IL-23, Janus kinase–signal transducers and activators of transcription signaling, and metalloproteinases are likely to confer an increased risk of mycobacterial disease. Although screening and preventive treatments have lowered the incidence of active tuberculosis, these tools are not applicable to patients with NTM disease. All patients receiving drugs associated with an increased risk of mycobacterial disease should be carefully monitored, and suspect lesions should undergo *Mycobacterium* culture. Further studies are needed to determine the prevalence of NTM disease in this setting, and to evaluate the safety of simultaneous anti-TNF and antimycobacterial treatment.

Review Criteria

Relevant publications for this Review were identified using the PubMed database. We reviewed English-language papers only. The following Medical Subject Heading (MeSH) terms were searched alone and in combination: "tuberculosis", "mycobacterium infections, atypical", "rheumatic disease", "tumor necrosis factor-alpha" and "safety".

Introduction

Patients with rheumatic disease are at a higher risk of infection than the general population. Studies have focused mainly on the infection risk associated with the treatment of rheumatic disease.[1] The advent of anti-tumor necrosis factor (TNF) therapy saw an increased incidence of active tuberculosis in patients undergoing antirheumatic therapy compared with the general population. Tuberculosis is among the most common severe infections seen in patients receiving anti-TNF treatment.[1]

The genus *Mycobacterium* comprises three main groups: the *M. tuberculosis* complex, *M. leprae*, which causes leprosy, and the nontuberculous mycobacteria (NTM). The most important causative agents of human tuberculosis are *M. tuberculosis*, *M. bovis* and *M. africanum*; *M. microti*, *M. caprae*, *M. canettii*, and *M. pinnipedii* are less frequent causative agents. The NTM include a highly divergent group of over 100 different species that are omnipresent in the environment (i.e. water and soil) and capable of causing opportunistic disease.[2] Interestingly, reports are emerging of NTM-associated disease in patients receiving anti-TNF treatment.[3,4] This Review focuses on the drug related risks, clinical presentation and new possibilities for screening and preventing mycobacterial disease in patients treated for rheumatic diseases.

Drug-related infection risk in antirheumatic therapy

The treatments currently available for rheumatic disease include six separate immunosuppressive drug classes, which all vary in their associated risk of infection. Steroids and methotrexate are the oldest classes in active use. Although the elevated infection risk associated with steroids has been well documented, this association remains unproven for methotrexate.[1]

Four new drug classes have been developed over the past 10 years, based on human or chimeric antibodies against cytokines or receptors with pivotal roles in the inflammatory pathways of immune mediated inflammatory disease. These agents are collectively referred to as 'biologics'; Table 1 shows all the biologic agents that are either currently approved for use or undergoing evaluation in clinical trials. Anti-TNF agents (the monoclonal antibodies etanercept, infliximab and adalimumab) pose the largest infection risk of all the biologics, and predispose patients to mycobacterial infection.[1] In the prescreening era, the estimated rate of tuberculosis infection in infliximab-treated patients with rheumatic disease in the US was 24.4 cases per 100,000 patients, compared with 6.2 cases per 100,000 patients who had not received infliximab.[5] This is not surprising, as TNF has an important role in granuloma formation and maintenance,[6] which is essential for host defense against mycobacteria.[5] Among the anti-TNF agents, infliximab and adalimumab are associated with a greater risk of infection than etanercept.[7]

In addition to the anti-TNF agents, three other new classes of drugs have become available for the treatment of rheumatic disease: human interleukin(IL)-1

Table 1: Currently approved biological agents and new drugs in the pipeline for the treatment of rheumatic diseases.

Drug class	Drug name	Developmental phase
Tumor necrosis factor antagonists	Etanercept, infliximab and adalimumab	Approved
	Golimumab and certolizumab pegol	Phase III
IL-1 receptor antagonists	Anakinra	Approved
$CD20^+$ B-cell antibodies	Rituximab	Approved
CD4-cell co-stimulation modulators	Abatacept	Approved
IL-6 receptor antibodies	Tocilizumab	Phase III
IL-17A antibodies	NA	Murine model / Phase I

Abbreviations: IL, interleukin; NA, not applicable

receptor antagonists, anti-CD20 antibodies and CD4+ T-cell costimulation modulators. Anakinra is the first IL-1 receptor antagonist to be approved by the US FDA. A meta-analysis of randomized controlled trials revealed a significantly increased risk of serious infection with higher doses of anakinra (≥100 mg/day) compared with lower doses or placebo. The pooled odds ratio (OR) for serious infection in patients treated with anakinra versus placebo was 2.75 (95% CI 0.90–8.35); however, no cases of mycobacterial infection were reported.[8] A single case of nonspecified NTM infection has been reported in a patient receiving anakinra treatment and concomitant prednisone and methotrexate.[9] For active tuberculosis, Brassard *et al.*[10] found 19 cases among 1,414 patients treated with anakinra in their cohort of 112,300 patients; however, the risk for tuberculosis was significantly increased only in past users of corticosteroids receiving anakinra (adjusted rate ratio 1.7, 95% CI 1.1–2.8). Additional case reports have described reactivation of previous tuberculosis infection in patients receiving anakinra monotherapy.[11]

Rituximab is the only available anti-CD20 antibody. A meta-analysis of three randomized controlled trials failed to demonstrate a significant increase in the rituximab-associated risk of serious infection (pooled OR 1.45, 95% CI 0.56–3.73).[8] However, the first cases of mycobacterial disease related to rituximab use have been reported this year.[4]

Abatacept is the only approved CD4+ T-cell costimulation modulator. A meta-analysis of five randomized controlled trials found no increase in the risk of serious infection associated with abatacept therapy (pooled OR 1.35, 95% CI 0.78–2.32).[8] However, the drug was shown to increase the infection risk in patients already using a biologic agent, and in patients with chronic obstructive pulmonary disease (COPD),[12] but did not impair the capacity to control *M. tuberculosis* infection in a murine model.[13]

New drugs and new indications

New indications for biologic agents - particularly for anti-TNF therapy - are currently under intense investigation. Anti-TNF agents have already proven to be effective in the treatment of various immune-mediated inflammatory diseases, including juvenile dermatomyositis, psoriasis, sarcoidosis and Behçet's disease.[14-17] These agents, however, have proven ineffective in other conditions that are characterized by locally increased levels of TNF, including multiple sclerosis and COPD.[18,19] Furthermore, although COPD is a risk factor for NTM disease, no NTM infections were observed during Phase III trials of anti-TNF therapy in patients with COPD.

The broadening spectrum of diseases treated with biologic agents stresses the need to implement mycobacterial infection screening strategies for patients who are candidates for anti-TNF treatment in all these settings. Current knowledge on the emergence of mycobacterial disease in patients treated with biologic agents needs to be disseminated to the users and prescribers of these new drugs.

As the indications diversify, so does the variety of biologic agents. Figure 1 displays the current and future targets for the development of antirheumatic drugs, together with their immunological functions against mycobacteria. Golimumab and certolizumab are novel monoclonal antibodies against TNF, and are effective in the treatment of rheumatoid arthritis (RA) and Crohn's disease, respectively. The infection risk associated with both of these agents is similar to that of other drugs in their class. Two trials, in which patients with evidence of previous or current tuberculosis were excluded, found one new case of pulmonary tuberculosis among 216 patients treated with certolizumab, despite tuberculin skin test (TST) screening, and no cases of mycobacterial disease among 137 patients who received golimumab therapy.[20,21]

IL-6 affects the function of T cells and B cells, and is overexpressed in the synovial tissue of patients with RA, making this cytokine a potential therapeutic target. In a 2008 Phase III trial, treatment with tocilizumab, a humanized monoclonalantibody that binds to the IL-6 receptor, was associated with an increased rate of infection, mainly of the respiratory tract, in patients with RA (101.9 and 98.7 per 100 patient years for high-dose and low-dose tocilizumab, respectively, compared with 96.1 per 100 patient years for placebo); no mycobacterial infections were noted.[22] The role of IL-6 in the immune response to mycobacteria is not completely understood. Although IL-6 proved to be important in the initial containment of *M. tuberculosis* infection in a mouse model, late containment occurred in the absence of IL-6,[23] suggesting that IL-6 is not essential for the host defense against tuberculosis. Other researchers have found that IL-6 inhibits the macrophage activation necessary for infection eradication in murine tuberculosis.[24] The issue of whether or not tocilizumab confers an increased risk of mycobacterial disease remains controversial, and demands careful investigation prior to approval of the drug.

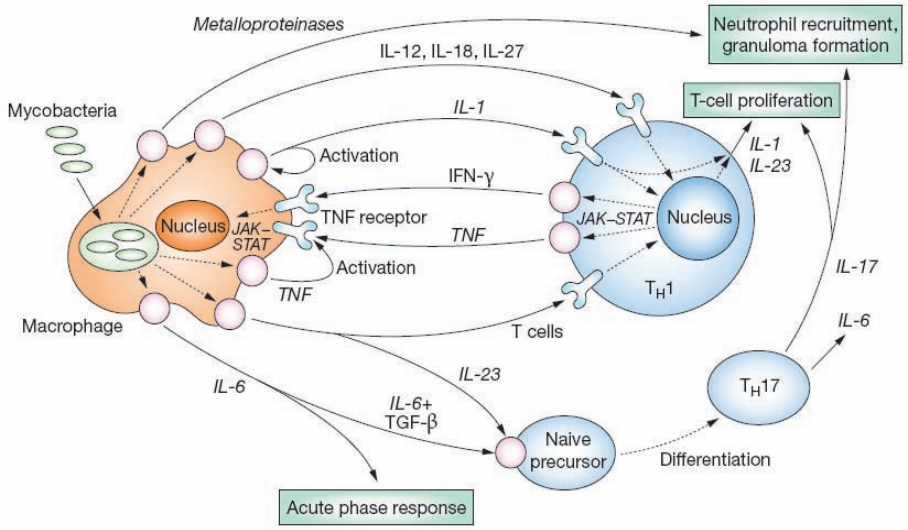

Figure 1: A basic representation of macrophage immune signaling after mycobacterial infection

Engulfing of mycobacteria by macrophages induces a strong T_H1 cell response. Macrophages activate T_H1 cells by secretion of various interleukins, and induce their own activity via TNF and IL-1 secretion. IL-6 and IL-23 are secreted to induce differentiation of precursor cells to T_H17 cells and to elicit the acute phase response. Activated T_H1 cells, in turn, stimulate the macrophages by secretion of IFN-γ and TNF. This, combined with metalloproteinase secretion, leads to neutrophil recruitment, T-cell proliferation and finally granuloma formation. JAK-STAT signaling mediates the activation of macrophages and T_H1 cells. The current and future targets for antirheumatic drugs are shown in italics.
Abbreviations: IFN, interferon; IL, interleukin; JAK, Janus kinase; STAT, signal transducer and activator of transcription; T_H, T helper; TNF, tumor necrosis factor.

IL-17 and IL-23 also represent promising novel targets.[25,26] IL-17 has the capacity to induce chronic destructive arthritis independent of IL-1 and TNF. Anti-IL-17A cytokine therapy was successful in mouse models of arthritis, but awaits clinical trials in humans.[25] Both IL-17 and IL-23 have important roles in all stages of the immune response against mycobacterial infection, from neutrophil recruitment in the earliest phase, to granuloma formation and maintenance in later stages (Figure 1).[27] Therapeutic agents that influence these cytokines, therefore, are likely to alter a patient's susceptibility to mycobacterial disease, or could reactivate latent disease.

Modification of proinflammatory cytokine signaling pathways in the brain is a proposed mechanism of action for a new generation of antidepressants. These include the stress-activated/mitogen-activated protein kinases and Janus kinase-signal transducers and activators of transcription (JAK-STAT) signaling pathways, and could be useful in ameliorating peripheral inflammation in RA.[28] Similar to alterations in the interferon-γ and IL-12 signaling, however, JAK–STAT signaling is an essential step in mycobacterial immunity, and modification leads to increased susceptibility to mycobacterial disease in humans.[29]

Another potential antirheumatic strategy is inhibition of the metalloproteinases, either directly or indirectly by modulating gene expression or preventing protein activation.[30] In an animal model of pulmonary tuberculosis, metalloproteinase-9 was required for macrophage recruitment and granuloma formation;[31] therefore, similar to the anti-TNF agents, metalloproteinase inhibition might disrupt granuloma formation and thus perturb the host defense against mycobacteria.

Mycobacterial disease in patients undergoing anti-TNF therapy

In most patients, tuberculosis represents reactivation of a previous infection, presenting after a median of 12 weeks of infliximab therapy, or after 12 months of treatment with etanercept.[6,32-34] Diagnosing mycobacterial disease in patients treated with biologic agents is complicated. Patients often present with extrapulmonary mycobacterial disease,[3,32,33] and require invasive diagnostic techniques. Extrapulmonary tuberculosis disease types are generally paucibacillary infections, which hampers confirmation of the diagnosis by *Mycobacterium* culture.

Few data on the emergence of NTM disease in patients undergoing anti-TNF therapy have been published. Wallis *et al.*[34] reported 29 cases of unspecified NTM infections associated with anti-TNF therapy in 622 patients with granulomatous infectious diseases. Whether these patients had occasional NTM isolates or disease according to the American Thoracic Society diagnostic criteria (summarized in Box 1)[2] cannot be substantiated from the report. Cases of NTM disease classified according to the American Thoracic Society criteria are described in separate case reports. Psoas muscle abscesses caused by *M. avium*,[3] *M. xenopi* pulmonary disease,[35] *M. xenopi* spondylodiscitis,[36] *M. marinum* tenosynovitis[37] and fatal pulmonary *M. abscessus* disease[38] have all been recorded in patients treated with etanercept. Furthermore, infliximab treatment has been associated with *M. abscessus* skin infection, disseminated *M. avium* complex disease, *M. peregrinum* pulmonary disease and *M. fortuitum* hepatitis,[3] and there has been one reported case of *M. szulgai* pulmonary disease during adalimumab therapy.[39]

The number of patients with tuberculosis associated with biologic therapy is thought to outnumber that of NTM disease, although a recent report, based on the Emerging Infections Network of the Infectious Diseases Society of America, showed the opposite.[3] Extrapulmonary and disseminated NTM disease seem to be the most common forms.[3,35-37] Pulmonary disease might arise specifically in patients with additional risk factors, including bronchiectasis and COPD.[35,39]

The range of NTM species involved in anti-TNF-associated mycobacterial disease is intriguing, as it includes species of both high pathogenicity (e.g. *M. marinum*, *M. xenopi* and *M. szulgai*)[2,40,41] and low pathogenicity (e.g. *M. fortuitum*, *M. peregrinum*).[2] This might reflect the importance of host factors in these infections.

Box 1: Summary of the American Thoracic Society diagnostic criteria for nontuberculous mycobacterial infection[2]

Clinical.
1. Pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules.
and
2. Appropriate exclusion of other diagnoses.

Microbiological.
1. Positive culture results from at least two separate expectorated sputum samples. (If the results from the initial sputum samples are nondiagnostic, consider repeat sputum AFB smears and cultures.)
or
2. Positive culture results from at least one bronchial wash or lavage.
or
3. Transbronchial or other lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM or biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.
4. Expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination.
5. Patients who are suspected of having NTM lung disease but who do not meet the diagnostic criteria should be followed until the diagnosis is firmly established or excluded.
6. Making the diagnosis of NTM lung disease does not, *per se*, necessitate the institution of therapy, which is a decision based on potential risks and benefits of therapy for individual patients.

Abbreviations: AFB, acid-fast bacilli; NTM, nontuberculous mycobacteria

According to the WHO, one-third of the world's population is thought to have a latent tuberculosis infection (LTBI). However, there is no evidence of a latent phase in NTM infections, which, in part, explains the low frequency of NTM disease compared with tuberculosis reactivation in patients undergoing anti-TNF treatment. Furthermore, NTM infections usually present after at least 12 months of anti-TNF treatment and are, therefore, considered to be new infections. The generally lower pathogenicity of NTM species, as opposed to *M. tuberculosis*, could further explain the lower frequency of anti-TNF-associated NTM disease. Moreover, underdiagnosis of NTM disease due to insufficient awareness of the pathogenic potential of NTM species is a recognized problem.[42] The prominent role of TNF in the pathogenesis of NTM infection was proven in mouse models, where anti-TNF treatment increased the mycobacterial burden.[43]

Screening and prevention of mycobacterial disease

If neglected, LTBI can progress rapidly to active and potentially fatal disease during immunosuppressive treatment, highlighting the importance of screening patients for deciding whether or not they should receive such therapy. Both the

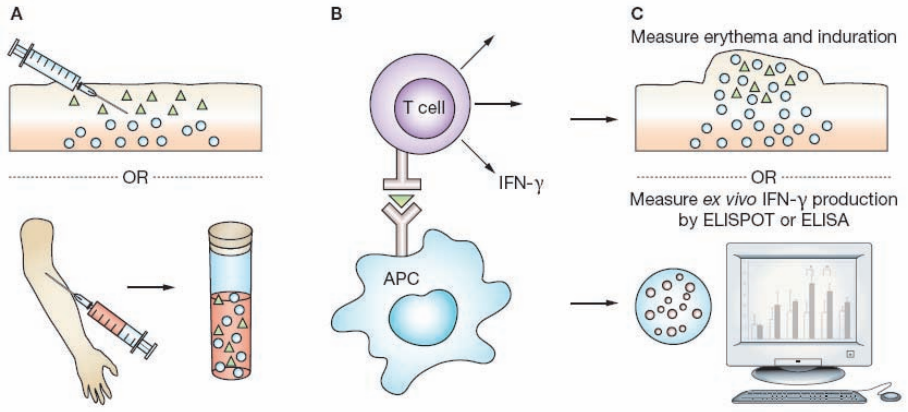

Figure 2: Immunodiagnosis of latent tuberculosis is based on antigen recognition by previously sensitized T cells

(**A**) Antigens are delivered intradermally for the tuberculin skin test, and exposure takes place in sampled blood *ex vivo* for the IFN-γ release assays. (**B**) T cells produce many cytokines, including IFN-γ, upon recognition of the *Mycobacterium tuberculosis* complex antigens. (**C**) In the skin, T-cell recognition triggers a local inflammatory reaction leading to erythema and induration; IFN-γ production is analyzed by ELISPOT or ELISA in the IFN-γ release assay.
Abbreviations: APC, antigen presenting cell; ELISA, enzyme linked immunosorbent assay; ELISPOT, enzyme-linked immunosorbent spot; IFN, interferon.

US Centers for Disease Control and Prevention and the British Thoracic Society have produced recommendations for LTBI screening in patients who are eligible for anti-TNF therapy. These guidelines include an assessment of the infection risk through focused history taking, TST (Figure 2) and chest radiography.[6,44,45] However, interpretation of a TST is difficult in patients who are already receiving immunosuppressive treatment.[44,45] The latest additions to these tools are the T-cell interferon-γ release assays (TIGRAs). These tests are based on the detection of interferon-γ production by T cells, stimulated by the recognition of mycobacterial antigens (Figures 1 and 2), from a whole blood sample.[46] Although calculating the sensitivity and specificity of TIGRAs is hampered by the lack of an accurate 'gold standard' diagnostic test for LTBI, the assays have demonstrated a better diagnostic performance than TST for LTBI screening in candidates for anti-TNF therapy.[47,48] Hence, these tests are increasingly incorporated into national screening guidelines, for instance in Switzerland.

As there is no evidence of the existence of a latent phase in NTM disease, screening for NTM before initiating immunosuppressive treatment might not be feasible. Thus, early detection during immunosuppressive therapy should be the goal in this setting. Currently, there are no specific tests for the detection of NTM infection. TSTs are mostly negative in patients with NTM infections, and chest radiography shows diverse, partly species-specific patterns;[2] moreover, these features represent active NTM disease and cannot be used to identify

early infection. TIGRAs are probably of no use for detecting NTM disease, as only a limited subset of NTM, including *M. kansasii*, *M. szulgai* and *M. marinum*, have antigens similar to those used in the TIGRAs, namely ESAT-6 and CFP-10.[49] The prevalence of TIGRA positivity in patients infected by these NTM is a focus of ongoing investigations in our institutes. Thus, in the absence of a specific test for early NTM disease, especially in patients with additional risk factors (such as COPD and bronchiectasis), sputum should be repeatedly cultured for mycobacteria.[39,42] In patients suspected of such predisposing pulmonary diseases, chest radiography or CT could be considered. In addition, extrapulmonary disease should be carefully investigated, including staining of biopsy samples for acid-fast bacilli and performing mycobacterial cultures.
Preventive treatment, usually 6-9 months of isoniazid therapy, for those with assumed LTBI is now advocated,[1,6,43,44] and has been shown to be successful.[1,33] The first cases of anti-TNF-associated tuberculosis in patients who received preventive treatment have already been published: some could be related to noncompliance, and some are probably newly acquired infections based on the long interval (up to 35 months) between the initiation of anti-TNF therapy and disease onset, although this has not been proven by DNA-typing.[50] Paradoxically, isoniazid is used for tuberculosis prophylaxis, whereas it is more effective against actively replicating bacilli than the dormant bacilli of LTBI.[51]

Treatment of mycobacterial disease during anti-TNF therapy

The appropriate treatment of LTBI before initiation of anti-TNF therapy is the same as that for standard LTBI. Patients in whom active disease is encountered during pretreatment screening should complete their tuberculosis treatment before starting anti-TNF therapy. If necessary, anti-TNF therapy can begin in these patients after the first 2 months of tuberculosis treatment (the intensive phase), in case of infection by a fully susceptible tuberculosis strain.[43,44] No specific recommendations exist for antimycobacterial treatment in the event of active tuberculosis disease during anti-TNF treatment; thus, the recommendations from the American Thoracic Society/ Centers for Disease Control and British Thoracic Society for treatment duration according to localization of disease apply in this setting. The experience of treating NTM disease in the setting of anti-TNF therapy is limited. Furthermore, the treatment of these patients is complicated because different regimens exist for the different bacterial species of NTM, based mostly on their varying growth rates. For disease caused by slow-growing NTM, the American Thoracic Society advocates that treatment with rifampicin and ethambutol should be continued until 12 months after culture conversion, with the addition of macrolides for *M. avium* complex disease.[2] The results of a recent trial by the British Thoracic Society, however, demonstrated no additional effect of macrolides in this setting;[52] hence, they still consider 2 years of rifampicin and ethambutol to be the optimal treatment approach in these patients.[52]

For bacteria with a rapid growth rate, such as *M. fortuitum* and *M. abscessus*, a combination of macrolides, parenteral agents, quinolones or other broad-spectrum antibiotics based on *in vitro* susceptibility of the isolate should be used.[2] However, the outcome of NTM treatment is often disappointing,[2,52] and expert consultation should always be sought in the setting of NTM disease during immunosuppressive therapy.[2]

An increased risk of paradoxical response to antimycobacterial therapy, characterized by development of previously nonexistent tuberculosis lesions or worsening of pre-existing lesions during antituberculosis treatment in patients who initially improved, has been reported in infliximab-treated tuberculosis patients and in one case of NTM disease.[3,53] Garcia Vidal *et al.*[53] suggested that concurrent low-dose anti-TNF treatment might produce immunological regulation that is beneficial for this group of patients.[53] Furthermore, the disruption of granuloma formation by anti-TNF agents could increase exposure of the bacteria to antimycobacterial drugs, resulting in improved outcomes for the infection.[54] A 2008 report observed no complications in three patients who restarted anti-TNF treatment immediately after antituberculosis treatment.[55] Evidence that restarting anti-TNF therapy during antimycobacterial therapy is safe and effective for the treatment of mycobacterial infection first emerged from a case reported by Matsumoto and collegues,[56] where infliximab therapy was safely restarted after 2 months of antituberculosis treatment. In one patient with pulmonary *M. szulgai* infection, anti-TNF treatment was continued during antimycobacterial therapy, which lead to a favorable outcome of the patient's infection and control of his rheumatological complaints without unexpected reactions.[39] However, in the absence of sufficient safety data, whether or not anti-TNF treatment can be continued during antimycobacterial treatment remains a matter of debate. The most recent American College of Rheumatology recommendations consider active tuberculosis infection prior to completing a standard regimen of antituberculosis therapy to be a contraindication for treatment with biologic agents; no information is available for NTM disease.[57]

Conclusions

Mycobacterial disease is a major cause of severe infection in patients receiving anti-TNF therapy, and is associated with anti-IL-1 and anti-CD20 antibody treatment for rheumatic disease. Although tuberculosis is the most frequently observed mycobacterial disease, NTM infection is an increasingly recognized condition in this setting. Among the drug classes currently in development for antirheumatic therapy, anti-IL-17 and anti-IL-23 antibodies, agents that modify JAK-STAT-signaling, and metalloproteinase inhibitors are likely to confer an increased risk of mycobacterial infection. The treatment of NTM disease is difficult, and the outcome is often disappointing. Screening and preventive treatments for LTBI have lowered the incidence of anti-TNF-related tuberculosis; however, these treatments cannot be applied to NTM

disease. All patients receiving drugs that are associated with an increased risk of mycobacterial infection should be carefully monitored. Monitoring should include invasive techniques to diagnose extrapulmonary disease and repeated mycobacterial culture of pulmonary samples, especially in patients with a history of pulmonary disease. The prevalence of NTM disease and the possibility of simultaneous anti-TNF and antimycobacterial treatment in this setting should be the focus of future studies.

Supplementary information in the form of a Box is available on the *Nature Clinical Practice Rheumatology* website.

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Chapter 4

How can we improve treatment outcome in nontuberculous mycobacterial disease?

4.1 *In vitro* drug susceptibility of 2275 clinical nontuberculous *Mycobacterium* strains of 49 species in the Netherlands.
In Revision

4.2 *In vitro* activity of thioridazine against mycobacteria
Int J Antimicrob Agents: 2009 Aug;34(2): 190-1.

4.3 Surgery for nontuberculous mycobacterial lung disease: Strike in time
Int J Tuberc Lung Dis: In Press

In vitro drug susceptibility of 2275 clinical nontuberculous *Mycobacterium* isolates of 49 species in the Netherlands

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Abstract

Objectives: To compile results of drug susceptibility testing on a wide variety of nontuberculous mycobacteria isolated from clinical samples in the Netherlands and discuss their implications for treatment regimens.

Methods: We subjected 2275 strains of 49 different species of nontuberculous mycobacteria, isolated from 2072 patients in the Netherlands between 2000 and 2007, to drug susceptibility testing with the 25 wells Middlebrook 7H10 agar dilution method. Isoniazid, rifampicin, rifabutin, ethambutol, clarithromycin, ciprofloxacin, cycloserine, prothionamide, amikacin, clofazimine and streptomycin were included in the test panel.

Results: The patterns of drug susceptibilities and minimum inhibitory concentrations were found conserved within species and differed significantly between species, revealing their value in taxonomy. Most nontuberculous mycobacteria were susceptible to clarithromycin. Susceptibility to ciprofloxacin and amikacin was less frequent and limited to *Mycobacterium kansasii*, *M. xenopi*, *M. fortuitum* and species phylogenetically related to these three species. Susceptibility to first-line anti-tuberculosis drugs was rare, except for *M. kansasii* and phylogenetically related species. Slowly growing nontuberculous mycobacteria were susceptible to second-line anti-tuberculosis drugs such as rifabutin, cycloserine, clofazimine and prothionamide; the latter also had activity against *M. fortuitum* and related rapid growers.

Conclusions: Clarithromycin and rifabutin are most active against nontuberculous mycobacteria. The activity of other second-line anti-tuberculosis drugs is not supported by clinical data. To improve the utility of drug susceptibility testing, the selection of drugs should be changed to more drugs with proven clinical efficacy, correlating with *in vitro* susceptibility.

Introduction

There is an increasing number of clinical isolates of nontuberculous mycobacteria (NTM) in many countries and growing awareness of their ability to cause disease.[1,2] Nontuberculous mycobacteria are opportunistic pathogens that occasionally can cause severe disease, usually in patients with pre-existent pulmonary disease or systemic impairment of immunity.[1] Treatment of NTM disease is time consuming and often complicated. Macrolide-based multi-drug regimens are currently advocated by the American Thoracic Society (ATS).[1] In a recent trial of the British Thoracic Society (BTS) no beneficial effect of macrolides to treatment of patients with pulmonary *M. avium* complex (MAC), *M. malmoense* and *M. xenopi* disease was found.[3] Overall, the degree of evidence to support the choice of treatment is limited since few clinical trials have been conducted, especially for disease due to less prevalent NTM species; current guidelines are mainly based on case reports and clinical experience.

Although there is a lack of correlation between *in vitro* drug susceptibility testing (DST) results and *in vivo* treatment outcome, DST is nevertheless valuable, especially in patients with no response to first line treatment, or with a relapse of prior NTM disease.[1] For many of the infrequently isolated NTM species, no DST results have been published and there is little basis to predict a potentially successful treatment regimen.

A variety of DST methods have been applied to NTM. The Clinical and Laboratory Standards Institute (CLSI) currently recommends broth-based methods for MAC and related slowly growing NTM, with the broth micro-dilution technique considered also suitable for rapid growers.[4] Application of these techniques has highlighted the therapeutic potential of the macrolides, linezolid and tigecycline for disease due to both slow and rapid growing NTM.[1,4-6]

In the Netherlands, a Middlebrook 7H10 agar dilution method has been used for over a decade with favorable results for *Mycobacterium tuberculosis* complex isolates.[7] This study reports the DST results on a wide variety of nontuberculous mycobacteria isolated from clinical samples in this country and discusses the implications of these findings for NTM treatment regimens.

Materials and Methods

We collected DST results of all clinical NTM isolates subjected to laboratory diagnosis at the Dutch National Institute for Public Health and the Environment (RIVM), which serves as the national mycobacteria reference laboratory, in the period between January 2000 and January 2007.

Identification to the species level was performed by first ruling out membership of the *M. tuberculosis* complex, using the GenoType MTBC line-probe assay (Hain Lifesciences, Nehren, Germany), followed by

application of the INNO-LiPA MYCOBACTERIA v2 reverse line-blot (Innogenetics NV, Ghent, Belgium) for the more common NTM species. If identification to species level was not possible by these methods, 16S rDNA sequencing (151bp hypervariable region A) was performed. Its result was compared with the BLAST (National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov) sequence database. Prior to 2004, 16S sequencing was performed for all isolates after ruling out the *M. tuberculosis* complex, *M. avium* complex and *M. avium* by the respective Accuprobe kits (GenProbe, San Diego, US).

Drug susceptibility testing was performed using the 25 wells agar dilution method, as recently published.[7] In short, dilutions of antimycobacterial drugs are mixed in liquefied 7H10 agar and filled out in 25-well plates. After inoculation and incubation, the bacterial growth at different concentrations of the antimycobacterial drugs is compared to that on agar in a well without the drug and inoculated with 1/100 of the inoculum. The minimum inhibitory concentration (MIC) is the lowest concentration of antimycobacterial drugs that inhibits more than 99% of the growth of the mycobacterial inoculum. Dilutions of antimycobacterial drugs in 7H10 agar are prepared with the following concentrations: 0.1, 0.2, 0.5, 1, 2, 5, 10 and 20 mg/L isoniazid (Sigma Chemical Co., St. Louis, MO); 0.1, 0.2, 0.5, 1, 2, and 5 mg/L rifampicin (Sigma); 1, 2, 5, 10, and 20 mg/L streptomycin (Sigma); 1, 2, 5, 10, and 20 mg/L ethambutol (Sigma); 1, 2, 5, 10 and 20 mg/L amikacin (ICN Biomedicals, Inc., OH); 1, 2, 4, 8 and 16 mg/L ciprofloxacin (Bayer, Mijdrecht, NL); 2, 4, 8, 16 and 32 mg/L for clarithromycin (Abbott, North Chicago, IL); 2, 5, 10, 20 and 50 mg/L cycloserine (Sigma); 1, 2, 5, 10 and 20 mg/L prothionamide (Sanavita, Werne, Germany); 0.5, 1, 2, 5 and 10 mg/L clofazimine (Novartis Pharma, Breda, NL); and 0.2, 0.5, 1, 2 and 5 mg/L rifabutin (Pharmacia, Capelle a/d IJssel, NL).

The breakpoint concentrations are 1 mg/L for isoniazid and rifampicin, 5 mg/L for ethambutol, streptomycin, prothionamide and amikacin, 2 mg/L for ciprofloxacin, rifabutin and clofazimin, 16 mg/L for clarithromycin and 50 mg/L for cycloserine.[4]

Growth at the breakpoint concentration is reported as susceptible, and growth at higher concentrations of the drug is considered resistance. Isolates with MICs of 0.2 or 0.5 mg/L for isoniazid, or 5 or 10 mg/L for ethambutol and streptomycin, are reported as "intermediate resistant".

For each series of isolates inoculated on DST plates, one *Mycobacterium gordonae* strain, one *Mycobacterium avium* strain and three *M. tuberculosis* control strains are included as internal controls. As a secondline quality control, strains are exchanged in a blind fashion with a peripheral laboratory and retested to test the reproducibility.

Table 1: Qualitative *in vitro* drug susceptibility testing results, per species

Species/ drug	n	INH	RIF	ETH	STR	RIB	AMI	CIP	CLA	CYC	CLO	PRO
M. avium	688	R 100	R 99	R 49	R 100	**S 86**	R 100	R 95	**S 80**	**S 95**	**S 93**	**S 96**
MAC	118	R 99	R 96	R 75	R 99	**S 86**	R 98	R 92	**S 78**	**S 98**	**S 92**	**S 97**
MAC-X	29	R 93	R 79	I 48	R 86	**S 96**	R 97	R 90	**S 96**	**S 97**	**S 100**	**S 76**
M. intracellulare	201	R 99	R 95	I 56	R 99	**S 94**	R 98	R 99	**S 96**	**S 85**	**S 98**	**S 91**
M. paraffinicum	8	R 100	R 88	R 50	R 100	**S 100**	R 100	R 100	**S 100**	**S 100**	**S 100**	**S 75**
M. scrofulaceum	8	R 100	R 100	R 75	R 100	**S 100**	R 100	R 100	**S 100**	**S 100**	**S 100**	**S 100**
M. haemophilum	49	R 100	R 96	R 100	R 65	**S 96**	R 71	**S 88**	**S 94**	**S 78**	R 92	**S 61**
M. bohemicum	8	R 100	**S 100**	R 75	R 88	**S 100**	R 100	R 88	**S 100**	**S 100**	**S 100**	**S 100**
M. malmoense	90	R 100	**S 68**	R 57	R 80	**S 99**	R 79	R 74	**S 97**	**S 96**	**S 99**	**S 98**
M. interjectum	12	R 100	**S 58**	I 58	R 67	**S 100**	R 100	**S 83**	**S 100**	**S 100**	**S 100**	**S 100**
M. lentiflavum	11	I 64	R 100	R 64	R 82	**S 100**	R 100	**S 91**	**S 91**	**S 91**	**S 91**	**S 91**
M. simiae	29	R 100	R 97	R 97	R 100	R 97	R 100	R 62	R 75	**S 100**	**S 90**	**S 66**
M. marinum	61	R 100	**S 97**	**S 95**	**S 93**	**S 97**	**S 95**	**S 88**	**S 97**	**S 97**	**S 100**	**S 97**
M. kansasii	262	I 92	**S 98**	**S 92**	**S 85**	**S 100**	R 54	**S 85**	**S 99**	**S 99**	**S 100**	**S 100**
M. szulgai	23	I 91	**S 96**	**S 91**	**S 91**	**S 100**	**S 87**	**S 74**	**S 100**	**S 87**	**S 100**	**S 100**
M. terrae	11	R 100	R 82	**S 91**	R 82	**S 100**	R 91	R 82	**S 91**	**S 100**	R 64	**S 73**
M. xenopi	50	I 84	**S 82**	R 56	**S 100**	**S 100**	**S 94**	**S 100**	**S 100**	**S 98**	**S 100**	**S 100**
M. noviomagense	10	I 100	R 90	I 60	**S 100**	**S 100**	**S 100**	**S 100**	**S 100**	**S 100**	**S 100**	**S 100**
M. celatum	15	R 53	R 100	I 47	**S 93**	R 93	**S 60**	R 80	**S 93**	**S 87**	**S 93**	**S 100**
M. gordonae	278	R 81	**S 98**	**S 96**	**S 85**	**S 100**	**S 85**	**S 99**	**S 100**	**S 99**	**S 100**	**S 100**
M. abscessus	82	R 100	R 100	R 99	R 100	R 93	R 95	R 93	**S 62**	R 99	R 90	R 93
M. chelonae	54	R 98	R 98	R 100	R 96	R 94	R 96	R 59	**S 81**	R 92	R 80	R 67
M. mageritense	6	R 100	R 100	R 33	R 83	R 100	R 83	**S 100**	R 83	R 100	R 83	R 100
M. fortuitum	46	R 96	R 98	R 67	R 96	R 83	**S 56**	**S 96**	R 83	R 96	**S 54**	**S 67**
M. peregrinum	23	R 100	R 96	R 52	R 70	**S 78**	**S 96**	**S 100**	**S 95**	R 91	**S 70**	**S 65**
MFC	42	R 100	R 95	R 43	R 71	R 62	**S 90**	**S 95**	R 58	R 93	R 60	**S 69**
M. mucogenicum	15	R 93	R 73	**S 60**	R 73	**S 67**	**S 80**	**S 67**	**S 93**	R 73	R 53	R 60
M. alvei	5	R 100	R 100	I 60	R 60	**S 60**	**S 100**	**S 100**	**S 60**	R 80	**S 80**	R 60
M. holsaticum	6	I 83	R 100	**S 83**	**S 100**	**S 100**	**S 100**	**S 100**	**S 100**	**S 100**	**S 83**	**S 100**

Rx: resistant; Sx: susceptible; Ix: intermediate susceptibility; x: percentage of strains with the qualitative test result

Results

We performed DST on 2275 isolates of 49 different NTM species or species complexes, isolated from 2072 patients. The results obtained for published species of which five or more isolates were tested (2240 isolates of 29 species or complexes) are detailed qualitatively in Table 1. The median MIC's measured for these isolates are recorded in Table 2. The results obtained in the least frequent (n<5) species are recorded in Table 3.

We found isolates of most species to be susceptible to the macrolide clarithromycin, most notably among the slow-growing NTM. Resistance to clarithromycin was only noted in the majority of *M. simiae*, *M. mageritense* and *M. fortuitum* isolates (Table 1 & 2).

Clarithromycin susceptibility was variable among *M. avium* complex and *M. fortuitum* complex strains. Most *M. avium* strains had MICs around the breakpoint (16 mg/L; Table 2), with a separate grouping (20%) exhibiting true clarithromycin-resistance (MIC>32 mg/L). For separate *M. intracellulare* sequevars and MAC-X (MAC unidentified by the Inno-Lipa Mycobacteria v2 reverse line blot) median MICs were lower (Table 2). Within the *M. fortuitum* complex, *M. fortuitum* and *M. peregrinum* could be separated as *M. fortuitum* is resistant to clarithromycin and rifabutin, whereas *M. peregrinum* was found susceptible (Table 1).

Resistance to the fluoroquinolone ciprofloxacin was more frequent and noted in the majority of *M. avium* complex, *M. chelonae* and *M. abscessus*, the phylogenetically related *M. malmoense* and *M. bohemicum,* as well as in *M. simiae*, *M. terrae* and *M. celatum* isolates (Table 1).

Ciprofloxacin susceptibility was variable among *M. simiae*, *M. terrae*, *M. malmoense* and *M. mucogenicum* isolates. The MICs were either around the breakpoint concentration (*M. terrae*, *M. malmoense*; Table 2), or there were separate strains with low and high MICs (*M. simiae*, *M. mucogenicum*).

We found *in vitro* susceptibility to first line anti-tuberculosis drugs, especially isoniazid and rifampicin, to be rare among NTM. However, susceptibility to rifabutin was frequent (Table 1). The phylogenetically related *M. kansasii*, *M. szulgai* and *M. marinum* are susceptible to most classes of antimycobacterial drugs, as are the phylogenetically more distant *M. xenopi* and *M. gordonae*.

The *M. marinum*, *M. kansasii*, *M. szulgai* group and *M. xenopi*, *M. noviomagense* and *M. celatum* were susceptible to amikacin. Susceptibility to the second line anti-tuberculosis drugs (clofazimin, cycloserine and prothionamide) was common, especially among slow-growing NTM. Slow and rapid growing NTM are divided by cycloserine susceptibility, with *in vitro* resistance restricted to the rapid growers, except for *M. holsaticum*.

Serial isolates were available for 237 patients (n=587; 2.5 per patient). For serial isolates, two-fold changes in MICs for any drug were common. Four-fold changes in MICs for any drug occurred in 5% of patients. This percentage was consistent among the different drugs tested.

Table 2: Median MIC (mg/L) of all drugs included in the panel, per species

Species/ drug	n	INH	RIF	ETH	STR	RIB	AMI	CIP	CLA	CYC	CLO	PRO
M. avium	688	5	>5	10	>20	2	>20	>16	16	20	1	=<1
MAC	118	5	>5	20	>20	2	>20	>16	16	50	1	2
MAC-X	29	5	>5	10	>20	1	>20	>16	4	20	=<0.5	=<1
M. intracellulare	201	5	>5	10	>20	1	>20	>16	8	50	=<0.5	2
M. paraffinicum	8	5	>5	20	10	1	>20	4	4	20	=<0.5	=<1
M. scrofulaceum	8	10	5	20	20	1	>20	>16	4	20	1	=<1
M. haemophilum	49	>20	=<0.1	>20	10	=<0.2	>20	=<1	=<2	50	=<0.5	2
M. bohemicum	8	>20	0.5	20	20	=<0.2	>20	16	=<2	=<2	=<0.5	2
M. malmoense	90	5	1	20	>20	=<0.2	>20	4	=<2	50	=<0.5	=<1
M. interjectum	12	5	0.5	10	10	=<0.2	10	=<1	=<2	10	=<0.5	=<1
M. lentiflavum	11	1	>5	20	10	1	20	=<1	16	=<5	1	=<1
M. simiae	29	10	>5	20	>20	>5	>20	2	>32	50	1	5
M. marinum	61	10	0.2	2	2	=<0.2	2	=<1	=<2	10	=<0.5	=<1
M. kansasii	262	1	0.2	5	5	=<0.2	10	2	=<2	10	=<0.5	=<1
M. szulgai	23	1	0.5	5	5	0.5	5	2	=<2	50	=<0.5	=<1
M. terrae	11	>20	>5	5	20	1	>20	8	=<2	50	5	5
M. xenopi	50	0.5	0.5	20	=<1	=<0.2	5	=<1	=<2	50	=<0.5	=<1
M. noviomagense	10	0.5	2	10	=<1	=<0.2	2	=<1	=<2	50	=<0.5	=<1
M. celatum	15	2	>5	10	5	>5	5	8	=<2	50	1	=<1
M. gordonae	278	2	0.2	2	5	=<0.2	5	=<1	=<2	20	=<0.5	=<1
M. abscessus	82	>20	>5	>20	>20	>5	>20	8	=<2	>50	5	>20
M. chelonae	54	>20	>5	>20	>20	>5	>20	4	=<2	>50	5	>20
M. mageritense	6	>20	>5	10	>20	5	>20	=<1	>32	>50	5	>20
M. fortuitum	46	5	>5	20	>20	>5	5	=<1	>32	>50	2	2
M. peregrinum	23	2	>5	20	20	2	=<1	=<1	=<2	>50	2	2
MFC	42	>20	>5	10	>20	>5	5	=<1	>32	>50	5	2
M. mucogenicum	15	>20	>5	2	20	0.5	5	=<1	=<2	>50	5	>20
M. alvei	5	>20	>5	10	20	2	2	=<1	=<2	>50	2	>20
M. holsaticum	6	1	>5	=<1	=<1	1	=<1	=<1	=<2	20	1	2

Acquired resistance, defined as a resistant follow-up isolate after a susceptible primary isolate, was noted in 28 patients; acquired macrolide resistance was most frequent (22 patients; 11 *M. avium*, 3 *M. intracellulare*, 2 MAC, 3 *M. abscessus*, 1 *M. chelonae*, 1 *M. kansasii* and 1 *M. malmoense*), followed by acquired rifampicin resistance (n=5; 4 *M. malmoense*, 1 *M. szulgai*) and acquired ciprofloxacin resistance (n=3; 1 *M. malmoense*, 1 *M. kansasii* and 1 *M. szulgai*)

Discussion

Analyzing the results of seven years of drug susceptibility testing for NTM using the 25 wells agar dilution method, it is evident that the results yield important clues for optimization of NTM species-specific therapy.

The debate on the role of DST in management of NTM disease is ongoing, mainly because of the observed discrepancies between *in vitro* susceptibility and *in vivo* response to treatment. These discrepancies need further study, especially for the newer antimicrobial drugs and less frequently isolated NTM species. Baseline testing for specific drugs such as rifampicin for *M. kansasii* and clarithromycin for *M. avium* complex isolates helps to understand treatment failure or relapse and to thereafter select second line treatment regimens.[1]

Our *in vitro* results confirm the potential efficacy of the macrolides in the treatment of NTM disease. Only *M. fortuitum*, *M. mageritense* and *M. simiae* isolates are usually macrolide resistant. Clinical trials of macrolide based treatment for *M. avium* complex disease in the United States have demonstrated superior results,[8] compared to previous European trials using exclusively rifampicin and ethambutol only.[9] However, recently published results of the BTS on a comparative trial of 2 years rifampicin, ethambutol and clarithromycin or ciprofloxacin for pulmonary MAC, *M. malmoense* and *M. xenopi* disease were unable to demonstrate superiority of either triple-drug regimen drug over a rifampicin and ethambutol only regimen[3] tested previously in a similar trial setting.[9] Within this recent trial, *in vitro* susceptibility to ciprofloxacin or clarithromycin was not assessed.[3] We found frequent ciprofloxacin resistance among MAC (92-99%) and *M. malmoense* (74%; Table 1) strains. While casting doubt on the efficacy of ciprofloxacin, our results offer no explanation for the limited results in the patients receiving a macrolide based regimen in the BTS study.[3] This illustrates the necessity to acquire more clinical data that establish the correlation between *in vitro* and *in vivo* activity. There could be a role for macrolides in case of first-line treatment failure or relapse with susceptible strains.

Although clarithromycin resistance in primary isolates was rare, 9% (22/237) of our patients with serial isolates acquired clarithromycin resistance during treatment, or experienced a relapse with acquired clarithromycin resistance. *In vitro* resistance to macrolides has already been related to poor *in vivo* treatment outcome; for the first line anti-tuberculosis drugs this relationship has not been established.[1]

For the macrolide resistant species in our study, *in vitro* susceptibility to ciprofloxacin indicates a possible role for the fluoroquinolones. Most *M. simiae* isolates, however, are also resistant to ciprofloxacin (Table 1). Treatment results in *M. simiae* disease are, probably as a result, poor.[10]

The common *in vitro* resistance to ciprofloxacin (Table 1) may explain its limited *in vivo* activity in the latest BTS trial.[3] Moxifloxacin is more active *in vitro* than ciprofloxacin[1] and may be active against isolates that have median MICs just above the ciprofloxacin breakpoint (*M. malmoense*, *M. chelonae*). Combination therapy of moxifloxacin and clarithromycin may not be advisable, as fluoroquinolones were recently demonstrated to attenuate clarithromycin activity in a murine model of *M. avium* complex disease.[11] How this translates to human disease remains to be determined.

The widespread amikacin resistance is contrary to results in available literature, at least for *M. avium* and *M. abscessus*.[1,4] The discrepancies, which may result from the test methods or the applied breakpoint concentrations, are cumbersome since a recent trial of multi-drug MAC therapy demonstrated better sputum conversion rates in patients receiving an aminoglycoside as adjunctive therapy.[12] Hence, reporting false amikacin resistance may negatively affect therapy choice and outcome.

In vitro susceptibility to cycloserin, prothionamide and clofazimine was common, especially among slow-growing NTM. *In vitro* susceptibility of *M. avium* isolates to clofazimine and cycloserin, similar to our findings, was also found in patients in the USA with disseminated infections at the start of the HIV/AIDS epidemic.[13] However, clinical efficacy of these drugs in NTM treatment has not been sufficiently proven and they are limited by their toxicity.[1] Studies in murine models in the 1980s showed limited efficacy of 3-5 drug combination therapy that included these drugs.[14, 15]

Similar DST results may hint at a phylogenetic relationship between various species, for example *M. kansasii*, *M. szulgai* and *M. marinum* (Table 1 & 2). The susceptibility to first line antituberculosis drugs, except isoniazid, observed in these three species hints at their close phylogenetic relationship with the *M. tuberculosis* complex, also observed by Devulder *et al.* in their multi-gene taxonomical model.[16].

The currently available technique and the range of drugs tested offer too little therapy guidance for clinicians and need improvement. Our results call for a reconsideration of the drugs to be included in the test-panel and illustrate the necessity to acquire more clinical data that establishes the correlation between *in vitro* and *in vivo* information. There should be separate test panels for slow and rapid growing NTM and new, promising antimycobacterial drugs, such as moxifloxacin, linezolid, the carbapenems and tigecyclin should be included in test panels and their breakpoints established. Standardized control strains should be used to allow inter-laboratory comparisons. Application of liquid culture systems for DST may decrease the turnaround time. Recently,

Table 3: DST results of seldom encountered NTM

Species	n	INH	RIF	ETH	STR	RIB	AMI	CIP	CLA	CYC	CLO	PRO
M. conspicuum	4	R	R	S	S	S	R	S	S	S	S	S
M. palustre	4	R	S	I	R	S	R	S	S	S	S	S
M. gilvum	2	R	S/R	S	S	S	S	S	S	S	S	S
M. hiberniae	2	R	R	S	S	S	S	S	S	S	S	S
M. cosmeticum	1	R	R	I	R	R	S	R	S	S	R	R
M. florentinum	1	R	R	I	R	S	R	R	S	S	R	S
M. hassiacum	1	I	S	S	S	S	S	S	S	S	S	S
M. neoaurum	1	R	S	R	S	S	S	S	S	R	S	R
M. nonchromogenicum	2	R	R	S	S	S	R	S	S	S	S/R	S
M. novocastrense	1	I	R	S	S	S	S	S	S	S	S	S
M. obuense	2	R	R	R	S	S	S	S	S	R	S	S/R
M. phlei	3	R	R	S	S	S	S	S	S	S	R	R
M. porcinum	1	R	R	R	R	R	S	S	R	R	R	R
M. shimoidei	1	I	R	S	S	S	S	S	S	S	S	S
M. smegmatis	1	R	R	S	S	R	S	S	R	R	S	R
M. sphagni	1	R	R	S	S	S	S	S	S	S	S	R
M. vanbaalenii	1	R	R	S	S	S	S	S	S	S	S	S
M. wolinskyi	2	R	R	S	S/R	S	R	S	R	R	S	R
M. nebraskense	1	R	S	S	R	S	R	S	S	S	S	R
M. heidelbergense	3	I	R	I	S	S	R	S	S	S	S	S

R: resistant; S: susceptible; I: intermediate susceptibility

susceptibility testing for *M. tuberculosis* complex bacteria in automated liquid culture systems has been standardized.[17]

In summary, the 25 wells agar dilution method is an inexpensive and reliable method for drug susceptibility testing of nontuberculous mycobacteria. Results for the macrolides and fluoroquinolones appear useful for therapy guidance. DST can assist in selecting second line treatment regimens in cases of treatment failure or relapse. Most NTM species show *in vitro* susceptibility to macrolides. Against the background of the conflicting results of the clinical trials it is not clear what this means for clinical practice. This illustrates the necessity to acquire more clinical data that establish the correlation between *in vitro* and *in vivo* activity. One could defend a recommendation to use macrolides in case of treatment failure or relapse with susceptible strains. Acquired resistance, mainly to the macrolides, is a significant issue and was also recorded in our study. A reconsideration of the drugs included in the test is needed to improve its utility, especially for rapid growing mycobacteria. The utility of first and second line antituberculosis drug testing is very limited and this should be substituted for newer drugs with proven clinical efficacy correlating with *in vitro* susceptibility. The DST results have additional taxonomical value.

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In vitro activity of thioridazine against mycobacteria

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Thioridazine is an antipsychotic drug that is active against mycobacteria by targeting the type II NADH dehydrogenase, succinate dehydrogenase, and the binding of calcium to proteins as well as disruption of aerobic respiration under microaerobic conditions.[1]

The activity of the thioridazine against mycobacteria has gained renewed attention due to the ongoing spread of multidrug-resistant tuberculosis (MDR-TB).[2] The recent emergence of extensively drug-resistant (XDR-) TB only strengthened the search for new antimycobacterial drugs. Even aggressive treatment regimens including high numbers of drugs at maximum tolerable doses and adjunctive surgery result in cure rates of only 60% in XDR-TB.[3]

Although tuberculosis is a major threat to public health, cure rates for nontuberculous mycobacterial (NTM) disease are comparable to those in XDR-TB. In a recently published trial by the British Thoracic Society (BTS), cure rates after 5 years' follow-up were only in the 20-40% range.[4] These sobering rates are in part due to the pre-existing conditions that increased these patients' susceptibility to NTM disease, e.g. chronic obstructive pulmonary disease. In MDR and XDR-TB, as well as in NTM disease, new drugs are needed to improve cure rates.[3-5] However, most of the drugs currently in the pipeline are tested exclusively for their activity against *Mycobacterium tuberculosis*.

Thioridazine has a well-known safety profile owing to its extensive use in psychiatry and may represent a good candidate for drug trials for MDR-TB, XDR-TB and NTM disease. To establish its possible efficacy, minimum inhibitory concentrations (MICs) of thioridazine were determined for clinical isolates of *M. abscessus* (n=3), *M. avium* (4), *M. kansasii* (1), *M. malmoense* (1), *M. simiae* (1), *M. xenopi* (1), *M. gordonae* (2), *M. fortuitum* (1), *M. szulgai* (2) and *M. tuberculosis* (8), applying a previously described 25-well agar dilution method.[6] The *M. tuberculosis* isolates were four pan-susceptible isolates, three MDR isolates and one XDR isolate.

Susceptibility to thioridazine was tested at concentrations of 1, 2, 4, 8, 16, 32, 64 and 128 mg/L. The MIC was defined as the concentration able to reduce bacterial growth by >99%; Growth on thioridazine-enriched Middlebrook 7H10 medium was compared with an undiluted and a 1/100 diluted standardized bacterial suspension on drug free medium.
The MICs and the time of reading the plates are given in Table 1 for all isolates tested. For the rapid growers (*M. abscessus* and *M. fortuitum*) sufficient growth was noted after 4 days of incubation; most slow growers required 8 or 11 days. Thioridazine is most active against *M. tuberculosis*. All *M. tuberculosis* isolates had MICs of 4 mg/L, regardless of their susceptibility to first- or second-line drugs. In humans, 0.5 mg/L is the acceptable maximum plasma concentration of thioridazine.[2] However, *M. tuberculosis* bacteria residing in macrophages are

Table 1: MICs (mg/L) and duration of incubation to obtain sufficient growth

Strain nr.	Species	MIC (mg/L)	Incubation (days)
NLA000600658	*M.avium*	32	4
NLA000800431	*M. avium*	32	11
NLA000800441	*M. avium*	16	4
C687	*M.avium*	32	11
NLA000600659	*M. avium* complex	16	8
NLA000800050	*M. malmoense*	32	11
NLA000800280	*M. kansasii* type I	2	11
NLA000701790	*M. szulgai*	16	8
NLA000800062	*M. szulgai*	16	8
NLA000800549	*M. xenopi*	8	11
NLA000800295	*M. simiae*	16	8
NLA000600643	*M. gordonae*	16	11
C809	*M. gordonae*	16	8
NLA000600203	*M. abscessus*	64	4
NLA000800454	*M. abscessus*	64	4
NLA000800448	*M. abscessus* sv	16	4
NLA000800494	*M. fortuitum*	32	4
NLA000600443	XDR-*M. tuberculosis*	4	11
NLA000500538	MDR-*M. tuberculosis*	4	11
NLA000501371	MDR-*M. tuberculosis*	4	11
NLA000601463	MDR-*M. tuberculosis*	4	11
NLA000600523	*M. tuberculosis*	4	8
C100	*M. tuberculosis*	4	11
C2147	*M. tuberculosis*	4	11
C937	*M. tuberculosis*	4	11

sv, smooth colony variant; X/MDR, extensively/multi-drug resistant

susceptible to thioridazine even at 0.1 mg/L, since macrophages concentrate thioridazine in the vacuoles where *M. tuberculosis* resides.[2]

Thioridazine is less active against NTM, especially against rapid growers. Results were consistent among isolates of the same species, or differed by a single two-fold step, as for *M. avium*. MICs in the range of 16 to 32 mg/L, the most common MICs for NTM, appear to preclude a role for thioridazine in therapy of NTM disease. The low level of activity against *M. abscessus* is especially unfortunate. Bacteria of this species can cause severe disease and their level and extent of drug resistance demands new, active compounds.[5] Whether the concentrating effect within macrophages will lead to local drug concentrations capable of killing NTM remains to be established. Based on our *in vitro* results this seems less likely.

Although its cardiotoxicity is a significant drawback,[7] thioridazine may add to the activity of regimens for MDR- or even XDR-TB, for which very few active drugs are currently available. A potential role for thioridazine in the treatment of latent TB, due to its disruption of aerobic respiration under microaerobic conditions, may warrant separate investigation.

Thioridazine appears less likely to add activity to the regimens for pulmonary NTM disease propagated by the American Thoracic Society and BTS.[4,5]

In summary, thioridazine is active *in vitro* against susceptible and MDR or XDR *M. tuberculosis*. Due to a concentrating effect within macrophages, there may be a role for thioridazine in the treatment of MDR- or even XDR-TB. With MICs at least four- to eight-fold higher than those observed for *M. tuberculosis*, its activity against NTM is significantly lower, thus a role in treatment of NTM disease is less likely.

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Surgical treatment of nontuberculous mycobacterial lung disease: Strike in time

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Abstract

Background: Drug therapy for nontuberculous mycobacterial (NTM) lung disease yields limited results. Adjunctive surgery is beneficial in selected cases, though its indication and timing are not well described in literature. Therefore, we describe our experiences with the adjunctive role and benefits of surgery for pulmonary NTM disease, specifically addressing its indications and timing.

Methods: We performed a retrospective medical file review of eight patients who underwent surgical treatment for NTM lung disease in the period of January 2000 to January 2009, as well as a review of available literature.

Results: Therapy-resistant cavitary NTM disease was the most frequent indication for surgery; two patients underwent pneumonectomy for an infected destroyed lung. *Mycobacterium avium* was the most common causative agent. Surgery resulted in culture conversion in seven patients; one patient died two months after pneumonectomy. No relapses have been noted in the other seven after an average of 19 months of follow-up.

Conclusions: Adjunctive surgical treatment for NTM lung disease yields encouraging results, similar to previously published case series. Careful patient selection, based on extent and type of disease as well as on cardiopulmonary fitness, is important. Potential benefits of surgery should be considered for every individual patient in whom NTM lung disease is diagnosed and re-evaluated after six months of treatment. Where possible, surgery should be pursued and timely conducted.

Introduction

Pulmonary disease due to nontuberculous mycobacteria (NTM) is a condition that has many faces, ranging from hypersensitivity pneumonitis to nodular-bronchiectatic disease in previously healthy women to the classical cavitary disease type that mostly affects males with pre-existing pulmonary disease.[1] Cavitary disease is the most frequent disease type in the Netherlands. It is one of the factors contributing to a poor response to therapy, alongside previous treatment and a history of chronic obstructive lung disease or bronchiectasis.[2]

Drug therapy of NTM disease is long-term, cumbersome because of its side effects, and often yields poor results. In a recent trial by the British Thoracic Society (BTS), only 36% of 371 patients with mostly cavitary disease who received 24 months of multi-drug therapy for pulmonary NTM disease were alive and cured after five years of follow-up. Fifteen percent of the patients had treatment failure, relapsed or died due to NTM disease.[3] Similar results have been observed recently in retrospective studies in the Netherlands.[4] These data show striking similarities with those for multidrug-resistant tuberculosis and confirm that treatment needs to be improved. Previous authors have, for both conditions, suggested adjunctive surgical treatment, although important caveats are made.[5-7] Surgery should be an adjunctive treatment modality, to be performed in a multidisciplinary program on a select category of patients.[5,7]

The University Lung Centre Dekkerswald is a third-line reference hospital for mycobacterial disease, and part of the Radboud University Nijmegen Medical Centre in Nijmegen, the Netherlands. Dekkerswald is the only centre in the Netherlands with experience in the field of surgery for pulmonary NTM disease and tuberculosis. In this study we explore the potential benefits, timing and in- and exclusion criteria of surgery for pulmonary NTM disease based on our experience in recent years and a review of available literature.

Methods

We reviewed the medical files of all patients who underwent surgical treatment for pulmonary NTM disease at the University Lung Centre Dekkerswald, in the January 2000 - January 2009 period. From the medical files we extracted demographic and clinical data, focusing on the timing of surgery, pre- and post-surgery drug therapy and final outcome.

Nontuberculous mycobacterial disease was diagnosed using the American Thoracic Society (ATS) diagnostic criteria available at the time of patient referral.[1,8]

Identification of the NTM isolates was performed at the Dutch National Mycobacteria Reference Laboratory, by the INNO-LiPA MYCOBACTERIA v2 reverse-line blot. Drug susceptibility testing was performed using the agar dilution method.[9]

No ethical approval was required for the current study.

Preoperative assessment

Patients' eligibility for surgery was discussed in multidisciplinary meetings with anaesthesiologists, thoracic surgeons and respiratory physicians. In absence of specific criteria for patient selection, the BTS and the American College of Chest Physicians' (ACCP) guidelines for patient selection for lung cancer resection were followed,[10,11] as suggested in the recent ATS statement on NTM disease.[1] Thus, patients were evaluated by maximal cycle ergometry, ventilation perfusion scanning and spirometry, to estimate postoperative FEV1 and DLCO, as well as by body mass index (BMI) measurement, nutritional assessment, with dietary supplementation when indicated, and exclusion of significant cardiac disease.

Peroperatively, all patients received three intravenous doses of amoxicillin/clavulanic acid 1000/200mg.

Results

In the study period eight patients underwent surgical treatment at Dekkerswald. The baseline characteristics of the patients are outlined in Table 1. Six patients were referred to our hospital because of therapy resistant cavitary NTM disease, two for NTM infection of a destroyed lung due to prior tuberculosis (Figures 1 and 2);

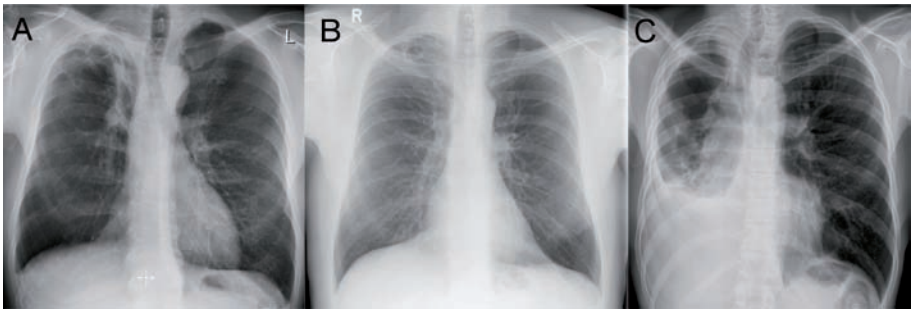

Figure 1: Chest radiographs of patients who underwent surgical treatment

A&B: Right upper lobe cavitary disease caused by *M. avium* (Table 1: patient 2) and *M. xenopi* (patient 5); C: Destroyed right lung caused by prior tuberculosis, super-infected by *M. avium* (patient 1)

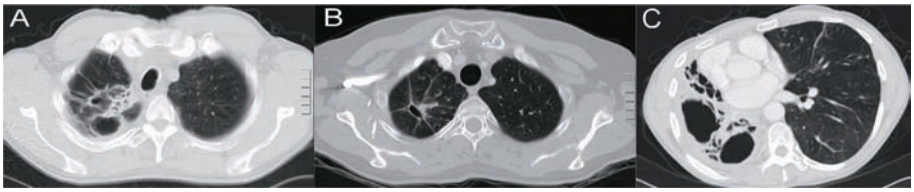

Figure 2: Computed Tomography images of patients who underwent surgical treatment

A&B: Right upper lobe cavitary disease caused by *M. avium* (Table 1: patient 2) and *M. xenopi* (patient 5); C: Destroyed right lung caused by prior tuberculosis, super-infected by *M. avium* (patient 1)

none had disease related complications necessitating surgery, e.g. hemoptysis. *Mycobacterium avium* was the causative agent of disease in six patients; two patients had clarithromycin resistant isolates. *M. xenopi* and *M. avium* complex, other than *M. avium* or *M. intracellulare*, bacteria were causative agents in one patient each. All patients were HIV-negative and had intact interferon-γ/IL-12 pathways.

The average duration of treatment at the time of surgery was 22 months, although three patients had received previous cycles of treatment for NTM disease (Table 1). The four patients that are currently alive and completed post-surgical chemotherapy received an average of 9 months of treatment after surgery. The average duration of follow-up is 19 months.

The procedures performed are detailed in Table 1. Two pneumonectomies were performed, both in patients who did not meet the BTS/ACCP eligibility criteria (Table 1). Surgery and post-surgical chemotherapy resulted in persistent culture-negative status in all but one patient (88%). This patient was oxygen-dependent and had a FEV1 of 0.58L (23% predicted), far below the 2L limit for pneumonectomy.[10,11] After right pneumonectomy, the patient developed respiratory failure and was temporarily re-admitted to the intensive care unit for non-invasive positive pressure ventilation; she died of pulmonary embolism two months after surgery and was not sampled for *Mycobacterium* culture (Table 1, patient 4).

Five out of eight patients experienced a complication of surgery (63%), mostly persisting pneumothorax that could be treated conservatively (n=3; 38%). One case of respiratory failure, one case of pneumonia and one case of collapse of the right lower lobe, due to stasis of pulmonary secretions and resolved by suction during bronchoscopy, were noted (Table 1).

Discussion

Surgical treatment adjunctive to multi-drug therapy resulted in culture conversion in seven of eight complicated cases of pulmonary NTM disease. No relapses have yet been noted. Our results, based on a small series of patients, are in line with the few case series published from other centres and recorded in Table 2.[7,12-22] Conversion rates are generally very high (90-100%) and few relapses are noted, marking the efficacy of combined medical and surgical treatment.[7,12-22] Moreover, the current results compare positively to the 67% overall and 50% culture conversion in *M. avium* disease observed after medical treatment in a recent study that included many patients from our centre.[4] Similar outcomes

M, male; F, female; BMI, body mass index; #, with persistent obstruction; Pulm. TB, pulmonary tuberculosis; PE, pulmonary embolism; COPD, chronic obstructive pulmonary disease; ND, not determined; L, left; R, right; UL, upper lobe; LL, lower lobe; MLE, mediastinal lymphnode enlargement; x(H)RECla, (isoniazid+) rifampicin+ethambutol+ clarithromycin regimen of x months duration; Rb, rifabutin; Mox, moxifloxacin; Pro, prothionamide; Ami, amikacin; Z, pyrazinamide; ~x, treatment ongoing, x months completed at the time of data collection.

Table 1: Clinical characteristics of all patients who underwent surgery for pulmonary disease caused by nontuberculous mycobacteria

pt	Sex, age	Species	Pre-existing disease	Pulmonary function	Radiographic features	Pre-treatment	AFB/ Culture at surgery	Surgical procedure	Complications	Post-surgery treatment	Follow-up (months)	Outcome
1	M, 54	*M. avium*	Pulm. TB, 1978 BMI: 18	FEV1: 1.26L VO2max: 19.7 ml/min/kg	Destroyed R lung, nodular lesions L, fibrotic scars L, bronchiectasis LUL	15RECla	- / +	Pneumonectomy R	-	~9RECla	~9 mo	Culture conversion
2	M, 57	*M. avium*	COPD BMI: 23	FEV1: 2.42L VO2max: 35.5 ml/min/kg	Cavitary lesions RUL and apex RLL	34RECla	+ / +	Lobectomy RUL, segmentectomy apex RLL	Pneumonia L	10RE	50 mo	Cured
3	F, 50	*M. avium* Cla-Resistant	Asthma# BMI: 21	FEV1: 1.73L VO2max: 28.9 ml/min/kg	Cavitary lesion LUL, nodular lesions in all lobes L+R	31RECla, previous treatment cycles	+ / +	Lobectomy LUL, wedge excision of pleural lesion	-	12RECla	26 mo	Cured
4	F, 50	*M. avium* Cla-Resistant	Pulm. TB, 1973 BMI: 20	FEV1: 0.58L VO2max: ND	Destroyed R lung, cavity and bronchiectasis LUL, nodular lesions LUL and LLL, MLE	15HRE4RbECl aMoxPro	- / +	Pneumonectomy R	Respiratory failure	2RbEClaM oxProAmi	2 mo	Death due to PE
5	M, 53	*M. xenopi*	COPD BMI: 27	FEV1: 3.25L VO2max: 24.1 ml/min/kg	Cavitary lesion RUL	14HRECla, previously 12HRZE	- / +	Wedge excision apico-dorsal RUL	Persistent pneumothorax	4HRECla	28 mo	Cured
6	M, 41	*M. avium*	COPD BMI: 17	FEV1: 3.45L VO2max: 27.2 ml/min/kg	Cavitary lesion LUL	26RECla	- / +	Lobectomy LUL, wedge excision LLL	Persistent pneumothorax	12RECla	29 mo	Cured
7	M, 48	*M. avium* complex	COPD BMI: 20	FEV1: 2.45L VO2max: 24.2 ml/min/kg	Cavities and bronchiectasis LUL and RUL	18RECla, previous treatment cycles	- / +	Staged Bilobectomy RUL & LUL	Collapse RLL, persistent pneumothorax	~8RECla	~8 mo	Culture conversion
8	M, 59	*M. avium*	Silicosis BMI: 25	FEV1: 3.69L VO2max: 24.7 ml/min/kg	Cavitary lesion RUL	20RECla	- / +	Lobectomy RUL	-	~6RECla	~6 mo	Culture conversion

have been observed in series of adjunctive surgical treatment for multidrug-resistant tuberculosis.[5] Still, retrospective case series are inadequate to determine optimal therapies and comparative trials of drug treatment versus drug and surgical treatment are needed to establish its true benefit. Moreover, culture conversion alone may not be the appropriate endpoint in these complex patients with pre-existing pulmonary conditions. Quality of life and all-cause mortality should be included as endpoints in future trials.

The most critical issues in the decision on this intervention are the selection of patients eligible for surgery and its exact timing. There are no published patient selection criteria for this type of surgery[1] and available case series generally have not reported pulmonary function test results or other physiological parameters such as BMI of their patients.

Timing is essential to prevent disease progression into a stage where safe and effective surgery is no longer possible. Development of cavitary lesions in the contralateral lung, deterioration of patient condition and acquisition of further drug resistance may render adjunctive surgery and post-operative chemotherapy ineffective, as we have noted in patient 4 (Table 1). Mitchell and co-workers considered surgery either at the initial consultation, if evidence of irreversible, focal parenchymal injury emerged during radiologic evaluation, or after failure of adequate therapy.[7] They operated on 236 patients after 2 to 6 months of multi-drug therapy, similar to the three months advocated by Shiraishy and the 6 months advocated by Watanabe.[7,19,22] Clinical improvement and culture conversion are to be expected within 6 months of treatment with macrolide-based regimens.[23] Expert consultation, including assessment of the potential role of surgery, should be sought in this time-frame. In our patients, however, this was not the case. Our patients had already received an average of 22 months of multi-drug therapy, mostly at their regional hospital. Expert consultation is strongly encouraged in the ATS statement on NTM disease[1] and should be commonplace, especially in case of possible treatment failure. Our reference hospital was only consulted after these, sometimes multiple, prolonged treatment courses had failed. Early expert consultation needs to be propagated in the Netherlands. Surgery in an earlier phase of the disease offers the advantage that lesions may be smaller and easier to resect,[15,16,19,22] further lung tissue damage may be prevented and the duration of antimycobacterial drug exposure, with its inherent risks, may be limited.

The possible role of surgery must be considered, in communication with a specialized centre, for every patient who is about to start multi-drug therapy. Even if patients show an initially favorable response to treatment, lesions of their pre-existing pulmonary disease or the cavitary lesions resulting from NTM disease may provide a niche for NTM and eradication by chemotherapy alone may be impossible. The study by Lam *et al*. clearly demonstrated the negative impact of cavitary lesions on culture conversion (hazard ratio 4.00; 95% CI 1.74-9.19; p=0.001);[2] cavitary disease is the most prevalent pulmonary

NTM disease type in the Netherlands.[4] Drug concentrations inside inside cavities may be sub-therapeutic, as within tuberculous pleural effusions and psoas abscesses.[24] Compliance with chemotherapy may decrease during the long treatment of NTM disease. Surgery may thus be best performed when a patient is still motivated and compliant with therapy.

To evaluate a patient's eligibility for surgery, two main aspects are relevant. The first is the localization and extent of disease. Previous studies have proposed cavitary disease, destroyed lung tissue and continued sputum positivity despite maximal drug therapy as indications for surgery.[7,14,16,19,20] Lobectomy or bilobectomy is a possibility if cavitary lesions in an upper lobe are accompanied only by minor nodular lesions. In eligible patients who present with a destroyed lung, either due to NTM or super-infected by NTM, pneumonectomy is the procedure of choice (Table 1).[7]

The second aspect influencing patient eligibility for NTM surgery is the physical condition of the patient. This must be assessed through lung function measurements and radiographic assessment of remaining lung volumes. Moreover, patients should be motivated and their expectations of surgery should be clear.

Extrapolation of the BTS and ACCP guidelines for patient selection for lung cancer resection is reinforced by our single patient who underwent pneumonectomy despite insufficient pulmonary function; this patient (Table 1, patient 4) experienced severe complications (respiratory failure) and died 2 months post-surgery. With the benefit of hindsight, this patient should not have been operated. Besides the clinical aspects of eligibility for surgery, surgical treatment must be performed in a centre that has thoracic surgeons, infectious disease specialists and pulmonary physicians with experience in

Table 2: Outcome of surgical treatment for pulmonary NTM disease in previous reports. *other species were present

Publication	No. of patients	Predominant species	Sputum culture conversion rate	Long term relapse rate
Corpe et al. 1981 11	124	*M. avium* complex	93%	5%
Moran et al. 1983 12	37	*M. intracellulare*	94%	5%
Pomerantz et al. 1991 13	38	*M. avium* complex*	84%	0%
Ono et al. 1997 14	8	*M. avium* complex	100%	13%
Shiraishi et al. 1998 15	33	*M. avium* complex	94%	6%
Nelson et al. 1998 16	28	*M. avium* complex	90%	4%
Lang-Lazdunski et al. 2001 17	18	*M. xenopi*	89%	0%
Shiraishi et al. 2002 18	21	*M. avium* complex	100%	10%
Shiraishi et al. 2004 19	11	*M. avium* complex*	100%	9%
Sherwood et al. 2005 20	26	*M. avium* complex*	82%	0%
Watanabe et al. 2006 21	22	*M. avium* complex	100%	5%
Mitchell et al. 2008 6	236	*M. avium* complex*	100%	0%

Box 1: Proposed indications for adjunctive surgery in patients with pulmonary disease caused by nontuberculous mycobacteria

For patients culture positive after 6 months of optimal multidrug therapy, surgery should be considered if

the disease is

1. limited to one lung, or
2. a maximum of one lobe in each lung, with only nodules in other lobes,

while the patient is

1. in a cardiopulmonary condition that allows for pulmonary surgery and
2. in a nutritional state that allows for surgery (BMI) and
3. motivated

and there is effective post-operative

1. multi-drug treatment possible, as well as
2. care by experienced physicians in a centre that can offer long-term follow-up

NTM disease as well as general pulmonary surgery and capable of providing long-term follow-up. The impact of experience is shown by Mitchell *et al*. who demonstrated a decrease in mortality rate associated with an increase in case volume.[7] In Box 1, we have translated the three eligibility criteria into proposed indications for adjunctive surgery in patients with pulmonary NTM disease.

Our overall complication rate is high, compared to the 50% (19/38) in the case series by Pomerantz *et al*.,[14] 44% (4/9) reported by Lang-Lazdunski *et al*.,[18] 28.6% (6/21) reported by Shiraishi *et al*.,[20] or the 18.5% (49/236) reported by Mitchell *et al*.[7] The predominance of persisting pneumothorax, which could be treated conservatively in our patients, is also recorded by other authors.[7,14,16,18] Interestingly, we have noted no bronchopleural fistula, the most severe complication associated with right pneumonectomy.[7,14,16,18,20] Surgery in an earlier phase of NTM disease may limit the number and severity of complications.[16,19,22]

There are no trials on the duration of post-surgical drug treatment; previous studies recorded good results using 6-12 months of post-surgery drug treatment with macrolide based regimens.[19,22] The ATS currently advocates treatment for twelve months after culture conversion.[1] Thus, if culture conversion is only obtained after surgery, 12 months of post-surgical treatment is conceivable.

It is striking that surgical series predominantly feature patients with pulmonary MAC or, to a lesser extent, *M. xenopi* disease.[7,12-22] This may be related to the fact that these two species are most difficult to eradicate by drug therapy, as demonstrated in the recent BTS trial.[3] Whether this results from host or pathogen factors remains unknown.

In conclusion, a select category of patients can benefit from adjunctive surgical treatment for pulmonary NTM disease. The predominance of cavitary pulmonary NTM disease in Western Europe and its poor response to drug therapy stresses the potential role for surgical treatment. Careful patient selection and expert consultation is of the utmost importance. Possible benefits

of surgery should be considered for every patient in whom pulmonary NTM disease is diagnosed and re-evaluated after six months of treatment. Where possible, surgery should be pursued; Dekkerswald aspires to provide this type of surgery for the Netherlands.

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Chapter 5

The burden of unidentifiable mycobacteria in a national reference laboratory

5.1 Re-analysis of 178 previously unidentifiable *Mycobacterium* isolates in the Netherlands from the 1999-2007 period.
Submitted

5.2 *Mycobacterium noviomagense* sp. nov.; clinical relevance evaluated in 17 patients. *Int J Syst Evol Microbiol 2009; 59(4): 845-9.*

5.3 Proposal to elevate *Mycobacterium avium* complex ITS sequevar MAC-Q to *Mycobacterium vulneris* sp. nov.
Int J Syst Evol Microbiol 2009; 59(9): 2277-82.

5.4 *Mycobacterium mantenii* sp. nov.; A novel pathogenic slowly growing scotochromogenic *Mycobacterium* species.
Int J Syst Evol Microbiol. Epub July 22nd, 2009

Re-analysis of 178 previously unidentifiable *Mycobacterium* isolates in the Netherlands from the 1999-2007 period

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Abstract

Nontuberculous mycobacteria that can not be identified to the species level by reverse hybridization assays and sequencing of the 16S rDNA gene are a challenge for reference laboratories. However, the number of 16S gene sequences added to online public databases is growing rapidly and so is the number of *Mycobacterium* species. Therefore, we re-analyzed 178 *Mycobacterium* isolates with 53 previously unmatched 16S rDNA gene sequences, submitted to our national reference laboratory in the 1999 tot 2007 period. We repeated molecular identification using two commercially available identification kits, targeting separate genetic loci, and repeated 16S rDNA gene sequence comparisons using the GenBank database.

Ninety-three out of 178 isolates (52%) with 20 different 16S rDNA sequences could now be assigned to validly published species. The two reverse hybridization assays provided false identifications for five recently described species and we recorded discrepancies in identification results between the two reverse hybridization assays.

Identification by reverse hybridization assays underestimates the genetic heterogeneity among NTM. This heterogeneity can be clinically relevant as particular sub-groupings of species can cause specific disease types. Therefore, sequence-based identification is preferable, at least at reference laboratory level. The targets needed for clinically useful results remain to be established. The number of NTM species in the environment is probably so high that unidentifiable clinical isolates should be designated a separate species status only if this is clinically meaningful.

Introduction

The isolation frequency of nontuberculous mycobacteria (NTM) increases in many countries where the incidence of tuberculosis is in decline.[1,2] The NTM are ubiquitous in the environment and their presence in clinical samples does not necessarily indicate NTM disease. Assessment of the clinical relevance of these isolates is not straightforward. As an important support, the American Thoracic Society (ATS) has published statements to aid in this assessment.[3]
Clinical relevance differs significantly by species, which makes species identification crucial.[3] NTM are nowadays mostly identified using molecular tools, such as species-specific probes, often incorporated in commercial line probe assays, or direct sequencing of semi-conserved genes with proven taxonomic values. Among these, the 16S rDNA, *rpoB* and *hsp65* genes and 16S-23S internal transcribed spacer (ITS) region are most commonly used.[4-7]
Not all clinical NTM isolates can be convincingly identified by molecular identification tools. The ongoing increase in the number of newly recognized species is testimony to this phenomenon.[8]
To establish the magnitude of the problem of unidentifiable NTM, we re-investigated all NTM, submitted to the national reference laboratory in the Netherlands in the 1999 tot 2007 period that could previously not be convincingly identified by partial 16S rDNA gene sequencing. All 16S sequences were reanalyzed and the isolates re-identified applying two commercially available identification kits, targeting two separate genetic loci.

Methods

At the national mycobacteria reference laboratory (National Institute for Public Health and the Environment [RIVM]), NTM are identified using the INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Ghent, Belgium) reverse-line blot assay, according to the manufacturer's instructions. If no identification to the species level is obtained, additional sequencing of the hypervariable region A (151 base pairs) of the 16S rDNA gene is performed, but only upon request by the referring clinician. Prior to 2004, we used the AccuProbe (GenProbe, San Diego, USA) assays as a first line of NTM identification.
If the 16S sequences yielded a less than 100% match in the GenBank (National Center for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov) sequence database or a match with a species not validly published at the time of referral, the respective isolates were designated 'Unknown *Mycobacterium* Species (UMS)' and numbered consecutively. We extracted these sequences from our database and subjected these to a new comparison with the GenBank sequence database in June 2008. We performed multi-sequence alignment with sequences of reference strains of the closest related mycobacteria using Clustal X software.[9] The resulting topology and tree, inferred by neighbour joining and visualized using the MEGA software package,[10] were evaluated by bootstrap analyses based on 1000 re-samplings.

Table 1: Identification results of previously unidentifiable NTM species.

UMS	n	16S hypervariable region A (GenBank)	GenoType CM/AS	Inno-LiPA	Interpretation
1	18	**100% M. noviomagense NLA000500338^T**	Myc	Myc	M. noviomagense
2	2	99% M. intracellulare W249st	*MSC*	MAIS	MAC
3	2	**100% M. nonchromogenicum FI-06254**	Myc	Myc	M. nonchromogenicum
7	9	**100% M. arupense DSM 44942**	Myc	Myc	M. arupense
10	6	99% M. colombiense CIP108962	*MINT*	MAIS	MAC
11	3	99% M. interjectum DSM 44064	MIJ	*MAIS*	M. interjectum sqv.
12	4	**100% M. saskatchewanense 00-250**	*MINT*	Myc	M. saskatchewanense
13	4	**100% M. seoulense 03-19**	*MSC*	*MAIS*	M. seoulense
14	1	96% M. pyrenivorans DSM 44605	Myc	Myc	RGM
15	3	**100% M. pulveris CIP 106804**	Myc	Myc	M. pulveris
16	5	98% M. saskatchewanense 00-250	*MSC*	Myc	MAC
17	2	99% M. smegmatis ATCC 700504	MSM	MSM	M. smegmatis sqv
18	3	**100% M. holsaticum 1406**	Myc	*MGO*	M. holsaticum
19	4	**100% M. hiberniae DSM44241**	Myc	Myc	M. hiberniae
20	2	95% M. nonchromogenicum ATCC 19530	Myc	Myc	M. terrae related sp.
22	4	98% M. szulgai CIP 104532	Myc	Myc	SGM
23	6	99% M. chlorophenolicum CIP 104189	Myc	Myc	RGM
24	9	99% M. kumamotonense CCUG 51961	Myc	Myc	M. terrae related sp.
25	2	98% M. fallax ATCC 35219	*MGO*	Myc	RGM
26	4	95% M. botniense DSM 44537	*MFO2*	Myc	M. xenopi related sp.
27	24	**100% M. gordonae FI-06271**	MGO	MGO	M. gordonae
28	5	**100% M. fortuitum ATCC 49403**	MFO2	MFO	M. fortuitum
29	1	96% M. doricum DSM 44339	Myc	*MFO*	RGM
30	3	98% M. mucogenicum ATCC 49650	MFO2	MFO	M. fortuitum complex
31	1	**100% M. avium 104**	MAV	MAV	M. avium
32	4	99% M. terrae ATCC 15755	Myc	Myc	M. terrae related sp.
33	4	99% M. mucogenicum ATCC 49650	MMC	Myc	RGM
34	4	98% M.gordonae CIP 104529	MGO	MGO	M. gordonae related sp.
35	1	**100% M. triviale ATCC 23290**	Myc	Myc	M. triviale
36	1	98% M. simiae CIP 104531	*MLE*	Myc	M. simiae related sp.
37	1	98% M. simiae CIP 104531	Myc	*MAIS*	M. simiae related sp.
38	4	**100% M. palustre DSM 44572**	M. palustre	Myc	M. palustre
39	1	**100% M. lentiflavum CIP 105465**	MLE	Myc	M. lentiflavum
40	1	99% M. gordonae CIP 104529	MGO	MGO	M. gordonae sqv.
41	1	98% M. asiaticum DSM 44297	*MGO*	Myc	M. gordonae related sp.
42	4	99% M. holsaticum 1406	Myc	Myc	SGM
43	3	**100% M. branderi CIP 104592**	Myc	Myc	M. branderi
44	2	**100% M. nebraskense DSM 44803**	Myc	*MAIS*	M. nebraskense
45	2	**100% M. aurum N196**	Myc	Myc	M. aurum
46	1	98% M. avium ATCC 25291	MSC	Myc	MAC
48	2	94% M. branderi CIP 104592	Myc	Myc	SGM
49	1	99% M. scrofulaceum CIP 105416	MSC	*MINT1*	MAC
50	1	**100% M. cosmeticum CIP 108169**	*MFO*	*MFO*	M. cosmeticum
52	1	98% M. sphagni DSM44076	MPE	MFO	M. fortuitum complex
53	1	97% M. doricum DSM 44339	Myc	Myc	RGM
54	3	99% M. fortuitum ATCC 49404	MPE	MFO	M. fortuitum complex
59	2	99% M. sphagni DSM 44076	*MMC*	Myc	M. fortuitum complex
60	1	94% M. tusciae CIP106367	Myc	Myc	RGM
61	1	**100% M. florentium DSM 44852**	Myc	Myc	M. florentinum
62	1	98% M. porcinum CIP 105392	*MMC*	Myc	M. fortuitum complex
63	1	**100% M. monacense B9-21-178**	Myc	Myc	M. monacense
64	1	99% M. interjectum DSM 44064	MIJ	Myc	M. interjectum sqv.
65	1	98% M. celatum CIP 106109	Myc	Myc	M. terrae related sp.

Myc: *Mycobacterium* species; MSC: *M. scrofulaceum*; MAIS: *M. avium-intracellulare-scrofulaceum* complex; MINT: *M. intracellulare*; MIJ: *M. interjectum*; MSM: *M. smegmatis*; MGO: *M. gordonae*; MFO: *M. fortuitum*; MAV: M. avium; MPE: *M. peregrinum*; MMC: *M. mucogenicum*; MLE: *M. lentiflavum*; MAC: *M. avium* complex; RGM: Rapid-growing *Mycobacterium*; SGM: Slow-growing *Mycobacterium*; sqv.: sequevar; sp.: species. **Bold face** indicates that identification to species level was obtained after re-analysis of the 16S gene sequence. *Italics* indicate identification by hybridization assays not in accordance with partial 16S sequence result.

To assess the impact of (reverse) hybridization assays on the disclosure of genetic diversity among NTM, at least one isolate from each UMS was subjected to additional identification using the GenoType Mycobacterium CM/AS (Hain Lifescience, Nehren, Germany) and INNO-LiPA MYCOBACTERIA v2 identification kits, used according to the manufacturer's instructions.
We considered UMS with a >1% sequence difference from a species type strain or with a reverse line blot identification conflicting with the 16S sequence result as related to that species. UMS with 16S sequences 1% divergent from established species were considered sequevars of that species, unless the reverse line blot identification results was discordant.

Results

We found 178 clinical isolates with 53 different 16S rDNA gene sequences, not matching with sequences of validly published species available in the GenBank database at the time of referral to the RIVM. These made up 4% of the 4481 NTM isolates referred to the RIVM in the studied period. During this period, another 913 NTM isolates (20%) were no further identified than as *M. avium* complex, other than *M. avium* or *M. intracellulare* (n=392), *M. fortuitum* complex (104), NTM not reacting with the *M. avium* complex and the *M. tuberculosis* complex AccuProbe kits (347) or NTM reacting only with the *Mycobacterium* species probe of the INNO-LiPA assay (70). More detailed identification for these 913 isolates was not requested by the referring clinicians and thus not conducted.
The isolation frequency of the UMS and results of the new identification efforts are detailed in Table 1. Multi-sequence alignment of the partial 16S rDNA gene sequences resulted in the phylogenetic tree visualized in Figure 1.
Based on our re-analysis of the partial 16S gene sequence, 20 UMS (yielding 93 isolates; 52%) could be assigned to validly published species, including our recently described *Mycobacterium noviomagense* (UMS1; Table 1).[11] The remaining 85 isolates, making up 33 UMS, were related to the *M. avium* complex (n=10), *M. fortuitum* complex (n=7), *M. xenopi* (n=3), *M. terrae* complex (n=4), *M. gordonae* (n=3), *M. simiae* (n=2), *M. interjectum* (n=2) or were assigned to the slow or rapid growers, distantly related to established species (Table 1 & Figure 1).
The GenoType CM/AS assay identified 28 of the 53 UMS (53%) to species (n=24) or complex level (*M. fortuitum* complex; n=4). Twelve of these identifications were not in accordance with species or complex identifications based on the partial 16S gene sequence (Table 1, italics). The Inno-LiPA assay identified 19 UMS (36%) to species (n=7) or complex level (n=12; 6 *M. avium* complex, 6 *M. fortuitum* complex). Eight identification results were discordant with species or complex identifications based on the partial 16S gene sequence (Table 1, italics). Identifications as *M. fortuitum* complex or *M. gordonae* by the two assays were especially frequent.

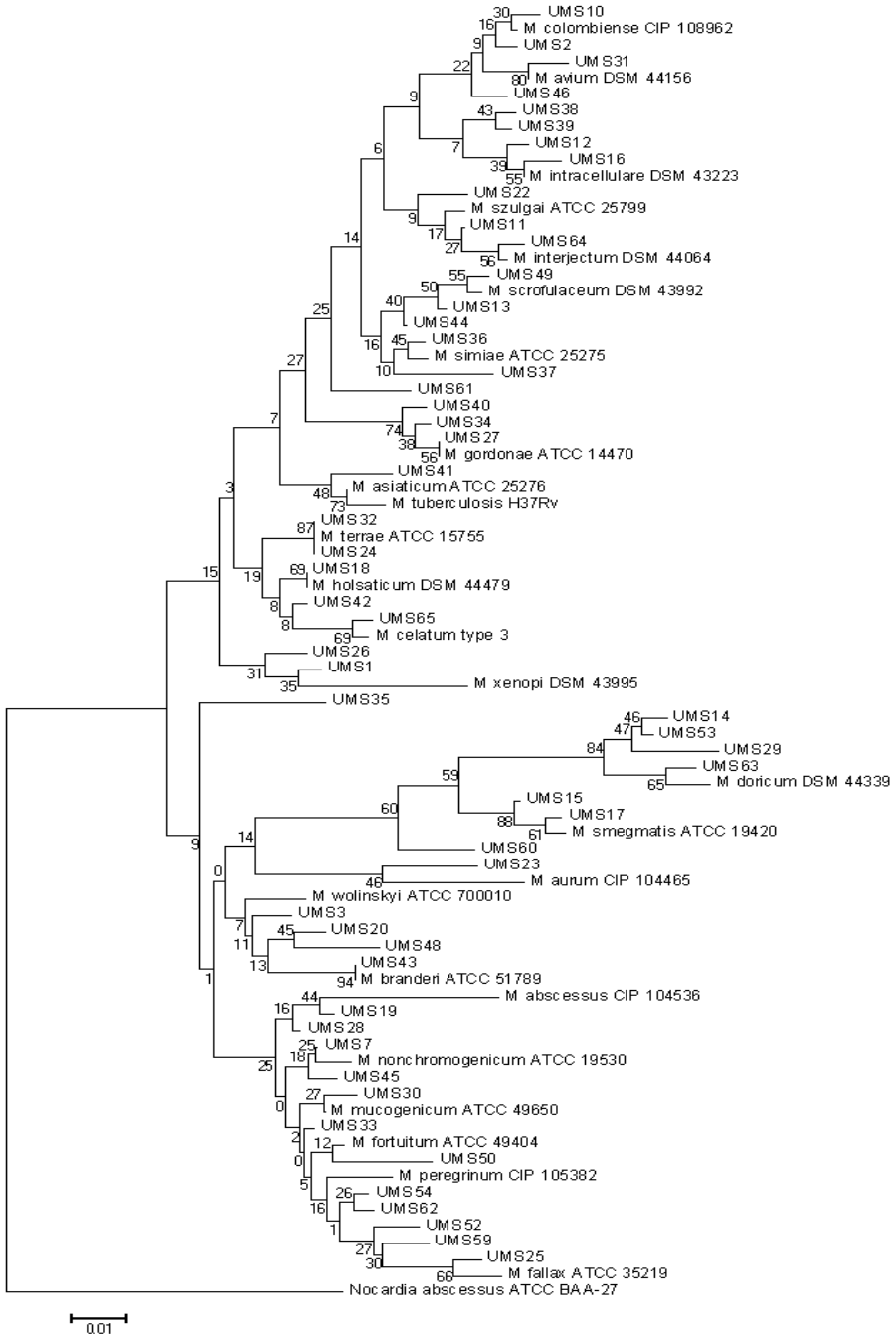

Figure 1: Phylogenetic relationships of the UMS and related species of *Mycobacterium*, based on partial 16S rDNA gene sequences.

Neighbour-joining (NJ) tree was created and bootstrapped 1000x with CLUSTAL X[9] and visualized with MEGA 4.0.[10] Bootstrap values are indicated at the nodes.

Discordance between species or complex identifications by the two hybridization assays was noted in 6 different UMS (UMS 2, 10, 11, 13, 49 and 64, Table 1). This mainly involved isolates related to the *M. avium* complex, *M. interjectum* or *M. scrofulaceum*.

Twenty six (49%) of the UMS were only encountered once; the average number of isolates considering all UMS was 3.4 (range 1-24). Most UMS isolates were cultured from respiratory samples (n=162; 129 sputa, 33 broncho-alveolar lavage fluid samples; 91%). Sixteen UMS isolates (9%) were cultured from normally sterile samples, including bone marrow (n=1), lung (2), lymph node (4), pleura (1) and joint biopsies (1), as well as urine (4), gastric aspirate (2) and maxillary sinus lavage fluid (1).

Discussion

Unidentifiable NTM are a significant phenomenon, making up at least 4% of all NTM submitted to our national reference laboratory. This is well above the 1% estimated by Tortoli and co-workers.[12] Still, we most likely underestimate the number of UMS isolates. For isolates identified to species or complex level by the hybridization assays, additional 16S rDNA gene sequencing is not free of charge and therefore not routinely performed. Yet, this may reveal novel 16S rDNA sequences and thus UMS. In our situation, this seems most prominent in isolates identified by hybridization assays as *M. fortuitum* complex, *M. avium* complex or *M. gordonae*. In the 20% of all submitted isolates that were not identified to species level many additional UMS may be identified.

Reanalysis of the 16S rDNA sequence identified 20 of our 53 UMS as validly published NTM species. Forty-eight percent of the unidentifiable isolates (n= 85) represent 33 novel species or variants of established species.

UMS encountered in this study are clustered in specific parts of the phylogenetic tree of mycobacteria. Referral to our reference lab may be more likely for strains considered possible pathogens and this creates a potential selection bias. Still, this implies that in specific branches of the phylogenetic tree, such as within the *M. avium* and *M. fortuitum* complexes, more species exist than are currently described.

Most of the UMS were single pulmonary isolates, which may reflect limited clinical relevance, i.e. patients failed to meet the ATS diagnostic criteria for pulmonary NTM disease,[3] or a reluctance to submit further isolates for identification. In nine cases, UMS were isolated from normally sterile sites, thus likely causative agents of true NTM disease.

The current hybridization assays can only recognize a limited number of species. For manufacturers, it is a matter of choice to decide whether recently described species should be added or replace species currently included in the assay. Moreover, the hybridization assays are based on the detection of short DNA sequences. This mono-phasic approach precludes a high degree of genetic variation among identically identified strains and thus misses

the distinction between related (sub)species. Our results demonstrate that hybridization assays provide false identifications for recently described species including *M. monacense, M. florentinum, M. holsaticum*, *M. cosmeticum* and *M. saskatchewanense* (Table 1). Importantly, we also recorded differences in identification results between the two hybridization assays tested. Since clinical relevance and drug susceptibility of NTM species differs, correct identification is important.[3,8,11-13] Sequence based identification is more reliable than the limited approach of hybridization assays, although this requires sophisticated and expensive laboratory equipment and may be most suitable for reference laboratories.

For UMS, the 16S sequence similarity to an established species does not provide guidance for clinicians. Our previous identification of *M. noviomagense* pointed out that close genetic relationships with *M. xenopi* were associated with very different phenotypical features, drug susceptibility and clinical relevance.[11] Genetic relationships, based on a partial single target should therefore be interpreted with caution.

Identification based on DNA sequence analysis of a (partial) single gene disregards genetic variation in the rest of the genome. Multi-gene identification may improve our understanding of mycobacterial taxonomy and result in clinically relevant distinctions within species. *Mycobacterium kansasii* is a good example in this respect, as five subtypes have been described based on multiple genetic targets; one subtype causes pulmonary disease, one is a causative agent of HIV-related disseminated disease, while the three remaining types are environmental bacteria, not associated with human disease.[14] The maximum resolution of genetic identification will only be achieved after the introduction of routine sequencing of whole genomes of all available *Mycobacterium* isolates. This will lead to a robust phylogenetic tree that can be enriched with clinical data as a self-learning model to improve our understanding of mycobacterial virulence. It is conceivable that this will also lead to a complete re-consideration of the ever growing list of new species that are described on basis of limited variation on semi-conserved genes and some degree of phenotypic variation.

It is questionable whether our UMS isolates with novel partial 16S rDNA sequences represent new species or variants of established species. Heterogeneity within the 16S rDNA gene has been described for multiple species including *M. gordonae*.[15] Conversely, among rapid growers, new species have been described which share identical 16S rDNA gene sequences but differ in other genetic and biochemical traits.[8,16] With regard to this, *rpoB* sequences are increasingly used to define novel species.[16]

What should make up a new species? A unique 16S rDNA gene sequence remains the gold standard, although an exact cut-off point indicating distinct taxa has not been established for mycobacteria. A separate species status based entirely on unique 16S rDNA sequences would result in hundreds, if not thousands, of new species; it is doubtful whether this would serve clinicians

or only add to the confusion. Our results demonstrate the presence of a large number of potentially new species. Moreover, human isolates represent only the tip of the 'NTM-iceberg'.[8] Here, we agree with Telenti, who has proposed that "clinical meaningfulness should be the key to taxonomic precision".[17]
Amidst an ever increasing number of species, a classification of NTM based on virulence factors, not unlike Runyon's classification based on growth rate and pigmentation,[18] may be a future strategy. Such a classification could begin if more NTM have their entire genomes sequenced.[19]
In conclusion, four percent of NTM isolates submitted to our reference laboratory are unidentifiable *Mycobacterium* species. A minority is isolated from normally sterile sites and may be causative agents of human disease. Periodical reanalysis of UMS is warranted to reclassify them. Identification by hybridization assays underestimates the genetic heterogeneity among NTM. This heterogeneity can be clinically relevant as specific (sub)species can cause specific disease types. Sequence based identification is preferable, at least at reference laboratory level, although the exact target and number of targets needed for clinically useful results remains to be established. The number of NTM species in the environment is probably so high, that clinical UMS isolates should be analyzed and designated a separate species status only if this is clinically meaningful.

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Mycobacterium noviomagense sp. nov.; clinical relevance evaluated in 17 patients

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Summary

Eighteen isolates of a nonchromogenic, slowly growing, non-tuberculous species of the genus *Mycobacterium* were cultured from respiratory specimens obtained over the last eight years from 17 patients in the Netherlands. These isolates were grouped because they revealed a unique 16S rDNA gene sequence and were related to *Mycobacterium xenopi*. None of the 17 patients met the American Thoracic Society diagnostic criteria for non-tuberculous mycobacterial disease, which distinguishes the novel isolates from the related species *M. xenopi*. A polyphasic taxonomic approach, including identification by biochemical and phenotypical analysis, *hsp65* gene sequencing and PCR restriction enzyme pattern analysis, and sequence analyses of the *rpoB* gene and 16S-23S internal transcribed spacer supported the separate species status of the novel isolates. The name *Mycobacterium noviomagense* sp. nov. is proposed for the novel strains. The type strain is NLA000500338^T (=DSM 45145^T =CIP 109766^T). A more distinctive taxonomy of NTM is a prerequisite for the assessment of their clinical relevance.

Improved detection and identification techniques have triggered renewed interest in non-tuberculous mycobacteria (NTM) and their role as opportunistic pathogens. Polymerase chain reaction (PCR) techniques and 16S rDNA gene sequence analysis have brought to light a series of novel NTM species. However, the clinical relevance of these species is not always clear.[1-3]

NTM infections present predominantly as chronic pulmonary disease, although extrapulmonary and disseminated infections have also been described.[3] Local immunosuppression due to pre-existing pulmonary disease and systemic immunosuppression, e.g. in haematological malignancy, immunosuppressive medication and HIV/AIDS, have been identified as predisposing factors for NTM infections.[3] Infection has to be differentiated from contamination and pseudo-infection, characterised by single NTM isolates from the respiratory or digestive tract without signs of disease.[3,4] Their ubiquitous presence in the environment, survival in flowing water systems and resistance to disinfectants implies that NTM often represent laboratory or medical equipment contamination.[3-5] The diagnostic criteria proposed in a Statement by the American Thoracic Society (ATS) are designed to differentiate between true infection and pseudo-infection or contamination, based on clinical, radiological and microbiological features.[3]

This study describes the grouping of 18 previously unknown *Mycobacterium* isolates with identical 16S rDNA sequences and with a high degree of gene sequence similarity to strains of *M. xenopi*. As other features of these bacteria were highly distinct from *M. xenopi* and the clinical relevance differed significantly between the newly recognised bacteria and *M. xenopi*, the 18 isolates are proposed to represent a novel species of the genus *Mycobacterium*.

The 18 novel isolates were acquired from pulmonary samples (13 from sputum, 4 from broncho-alveolar lavage fluid and 1 from a post-mortem lung biopsy) of 17 patients in the Netherlands between January 1999 and January 2007. To determine clinical relevance, we examined the medical records of all 17 patients; their baseline characteristics are displayed in Table 1. The predominance of male patients, mean age and history of chronic pulmonary disease are comparable with previous NTM studies.[3,6] None of the patients had clinical and radiographic features suggestive of mycobacterial lung disease; one was systemically immunocompromised due to HIV co-infection. The post-mortem lung biopsy sample showed histological features of bronchopneumonia and invading bacteria, without features of mycobacterial disease such as granuloma formation. All patient samples were negative for acid-fast bacilli (AFB) on direct microscopy which suggests the presence of a low number of NTM in the original sample or contamination after sample division for culture and microscopy. Although follow-up sputum cultures were performed for 16 patients, only one patient produced another culture that was positive for the novel strains. All others had a single positive culture. Based these findings, none of the patients from whom the novel strains were isolated met the American Thoracic Society criteria for

Characteristic	No. of patients
Male	13 (77%)
Mean age (yr) (range)	53 (29-86)
Dutch origin	16 (94%)
Pre-existing pulmonary disease	15 (88%)
COPD	8 (47%)
Lung cancer	4 (24%)
Healed Tuberculosis	2 (12%)
Recurrent pulmonary infection*	3 (18%)
Bronchiectasis	2 (12%)
Current smoker	5 (29%)
Past smoker	3 (18%)
Alcohol abuse	3 (18%)
HIV infection	1 (6%)

Table 1:
Characteristics of the patients in the study group

The total number of patients was 17. COPD: chronic obstructive pulmonary disease
* >3 in 6 months prior to sampling

pulmonary NTM disease.[3] Good clinical response to non-antimycobacterial regimes, the subsequent establishment of an alternative diagnosis and the observed spontaneous conversion to negative cultures suggested that the novel strains were not the causative agent of these patients' symptoms. The novel strains therefore seem to have no clinical relevance, which distinguishes them from phylogenetically related, but more pathogenic species such as *M. xenopi* and *M. heckeshornense*.[3,7,8]

Two patients received antituberculosis treatment for an average period of 10 weeks, after a presumptive diagnosis of TB. Treatment of patients not meeting the ATS diagnostic criteria is potentially harmful to patients in terms of adverse effects and costs.[9]

Geographical clustering of the patients was observed, favouring the South-east of the Netherlands, in adjacent areas of the Limburg (7 cases), Gelderland (5 cases) and Noord-Brabant (2 cases) provinces. This clustering suggested the presence of the novel strains in specific local environments or tap water. Since the clustering was seen geographically, but not over time, contamination from medical machinery or the laboratories involved seemed less likely.

All of the novel isolates were subjected to laboratory diagnosis by the National Mycobacteria Reference Laboratory of the National Institute for Public Health and the Environment (RIVM). The RIVM provides molecular identification, drug susceptibility testing and epidemiological typing of mycobacterial isolates for all healthcare institutions in the Netherlands.

Biochemical identification and high performance liquid chromatography (HPLC) analysis of cell wall mycolic acid content were performed using

Table 2: Biochemical identification results for the novel isolates and closely related species

Characteristic	1	2	3	4
Growth at 45°C	-	+	+	+
Morphology	NC	SC	SC	SC
Colony size	Small	Large	NP	Small
Tolerance to (in LJ medium):				
Isoniazid 10 µg/ml	-	-	-	NP
Thiophen-2-carboxylic acid	+	+	NP	NP
Hydroxylamine 250 µg/ml	Variable	+	NP	NP
Para-nitrobenzoate 500 µg/ml	+	Variable	NP	NP
5% NaCl	-	-	-	-

Taxa: 1, *M. noviomagense* sp. nov.; 2, *M. xenopi*; 3, *M. heckeshornense*;[7] 4, *M. botniense*.[12] +, positive; -, negative; NC, nonchromogenic; SC, scotochromogenic; NP, not published

previously described approaches.[10,11] Eight isolates (4 *M. xenopi*, 4 of the novel strains) were subjected to biochemical testing. The results are detailed in Table 2. Morphologically, two patterns were clearly discernible; on Middlebrook 7H10 media, the *M. xenopi* isolates were scotochromogenic, showing yellow pigmentation, whereas the colonies of the novel species were smaller and nonchromogenic. All *M. xenopi* isolates grew at 45°C, as previously reported,[12] but the novel strains were unable to grow at this temperature (Table 2). Biochemically, the novel isolates were indistinguishable from the cluster comprising *M. xenopi*, *M. botniense* and *M. heckeshornense*, with negative results for urease, Tween 80 hydrolysis, niacin production, nitrate reductase, acid phosphatase and semi-quantative catalase. HPLC of the novel isolates revealed a pattern characterized by one early and one late cluster of peaks, a profile similar to that of *M. xenopi*, *M. heckeshornense* and *M. botniense* (Figure 1). The HPLC mycobacterium library (available online at http://www.MycobacToscana.it) was used for this comparison.

Susceptibility testing was performed for eleven of the novel isolates from eleven patients using the agar dilution method.[13] For the novel isolates, we recorded *in vitro* resistance to rifampicin (MIC 2mg/L), resistance or intermediate susceptibility to ethambutol (MIC 10-20 mg/L) and intermediate susceptibility to isoniazid (MIC 0.5-1mg/L). The novel species proved susceptible *in vitro* to streptomycin, cycloserine, prothionamide, amikacin, ciprofloxacin, clofazimine, clarithromycin and rifabutin. The drug susceptibility pattern for the novel isolates differed slightly from clinical isolates of *M. xenopi* tested at RIVM which were mostly susceptible, *in vitro*, to rifampicin (MIC 0.5-1 mg/l).[8] These results for *M. xenopi* are in accordance with previous reports.[14]

For molecular identification, sequencing of the full 16S rDNA gene and 16S-23S internal transcribed spacer (ITS), partial *rpoB* and *hsp65* gene,

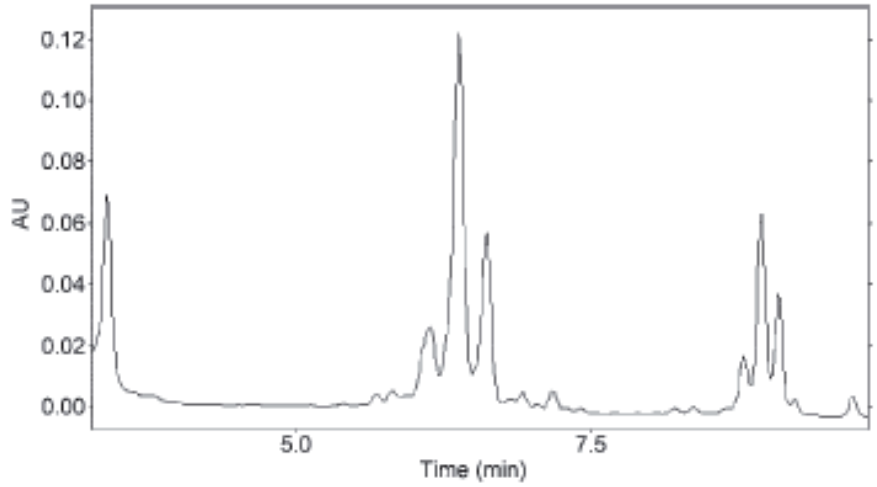


Figure 1: Mycolic acid patterns of *M. noviomagense* sp. nov. obtained by HPLC analysis. One early and one late cluster of peaks are present, similar to *M. xenopi*. The first (left) and last (right) peaks are the low and high weight molecular standards

and PCR restriction enzyme pattern analysis (PRA) of the *hsp65* gene were performed, using previously published methods.[15-18] The sequences obtained were compared with the GenBank/EMBL (National Centre for Biotechnology Information, NCBI, http://www.ncbi.nlm.nih.gov) gene sequence databases.

The full 16S rDNA gene sequence for the new species showed 96% sequence similarity with that of *M. xenopi* and was 97% similar to those of *M. heckeshornense* and *M. shimoidei*. A sequence difference of 1% has been proposed in the literature as the threshold for the designation of a novel species.[1,19]

The full 16S rDNA gene sequence of the of the novel strains was aligned with those of reference strains of the closest related mycobacteria using Clustal X software.[20] The resulting topology and tree, inferred by neighbour-joining and visualized using the LOFT software package[21] were evaluated by bootstrap analyses based on 1000 resamplings (Figure 2).

Analysing only the hypervariable region A of the 16S rDNA gene (151 bp), we found a 100% match in the GenBank/EMBL database, to a strain designated "most closely resembling *M. xenopi*", reported by Hall *et al.*[19] Analysis of phenotypic and genetic characteristics of the new species (Table 2, Figure 1, 2 and 3) demonstrates that very subtle 16S rDNA gene sequence differences can be associated with extensive divergence from the closest related species. This brings into question the use of monophasic identification methods.

Analysis of the 16S-23S ITS region revealed mixed sequence patterns for all 18 novel isolates, even from single colony cultures, and necessitated cloning. For cloning of the ITS, amplicons were generated using primers provided with the Inno-LiPA Mycobacteria v2 kit (Innogenetics), and cloned in the PGEM-T Easy vector (Promega, Leiden, The Netherlands) according to the manufacturer's

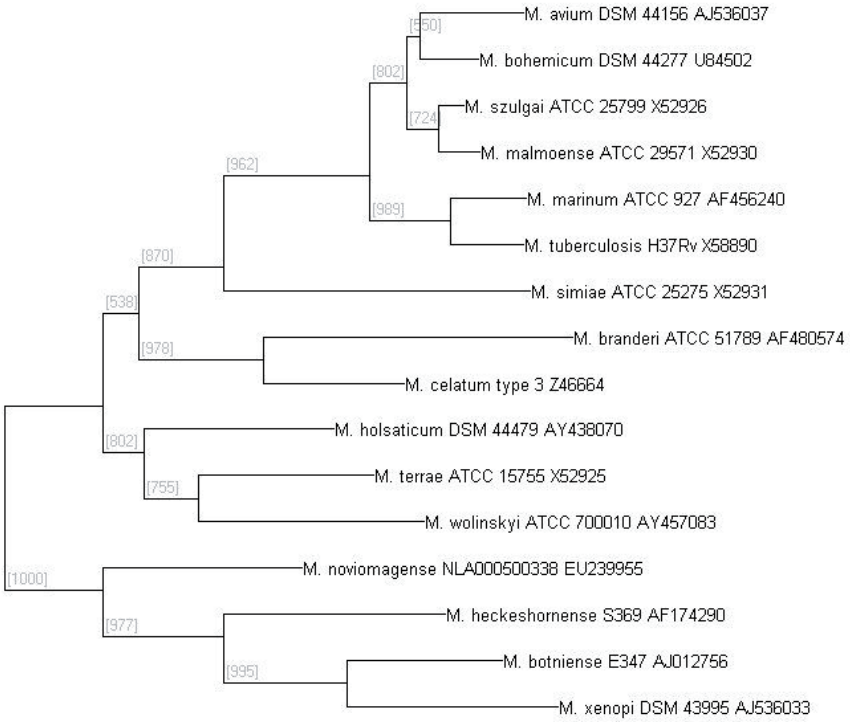


Figure 2: Phylogenetic relationship of the type strain of the novel species, *M. noviomagense* sp. nov., and related species of the genus *Mycobacterium*, based on 16S rDNA gene sequences. The neighbour-joining tree was created and bootstrapped 1000 times with CLUSTAL X[20] and visualized with LOFT (Levels of Orthology through Phylogenetic Trees).[21] Bootstrap values are indicated at the nodes.

instructions. Thirty colonies of transformed *Escherichia coli* strain DH5F were picked for each sample, cultured, and used to prepare plasmid DNA using the QIAprep 96 Turbo BioRobot Kit (Qiagen, Venlo, the Netherlands). For sequencing of the ITS region cloned into the pGEM-T vector, the universal vector primers M13(-21) and M13rev were used on the plasmid preparation as target. Cloning resulted in recognition of two distinct copies of the ITS, both with <68% sequence similarity to *M. xenopi*. A 93% sequence similarity was noted between the two ITS copies. The presence of multiple copies of the 16S-23S ITS region, thus possibly multiple rRNA operons, is unexpected. This phenomenon has not been described for *M. xenopi* or closely related slow-growing NTM species and thus supports the separate species status of the novel isolates.

The *rpoB* sequence was 95% similar to that of the recently described species *M. seoulense* and only 92% similar to that of *M. xenopi*. For the *hsp65* sequence, the most closely related sequences (95%) were found among members of the *M. avium* complex and *M. branderi*, with <93% similarity to *M. xenopi*. The considerable divergence in these two targets from the otherwise related

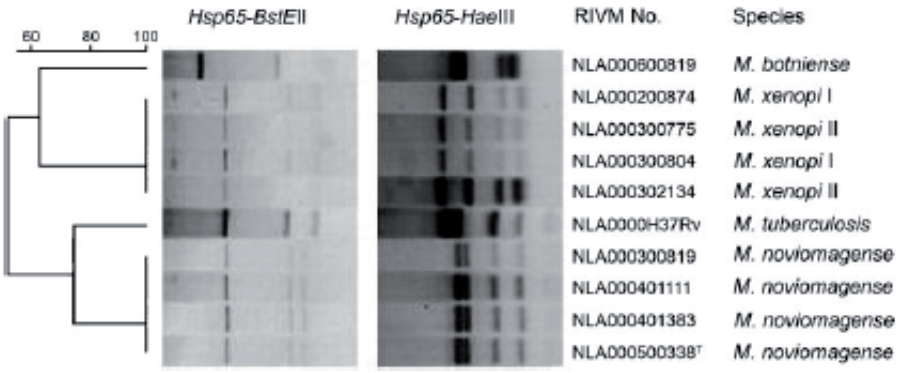


Figure 3: PRA typing results for the hsp65 gene. Different fragment length patterns are observed for *M. botniense* (lane 1), *M. xenopi* (lanes 2–5), *M. tuberculosis* H37Rv^T (lane 6) and strain NLA000500338^T (*M. noviomagense* sp. nov.; lanes 7–10)

cluster comprising *M. xenopi*, *M. botniense* and *M. heckeshornense* supports the separate species status of the novel isolates. The *hsp65* and *rpoB* sequences were aligned with those of related mycobacterial species, as for the 16S rDNA sequence. The resulting topologies and trees are available as Supplementary Figures S1 and S2 (in IJSEM Online).

The *hsp65* PRA results for the novel isolates, *M. xenopi*, *M. tuberculosis* H37Rv^T and *M. botniense* ATCC 700701^T are shown in Figure 3. For the novel isolates, digestion with *Bst*EII resulted in fragments of 240/120/100 bp, digestion with *Hae*III gave fragments of 130/10/70/45 bp. A PRAsite (http://app.chuv.ch/prasite/index.html) comparison showed this to be a unique fragment length combination. Isolates of *M. xenopi* and *M. tuberculosis* tested simultaneously were correctly identified using the PRAsite database; no entry was found for *M. botniense*.

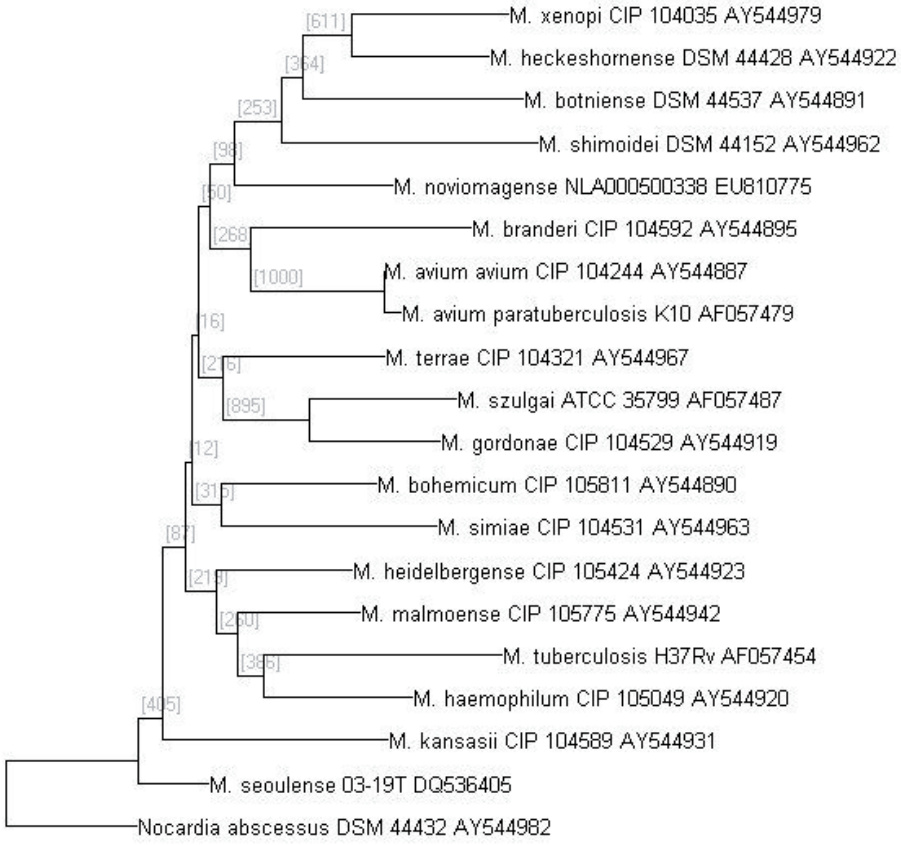
Description of *M. noviomagense* sp. nov.

Mycobacterium noviomagense (no.vi.o.ma.gen'se. N.L. neut. adj., pertaining to Noviomagus, the Roman name of the major city in the endemic region in the Netherlands and the location of the reference hospital; current name: Nijmegen). Acid-fast and Gram-positive rods. Colonies are nonchromogenic and appear after 4 weeks of culture at 37°C, no growth occurs at 45°C. Negative in tests for urease, Tween 80 hydrolysis, niacin production, nitrate reductase, acid phosphatase and semi-quantative catalase. Can be readily identified by its unique rDNA sequences.

The type strain, NLA000500338^T (=CIP 109766^T; =DSM 45145^T), was recovered from sputum.

Acknowledgements
We respectfully thank Dr. Pirjo Torkko of the Laboratory of Environmental Microbiology, National Public Health Institute, Kuopio, Finland, for providing us with *M. botniense* ATCC 700701 ^{T} for comparative analysis. We thank Rebecca Millecamps at Innogenetics, Gent, Belgium, for assistance with the ITS sequencing and Anita Schuerch for assistance with the phylogenetic analyses.

Note: GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, 16S-23S ITS 1 and 2, *hsp65* and *rpoB* gene sequences are EU239955, EU439248, EU439249, EU600390 and EU810775. Phylogenetic trees based on *hsp65* and *rpoB* sequences of selected mycobacterial species are available as supplementary material in IJSEM Online.



Supplementary Figure S1: Phylogenetic relationship of the type strain of the novel species, *M. noviomagense* sp. nov., and related species of the genus *Mycobacterium*, based on *rpoB* gene sequences. The neighbour-joining tree was created and bootstrapped 1000 times with CLUSTAL X[20] and visualized with LOFT (Levels of Orthology through Phylogenetic Trees).[21] Bootstrap values are indicated at the nodes.

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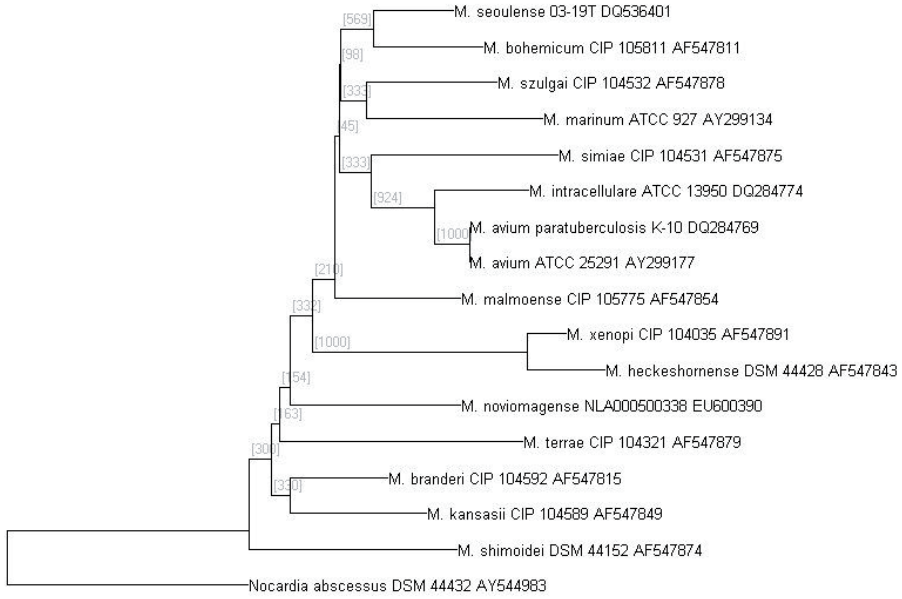

Supplementary Figure S2: Phylogenetic relationship of the type strain of the novel species, *M. noviomagense* sp. nov., and related species of the genus *Mycobacterium*, based on *hsp65* gene sequences. The neighbour-joining tree was created and bootstrapped 1000 times with CLUSTAL X[20] and visualized with LOFT (Levels of Orthology through Phylogenetic Trees).[21] Bootstrap values are indicated at the nodes.

Proposal to elevate *Mycobacterium avium* complex ITS sequevar MAC-Q to *Mycobacterium vulneris* sp. nov.

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Summary

The *Mycobacterium avium* complex consists of four validly published species, *M. avium*, *M. colombiense*, *M. intracellulare* and *M. chimaera*, and a variety of other strains that may be members of undescribed taxa. We report on two isolates of a scotochromogenic, slowly growing non-tuberculous *Mycobacterium* species within the *M. avium* complex from a lymphnode and an infected wound after a dogbite of separate patients in the Netherlands. The extrapulmonary infections in immunocompetent patients suggest a high level of virulence. These isolates are characterized by a unique nucleotide sequence in the 16S rDNA gene, 99% similar to *Mycobacterium colombiense*, and the MAC-Q 16S-23S internal transcribed spacer sequence. Sequence analyses of the *hsp65* gene revealed 97% similarity to *M. avium*. The *rpoB* gene sequence was 98% similar to *M. colombiense*. Phenotypically, the scotochromogenicity, positive semi-quantitative catalase and heat-stable catalase tests, negative tellurite reductase and urease tests and susceptibility to hydroxylamine and oleic acid set these isolates apart from related species. High performance liquid chromatography analysis of cell wall mycolic acid content revealed a unique pattern, related to that of *M. avium* and *M. colombiense*. Combined, these findings supported a separate species status within the *Mycobacterium avium* complex.

We propose elevation of scotochromogenic *M. avium* complex strains sharing this 16S gene and MAC-Q internal transcribed spacer sequence to separate species status under the name *Mycobacterium vulneris*. The type strain is NLA000700772^T (=DSM 45247^T; =CIP 109859^T).

Mycobacterium avium complex (MAC) bacteria are the most frequently isolated nontuberculous mycobacteria (NTM) worldwide and are capable of causing a wide spectrum of clinical disease. Pulmonary disease, mostly in patients with pre-existent pulmonary diseases, is most common, followed by lymphadenitis in immunocompetent children and disseminated disease in systemically immunocompromised patients.[1]

The taxonomy of the MAC has been a subject of debate for decades. The MAC was long divided into two species, *M. avium* and *M. intracellulare*, and a number of unnamed bacteria not belonging to these two taxa. Frothingham and Wilson noted that sequencing of the 16S-23S internal transcribed spacer (ITS) revealed a wide range of genetic diversity among reference strains of those unnamed MAC bacteria, suggesting the presence of several as yet undefined species.[2] Two such groups have recently been elevated to species rank, *M. chimaera* and *M. colombiense*.[3,4] Within this study, we report on a novel *M. avium* complex member, related to *M. colombiense*, which was isolated from two patients in the Netherlands.

Case reports

A previously healthy, 42 year old woman presented with a painful wound in the left lower leg seven weeks after a dogbite. Several small white lesions with limited ulceration were noted. Surgical wound excision and oral amoxicillin and clavulanic acid had limited success. An abscess with fistula to the skin and spontaneous wound rupture prompted renewed surgical debridement, *Mycobacterium* culture, and vacuum therapy. The cultures yielded a nontuberculous *Mycobacterium*. A third wound debridement and vacuum therapy eventually led to symptomatic improvement and wound closure. *Mycobacterium* cultures from samples of the third debridement remained negative.

A previously healthy, two year old girl presented at another hospital with painless swelling of a right cervical lymph node and violaceous overlying skin. No other symptoms were reported. A biopsy revealed granulomatous inflammation, but no acid-fast bacilli were visible on direct microscopy. Cultures of the biopsy material yielded a nontuberculous *Mycobacterium* and surgery was successfully performed. No relapse has been noted since.

Both isolates were sent to the National Institute for Public Health and the Environment (RIVM), Bilthoven, the Netherlands. The RIVM is the national mycobacteria reference laboratory, which provides identification, typing and drug susceptibility testing.

We investigated biochemical and phenotypical features and performed high performance liquid chromatography (HPLC) analysis of cell wall mycolic acid content, using previously described procedures.[5,6] We used the HPLC mycobacterium library (available online at http://www.MycobacToscana.it) for visual comparisons.

For primary identification we used the INNO-LiPA MYCOBACTERIA v2 (Innogenetics, Ghent, Belgium) and GenoType CM/AS (Hain Lifesciences, Nehren, Germany) reverse line-blots. To identify the isolates to the species level we sequenced the complete 16S rDNA gene, the 16S-23S internal transcribed spacer (ITS) region and partial *hsp65* and *rpoB* gene, using previously published primers and methods.[7-10] The DNA sequences obtained were compared with the GenBank/EMBL (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov) sequence database.

The 16S rDNA gene sequence of the *Mycobacterium* isolates of the two cases was aligned with those of reference strains in the *M. avium* complex using Clustal X software.[11] The resulting topology and tree, inferred by neighbour joining and visualized using the MEGA 4.0 software package[12] were evaluated by bootstrap analyses based on 1000 resamplings (Figure 1). The tree was rooted with *M. tuberculosis* H37Rv as an out-group.

We tested the presence of the IS*1245* element by amplification of a 427bp internal fragment, using P1 and P2 primers, as previously described.[13]

Drug susceptibility testing was performed using the 25 wells agar dilution method.[14] We included isoniazid, rifampicin, rifabutin, ethambutol, clarithromycine, ciprofloxacin, cycloserine, prothionamide, amikacin, clofazimine and streptomycin in the test panel.

On Middlebrook 7H10, Ogawa and Stonebrink media, the bacteria produce film-like growth with small, smooth, bright yellow pigmented colonies after 3 weeks of incubation at 36°C. Growth on Middlebrook 7H10 agar was only observed at 24, 30 and 36°C. Optimal growth occurred at 36°C. Colony morphology on Middlebrook 7H10 was similar at all temperatures.

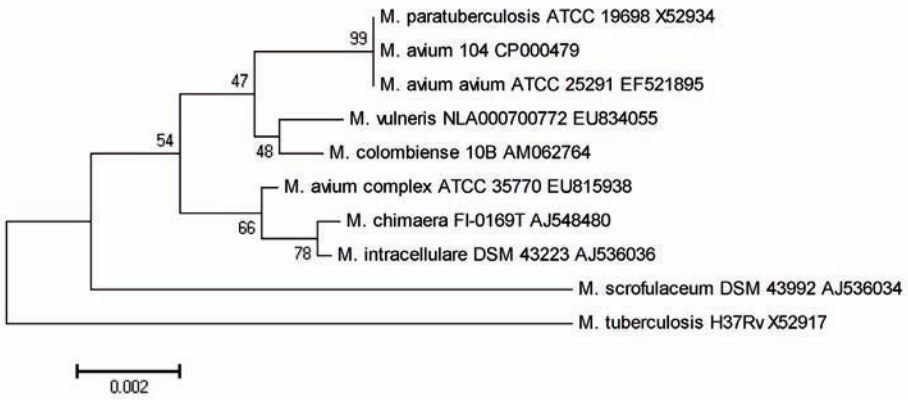

Figure 1: Phylogenetic relationship of the new species (*M. vulneris*) type strain and related species of *Mycobacterium*, based on 16S rDNA gene sequences.

Neighbour-joining (NJ) tree was created, bootstrapped 1000x and visualized with MEGA 4.0.[12] Bootstrap values are indicated at the nodes.

Table 1: Sequence comparison results for *M. vulneris* NLA000700772^T

Target	Results of GenBank/EMBL comparison
16S (1471 bp) GenBank EU834055	99% *M. colombiense* 10B^T (1436/1439 bp) 99% *M. chimaera* FI-0169^T (1432/1439 bp) 99% *M. avium* 104 (1463/1471 bp) 99% *M. paratuberculosis* ATCC 19698^T (1463/1471bp)
16S-23S ITS (281 bp) GenBank AF315833 MAC-Q	99% MAC-R, AF315834 (279/281bp) 98% MAC-E (ATCC 35847), L07852 (278/281 bp) 98% MAC-F, L07853 (277/281 bp) 97% *M. colombiense* 10B^T (275/281bp)
rpoB (726 bp) GenBank EU834057	98% *M. colombiense* CIP 108962^T (690/701 bp) 95% *M. chimaera* DSM 446232^T (669/701 bp) 94% *M. avium* 104 (678/714 bp) 94% *M. avium* ATCC 25291^T (665/701 bp)
hsp65 (424 bp) GenBank EU834054	97% *M. avium* ATCC 25291^T (415/424 bp) 97% *M. avium* 104 (415/424bp) 97% *M chimaera* CIP 107892^T (414/424 bp) 97% *M. intracellulare* ATCC 13950^T (412/424 bp)

The INNO-LiPA MYCOBACTERIA v2 assay revealed only reaction with the MAIS (*M. avium-intracellulare-scrofulaceum*) complex probe, thus identifying the isolates as *M. avium* complex members, different from *M. avium*, *M. intracellulare* and *M. scrofulaceum*. Analysis using the GenoType CM assay (which uses the 23S rDNA gene as its target) incorrectly identified the strain as *M. intracellulare*. This may reflect 23S gene sequence similarity of our isolates with *M. intracellulare*. Currently available commercial identification kits for nontuberculous mycobacteria do not have sufficient discriminative power to recognize particular sub-groupings among the MAC isolates. This may suffice in the clinical setting, as the treatment of MAC disease, so far, is independent of exact speciation results.[1] However, an improved recognition of clinically relevant sub-groupings within the complex may improve clinical management and eventually support research on the epidemiology and pathogenesis of MAC disease.

Sequencing of the complete 16S rDNA gene of both isolates revealed a unique sequence most closely related to *M. avium* complex bacteria (Table 1). The multisequence alignment results of the 16S rDNA gene sequence clarified its taxonomical position within the *M. avium* complex (MAC) as most closely related to *M. colombiense* (Figure 1). The 16S-23S internal transcribed spacer sequence of both strains was identical to the previously described MAC-Q ITS sequevar (GenBank: AF315833); it is 6bp different from the *M. colombiense* ITS sequevar (Table 1). The previously published MAC-Q strain[15] is the one isolated from patient two, the girl with lymphadenitis. We performed multisequence alignment of all published MAC ITS sequevars currently available in GenBank (Figure 2). Additional sequencing of the *hsp65* and *rpoB* genes revealed unique

sequences, detailed in Table 1. Sequences were, again, most similar to MAC members and related to *M. avium* rather than *M. intracellulare* (Table 1). Both strains had unique *hsp65* sequences, the difference being a G =>A substitution at position 140 (corresponding to codon 538 of the *M. tuberculosis* H37Rv *hsp65* gene). The *rpoB* sequence related the isolates specifically to *M. colombiense*, in line with the 16S results (Table 1). We aligned the *rpoB* and *hsp65* sequences with those of related *Mycobacterium* species, by the same methods as for the 16S sequence. In addition, we concatenated 16S, *hsp65* and *rpoB* sequences and aligned these with concatenated sequences of the related *Mycobacterium*

Figure 2: Phylogenetic tree based on ITS sequences showing the relationships of the new species (*M. vulneris*) type strain and other sequevars of MAC.

Each organism is indicated by the sequevar name and GenBank accession number**.** Neighbour-joining (NJ) tree was created, bootstrapped 1000x and visualized with MEGA 4.0.[12] Bootstrap values are indicated at the nodes.

species.[16] Resulting topologies and trees are available as supplementary material in IJSEM Online (Supplementary Figures S1 ,S2 and S3).

We were able to demonstrate, by PCR, the presence of the IS*1245* element in the genomes of the two isolates (results not shown). This supported its taxonomic position within the MAC, related to *M. avium* rather than *M. intracellulare*, although a minority of the *M. intracellulare* and other MAC strains are known to harbour this element.[15]

Phenotypical identification revealed a pattern generally similar to MAC strains, with negative tests for niacin accumulation, nitrate reduction, beta glucosidase, Tween 80 hydrolysis, 3-day arylsulfatase, urease and growth on MacConkey agar, but positive for 68° catalase. Our isolates are divergent in their positive semi-quantitave catalase test, susceptibility to hydroxylamine and oleic acid, as well as their scotochromogenicity. Positive semi-quantitative catalase tests are also noted for *M. colombiense*, which differs from our isolates in its negative urease test and colony morphology and pigmentation (Table 2).

Table 2: Biochemical identification results for *M. vulneris* sp. nov. and closely related species

Test item	*M. vulneris*	*M. colombiense*	*M. avium*	*M. intracellulare*	*M. chimaera*
Niacin accumulation	-	-	-	-	-
Nitrate reduction	-	-	-	-	-
68°C catalase	+	+/-	+/-	+/-	+
Catalase >45 mm	+	+	-	-	-
Beta glucosidase	-	NP	-	-	-
Tween 80 hydrolysis	-	-	-	-	-
Tellurite reduction	-	NP	+	+/-	+/-
3-day Arylsulfatase	-	+/-	-	-	-
Urease	-	+	-	-	-
Pigmentation	SC	NC	NC	NC	NC
Colony morphology	Smooth	Rough	Smooth	Smooth	Smooth
Growth rate	Slow	Slow	Slow	Slow	Slow
Growth at 25°C	+	+	+	+	+
Growth at 42°C	-	-	+/-	+/-	-
Growth on MacConkey agar	-	-	-	-	-
Tolerance to:*					
TCH 5µg/ml	+	NP	+	+	+
Thiacetazone 10µg/ml	+	NP	+	+	+
Isoniazid 1µg/ml	+	NP	+	+	+
p-Nitrobenzoic acid 500µg/ml	+	NP	+	+	+/-
Hydroxylamine 500µg/ml	-	NP	+/-	+/-	+/-
Oleic acid 250µg/ml	-	NP	+	+	+/-

+, positive; -, negative; +/-, variable; NP, not published; SC, scotochromogenic; NC, nonchromogenic; TCH, thiopen-2-carboxylic hydrazide. * all in Middlebrook 7H10 agar

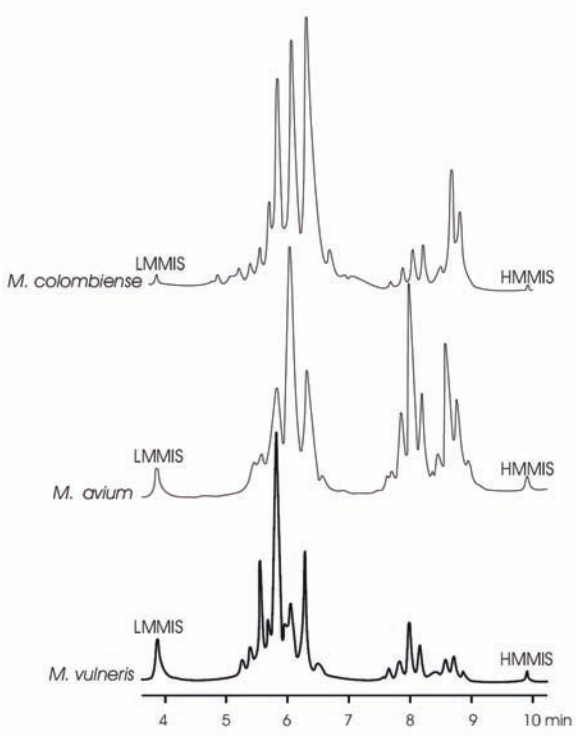

Figure 3:
Mycolic acid patterns of *M. vulneris* sp. nov., *M. avium* and *M. colombiense* obtained by HPLC analysis. The *M. vulneris* sp. nov. pattern is similar to that of other *M. avium* complex members, but does not share the increasing peak heights within the first cluster, typical for *M. colombiense*.
LMMIS, low molecular mass internal standard; HMMIS, high molecular mass internal standard

The HPLC pattern of the isolate comprises 3 clusters of peaks; the first cluster is the main one and includes four major peaks. The second and third emerge later and close to each other, presenting four and three peaks, respectively (Figure 3). This pattern is consistent among most strains included in the MAC, although the *M. colombiense* pattern is characterized by increasing peak heights within the first cluster and the second cluster is absent in *M. chimaera*.[3,4] The relative heights of the peaks in the second and third cluster vary within the MAC. *Mycobacterium avium* mostly presents lower peaks in the third cluster, as in our isolates. *Mycobacterium colombiense* usually present lower peaks in the second cluster (Figure 3).

Applying the 25-well agar dilution method for drug susceptibility testing,[14] we recorded resistance to isoniazid (MIC >20 µg/ml), ethambutol (MIC 20 µg/ml), streptomycin (MIC >20 µg/ml), amikacin (MIC 20 µg/ml) and ciprofloxacin (MIC >16 µg/ml), with susceptibility to rifampicin (MIC 1 µg/ml), rifabutin (MIC 1 µg/ml), clarithromycin (MIC 4 µg/ml), clofazimine (MIC =<0.5 µg/ml), cycloserin (MIC 20 µg/ml) and prothionamide (MIC 2 µg/ml).

Previous authors have suggested the presence of multiple species within MAC, based on various phenotypic and genetic traits.[2,17,18] Based on the phenotypic and genotypic features reported above, we believe that our isolates represent one such species. The extrapulmonary infections in immunocompetent patients suggest a high level of virulence.

Description of *Mycobacterium vulneris* sp. nov.

Mycobacterium vulneris (vu'lne.ris; L. gen. N., pertaining to the wound [vulnus] from which the type strain was recovered). The bacillus stains acid-alcohol fast. Cells are short rods, with occasional coccoid forms. No cording, spores or filaments are present. On Middlebrook 7H10, Ogawa and Stonebrink media, mature growth develops after 28 days of incubation at 36°C; growth is slower at 25 and 30°C and no growth occurs at 42°C. Colonies are small, scotochromogenic and bright yellow in appearance.

Isolates are negative for niacin accumulation, nitrate reduction, beta glucosidase, Tween 80 hydrolysis, tellurite reduction, 3-day arylsulfatase, urease and growth on MacConkey agar, but positive for 68° catalase and semi-quantitative catalase. Isolates are tolerant to isoniazid, TCH, p-nitrobenzoic acid and thiacetazone, but not to oleic acid and hydroxylamine.

Mycobacterium vulneris is readily identifiable by its unique HPLC pattern and 16S rDNA, *hsp65* and *rpoB* gene sequences. The 16S-23S ITS region was previously described as the MAC-Q ITS sequevar.

The type strain, recovered from a wound debridement specimen, is NLA000700772^T (=DSM 45247^T; =CIP 109859^T).

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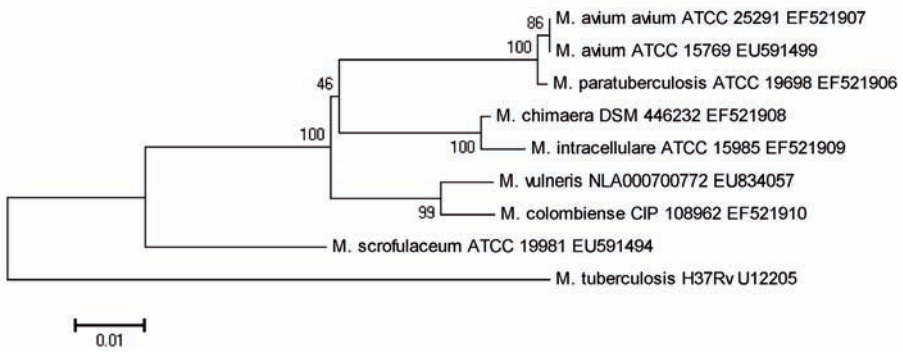
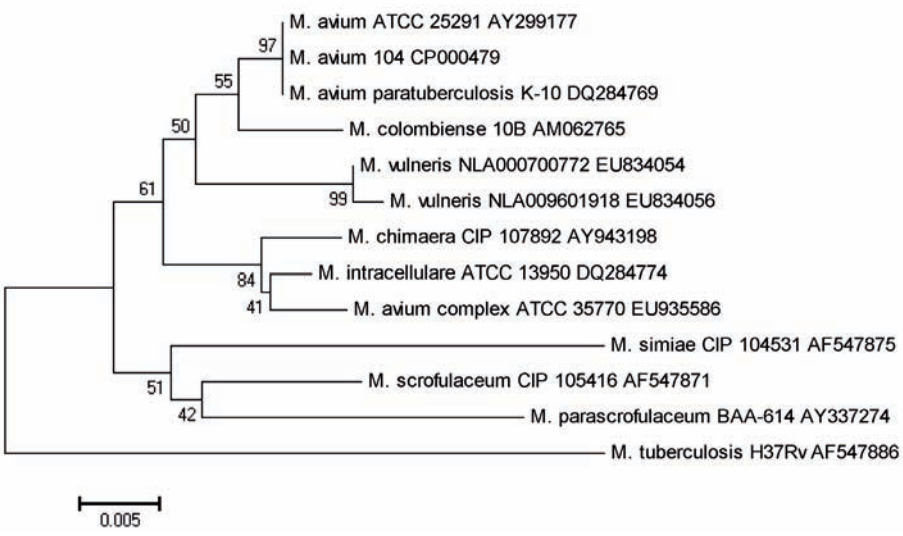
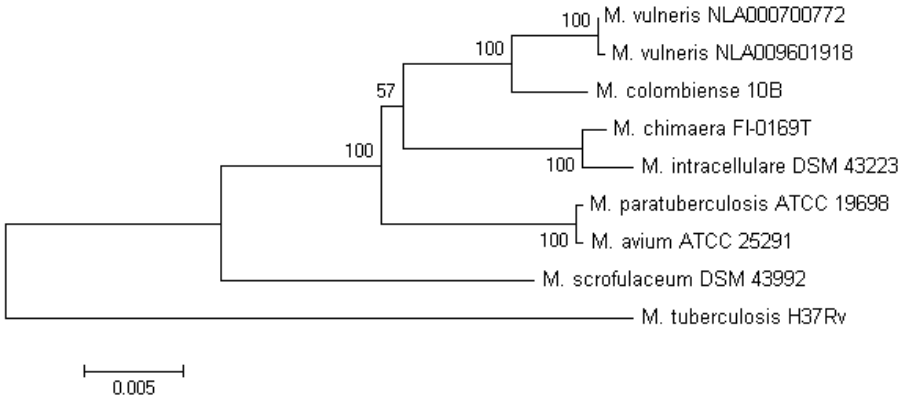
Note: The GenBank/EMBL/DDBJ accession number for the 16S rRNA and *rpoB* gene sequences of NLA000700772^T are EU834055 and EU834057. The GenBank/EMBL/DDBJ accession numbers for the *hsp65* gene sequences of strains NLA000700772^T and NLA009601918 are EU834054 and EU834056



Supplementary Figure S1: Phylogenetic relationship of the new species (*M. vulneris*) type strain and related species of *Mycobacterium*, based on *rpoB* gene sequences. Neighbour-joining (NJ) tree was created, bootstrapped 1000x and visualized with MEGA 4.0.[12] Bootstrap values are indicated at the nodes.



Supplementary Figure S2: Phylogenetic relationship of the new species (*M. vulneris*) strains and related species of *Mycobacterium*, based on *hsp65* gene sequences. Neighbour-joining (NJ) tree was created, bootstrapped 1000x and visualized with MEGA 4.0.[12] Bootstrap values are indicated at the nodes.



Supplementary Figure S3: Phylogenetic relationship of the new species (*M. vulneris*) strains and related species of *Mycobacterium*, based on concatenated 16S, *hsp65* and *rpoB* gene sequences.

Neighbour-joining (NJ) tree was created, bootstrapped 1000x and visualized with MEGA 4.0.[12] Bootstrap values are indicated at the nodes.

Mycobacterium mantenii sp. nov.; A novel pathogenic slowly growing scotochromogenic *Mycobacterium* species

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Summary

Slow growing, scotochromogenic bacteria of a novel *Mycobacterium* species were isolated from lymph node samples in two children, pulmonary samples in two elderly patients from different regions in the Netherlands, as well as from a surface water sample in Zambia. Its 16S rDNA gene, 16S-23S internal transcribed spacer, *hsp65* and *rpoB* gene sequences are unique in comparison to other mycobacteria. Phylogenetic analysis based on the 16S rDNA gene sequence most closely relates these micro-organisms to *Mycobacterium scrofulaceum* (8 bp difference; 0.6%). The *hsp65* sequence shows 96% similarity to that of *Mycobacterium saskatchewanense*, the *rpoB* sequence shows 95% similarity to that of *Mycobacterium chimaera*. The 16S-23S internal transcribed spacer (ITS) sequence places these micro-organisms within the *Mycobacterium avium* complex, as a novel ITS sequevar. This is not supported by the 16S, *hsp65* and *rpoB* gene sequences. Their scotochromogenicity, combined with mostly positive urease, positive semi-quantitative catalase and negative tellurite reduction tests set these isolates apart from related species. The mycolic acid patterns, obtained by high-performance liquid chromatography, are similar to that of *Mycobacterium scrofulaceum*, though the peak heights and distribution present minor differences. We propose the name '*Mycobacterium mantenii*'. The type strain, NLA000401474^T (=CIP 109863^T; =DSM 45255^T), was isolated from a lymph node biopsy sample.

Nontuberculous mycobacteria (NTM) are common in the environment and can be opportunistic pathogens. The NTM are capable of causing a wide spectrum of clinical disease. Pulmonary NTM disease, mostly in patients with pre-existent chronic pulmonary diseases, is most common, followed by lymphadenitis in immunocompetent children and disseminated disease in immunocompromised patients.[1] *Mycobacterium scrofulaceum*, first described by Prissick and Masson in 1956,[2] is mainly a causative agent of paediatric cervicofacial lymphadenitis. The isolation frequency of *M. scrofulaceum* has decreased in the past decades, presumably due to competition with *M. avium* after chlorination of tap water became more commonplace.[1] On the other hand, several scotochromogenic slow growing mycobacteria isolated from children with cervicofacial lymphadenitis have been elevated to separate species status. These may have previously been falsely identified as *M. scrofulaceum*.[3] The International Working Group on Mycobacterial Taxonomy also found several isolates related to, but different from, *M. scrofulaceum*.[4]

We report on a group of five isolates with unique 16S rDNA, 16S-23S ITS, *hsp65* and *rpoB* gene sequences that represent a novel *Mycobacterium* species, related to *M. scrofulaceum*. The isolates were cultured from four patients, including two cases of paediatric cervicofacial lymphadenitis, in the Netherlands and from the Zambezi River, Zambia.

Case reports

In 2004, a previously healthy 2 ½-year-old girl presented with an 8 week history of right submandibular erythematous swelling, 3 cm in diameter with obvious fluctuation and a red skin. She was otherwise asymptomatic. A fine needle aspiration from the swelling was performed. The acid-fast stain of the material obtained was positive. A *M. avium* complex (MAC) strain was detected by real-time PCR, using the 16S-23S ITS as a target, and a scotochromogenic *Mycobacterium* grew in culture (isolate 04-1474). Antibiotic therapy with clarithromycin 15 mg/kg and rifabutin 5 mg/kg was started for a 12 week period. An open wound with persistent drainage developed and healed during therapy. After two years only a small scar remained.

The second patient was a previously healthy 18-month old girl who presented at another hospital in the Netherlands in 2007 with a fluctuating, painless left-sided submandibular swelling, 4 cm in diameter, with violaceous overlying skin. She had no other symptoms. A lymph node biopsy with drainage was performed and a scotochromogenic *Mycobacterium* was cultured from the biopsy material (isolate 07-937). Involution of the lymph node was noted during follow-up; no further therapy was necessary and after one year only a small scar remained.

The third patient was a 92-year-old Dutch woman, who was evaluated by a respiratory physician in a regional hospital in the North of the Netherlands in 2008. Her medical history included bronchiectasis. She presented with a

productive cough and slight dyspnea. A scotochromogenic *Mycobacterium* was isolated from one of three sputum samples (isolate 08-224). Her symptoms and radiographic features were not suggestive of mycobacterial disease and follow-up cultures remained negative, so this isolate was not considered clinically relevant, based on the American Thoracic Society diagnostic criteria.[1]
The fourth patient was a 68-year-old Dutch male, who reported with chronic cough and purulent sputum in 2008. His medical history included an α-1-antitrypsin deficiency and resulting pulmonary emphysema. A scotochromogenic *Mycobacterium* was isolated from a broncho-alveolar lavage (isolate 08-1102). In absence of radiographic features suggestive of pulmonary mycobacterial disease or positive follow-up cultures, this isolate was also not considered clinically relevant.
The fifth isolate was cultured from a water sample we took for other research purposes from the Zambezi river, Zambia, 150m upstream from the Victoria Falls in the year 2007.

All five isolates were subcultured on Middlebrook 7H10 and the egg-based Ogawa and Stonebrink solid media, as well in the Mycobacterial Growth Indicator Tube (MGIT) system. All media were incubated at 36°C; Middlebrook 7H10 slants were also incubated at 25°C, 30°C and 45°C.
The INNO-LiPA MYCOBACTERIA v2 reverse hybridization test was used for primary identification, according to the manufacturer's prescriptions. To obtain identification to the species level, we sequenced the full 16S gene, 16S-23S internal transcribed spacer (ITS) and, partially, the *hsp65* and *rpoB* genes, using previously published approaches.[5-8]
We compared the obtained sequences with the GenBank/EMBL sequence database. The full 16S rDNA gene sequences of the five isolates were aligned with those of reference strains of the closest related mycobacteria using Clustal X software.[9] The resulting topology and tree, inferred by neighbour-joining and visualized using the MEGA 4.0 software package[10] were evaluated by bootstrap analyses based on 1000 re-samplings (Figure 1). The tree was rooted with *M. tuberculosis* H37Rv^T as an out-group.
For biochemical and phenotypical identification we investigated colony morphology, ability to grow at temperatures ranging from 25 to 45°C, niacin accumulation, nitrate reduction, β-glucosidase, Tween 80 hydrolysis, 3-day arylsulfatase, urease, tellurite reduction, 68°C and semiquantitative catalase, growth rate, pigmentation, growth on MacConkey agar and tolerance to thiophen-2-carboxylic hydrazide (TCH) 5 μg/ml, oleate 250 μg/ml, p-nitrobenzoic acid 500 μg/ml, thiacetazone 10 μg/ml, hydroxylamine 500 μg/ml, and isoniazid 1 μg/ml, all in Middlebrook 7H10 agar, following previously published guidelines.[11] HPLC was used to investigate the cell wall mycolic acid composition according to previously reported methods.[12] We used the HPLC mycobacterium library (available online at http://www.MycobacToscana.it) for visual comparisons.

Susceptibility testing was performed using the Middlebrook 7H10 agar dilution method.[13] We tested susceptibility to rifampicin, rifabutin, isoniazid, ethambutol, streptomycin, amikacin, clarithromycin, ciprofloxacin, moxifloxacin, cycloserine, prothionamide, clofazimine and linezolid.

All five samples yielded small colonies with bright yellow pigmentation after 3 weeks of incubation on solid media at 36°C; growth was slower at 25°C and 30°C and no growth occurred at 45°C.

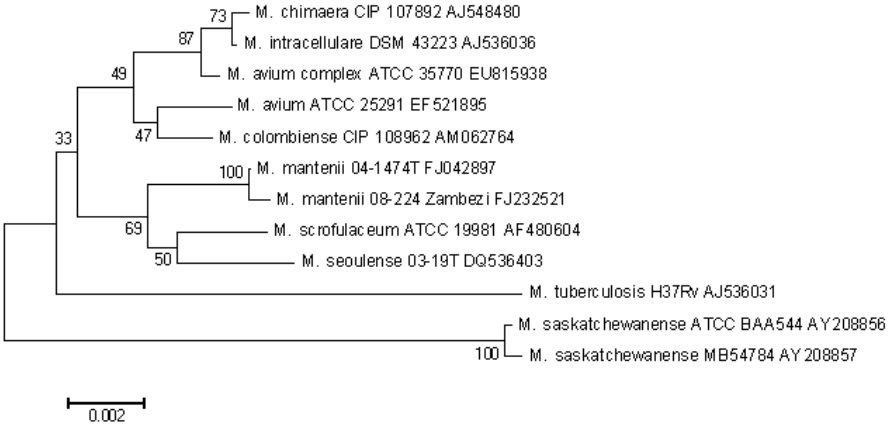

Figure 1: Phylogenetic relationship of the type strain and environmental isolate of *M. mantenii* sp. nov. and related species of the genus *Mycobacterium*, based on 16S rDNA gene sequences. Neighbour-joining tree was created, bootstrapped 1000x and visualized with MEGA 4.0.[10] Bootstrap values are indicated at the nodes.

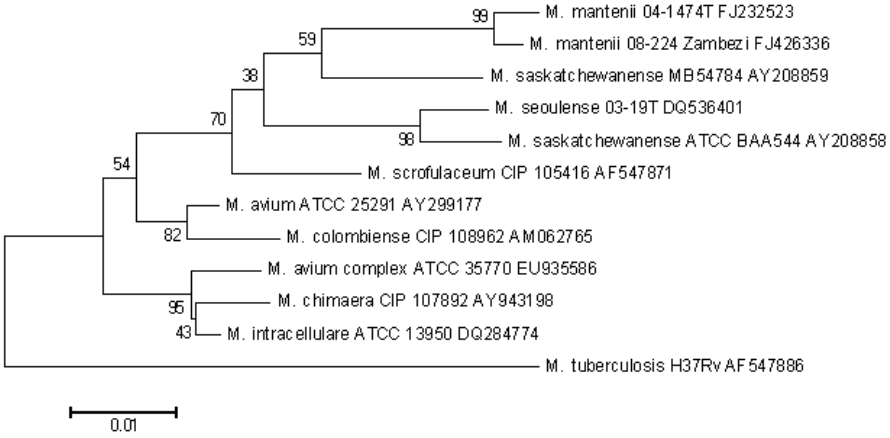

Figure 2: Phylogenetic relationship of the type strain and environmental isolate of *M. mantenii* sp. nov. and related species of the genus *Mycobacterium*, based on *hsp65* gene sequences. Neighbour-joining tree was created, bootstrapped 1000x and visualized with MEGA 4.0.[10] Bootstrap values are indicated at the nodes.

Table 1: Molecular identification results for *Mycobacterium mantenii* NLA000401474^T

Target	GenBank comparison results
16S (1437bp)	99% *M. scrofulaceum* ATCC 19981^T (1429/1437bp)
	98% *M. seoulense* 03-19^T (1422/1439bp)
GenBank: FJ042897	98% *M. avium* ATCC 25291^T (1422/1441bp)
	97% *M. saskatchewanense* ATCC BAA-544^T (1407/1440bp)
16S-23S ITS	96% "*M. vulneris* sp. nov." (MAC-Q) AF315833 (271/281bp)
281bp	96% *M. avium* complex ATCC 35847 (MAC-E) L07852 (270/281bp)
GenBank: FJ232522	95% M. sp. RiVM 9701605 (MAC-R) AF315834 (269/281bp)
	95% *M. avium* complex 5154-O'Connor (MAC-F) L07853 (269/281bp)
hsp65	96% *M. saskatchewanense* MB54784 (410/424bp)
424bp	96% *M. triplex* CIP 106108^T (410/424bp)
GenBank: FJ232523	96% *M. avium* ATCC 25291^T (408/424bp)
	95% *M. genavense* DSM 44424^T (408/424)
rpoB	95% *M. chimaera* CIP 107892^T (288/301bp)
301bp	95% *M. intracellulare* CIP 104243^T (288/301bp)
GenBank: FJ232524	95% *M. saskatchewanense* ATCC BAA-544^T (282/295 bp)
	94% *M. seoulense* 03-19^T (285/301 bp)

For all five isolates, the INNO-LiPA MYCOBACTERIA v2 reverse hybridization test revealed a reaction with the "*M. avium-intracellulare-scrofulaceum* complex" probe only. The full 16S rDNA gene (1437bp) revealed an 8bp difference (99.4% similarity) with *M. scrofulaceum* ATCC 19981 in all four clinical isolates (Table 1); the Zambezi river isolate had a single base pair difference with the four clinical isolates and differed 9 bp from *M. scrofulaceum*. All five isolates shared the 3 bp deletion in the hypervariable region B of the 16S rDNA gene, typical for *M. scrofulaceum* and absent in MAC species.

We recorded identical ITS, *hsp65* and *rpoB* gene sequences in all four clinical isolates; the GenBank comparison results are detailed in Table 1. The Zambezi river isolate sequences differed from the clinical isolates, the differences being 11 bp (4%) in the 16S-23S ITS, 3 bp (1%) in the *hsp65* and 5 bp (2%) in the *rpoB* sequence.

Based on the 1437bp 16S gene sequence the five isolates were most closely related to *M. scrofulaceum* and *M. seoulense* (Table 1 & Figure 1), which also are scotochromogenic slow-growing mycobacteria.[2,14] The *hsp65* and, to a lesser extent, *rpoB* sequences relate our isolates to *M. saskatchewanense*, another scotochromogenic slow growing NTM.[15] These, as well as the more distant relation to the MAC, were confirmed in the phylogenetic trees based on multi-sequence alignment of *hsp65* gene (Figure 2) and *rpoB* gene (Figure 3) sequences. In addition, we concatenated 16S, *hsp65* and *rpoB* sequences and aligned these with concatenated sequences of the related *Mycobacterium* species.[16] Resulting topology and tree are available as supplementary material in IJSEM Online (Supplementary Figure S1).

The ITS sequence is most closely related to the MAC (Table 1, Tree available as Supplementary Figure S2 in IJSEM Online). This matches the Inno-LiPA results, which uses ITS as its target. The different phylogenetic relationships arising from the different genetic targets, as well as the extent of the differences add to our view that these isolates make up a separate species.

The five studied isolates tested negative for niacin accumulation, nitrate reduction, β-glucosidase, Tween 80 hydrolysis, tellurite reductase, 3-day arylsulfatase and growth on MacConkey agar, but positive for urease (3/5), 68° catalase and semi-quantitative catalase. The isolates were tolerant to

Table 2: Biochemical features differentiating the five isolates from related species

Test item	**04-1474^T**	**07-937**	**07-1794**	**08-224**	**08-1102**	**MSC**	**MSE**	**MSK**	**MAV**
Nitrate reduction	-	-	-	-	-	-	+	-	-
68°C catalase	+	+	+	+	+	+	+	+	±
Catalase >45 mm	+	+	+	+	+	+	NP	±	-
Tellurite reduction	-	-	-	-	-	+	±	±	+
Urease	+	-	+	-	+	+	-	-	-
Pigmentation	SC	SC	SC	SC	SC	SC	SC	SC	NC
Growth at 45°C	-	-	-	-	-	-	-	-	±
Tolerance to hydroxylamine 500µg/ml*	-	-	-	-	-	NP	NP	NP	±

±, variable ; NP, not published; SC, scotochromogenic; NC, nonchromogenic; MSC, *M. scrofulaceum*; MSE, *M. seoulense*; MSK, *M. saskatchewanense*; MAV, *M. avium*; Comparative data extracted from references 14 (*M. seoulense*),15 (*M. saskatchewanense*) and 17 (*M. scrofulaceum* & *M. avium*). *in Middlebrook 7H10 agar

Table 3: Minimum inhibitory concentrations (µg/mL) in the agar dilution method

	04-1474	07-937	07-1794	08-1102
Isoniazid	>2	>2	>2	>2
Rifampicin	1	1	1	1
Ethambutol	10	20	10	5-10 (I)
Streptomycin	20	20	10	5
Rifabutin	=<0.2	=<0.2	=<0.2	=<0.2
Amikacin	20	>20	10	10
Ciprofloxacin	16	>16	16	16
Clarithromycin	=<2	8	4	4
Cycloserine	20	50	10	10
Clofazimine	=<0.5	5	=<0.5	=<0.5
Prothionamide	5	5	5	>20
Moxifloxacin	>2	>2	>2	>2
Linezolid	>2	>2	>2	>2

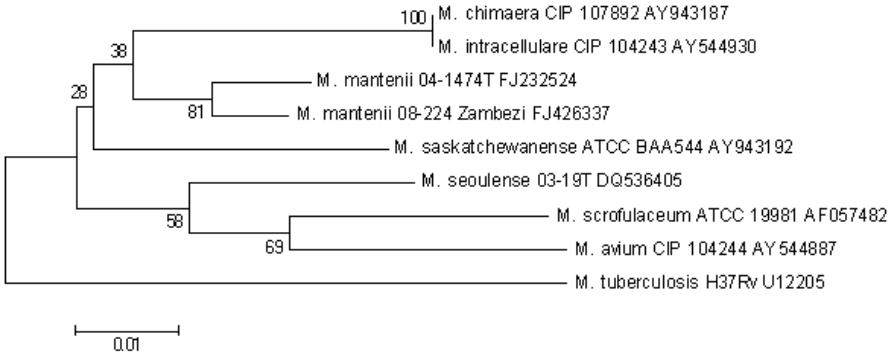

Figure 3: Phylogenetic relationship of the type strain and environmental isolate of *M. mantenii* sp. nov. and related species of the genus *Mycobacterium*, based on *rpoB* gene sequences. Neighbour-joining tree was created, bootstrapped 1000x and visualized with MEGA 4.0.[10] Bootstrap values are indicated at the nodes.

isoniazid, thiacetazone, para-nitrobenzoic acid, TCH and oleic acid, but not to hydroxylamine. The negative tellurite reduction tests and mixed urease results set the isolates apart from *M. scrofulaceum*, *M. saskatchewanense* and *M. seoulense*; our isolates also differ from the latter in their negative nitrate reduction tests. Their scotochromogenicity, combined with uniformly positive semi-quantitative and 68°C catalase tests, mostly positive urease and negative tellurite reduction tests and susceptibility to hydroxylamine set them apart from the MAC (Table 2).[17]

The HPLC profiles of NLA000401474 and the other clinical isolates are identical and overlap that typical of MAC, *M. scrofulaceum* and *M. seoulense*, i.e. three clusters of peaks, one early, large cluster and two late smaller clusters (Figure 4), though the peak heights and distribution present minor differences. This profile differs from the single, late emerging cluster of peaks characteristic for *M. saskatchewanense*.[15] The profile of the Zambezi river isolate is slightly divergent, with different peak heights in the first and higher peaks in the second cluster (Figure 4). A divergent HPLC profile in environmental isolates compared to clinical isolates has also been noted for *M. bohemicum*.[18]

The clinical isolates were only susceptible, *in vitro*, to rifampicin, rifabutin, clarithromycin, cycloserine, clofazimine and prothionamide; MICs are recorded in Table 3. The drug susceptibility pattern is similar to *M. scrofulaceum* and the MAC members, except for the remarkable *in vitro* susceptibility to the rifamycins, i.e. rifampicin and rifabutin (Table 3).

Although there is a 1bp difference in the 16S gene sequence, we consider the Zambezi river isolate and our four clinical isolates to belong to a single species, based on the minor genetic differences and overlapping biochemical and phenotypical features. Microheterogeneity in the 16S rDNA gene has been previously described in NTM.[19] The slight genetic differences may reflect the evolutionary divergence among nontuberculous mycobacteria.

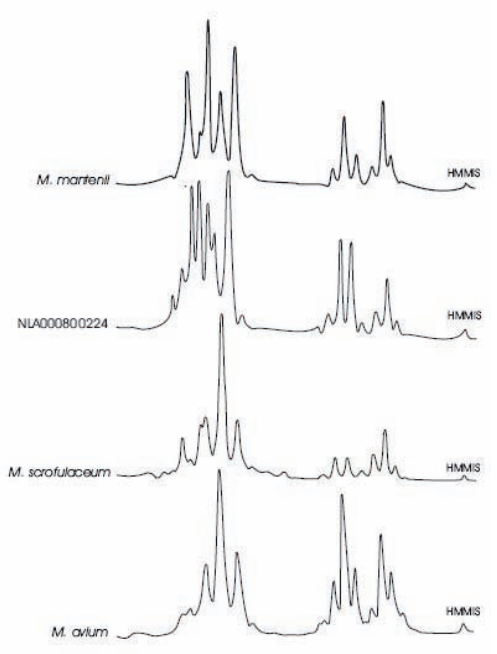

Figure 4:
Mycolic acid patterns of *M. mantenii*[T] and *M. mantenii* 08-224 (Zambezi), *M. scrofulaceum* and *M. avium* obtained by HPLC analysis.
One early and one late cluster of peaks are present in all three, although the peak heights and distribution are different. The Zambezi isolate is divergent, with a different peak distribution in the first cluster and a pronounced second cluster. HMMIS: high molecular mass internal standard

The five described isolates make up a new species, phylogenetically and phenotypically related to *M. scrofulaceum*, *M. seoulense*, *M. saskatchewanense* and, albeit more distantly, the MAC. Biochemical features, however, offer little distinction in the MAC and HPLC patterns in the MAC and *M. scrofulaceum* are known to overlap.[4] The 16S-23S ITS sequence places the five isolates inside the MAC, although bootstrap levels are low (Supplementary Figure S2). This does result in misidentification as a MAC species by the Inno-Lipa assay, not unlike the identification of *M. saskatchewanense* as MAC by the Accuprobe assay.[15] ITS sequences have been useful for taxonomic assignment within the MAC.[20-23] Our isolates comprise two distinct MAC ITS sequevars, although for a species not likely to be part of the MAC, naming them as such would be misleading. The spectrum of human disease of the new species, represented by the five described isolates, appears similar to that of *M. scrofulaceum*.[1,2]
Based on all available genetic, phenotypical and clinical data we conclude that the five isolates make up a new NTM species most closely related to *M scrofulaceum* and related to, but not part of, the MAC.

Description of *Mycobacterium mantenii* sp. nov.

Mycobacterium mantenii (man.ten'ii; N.L. gen. masc. n. *mantenii*, of Manten, in honour of Dr. A. Manten, microbiologist, who published the first cases of NTM disease in the Netherlands in 1957, as well as landmark reviews on the clinical relevance of NTM in the Netherlands). The bacillus stains acid-alcohol fast. Cells are short rods, with frequent coccoid forms. No cording, spores or filaments are

present. On Middlebrook 7H10, Ogawa and Stonebrink media, mature growth develops after 28 days of incubation at 25 to 36°C; no growth occurs at 45°C. Colonies are small, smooth, scotochromogenic and yellow in appearance.
It is negative for niacin accumulation, nitrate reduction, beta glucosidase, Tween 80 hydrolysis, tellurite reduction, 3-day arylsulfatase and growth on MacConkey agar, but positive for urease, 68°C catalase and semi-quantitative catalase. It is tolerant to isoniazid, thiacetazone, para-nitrobenzoic acid, TCH and oleic acid, but not to hydroxylamine.
Mycobacterium mantenii is readily identifiable by its unique 16S rDNA, 16S-23S ITS, *hsp65* and *rpoB* gene sequences. The type strain, recovered from a lymph node biopsy specimen, is NLA000401474^{T} (=CIP 109863^{T}; = DSM 45255^{T}).

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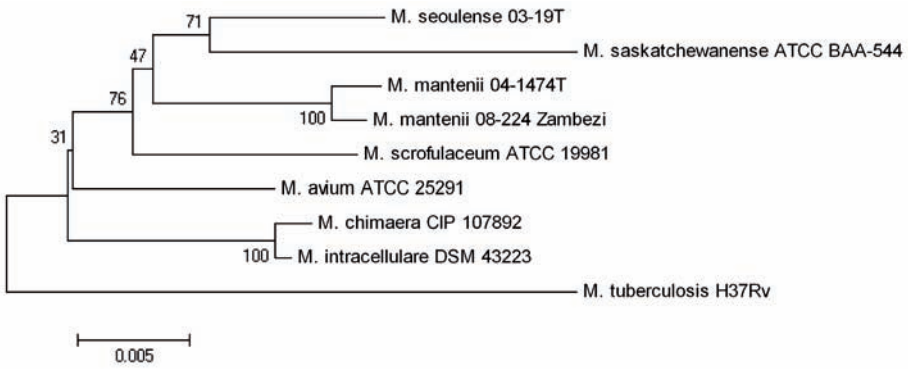
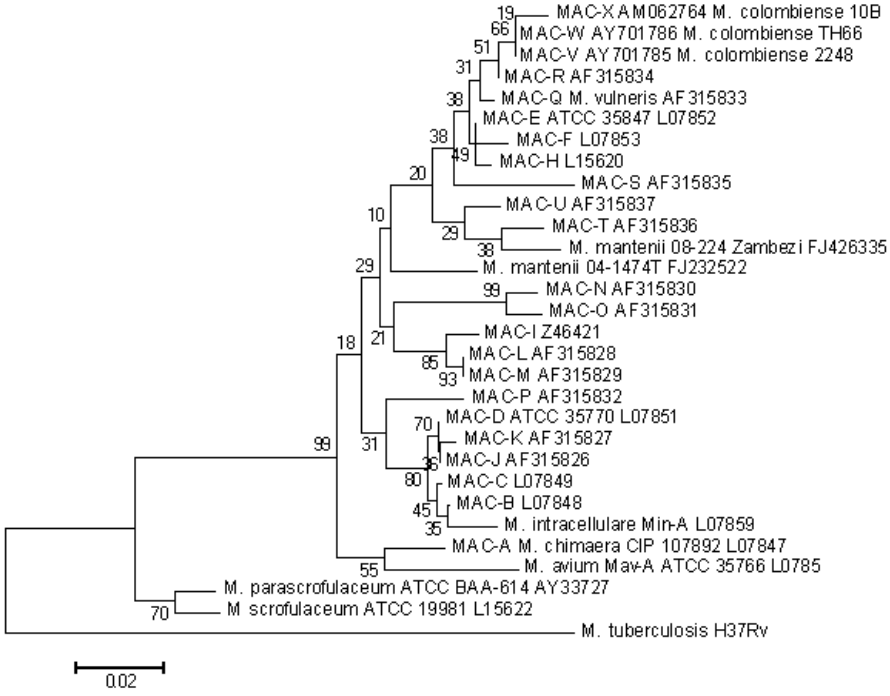
Note: The GenBank/EMBL/DDBJ accession numbers for the 16S rDNA, 16S-23S ITS, *hsp65* and *rpoB* genes of NLA000401474^{T} are FJ042897, FJ232522, FJ232523 and FJ232524. The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA, 16S-23S ITS, *hsp65* and *rpoB* gene sequences of NLA000800224 are FJ232521, FJ426335, FJ426336 and FJ426337.



Supplementary Figure S1: Phylogenetic relationship of the type strain and environmental isolate of *M. mantenii* sp. nov. and related species of the genus *Mycobacterium*, based on concatenated 16S, *hsp65* and *rpoB* gene sequences. Neighbour-joining tree was created, bootstrapped 1000x and visualized with MEGA 4.0.[10] Bootstrap values are indicated at the nodes.



Supplementary Figure S2: Phylogenetic relationship of the type strain and environmental isolate of *M. mantenii* sp. nov. and related species of the genus *Mycobacterium*, based on 16S-23S ITS sequences. Neighbour-joining tree was created, bootstrapped 1000x and visualized with MEGA 4.0.[10] Bootstrap values are indicated at the nodes.

Chapter 6

Inter- and intraspecies genetic divergence and its relation to mycobacterial taxonomy and virulence

6.1 Intraspecies genetic divergence in nontuberculous mycobacteria.
Submitted

6.2 The Region of Difference 1 in nontuberculous *Mycobacterium* species adds a phylogenetic and taxonomical character.
J Bacteriol. Epub July 17th, 2009

6.3 Presence of *esat-6* and *cfp-10* genes does not lead to phagolysosome translocation of *Mycobacterium szulgai*.
To be Submitted

Intraspecies genetic divergence in nontuberculous mycobacteria

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Abstract

The isolation frequency of nontuberculous mycobacteria (NTM) increases in many countries. The clinical relevance of NTM isolation is often unclear. For some species, subtypes with increased clinical relevance have been identified. To assess the intraspecies genetic divergence within several NTM species, we subjected two sets of strains to 16S rDNA, 16S-23S internal transcribed spacer, *hsp65*, *sodA*, *secA1* and *rpoB* gene sequencing. The first set consisted of clinical isolates of the *M. chelonae* complex (n=4), *M. avium* complex (n=4), *M. malmoense* (n=3), *M. kansasii* (n=3), *M. gordonae* (n=3) and *M. tuberculosis* H37Rv; the second part focused on *M. malmoense* (n=13) and *M. xenopi* (n=16) isolates with available clinical data. In the first set, concatenated sequence analysis revealed all unique sequences. In experiment two, we noted ten distinct types for *M. xenopi* and eight for *M. malmoense*. No subtype based on any of the six genetic targets was significantly associated with clinical disease or a specific source of isolation. In this study, we demonstrate that a substantial and not previously recognized intraspecies genetic divergence exists among NTM. The variance within the targeted genes has a mosaic rather than a stochastic pattern and its extent differs by species. The low number of *M. xenopi* and *M. malmoense* isolates in the current study hampered association of particular subtypes with increased clinical relevance. If this divergence can be extrapolated to genes associated with virulence, such associations are likely to exist.

Introduction

Nontuberculous mycobacteria (NTM) are mostly opportunistic, environmental pathogens[5]; their isolation frequency has increased in many countries where the incidence of tuberculosis is in decline.[6] The NTM are causative agents of pulmonary infections, mainly in patients with pre-existent pulmonary disease. Extrapulmonary NTM disease is rare and generally affects patients with impaired immunity.[6]

As the NTM are ubiquitous in the environment, a positive culture from a non-sterile body site, including the respiratory tract, may reflect occasional presence of or contamination by NTM rather than infection. To assist clinicians in the assessment of clinical relevance of NTM isolates from the respiratory tract, the American Thoracic Society (ATS) has established diagnostic criteria, which fit best with *Mycobacterium avium* complex, *M. kansasii* and *M. abscessus*.[6]

Several host factors are known to increase the susceptibility to NTM disease, including pre-existent pulmonary diseases and interferon-γ pathway deficiencies.[6] Bacterial virulence factors are mostly unknown. For *M. kansasii* and *M. xenopi*, subtypes with increased clinical relevance have been identified.[15,19] Still, the true extent of genetic divergence within NTM species and its association with clinical relevance has not been studied.

In the current study, we first explored genetic divergence within NTM species by applying multi-gene sequencing to clinical isolates of five NTM species. In a second experiment, we focused on *M. xenopi* and *M. malmoense*. For these two species, we have linked subgroups with clinical data, to explore differences in clinical relevance.

Methods

In the first experiment we subjected randomly selected clinical isolates of the *M. chelonae* complex (n=4), *M. avium* complex (n=4), *M. malmoense* (n=3), *M. kansasii* (n=3), *M. gordonae* (n=3), previously identified by 16S gene sequencing (151bp hypervariable region A)[11], as well as *M. tuberculosis* H37Rv to extensive multi-gene sequence analysis. We sequenced the forward and reverse strands of the 16S-23S internal transcribed spacer (ITS; 234-285bp) and the *hsp65* (424 bp), *sodA* (427 bp), *secA1* (660 bp) and *rpoB* gene (301 bp), using previously published primers.[1,10,12,18,21]

In the second experiment we subjected a larger number of clinical isolates of *M. malmoense* (n=13) and *M. xenopi* (n=16) to the same sequencing strategy; we selected isolates from pulmonary and extrapulmonary sources and different clinical relevance. Clinical data for the *M. xenopi* and *M. malmoense* isolates have been reviewed in two previous studies[8,20], in which the ATS diagnostic criteria for pulmonary NTM disease were applied.[6] Most extrapulmonary isolates were from cases of true NTM disease, with supportive clinical and histological features, whereas one *M. xenopi* isolate was from a faeces sample and not associated with true disease.

Table 1: Sequencing results for experiment 1

Strain	16S	ITS	*hsp65*	*sodA*	*secA1*	*rpoB*	identification
1010300199	MML	MML	*ML2	*MML2	*MML2	*ML2	MML
1010301186	MML	MML	MML	*MML1	*MML1	MML	MML
1010301752	MML	MML	MML	*MML3	*MML1	MML	MML
1010300601	MAV	Mav-A	Mav-A	*MAV2	*MAV3	MAV	MAV
1010300986	MAV	Mav-A	Mav-A	*MAV3	*MAV2	MAV	MAV
1010300987	MAV	Mav-B	Mav-A	MAV	MAV	MAV	Mav-B
1010400161	MAV	Mav-B	Mav-A	MAV	MAV	MAV	Mav-B
1010302078	MKA	Mka-I	Mka-I	Mka-I	*MKA2	Mka-I	MKA
1010302119	MKA	Mka-I	Mka-I	Mka-I	Mka-I	Mka-I	Mka-I
1010302197	MKA	Mka-II	Mka-II	Mka-II	*MKA3	*MKA2	MKA
1010301806	Mgo-II	Mgo-C	Mgo-II	*MGO2	*MGO3	-	MGO
1010301991	Mgo-I	*MGO2	Mgo-I	*MGO1	*MGO1	MGO	MGO
1010302187	Mgo-II	*MGO3	*MGO3	*MGO3	*MGO2	*MGO2	MGO
1010300639	MCC	MCH	MCH	MCH	*MCH	MCH	MCH
1010300866	MCC	*MAB2	MMS	MMS	*MMS	MMS	MMS
1010301670	MCC	Mab-A	MBO	*MBO	MBO	MBO	MBO
1010302073	MCC	Mab-A	MAB	MAB	MAB	MAB	MAB
H37Rv	MTB	MTB	MTB	MTB	MTB	MTB	MTB

MML, *Mycobacterium malmoense*; MAV, *M. avium*; MKA, *M. kansasii*; Mgo, *M. gordonae*; MCC, *M. chelonae* complex; MCH, *M. chelonae*; MAB; *M. abscessus*; MBO, *M. bolletii*; MMS, *M. massiliense*; MTB, *M. tuberculosis* complex; *sequence that does not match exactly with a GenBank entry

Obtained sequences were compared to the GenBank/EMBL sequence database (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov); where applicable, GenBank accession numbers are provided in brackets. Sequences unmatched in the GenBank/EMBL database were assigned a sequevar number, with the lowest number for the sequence most closely related to the type strain sequence.

We performed multi-sequence alignment using Clustal X software.[19] The resulting topology and tree, inferred by neighbour-joining and visualized using the MEGA 4.0 software package[17] were evaluated by bootstrap analyses based on 1000 re-samplings. For phylogenetic analyses, we constructed trees based on concatenated sequences of all targets.[14]

Results

Experiment 1: For the 18 isolates in experiment 1, 16S gene sequencing revealed intraspecies divergence only for *M. gordonae*; the DNA sequence of isolate 1010301991 was identical to that of the *M. gordonae* CIP 104529 type strain; the others were identical to clinical isolates from Italy (EU022513). Intraspecies divergence among *M. chelonae* complex isolates was not discerned (Figure 1). Results for all targets are recorded in Table 1.

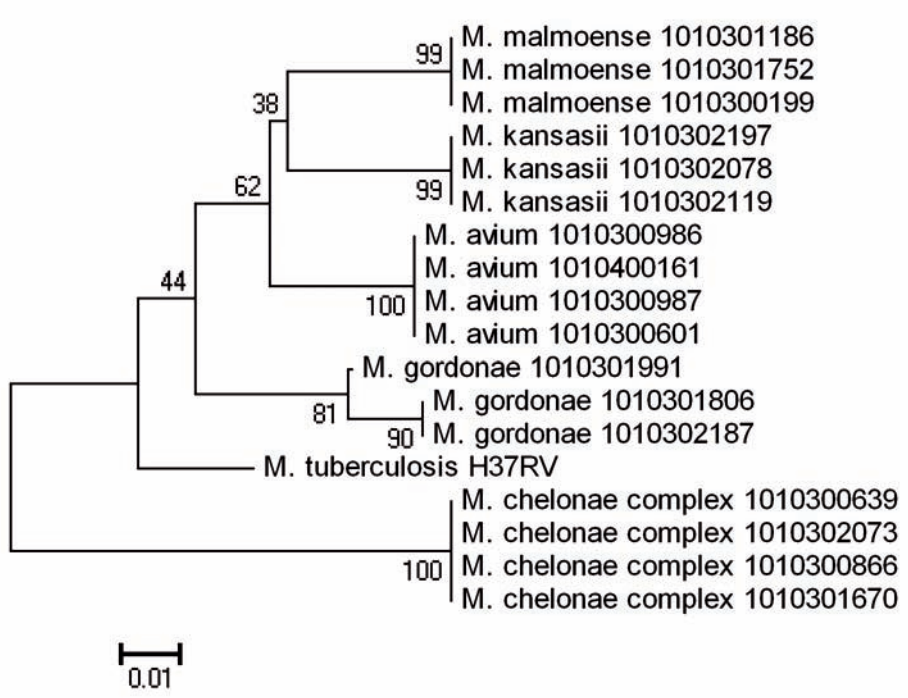

Figure 1: Experiment 1: Phylogeny based on 16S hypervariable region A sequences. Micro-heterogeneity is noted for *M. gordonae*; exact species identification within *M. chelonae* complex isolates is not possible.

M. kansasii

All secondary targets except *secA1* distinguished the *M. kansasii* isolates in two groups. Both 1010302078 and 1010302119 belonged to *M. kansasii* type I; 1010302197 belonged to *M. kansasii* type II. Of the *M. kansasii* type I isolates, 1010302119 had a *secA1* sequence identical to the *M. kansasii* ATCC 12478 type strain sequence; 1010302078 and 1010302197 differed only by 1bp and were both 2bp different from the *M. gastri* ATCC 15754 sequence, but 22bp (3%) from the *M. kansasii* ATCC 12478 type strain sequence.

M. avium

All four *M. avium* isolates had identical 16S, *hsp65* and *rpoB* sequences. Based on the ITS sequence, two previously published sequevars were discerned: 1010300601 and 1010300986 had a Mav-A sequevar, 1010400161 and 1010300987 had a Mav-B sequevar. Both Mav-B isolates had identical *sodA* (identical to that of ATCC 25291, EU409983) and *secA1* sequences. Both Mav-A isolates had unique *sodA* and *secA1* sequences; for 1010300601, the *sodA* sequence differed 1bp and the *secA1* sequence differed 10bp (2%) from the ATCC 25291 type strain sequence, for 1010300986 these differences were 3bp (*sodA*) and 5bp (*secA1*).

M. malmoense
The three *M. malmoense* isolates had identical ITS sequences. Based on *hsp65*, *secA1* and *rpoB* sequencing, 1010300199 was distinct from the other two isolates; the *secA1* sequences differed 2bp (1010300199) and 4bp (1010301186 and 1010301752) from the ATCC 25971 type strain sequence (AY724718). All three isolates had distinct *sodA* sequences; 1010301186 differed 2bp and 1010300199 and 1010301752 differed 6bp from the CIP 105775T type strain sequence.

M. gordonae
All three *M. gordonae* isolates had unique sequences for all the targets. Of the ITS sequences, one was unmatched in the GenBank database: the 1010301991 sequence differed 3bp from the Mgo-A sequevar (L42258); 1010301806 was identical to the Mgo-C sequevar (L42260) 1010302187 matched a clinical isolate from Italy (EU497913). For isolate 1010301991, the *hsp65* and *rpoB* sequences were identical to the ATCC 14470 type strain sequences (AF057468); for 1010302187 the *hsp65* sequence differed 15bp (4%) and the *rpoB* sequence was 8bp (3%) different; for 1010301806, the *hsp65* sequence was identical to previously published isolates (EF546780) and the *rpoB* gene repeatedly failed to amplify.
For the *sodA* 1010301991 differed 3bp (1%), 1010301806 differed 16bp (4%) and 1010302187 differed 26bp (6%) from the CIP 104529T type strain sequence. The *secA1* sequences of 1010301991 differed 11bp (2%), 1010302187 differed 24bp (4%) and 1010301806 differed 29bp (5%) from the ATCC 14470 type strain sequence.

M. chelonae complex
Three out of four *M. chelonae* complex members were identified to species level by ITS sequencing, one as *M. chelonae* (DQ986509), two as *M. abscessus* (Mab-A: AJ291580); 1010300866 had a sequence 2bp different from the Mab-A sequence and 2bp divergent from the *M. massiliense* CCUG 48898 type strain sequence (AY593978). This isolate was subsequently identified as *M. massiliense* by *hsp65* sequencing. The *hsp65* sequence of 1010301670 was 2bp different from *M. bolletii* CIP 108541T (EU266576). The *rpoB*, *sodA* and *secA1* gene sequences confirmed the identifications as *M. bolletii*, *M. massiliense*, *M. abscessus* and *M. chelonae*. All sequences were identical to type strain sequences, except for the 1010301670 *sodA* sequence which differed 1bp from the *M. bolletii* CIP 108541T sequence and the 1010300866 *secA1* sequence which differed 1bp from the *M. massiliense* CCUG 48898 type strain sequence. The *secA1* sequence for 1010300639 differed 13bp (2%) from the *M. chelonae* ATCC 35752 sequence.

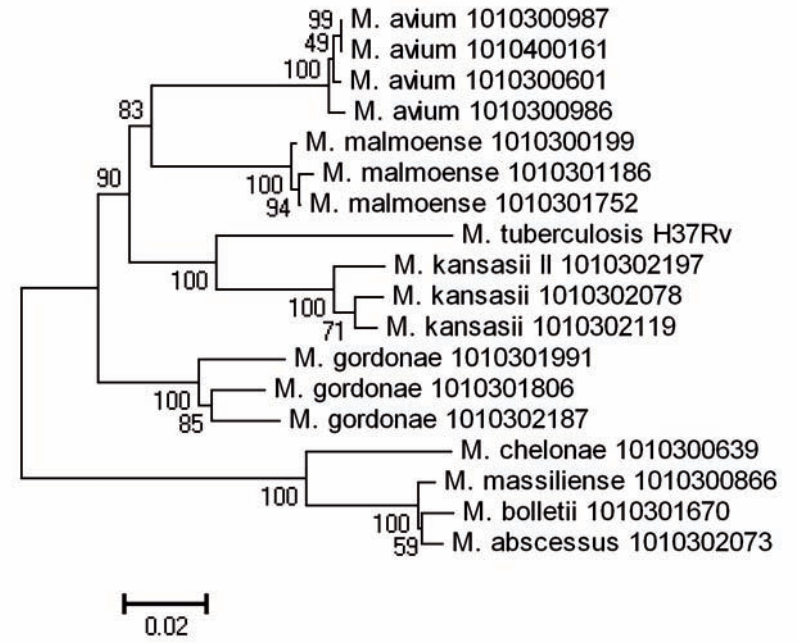

Figure 2: Experiment 1; concatenated 16S, ITS, *hsp65*, *sodA*, *secA1* and *rpoB* sequences. The two Mav-B ITS sequevars have identical sequences for all targets; all others have unique sequences, most notably *M. gordonae*.

The concatenated 16S, ITS, *hsp65*, *sodA*, *secA1* and *rpoB* sequences revealed mostly unique strains (Figure 2); only the two *M. avium* ITS sequevar Mav-B isolates had identical sequences for all targets. The highest level of intraspecies genetic divergence was noted for *M. gordonae* (Figure 2).

Experiment 2

In the second experiment we focused on two infrequently isolated species *M. xenopi* and *M. malmoense,* both often associated with clinical relevance. The sequencing results, sample origin and statements on clinical relevance are depicted in Table 2.

M. xenopi

The *M. xenopi* type I 16S sequence was identical to that of the CIP 104035 type strain (AF547982); type II differed in 1bp and matched multiple clinical isolates (AY215377). All three previously published ITS sequevars were also found in this study.[11] The *hsp65* sequences were all identical to the CIP 104035 type strain sequence (AF547891), all *secA1* sequences were identical, but differed 1bp from the ATCC 19250 type strain sequence (AY724734). Of the two *sodA* sequevars noted, type I is identical to the *M. xenopi* ATCC 19250 type strain sequence (X81391); type II differs 1bp. For *rpoB*, too, we noted two sequevars.

Table 2: Sequencing results for the *M. xenopi* and *M. malmoense* isolates

Strain	type	16S	ITS	*hsp65*	*sodA*	*secA1*	*rpoB*	Source	Disease#
1010100061	I	Mxe-I	Mxe-A	MXE	MXE	*MXE	MXE	P	Yes
1010100209		Mxe-I	Mxe-A	MXE	MXE	*MXE	MXE	EP	Yes
1010101945		Mxe-I	Mxe-A	MXE	MXE	*MXE	MXE	P	No
1010101975		Mxe-I	Mxe-A	MXE	MXE	*MXE	MXE	P	No
1010302059	II	Mxe-I	Mxe-A	MXE	MXE	*MXE	*MXE2	P	Yes
1010101526	III	Mxe-I	Mxe-B	MXE	MXE	*MXE	MXE	P	Yes
1010001068	IV	Mxe-I	Mxe-C	MXE	MXE	*MXE	MXE	P	Yes
1019901047	V	Mxe-I	Mxe-C	MXE	*MXE2	*MXE	MXE	P	Yes
1019900896	VI	Mxe-I+II	Mxe-A	MXE	*MXE2	*MXE	MXE	EP	No
1019900986	VII	Mxe-II	Mxe-A	MXE	*MXE2	*MXE	MXE	P	Yes
1010201356	VIII	Mxe-II	Mxe-B	MXE	MXE	*MXE	*MXE2	P	No
1019900581	IX	Mxe-II	Mxe-C	MXE	MXE	*MXE	MXE	EP	Yes
1019900792		Mxe-II	Mxe-C	MXE	MXE	*MXE	MXE	P	No
1010200040		Mxe-II	Mxe-C	MXE	MXE	*MXE	MXE	EP	Yes
1010200822	X	Mxe-II	Mxe-C	MXE	*MXE2	*MXE	MXE	P	Yes
1010201570		Mxe-II	Mxe-C	MXE	*MXE2	*MXE	MXE	EP	Yes
1010201707	I	MML	MML	MML	*MML1	*MML1	MML	P	Yes
1010401587		MML	MML	MML	*MML1	*MML1	MML	P	No
1010401612		MML	MML	MML	*MML1	*MML1	MML	P	No
1019901018	II	MML	MML	MML	*MML1	*MML2	MML	EP	Yes
1010401708		MML	MML	MML	*MML1	*MML2	MML	P	Yes
1010300497	III	MML	MML	MML	*MML2	*MML2	MML	P	Yes
1010401293	IV	MML	MML	MML	*MML3	*MML2	MML	P	Yes
1010400984	V	MML	MML	MML	*MML4	*MML2	MML	P	Yes
1019902232	VI	MML	MML	*Mml2	*MML1	*MML2	MML	EP	Yes
1010002047	VII	MML	MML	*Mml2	*MML1	*MML2	*MML2	EP	Yes
1019901374	VIII	MML	MML	*Mml2	*MML4	*MML2	MML	EP	Yes
1010400945		MML	MML	*Mml2	*MML4	*MML2	MML	P	Yes
1010500414		MML	MML	*Mml2	*MML4	*MML2	MML	P	Yes

MXE, *M. xenopi*; MML, *M. malmoense*; P, pulmonary; EP, extrapulmonary
#as defined by the American Thoracic Society diagnostic criteria[6]
*sequence that does not match exactly with a GenBank entry

The majority matched with the ATCC 19250 type strain sequence (AF057493); 1010302059 and 1010201356 differed in 1bp. All groupings based on single target sequevars were independent of other targets. No subtype based on any target was significantly associated with true NTM disease or pulmonary and extrapulmonary sources of isolation.

Based on the analysis of six concatenated genetic targets, the 16 *M. xenopi* isolates were divided into 5 clusters (Figure 3). Strains in the two major clusters are not all identical, but the differences were too subtle (below 0.1%) to form separate groupings. Table 2 illustrates that ten types were distinguished.

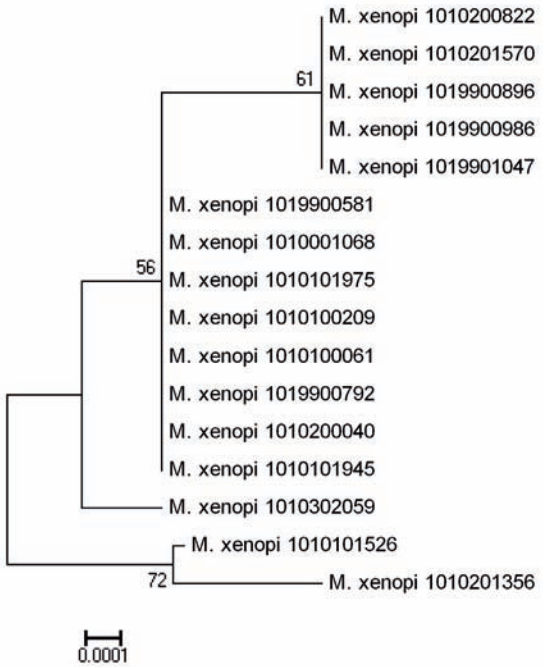

Figure 3:
M. xenopi; concatenated 16S, ITS, *hsp65*, *sodA*, *secA1* and *rpoB* sequences. The 16 *M. xenopi* isolates are divided into five clusters. Strains in the two major clusters are not identical, though differences are too subtle to form separate groupings.

M. malmoense

The 16S and ITS sequences were identical for all isolates and matched the ATCC 29571 type strain sequences. Two subtypes were discernable based on *hsp65* sequences: eight isolates matched the CIP105775 type strain sequence (AF547854), five had identical sequences which differed in 2bp from the CIP105775 type strain sequence. The *sodA* sequences divided the isolates in four groupings, unrelated to the *hsp65* types. None of these sequences matched the CIP105775 type strain sequence; type I (*MML1) differed in 1bp, *MML2 differed in 3bp, *MML3 differed in 6bp and *MML4 differed in 7bp (2%) from the CIP 105775 sequence. Two *secA1* sequence types were noted, not matching groupings based on the previous targets. The first group (*MML1) differed in 2bp from the ATCC 25971 type strain sequence (AY724718), the second differed in 4bp. All but one of our *M. malmoense* isolates matched the ATCC 29571 type strain *rpoB* gene sequence (AF057475); 1010002047 differed in 1bp. Again, no subtype based on any target was significantly associated with true NTM disease or pulmonary and extrapulmonary sources of isolation.

Based on the analysis of six concatenated genetic targets, the 13 *M. malmoense* isolates were divided into eight types (Figure 4; Table 2).

Discussion

In experiment 1 we revealed that genetic divergence exists in all NTM species tested in the current study. This extent of divergence has not been previously recorded. The variance within the targeted genes has a mosaic rather than a

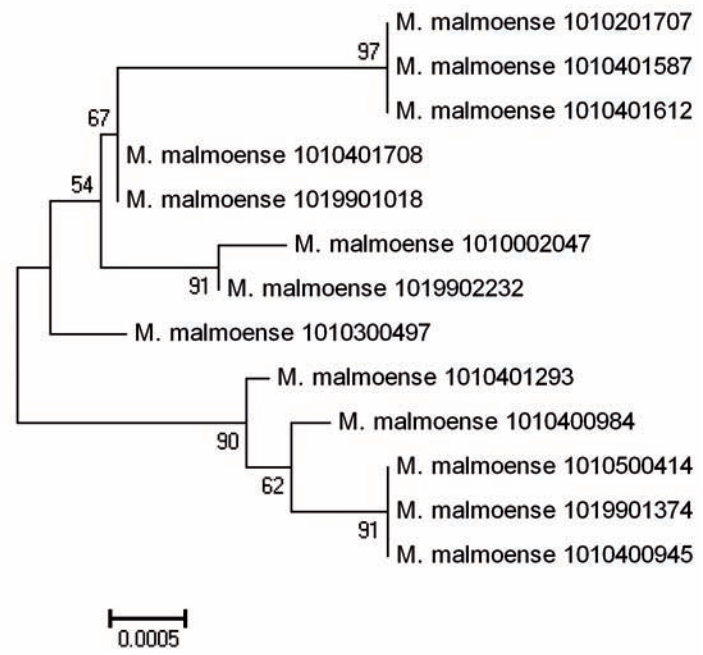

Figure 4:
M. malmoense; concatenated 16S, ITS, *hsp65*, *sodA*, *secA1* and *rpoB* sequences. The 13 *M. malmoense* isolates are divided into eight clusters.

stochastic pattern, i.e. groupings based on polymorphism in one gene were not reflected in groupings based on any of the other genes. Therefore, these sequence analyses presumably indicate a long term, whole genome evolution that can be used to subdivide strains within a species into separate groupings, representing different branches of the evolutionary tree.

For the two species studied in more detail, *M. xenopi* and *M. malmoense*, we noted an equal extent of genetic divergence. We could not link any subtype with pulmonary or extrapulmonary sources of isolation or, more importantly, with true NTM disease based on the American Thoracic Society diagnostic criteria.[6] In the current study only semi-conserved house keeping genes were analyzed, because they are believed to be most informative regarding the phylogeny of bacteria. However, virulence and pathogenicity may be based on highly subtle genetic differences between strains in genes essential in host-pathogen interactions. Therefore, it is conceivable that whole genome sequencing of multiple NTM isolates will be required to disclose associations between subtypes and clinical importance. In turn, these differences between subtypes may be utilized in relatively simple mono-target identification methods.

For *Mycobacterium kansasii*, subtyping based on ITS sequences is known to contribute to the daily practice of clinical laboratories as *M. kansasii* type I is a pulmonary pathogen, type II is a causative agents of HIV-associated infection, whereas the remaining types are typically avirulent environmental bacteria.[16] We have previously noted a similar association between *M. xenopi* 16S type II

and clinical disease.[20] The number of isolates tested per species in the current study may have been too low to permit sound statistical analysis. For *M. malmoense*, clinically non-significant isolates are very rare[7,8], which hampered our strain selection. This issue is also complicated by the emergence of intermediate types; we noted a *M. kansasii* type I isolate with a *secA1* sequence closely related to an *M. kansasii* II isolate, similar to the intermediate type described by Iwamoto and co-workers based on *hsp65* sequences.[9]

Most targets used in our study resulted in adequate species discrimination; only 16S sequencing revealed the well known lack of differentiation within the *M. chelonae* complex. In the group of isolates identified as *M. chelonae* complex by routine 16S rDNA gene sequencing, *rpoB* and *hsp65* sequencing revealed the existence of multiple subgroupings that were elevated to separate species status by previous researchers.[2] The presence of *M. bolletii* and *M. massiliense* has not been previously recorded in the Netherlands.

An important limitation of *sodA* and *secA1* sequencing is the paucity of sequence data available for comparison in GenBank. For *secA1* little data has been added since the publication of the type strain sequences by Zelazny and co-workers.[21] Aside from limited available data, the *sodA* sequences of *M. abscessus* CIP 104536T and *M. gadium* CIP 105388T stored in GenBank are identical. Based on their level of species recognition and availability of comparative data, 16S, ITS, *hsp65* and *rpoB* may be the most suitable targets for NTM identification, the latter being especially relevant for distinction of rapid growers.[2]

The divergence from published type strain sequences may reflect a geographically determined genetic divergence within NTM species and this may be important in the production of rapid hybridization assays for NTM identification. Such assays should ideally be based on sequences obtained from NTM isolated in the area where the assays will be used.

It is interesting that for *M. xenopi*, the divergence was essentially limited to the *rrn* operon (of which we examined 16S and ITS), whereas for *M. malmoense* the reverse was true. The background of these differences should be subject of future studies.

We also noted high (3-6%) levels of divergence among *M. gordonae* isolates in all targets and may result from long term evolutionary divergence; in the *rrn* operon this heterogeneity had been previously noted.[11] Among the rapid growers, new species have now been described based on >3% divergence from established species in the *rpoB* sequence.[2] An extrapolation of this cut-off value is likely to lead to the distinction of multiple species in the group now named *M. gordonae*. As *M. gordonae* is generally not of any clinical importance and, hence, the introduction of additional species designations may not be clinically meaningful, it may be more appropriate to introduce the term '*M. gordonae* complex' for these bacteria.

The mosaic rather than a stochastic pattern of intraspecies genetic divergence

may reflect the enormous genetic divergence among these environmental bacteria, only some of which are studied after recovering them from humans. On the other hand, the level of intraspecies divergence in the targets used in this study seems too low to be the result of lateral transfer of genetic information between NTM or between NTM and other taxa. There are indications of a role for lateral gene transfer in the evolution of *M. tuberculosis* and *M. marinum*, stemming from their whole genome sequences.[15] Despite our attempts, only whole genome sequencing will reveal the true extent of intra-(and in fact inter-) species genetic divergence among NTM. Currently, however, the vast majority of mycobacteria subjected to whole genome sequencing are *M. tuberculosis* complex members.[3]

There is an additional clinical utility for this intraspecies genetic divergence. A recent study revealed intraspecies divergence in a group of *M. mucogenicum* and *M. phocaicum* isolates which proved useful in an outbreak investigation.[4]

In summary, a previously unrecognized and substantial intraspecies genetic divergence exists among NTM. The variance within the targeted genes has a mosaic rather than a stochastic pattern and its extent differs by species. Taking our findings and previous studies together, it seems unavoidable to move into the direction of more extended DNA sequencing of NTM isolates. The extent of such analyses depends on the extent of genetic diversity, which will differ by species, as well as the clinical importance of a NTM species. To improve our understanding of the importance of genetic divergence, a structural collection of clinical information is essential. An international, integrative database combining DNA sequence data of NTM isolates and clinical aspects may be most beneficial in this respect. Such a database may also be helpful in refining the phylogenetic tree of NTM. It is conceivable that clinical importance will differ by (intraspecies) branch and once such a tree has been established, a reliable recognition of such branches by renewed, simple identification methods comes into sight.

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The Region of Difference 1 in nontuberculous *Mycobacterium* species adds a phylogenetic and taxonomical character

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Summary

The *esat-6* and *cfp-10* genes are essential for virulence in *Mycobacterium tuberculosis*. Among nontuberculous mycobacteria we found these genes only in *M. kansasii*, *M. szulgai*, *M. marinum* and *M. riyadhense*, with unique sequences. This adds a phylogenetic and taxonomical character and may represent a virulence factor for nontuberculous mycobacteria.

The 6 kDa early secretory antigenic target (ESAT-6) and 10 kDa culture filtrate protein (CFP-10) of *Mycobacterium tuberculosis* are potent T-cell antigens.[1,2] The genes encoding ESAT-6 and CFP-10 are situated within the "region of difference 1" (RD1) of *Mycobacterium tuberculosis*, but are also present in some nontuberculous mycobacteria (NTM).[1,2] Deletion of the RD1 in *M. tuberculosis* significantly decreases its virulence in animal models,[3] suggesting that genes residing in the RD1 are involved in the pathogenesis. In *M. tuberculosis* the RD1 proteins effect translocation to the cytosol, a mechanism for survival within macrophages.[4] Therefore, the RD1 in NTM may also play a crucial role in virulence.

To improve our understanding of the pathogenesis of NTM disease and the possible role of the RD1 in virulence, we screened a wide diversity of NTM for presence of the RD1.

From our laboratory database, we retrieved isolates of all 5 *M. kansasii* subtypes based on 16S-23S internal transcribed spacer sequencing (n=15), *M. szulgai* (4), *M. marinum* (4), *M. avium* (2), *M. conspicuum* (4), *M. genavense* (1), *M. bohemicum* (2), *M. interjectum* (2), *M. flavescens* (5), *M. xenopi* (2), *M. malmoense* (2), *M. riyadhense* (1) and *M. tuberculosis* H37Rv. The selection of strains was based on their phylogenetic relationship with the *M. tuberculosis* complex, in the multi-gene taxonomical model published by Devulder et al.[5]

To establish the presence of an RD1-like element and sequences of *esat-6* and *cfp-10* genes, we used Esa-12 CATGACAGAGCAGCAGTG and Esa-303 5'-GCCCTATGCGAACATCCC-3' primers for *esat-6* and opBR78 5'-GTAGCCCGGGATGGCAGAGATGAAGACCGATGCC-3' and opBR103 5'-TCAGAAGCCCATTTGCGAGGACAGC-3' primers for *cfp-10*.[6] The *M. smegmatis esat-6* and *cfp-10* gene sequences were extracted from the whole genome sequence in the GenBank database (accession number CP000480).

Using these primers, we were able to demonstrate an RD1 for *M. tuberculosis* H37Rv, as well as for all *M. kansasii* subtypes, *M. szulgai*, *M. marinum* and *M. riyadhense*. The PCR was repeatedly negative for isolates of the remaining species *M. avium*, *M. conspicuum*, *M. genavense*, *M. bohemicum*, *M. interjectum*, *M. flavescens*, *M. xenopi* and *M. malmoense*. For these species, we performed southern blotting and hybridized DNA membranes using the purified *M. tuberculosis* H37Rv and *M. kansasii* type I *esat-6* amplicon as a probe.[6] None of the PCR negative species hybridized with either probe (data not shown).

Presence of an RD1, characterised by an *esat-6*-like and *cfp-10*-like gene, is a phylogenetic character among the nontuberculous mycobacteria. It is found mainly only among slowly growing NTM species that are phylogenetically related to the *M. tuberculosis* complex, based on the multi-gene taxonomical model by Devulder et al.[5] and in the more distantly related rapid grower *M. smegmatis*. Possibly, presence of the RD1 reflects phylogenetic relationships to the *M. tuberculosis* complex.

Table 1: GenBank accession numbers of isolates sequenced in this study

Species	*esat-6*	*cfp-10*
M. szulgai	EU826486	FJ014490
M. marinum	EU826487	FJ014491
M. riyadhense	EU552926	EU552927
M. kansasii I	EU888292	FJ014492
M. kansasii II	EU888293	FJ014493
M. kansasii III	EU888294	FJ014494
M. kansasii IIIb	EU888295	FJ014495
M. kansasii IV	EU888296	FJ014496
M. kansasii V	EU888297	FJ014497
M. tuberculosis H37Rv	FJ014499	FJ014498

Previous authors have recorded presence of the RD1 in *M. flavescens*.[6] We were unable to demonstrate it in four reference strains (ATCC 23008, 23033, 23035 and 23039) and a clinical isolate. Thus, with its presence of an RD1 region, *M. smegmatis* still stands out among the rapid growing NTM.

Gey van Pittius *et al*. have demonstrated the ESX-5 locus, which they assume is a product of duplication of the RD1 and its secretion system (ESX-1), in most slow growing NTM species.[7] Our results seem to suggest that in many slow growers, after this duplication, the original ESX-1 was either lost or has undergone extensive mutation, barring hybridization.

All five subtypes of *M. kansasii* had distinct *esat-6* and *cfp-10* sequences; type three was subdivided in two separate lineages based on both *esat-6* and *cfp-10* sequences. Among *M. marinum* and *M. szulgai* strains, no difference was noted and the sequences obtained for *M. riyadhense* were unique. All obtained sequences are deposited in the GenBank database; accession numbers are detailed in Table 1. We aligned the *esat-6* and *cfp-10* gene sequences separately and concatenated using Clustal X software.[8] The resulting topology and tree, inferred by neighbor joining and visualized using the MEGA 4.0 software package,[9] were evaluated by bootstrap analyses based on 1000 re-samplings. Resulting trees are shown in Figure 1A (*esat-6*), B (*cfp-10*) and C (*esat-6* and *cfp-10* concatenated). From these trees, it is obvious that the slow growing RD1-positive NTM have *esat-6* and *cfp-10* sequences much closer related to those of *M. tuberculosis* than those of *M. smegmatis* (Figure 1A-C).

Thus, the presence of the RD1 in these slow growers marks a genetically closely related *Mycobacterium* grouping, and sequencing of the RD1 is a tool for (sub) species identification. Therefore, we propose that future introduction of new species phylogenetically related to the RD1-positive grouping should include an investigation of RD1 presence and gene sequences.

Deletion of the RD1 lowers the virulence of *M. tuberculosis* complex bacteria.[3,10,11] Although presence of an RD1 may thus be important for virulence, we were

not able to detect this genomic region in well known causative agents of disease in humans, including *M. avium* and *M. malmoense*.[1,12] In *M. smegmatis*, ESAT-6 and CFP-10 secretion has a role in conjugation, rather than translocation to the cytosol, by which *M. tuberculosis* survives within macrophages.[4,13] The remaining NTM species that harbour an RD1 are phylogenetically closer related to *M. tuberculosis* than to *M. smegmatis*,[5] which is also expressed in their *esat-6* and *cfp-10* sequences (Figure 1). Moreover, *M. kansasii*, *M. szulgai*

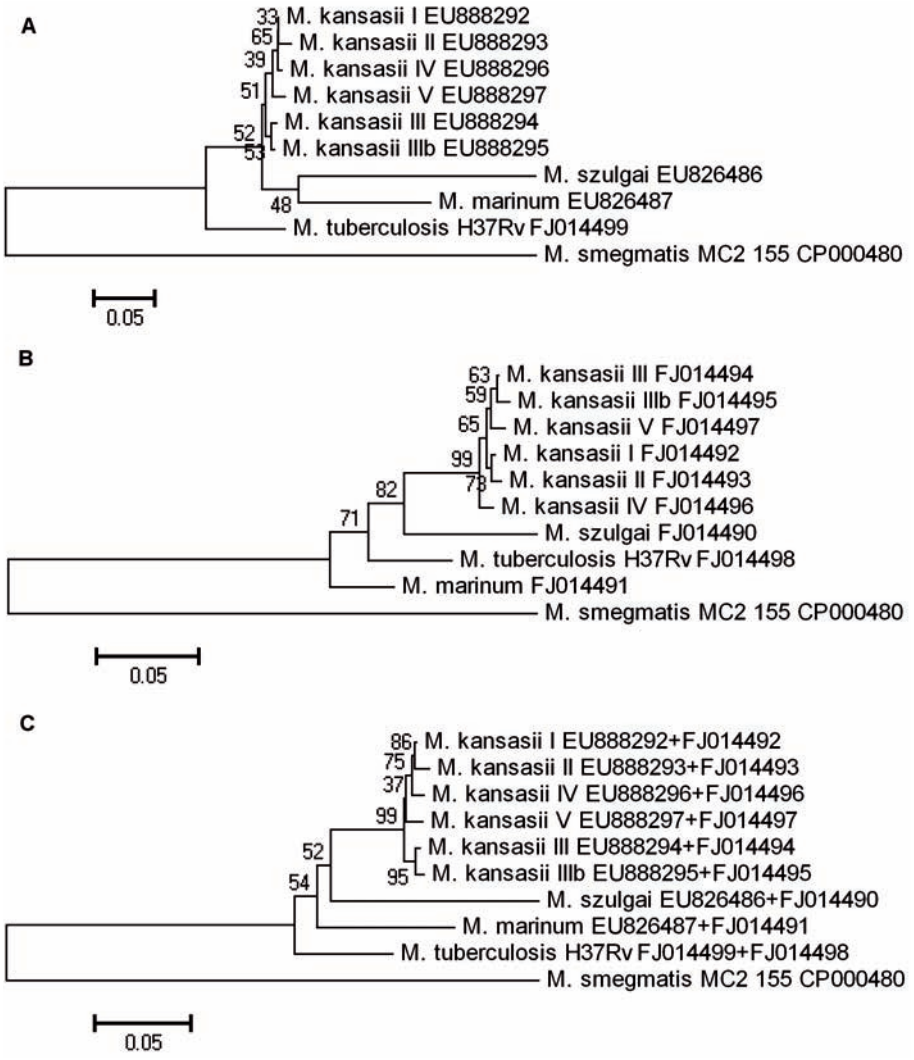

Figure 1: Phylogenetic trees based on multiple sequence alignment of *esat-6* (A), *cfp-10* (B) and concatenated *esat-6* and *cfp-10* (C) sequences.
Neighbour-joining (NJ) tree was created and bootstrapped 1000x with CLUSTALX and visualized with MEGA 4.0.[8,9] Bootstrap values are indicated at the nodes.

and *M. marinum* are considered most pathogenic among the NTM.[6,14,15] Therefore, the RD1 may play a role in virulence of these NTM.

The RD1 sequences differed between *M. kansasii* type I, an important causative agent of NTM disease, and the other types which are less or not involved in human disease.[6] Both clinically relevant and non-relevant *M. szulgai* isolates, determined using the American Thoracic Society diagnostic criteria,[14,15] shared identical sequences. The RD1 presence and gene sequences, thus protein structure, do not provide a complete explanation of the virulence of the different NTM. Presumably, host factors and pathogen factors other than RD1 presence are also important. *In vitro* infection experiments are necessary to clarify the role of the RD1 in slow growing NTM.

Demonstration and characterization of an RD1 in NTM has gained significance with the advent of the interferon gamma release assays (IGRAs) for the diagnosis of (latent) tuberculosis. These assays measure interferon-γ production and release by patients' T-lymphocytes after incubation with ESAT-6 and CFP-10 antigens of *M. tuberculosis*.[16] The presence of similar antigens in NTM, thus recognition of these antigens by patients infected by these NTM, theoretically lowers the specificity of the IGRAs in diagnosing latent tuberculosis.

In conclusion, an RD1 element, similar to *M. tuberculosis*, is present in *M. kansasii*, *M. szulgai*, *M. marinum* and *M. riyadhense*. Presence of an RD1 in general is a phylogenetic and taxonomical character of NTM, which hints at a phylogenetic relationship with the *M. tuberculosis* complex. The RD1 sequence analysis enables distinction to species or sub-species level. Future studies describing related new species should investigate RD1 presence and gene sequences. The role of the RD1 as a virulence factor and the impact of RD1-containing NTM on functioning of the IGRAs should be subject of further studies.

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Presence of *esat-6* and *cfp-10* genes does not lead to phagolysosome translocation of *Mycobacterium szulgai*

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Abstract

Mycobacterium tuberculosis secretes the 6 kDa early secretory antigenic target (ESAT-6) and 10 kDa culture filtrate protein (CFP-10), which effect translocation of these bacteria from the phagolysosome to the cytosol in macrophages; a crucial step for its virulence. *Mycobacterium szulgai*, a relatively virulent nontuberculous *Mycobacterium*, also harbors *esat-6* and *cfp-10* genes. To explore whether its virulence results from translocation from the phagolysosome to the cytosol in macrophages, we infected THP-1 cells with *M. szulgai* and *M. tuberculosis* to monitor their subcellular localization. Seventy-two hours after infection, no cytosolic *M. szulgai* bacteria were found, while 53% of the *M. tuberculosis* bacteria had translocated to the cytosol. Thus, while *M. szulgai* harbors *esat-6* and *cfp-10* genes, there is no translocation of the bacteria to the cytosol in THP-1 cells. ESAT-6 and CFP-10 structure or secretion could be critical factors. Translocation does not seem to be the underlying mechanism of virulence for *M. szulgai*.

Introduction

In *Mycobacterium tuberculosis*, the locus known as ESX-1 (ESAT-6 Secretion compleX 1) encodes a secretion system dedicated to secretion of the 6 kDa early secretory antigenic target (ESAT-6) and 10 kDa culture filtrate protein (CFP-10).[1,2] Genes encoding for ESAT-6 and CFP-10 like proteins are not limited to *M. tuberculosis* complex bacteria; the nontuberculous mycobacteria (NTM) *M. kansasii*, *M. marinum*, *M. szulgai* and *M. smegmatis* harbor similar genes.[3,4] In *M. tuberculosis* and *M. marinum* ESAT-6 and CFP-10 are crucial for translocation of the bacteria from the phagolysosome to the cytosol in myeloid cells, resulting in a direct access to the MHC class I antigen presentation pathway and thus CD8 T-cell activation. Translocation to the cytosol is not detected for the attenuated *M. bovis* BCG or *M. tuberculosis* ESX-1 knockout mutants. In *M. smegmatis*, ESAT-6 and CFP-10 have a role in conjugation.[2,5]

In a previous study, we found *M. szulgai* to be clinically highly relevant, i.e. 76% of the patients from whom *M. szulgai* is isolated have disease attributable to this organism.[6] We hypothesized that this level of virulence in humans, which is uncommon in NTM, may partially result from translocation from the phagolysosome to the cytosol in macrophages effected by ESAT-6 and CFP-10, as previously observed for *M. tuberculosis*. To explore this hypothesis, we performed an *in vitro* infection of human macrophages (THP-1) with *M. szulgai* to monitor the subcellular localization.

Methods

Isolates

We used a clinical *M. szulgai* isolate (NLA000701790) from a patient with severe, cavitary pulmonary disease. The isolate was identified by sequencing of the 16S rDNA gene. For comparison, we included a clinical *M. tuberculosis* isolate (NLA000200230) from a patient with pulmonary tuberculosis.

We sequenced the *esat-6* and *cfp-10* genes of the *M. szulgai* isolate using previously published primers.[7] The obtained sequences were aligned with those of *M. tuberculosis* H37Rv, *M. smegmatis* MC2 155 and previously investigated isolates of *M. szulgai*, *M. marinum* and *M. kansasii*, available in the GenBank/EMBL (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov) sequence database using Clustal X software.[8] The resulting topology and tree, inferred by neighbour joining and evaluated by bootstrap analyses based on 1000 re-samplings, was visualized using the MEGA 4.0 software package.[9]

Cell infections

THP-1 cells were cultured and maturation to macrophage like cells was induced by addition of PMA 24 hours before infection. Cells were infected with *M. szulgai* NLA000701790 and *M. tuberculosis* NLA000200230 cultures (Optical Density 0.5-1 McFarland) at a multiplicity of infection (MOI) of 10:1.

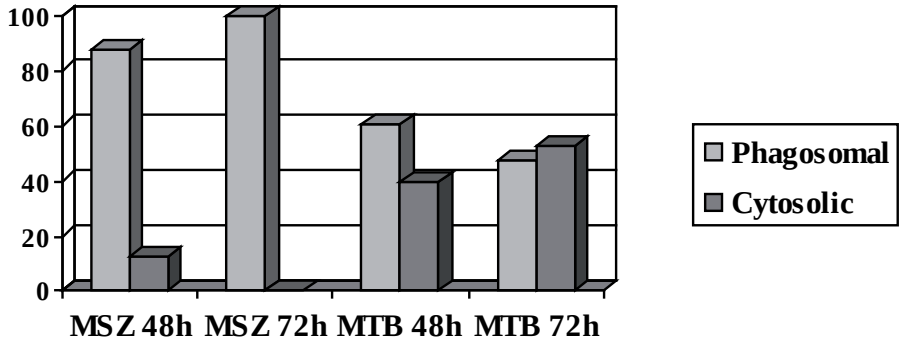

Figure 1: Percentage of phagosomal and cytosolic bacteria
MSZ: *Mycobacterium szulgai*; MTB: *M. tuberculosis*

After incubation for 2 hrs, extracellular bacteria were washed away. The cells were fixed at 48 and 72 hrs after infection and processed for cryo-immunogold electron microscopy, using previously published approaches.[2] We analyzed the intracellular localization of *M. szulgai* and *M. tuberculosis* using late endosomal markers cathepsin-D (clone 1C11, Zymed) LAMP-1 (clone H4B4, BD Biosciences) and CD63 (M1544, Sanquin, the Netherlands).

Results and Discussion

At 48 hrs after infection, the majority of *M. szulgai* bacteria were located within phagosomes, whereas 40% of the *M. tuberculosis* bacteria had translocated to the cytosol of the THP-1 cells. At 72 hrs, no cytosolic *M. szulgai* bacteria could be found, while 53% of the *M. tuberculosis* bacteria had translocated to the cytosol (Figures 1 and 2). Thus, despite presence of *esat-6* and *cfp-10* genes, translocation of *M. szulgai* from the phagolysosome to the cytosol of THP-1 cells could not be convincingly demonstrated. Although we noted a few cytosolic *M. szulgai* bacteria at 48 hrs after infection, these cytosolic bacteria were absent at 72 hrs post infection.

This finding suggests that presence of genes encoding for ESAT-6 and CFP-10-like proteins does not explain the high level of virulence of *M. szulgai*; at least not through translocation. From prior studies with *M. kansasii*[7] it is already known that the presence of ESAT-6 and CFP-10-like proteins can not explain the level of virulence. Conversely, this finding may explain the fact that *M. szulgai* disease is generally limited to hosts with an impairment of immunity or pre-existing tissue damage of affected organs.[6] The cell culture system here used most likely corresponds to an infection in immunocompetent persons rather than immunocompromised patients, specifically since THP1 cells represent matured macrophages, cells active in the immune responses.

Possibly, ESAT-6 and CFP-10 secretion, rather than presence of the respective genes, is the critical factor for virulence in an immunocompetent host.

Therefore, the ESX-1 secretion system of *M. szulgai* should be tested by 2D protein electrophoresis, to demonstrate the presence of ESAT-6 or CFP-10 in culture media on which *M. szulgai* is grown. ESAT-6 and CFP-10 secretion in host cells should also be monitored.

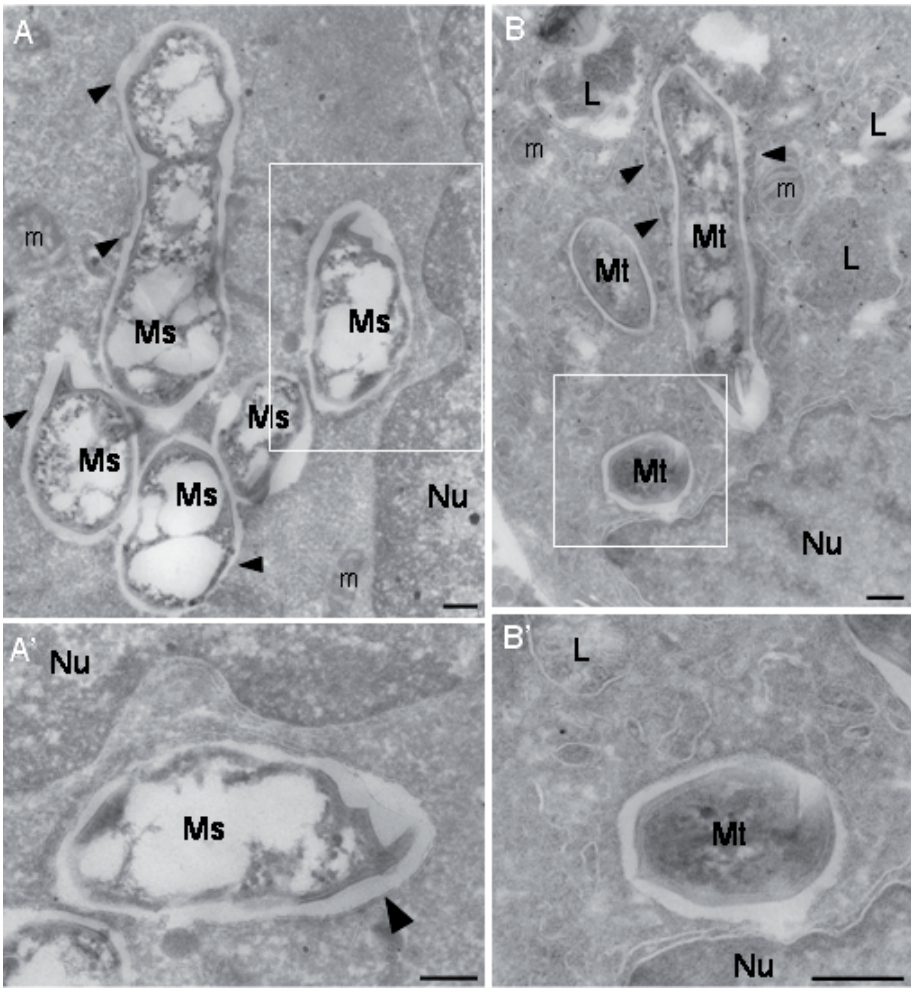

Figure 2: *M. szulgai* remains restricted in phagosome, while *M. tuberculosis* translocates into the cytosol of THP-1 cells.

THP1 cells were infected with *M. szulgai* (A) or *M. tuberculosis* (B) for 48 h, fixed and immunolabeled with lysosomal marker CD63 and 10 nm gold particles. Note that the magnification of A and B (all bars represent 200 nm) is the same. *Mycobacterium szulgai* bacteria are larger than *M. tuberculosis* and contain large electron lucent areas. Enlargement of the boxed region in A' demonstrated that *M. szulgai* remains restricted in a membrane enclosed compartment (arrowhead). *M. tuberculosis* is present both in the cytosol (B') and in membrane enclosed CD63 labeled compartments. Bars represent 200 nm; Ms: *M. szulgai*, Mt: *M. tuberculosis*, Nu: nucleus, L: lysosome, m: mitochondria, arrowheads: phagosomal membrane.

The *esat-6* and *cfp-10* gene sequences of the *M. szulgai* isolate were identical to those deposited in GenBank, EU826486 (*esat-6*) and FJ014490 (*cfp-10*). The *esat-6* sequence is 82% (236/289 bp) identical to that of *M. tuberculosis* H37Rv. These polymorphisms give rise to substitutions in 14 of 96 amino acids. The *M. szulgai cfp-10* sequence is 88% (267/303bp) identical to that of *M. tuberculosis* H37Rv, accounting for substitutions in 7 of 101 amino acids. The topology and tree resulting from the multi-sequence alignment are visualized in Figure 3.
These differences in the *esat-6* and *cfp-10* genes and their products in *M. szulgai* and *M. tuberculosis* are substantial and likely to result in changes in secondary and tertiary structure of ESAT-6 and CFP-10. The *esat-6* and *cfp-10* genes in *M. smegmatis* are very different from those in *M. tuberculosis* (figure 3) and their products have a very different role, i.e. in conjugation rather than translocation of the bacteria from the phagolysosome to the cytosol in myeloid cells.[5] Hence, the exact structure and thus functionality of these proteins in *M. szulgai* could also be different from those in *M. tuberculosis* and warrant further study.
Interestingly, the electron microscopic appearance of the *M. szulgai* bacteria was different from *M. tuberculosis*. The *M. szulgai* bacteria were large (average width 720 nm versus 500 nm for *M. tuberculosis*) and contained numerous large electron lucent areas (Figure 2).

In summary, while *M. szulgai* harbors *esat-6* and *cfp-10* genes, their products do not induce translocation of the bacteria from the phagolysosome to the cytosol in THP-1 cells to the extent known for *M. tuberculosis*. ESAT-6 and CFP-10 structure and functionality, as well as secretion could be the critical factors and should be addressed in future studies. The level of clinical relevance of *M. szulgai* cannot be explained by translocation of these bacteria to the cytosol.

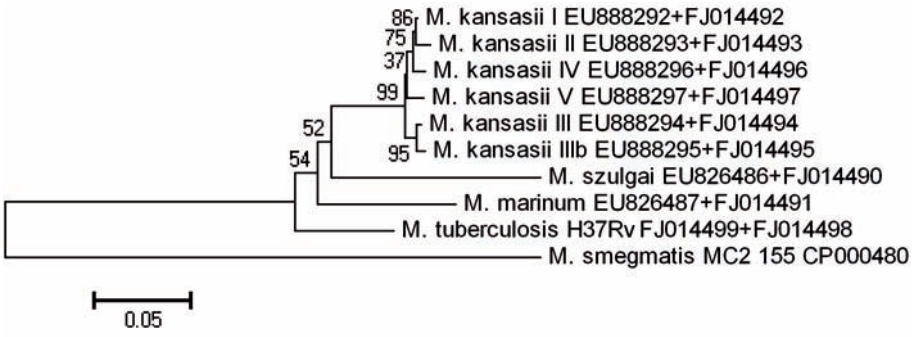

Figure 3: Phylogeny of ESX-1 harboring mycobacteria

The phylogeny is based on concatenated *esat-6* and *cfp-10* sequences, aligned using Clustal X software.[8] The Neighbour Joining tree was constructed and evaluated by bootstrap analyses based on 1000 re-samplings, using the MEGA 4.0 software package.[9] Bootstrap values are given at the nodes.

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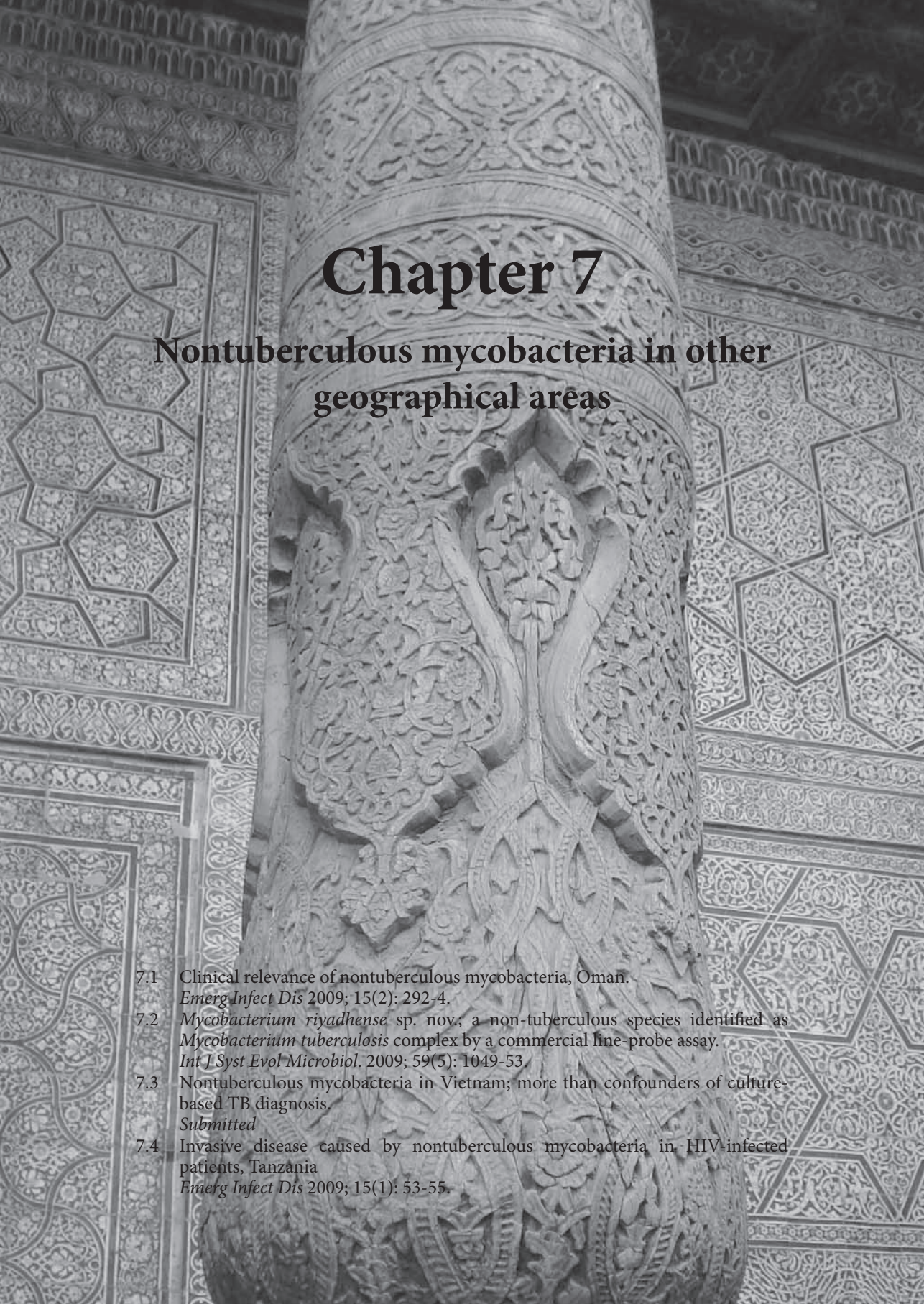

Chapter 7

Nontuberculous mycobacteria in other geographical areas

7.1 Clinical relevance of nontuberculous mycobacteria, Oman.
Emerg Infect Dis 2009; 15(2): 292-4.

7.2 *Mycobacterium riyadhense* sp. nov., a non-tuberculous species identified as *Mycobacterium tuberculosis* complex by a commercial line-probe assay.
Int J Syst Evol Microbiol. 2009; 59(5): 1049-53.

7.3 Nontuberculous mycobacteria in Vietnam; more than confounders of culture-based TB diagnosis.
Submitted

7.4 Invasive disease caused by nontuberculous mycobacteria in HIV-infected patients, Tanzania
Emerg Infect Dis 2009; 15(1): 53-55.

Clinical relevance of nontuberculous mycobacteria, Oman

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Summary

Little is known about the clinical relevance of nontuberculous mycobacteria in the Arabian Peninsula. We assessed the prevalence and studied a random sample of isolates at a reference laboratory in Muscat, Oman. Nontuberculous mycobacteria cause disease in this region and their prevalence has increased.

Introduction

Nontuberculous mycobacteria (NTM) are common inhabitants of the environment and have been cultured from water, soil and animal sources worldwide. The NTM are opportunistic pathogens, mostly affecting patients with pre-existing pulmonary disease such as chronic obstructive pulmonary disease or prior tuberculosis (TB), or those with systemic impairment of immunity. The latter group includes those with HIV-infection, those using immunosuppressive drugs and those with leukemia.[1]

Because NTM are common in the environment and resistant to commonly used disinfectants, they can be present in non-sterile patient material such as sputum and contaminate medical equipment (bronchoscope washers or samples in the laboratory) and consequently cause pseudo-infection.[1,2] To distinguish pseudo-infections from NTM disease and establish the clinical relevance of isolated NTM, the American Thoracic Society (ATS) has published diagnostic criteria.[1]

Studies on the clinical relevance of NTM have traditionally been restricted to western countries, where the incidence of TB is relatively low. More recently, research has been initiated in African and East Asian countries.[1-5] There have been no recent reports on isolation and clinical relevance of NTM on the Arabian Peninsula. We analyzed a random sample of NTM isolated from clinical samples in Oman by using molecular techniques. Their clinical relevance was retrospectively analyzed by applying the updated ATS diagnostic criteria for nontuberculous mycobacterial disease.[1]

The Study

Prevalence of NTM at the Central Public Health Laboratory (CPHL), the national TB reference laboratory of the Ministry of Health in Muscat, Oman, was assessed from the laboratory database. The CPHL Institutional Review Board reviewed and approved this study. The CPHL received 5,488 samples submitted for *Mycobacterium* spp. culture during 2006-2007. Samples were subcultured on Lowenstein-Jensen medium and automated Mycobacterial Growth Indicator Tubes (MGIT960; Becton Dickinson, Franklin Lakes, NJ, USA) liquid culture system and incubated at 36°C. A total of 491 (9%) samples yielded positive cultures. *Mycobacterium tuberculosis* complex bacteria were isolated from 445 (91%) samples, and NTM were isolated from 46 samples (9%). Most NTM were cultured from respiratory samples (sputum, n=36, 78%; bronchial wash, n=2, 4%), the remainder were from lymph nodes (n=3; 6%), urine (n=2; 4%) or other sources (n=3; 6%). The percentage of NTM increased from 7.6% (18/235) in 2006 to 10.9% (28/256) in 2007.

Thirteen samples were randomly selected from all NTM isolated at CPHL during January 2006-September 2007. Selected NTM were subjected to molecular identification at the Dutch National Mycobacteria Reference Laboratory (National Institute for Public Health and the Environment,

Bilthoven, the Netherlands). To identify NTM, after eliminating isolates of the *M. tuberculosis* complex by using the GenoType MTBC assay (Hain Lifesciences, Nehren, Germany), we used the INNO-LiPA MYCOBACTERIA v2 reverse line blot (Innogenetics, Ghent, Belgium). Both assays were used according to the manufacturer's instructions. If no species-specific result was obtained, additional sequencing of the hypervariable region A of the 16S rDNA gene was performed.[6]

The 13 samples were identified as nine *M. avium* complex (MAC; three *M. intracellulare* sequevars, three *M. chimaera*, one *M. colombiense*, one *M. avium*, and one untyped MAC), *M. marinum*, *M. kansasii*, *M. simiae* and *M. flavescens* (Table). One sample could not be identified beyond the *M. avium-intracellulare-scrofulaceum* complex level because insufficient DNA was available for further analyses. Because our molecular identification method does not distinguish *M. marinum* from *M. ulcerans*, we performed a light exposure test, which prompted yellow colony pigmentation, typical for *M. marinum*. One sample yielded *M. tuberculosis* and *M. intracellulare* (Table 1).

We assessed the clinical relevance of isolates from 13 patients in a retrospective study, by applying ATS diagnostic criteria and scoring demographic and clinical data. Results are detailed in the Table. Seven (54%) of the patients were female (mean age 43 years). Eight (62%) of thirteen patients met the ATS diagnostic criteria and were thus likely to have NTM disease. Among nine patients with MAC isolates, six (67%) had MAC disease. Most (n=11; 85%) isolates were cultured from pulmonary samples. Fibrocavitary and nodular-bronchiectatic pulmonary NTM disease were noted, with a predominance of fibrocavitary disease (Table).

Information on predisposing conditions was not available for most patients, although a destroyed lung on chest radiographs suggested previous pulmonary disease. One patient was HIV-positive, and another patient had a relapse of pulmonary NTM disease.

Eight patients began treatment, mostly with first-line treatment for TB. Three patients with pulmonary MAC disease and one with *M. marinum* skin disease received regimens that included macrolides, fluoroquinolones or both. Therapy resulted in clinical improvement in all but one patient. Most patients are still receiving treatment.

Conclusions

A total of 9% of all *Mycobacterium*-positive cultures at CPHL in Muscat, Oman, yielded NTM. Although this conclusion is based on limited data, the prevalence of NTM seems to be increasing in Oman. Few studies are available, but this increase may be true for the entire Middle East region.

Eight of 13 patients met the ATS diagnostic criteria; this finding probably reflects a selection bias because CPHL is a reference laboratory. Nevertheless, these findings indicate that NTM isolation in Oman and throughout the

Table: Clinical and microbiological data for 13 patients with *Mycobacterium* spp. infections, Oman, 2006-2007*

Pt	Sex, Age	Species	AFB Smear	Positive cultures	Source	Predisposing conditions	Symptoms	Chest radiograph	2007 ATS criteria	Therapy	Outcome
1	F,64	*M. intracellulare*†	positive	Multiple	sputum	-	PC,F,WL,CP,M	NP	met	HRZE	Improved
2	M,29	MAIS complex	positive	Single	sputum	-	PC, Hp, WL	Cavities	met	HRZE	Improved
3	F,31	*M. chimaera*‡	negative	Multiple	sputum	-	none	RUL bronchiectasis	Not met	none	Stable
4	M,28	*M. chimaera*‡	positive	Multiple	sputum	-	PC,WL	R: multiple scars L: destroyed lung, abscesses, PT	Met	SClaCip	Failure
5	M,18	16S: *M. colombiense*	negative	Single	BAL	Heart disease	PC,Hp, CP	NP	Met	HRE	Improved
6	M,57	16S: *M. flavescens*	NP	Single	urine	-	Abdominal pain	Not performed	Not met	n.a	n.a.
7	F,54	*M. simiae*	negative	Single	sputum	HIV	PC	Patchy opacities in LUL and lingula	Met	HRE	Improved
8	F,12	*M. kansasii* III/IV/V	negative	Single	sputum	-	none	normal	Not met	n.a.	n.a.
9	M,43	*M. tuberculosis* & *M. intracellulare*†	positive	Multiple	sputum	-	PC,F,WL,M	Cavities	n.a.	HRZES	Failure
10	F,7	*M. marinum*	negative	Single	Skin		Skin lesion	NP	Met	ECla	Improved
11	F,65	*M. chimaera*‡	positive	Multiple	sputum	-	PC, Back ache	Destroyed L lung	Met	ClaCip	Improved
12	F,83	*M. avium*	negative	single	sputum	-	none	RUL infiltration	Not met	n.a.	n.a.
13	M,63	*M. intracellulare*†	positive	single	sputum	Prior pulmonary NTM disease	PC,WL,M	L +R bronchiectasis, parenchymal infiltration	Met	RECip	Improved

*AFB, Acid-fast bacilli; ATS, American Thoracic Society; MAIS, *Mycobacterium avium-intracellulare-scrofulaceum*; BAL, broncho-alveolar lavage fluid; PC, productive cough; Hp, hemoptysis; F, fever; WL, weight loss; CP, chest pain; M, malaise/fatigue; L, left; R, right; UL, upper lobe; PT, pleural thickening; H, isoniazid; R, rifampicin; Z, pyrazinamide; E, ethambutol; S, streptomycin; Cla, clarithromycin; Cip, ciprofloxacin; n.a.; not applicable; NP, not performed;
†Reaction with the MIN-1 probe; *M. intracellulare* sequevar Min-A, -B, -C or -D.
‡Reaction with the MIN-2 probe; *M. intracellulare* sequevar MAC-A, recently elevated to species level (*M. chimaera*).[7]

Middle East region is a serious issue that requires attention by clinicians and microbiologists.

Most isolates were MAC members (9/13; 69%), a predominance also noted in previous studies in the USA, European and west Asian countries (25%),[1,8] as well as in a recent study from South Korea (48%).[9] MAC isolates from Oman were mostly *M. intracellulare* sequevars. Infrequent isolation of *M. avium* is noteworthy, despite the small number of isolates in the random sample. Previous North American studies have suggested that *M. intracellulare* is the more common pulmonary pathogen within the MAC.[1] We identified three pulmonary samples as MAC-A strains, recently elevated to species rank and named *M. chimaera*.[7] Two samples were clinically relevant. Although *M. chimaera* has been assumed to be highly virulent,[7] a recent study in Germany found only 3.3% of 90 *M. chimaera* isolates to be clinically relevant.[10] *Mycobacterium colombiense* was first described as a causative agent of mostly disseminated disease in HIV patients from Colombia[11] and was recently isolated from a child with lymphadenopathy in Spain.[12] Its isolation in other countries and from respiratory samples in HIV-negative patients has not been previously reported. Isolation of *M. simiae* is of interest, as this species has been reported to be especially prevalent in the Middle East,[1] and HIV-associated disease has been reported in the region.[13]

Most patients in our study received standard treatment for TB. Although treatment should be prolonged and pyrazinamide discontinued because of natural resistance to pyrazinamide in NTM, the choice of first-line treatment for TB, without companion drugs such as macrolides or fluoroquinolones, is supported by a recently published trial of the British Thoracic Society.[14]

In summary, NTM are a serious issue in Oman and their prevalence may be increasing. Our random sample demonstrates that MAC isolates are most frequently isolated. True NTM disease, on the basis of ATS diagnostic criteria, was diagnosed in 62% of the patients assessed. Isolation of NTM is clinically relevant on the Arabian Peninsula and warrants further study.

Acknowledgement:
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Mycobacterium riyadhense sp. nov.; a non-tuberculous species identified as *Mycobacterium tuberculosis* complex by a commercial line-probe assay

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Summary

A non-chromogenic, slowly growing *Mycobacterium* strain was isolated from a maxillary sinus lavage from a symptomatic patient in Riyadh, Saudi Arabia. It was initially identified as a member of the *Mycobacterium tuberculosis* complex by a commercial line-probe assay. Its 16S rDNA, *hsp65* and *rpoB* gene and 16S-23S internal transcribed spacer sequences were unique; phylogenetic analysis based on the 16S rDNA sequence groups this organism close to *M. szulgai* and *M. malmoense*. Its unique biochemical properties and mycolic acid profile support separate species status. We propose the name *Mycobacterium riyadhense* sp. nov. to accommodate this strain. The type strain is NLA000201958^T (=CIP 109808^T; =DSM 45176^T).

A 19-year-old male reported to the otolaryngology department of the King Faisal Hospital with pain and swelling of the left side of his face with protrusion of the left eye after blunt trauma. A computed tomography scan of the sinuses was performed, which revealed a tumor in the left maxillary sinus. The tumor involved the nasal septum, extended into the left orbit and infiltrated the medial and inferior rectus as well as the optic nerve. A chest radiograph revealed no abnormalities. A lavage of both maxillary sinuses was performed for diagnosis. Mycobacterial cultures were performed on Lowenstein-Jensen (LJ) media and yielded an isolate with unusual colony morphology after 3 weeks of incubation at 36°C.

The patient was presumed to have bone tuberculosis (TB) and started a nine month anti-tuberculosis regimen. The patient improved both clinically and radiologically. He has not suffered a relapse since. The isolated strain was sent to the Dutch National Mycobacteria Reference Laboratory (RIVM, Bilthoven, Netherlands) for identification, as part of a second-line quality control program. Identification of the strain was first attempted using three commercial line-probe assays, GenoType MTBC and GenoType CM/AS (Hain Lifescience GmbH, Nehren, Germany) and INNO-LiPA MYCOBACTERIA v2 (Innogenetics NV, Ghent, Belgium), all used according to manufacturer's instructions.

Table 1: Sequence comparisons between strain NLA000201958^T and its closest relatives

Gene/region	GenBank	RIDOM
16S rDNA (full)	*M. malmoense* (99%) *M. szulgai* (99%) *M. bohemicum* (98%)	*M. szulgai* DSM44166^T (99.1%) *M. avium* complex ATCC35770 (98.4%) *M. intracellulare* ATCC35772 (98.4%) *M. haemophilum* ATCC29548^T (98.4%)
16S-23S ITS (273bp)	*M. szulgai* (91%) *M. kansasii* (91%) *M. marinum* (91%)	*M. kansasii* DSM44162^T (92%) *M. gastri* DSM43505^T (91%) *M. marinum* DSM44344^T (90%)
23S rDNA (full)	*M. kansasii* (97%) *M. avium* (97%) *M. ulcerans* agy99 (97%)	
rpoB (472bp)	*M. avium* 104 (93%) *M. paratuberculosis* K-10 (93%) *M. tuberculosis* H37Rv^T (91%)	
hsp65 (421bp)	*M. genavense* DSM44424^T (95%) *M. bohemicum* CIP105811^T (95%) *M. malmoense* CIP105775^T (95%)	
esat-6	*M. kansasii* (89%) *M. tuberculosis* H37Rv^T (87%) *M. szulgai* (85%)	
cfp-10	*M. tuberculosis* H37Rv^T (88%) *M. marinum* M (85%) *M. ulcerans* (84%)	

Note: Where no strain is indicated, multiple highly similar sequences are deposited in GenBank

To obtain identification to the species level, we sequenced the 16S rDNA gene, 16S-23S internal transcribed spacer (ITS), and *rpoB* and *hsp65* genes, using previously described approaches.[1-4] The DNA sequences obtained were compared with RIDOM (Ribosomal Differentiation of Medical Microorganisms: http://rdna.ridom.de) and GenBank (National Center for Biotechnology Information: http://www.ncbi.nlm.nih.gov) databases.
To establish the presence of a region of difference 1 (RD1)-like element and the DNA sequences of the *esat-6* and *cfp-10* genes, we used primers from a previous study.[5]

The most commonly investigated biochemical and phenotypical features (colony morpholgy, ability to grow at temperatures ranging from 24 to 45 °C, niacin accumulation, nitrate reduction, β-glucosidase, Tween 80 hydrolysis, 3-day arylsulfatase, urease, tellurite reduction, 68 °C and semiquantitative catalase, growth rate, pigmentation, growth on MacConkey agar and tolerance to 5% NaCl [on LJ medium], thiophene-2-carboxylic hydrazide [TCH] 5 µg/ml, oleate 250 µg/ml, p-nitrobenzoic acid 500 µg/ml, thiacetazone 10 µg/ml, hydroxylamine 500 µg/ml, and isoniazid 1 µg/ml [all in Middlebrook 7H10 agar]) as well as high performance liquid chromatography (HPLC) analysis of cell wall mycolic acid content were tested by the Regional Reference Center for Mycobacteria (Careggi Hospital, Florence, Italy), using standard procedures described previously.[6,7]
Drug susceptibility testing was performed using the 25-well agar dilution method.[8] We included isoniazid, rifampicin, rifabutin, ethambutol, clarithromycine, ciprofloxacin, cycloserine, prothionamide, amikacin, clofazimine and streptomycin in the test panel.

Applying the GenoType MTBC assay, a non-specific reaction was noted with hybridization of the *M. tuberculosis* complex band only (banding pattern 1, 2, 3). The InnoLipa MYCOBACTERIA v2 line-probe assay yielded a *Mycobacterium* genus probe reaction, though no species-specific result. The GenoType CM (Common Mycobacteria) kit identified the strain as *M. tuberculosis* complex based on a band 1,2,3,10,16 pattern. The supplementary AS (Additional Species) kit identified the strain as a non-specified *Mycobacterium* species, with banding pattern 1, 2, 3, 12. Sequencing of the full 23S rDNA gene, the assay's target, established its identity as a nontuberculous mycobacterium (Table 1). Based on these findings, IS*6110* and IS*1081* RFLP were performed to confirm its identity as *M. tuberculosis*.[9] No IS*6110* or IS*1081* element copies could be demonstrated (results not shown).
The sequencing results are listed in Table 1; the 16S, ITS, *rpoB* and *hsp65* gene sequences were all unique. Sequencing of the full 16S rDNA gene identified the bacterium as a *Mycobacterium szulgai*-like species (Table 1). The 16S rDNA gene sequence was aligned with those of reference strains of the closest related mycobacteria using Clustal X.[10] The resulting topology and tree, inferred by

neighbour joining and visualized using the MEGA 4.0 software package,[11] were evaluated by bootstrap analyses based on 1000 resamplings (Figure 1). The tree was rooted with *Nocardia abscessus* ATCC BAA-279^T as an outgroup. Similar multisequence alignments and trees were created based on the ITS and *hsp65* sequences (Supplementary Figures S1 and S2, available in IJSEM online).
We were able to amplify an RD1 region, including an *esat*-6 (270bp) and cfp-10 (251bp) gene. Results of sequence comparisons for both genes are recorded in Table 1.

On Middlebrook 7H10, Ogawa and Stonebrink media, the strain produced small, rough, non-pigmented colonies after 28 days of incubation at 36 °C. After three days of exposure to light at ambient temperature, no pigmentation was observed. Growth on Middlebrook 7H10 agar was only observed at 24, 30 and 36 °C. Optimal growth occurred at 36 °C. Colony morphology on Middlebrook 7H10 was similar at all temperatures.
Generally, the biochemical profile of the isolate is unique, although it shares characterstics with *M. szulgai* and *M. malmoense*.[12] The isolate was negative for niacin accumulation, heat-stable catalase (pH 7, 68 °C), β-glucosidase, tellurite reduction, growth on MacConkey agar and tolerance to para-nitrobenzoic acid, hydroxylamine and oleic acid, but positive for nitrate reduction, semi-quantitative catalase, Tween 80 hydrolysis, 3 day arylsulfatase and urease activity and tolerance to TCH, thiacetazone and isoniazid. A comparison with profiles of *M. szulgai* and *M. malmoense* is recorded in Table 2.

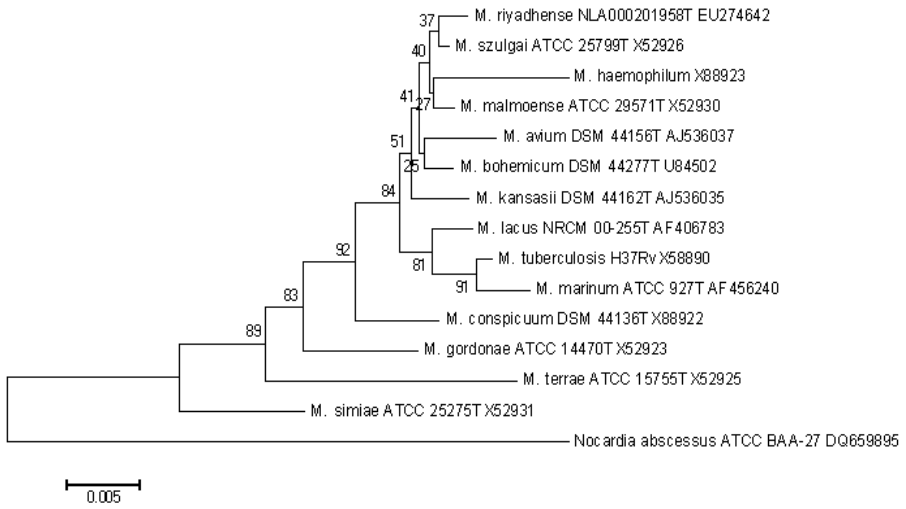

Figure 1: Phylogenetic relationship of NLA000201958^T (*Mycobacterium riyadhense* sp. nov.) and related species of *Mycobacterium*, based on 16S rDNA gene sequences. Neighbour-joining tree created and bootstrapped 1000 times with CLUSTAL X[10] and visualized with MEGA 4.0.[11] Bootstrap values are indicated at nodes.

Table 2: Biochemical identification results of strain NLA000201958^T and related species
* In Middlebrook 7H10 agar

Test	Strain NLA000201958^T	*M. szulgai*	*M. malmoense*
Nitrate reduction	+	+	-
68 °C catalase	-	+	+/-
Catalase >45 mm	+	+	-
β-glucosidase	-	+/-	-
Tween 80 hydrolysis	+	+/-	+
Tellurite reduction	-	+/-	+
3 day Arylsulfatase	+	+	-
Urease	+	+	-
Pigmentation	Absent	Photochromogen	Absent
Colony morphology	Rough	Smooth/rough	Smooth
Growth at 25°C	+	+	+/-
Tolerance to:*			
p-Nitrobenzoic acid 500 µg/ml	-	+	+
Isoniazid 1 µg/ml	+	+/-	+
Hydroxylamine 500 µg/ml	-	-	+/-

HPLC revealed a pattern characterized by a single, narrow, late-emerging cluster of peaks. A similar profile is presented by a limited number of mycobacteria (*M. brumae*, *M. fallax*, *M. triviale* and *M. tuberculosis* complex) with none of them fully overlapping the pattern of the strain characterized here (Figure 2). We used the HPLC mycobacterium library (http://www.MycobacToscana.it/english.htm) for this comparison.
Drug susceptibility testing revealed *in vitro* resistance to amikacin (minimum inhibitory concentration [MIC] 10 µg/ml) and para-amino-salicylate (MIC >1 µg/ml), intermediate susceptibility to isoniazide (MIC 1 µg/ml) and susceptibility to rifampicin (MIC 0.2 µg/ml), rifabutin (MIC ≤ 0.2 µg/ml), ethambutol (MIC 5 µg/ml), clarithromycine (MIC ≤ 2 µg/ml), ciprofloxacin (MIC 2 µg/ml), cycloserine (MIC 20 µg/ml), prothionamide (MIC ≤ 1 µg/ml), clofazimine (MIC ≤ 0.5 µg/ml) and streptomycin (MIC 5 µg/ml).

Although identification of *M. tuberculosis* complex is not the main use of the GenoType CM test, false-positive results may lead to incorrect diagnoses of TB and unwarranted TB treatment. Aside from the identification as *M. tuberulosis* by a line probe assay, which sparked this study, both the molecular and HPLC analyses of the novel strain suggest a phylogenetic relationship with *M. tuberculosis*; the presence of an RD1 region strengthens this assumption. Its identity as a nontuberculous mycobacterium is easily proven by sequencing of the 16S rDNA, *rpoB*, *hsp65*, or RD1 genes and the absence of IS*6110* and IS*1081* elements. The novel strain seems closely related to *M. szulgai*, based on 16S rDNA gene and ITS sequences (Figure 1 and Supplementary Figure S1),

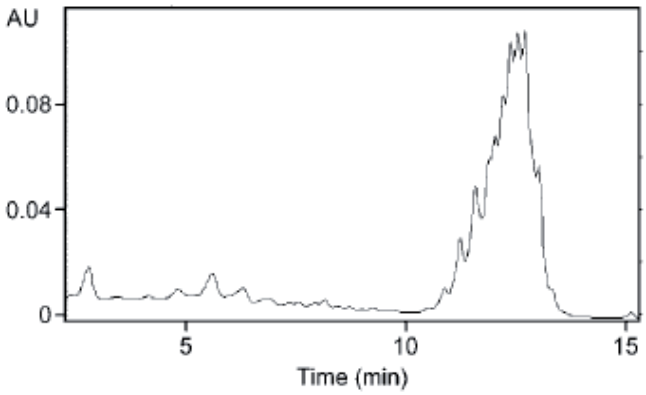

Figure 2: Mycolic acid pattern of strain NLA000201958^T (*M. riyadhense* sp. nov.) obtained by HPLC analysis. The single, narrow, late-emerging cluster of peaks is similar, though not identical, to results for *M. brumae*, *M. fallax*, *M. triviale* and *M. tuberculosis* complex.

although it is related more distantly to *M. szulgai* based on *rpoB* and *hsp65* sequences (Table 1 and Supplementary Figure S2). Its nonchromogenicity, however, is distinct from the photochromogenic *M. szulgai*.

Retrospectively, this isolate seems clinically relevant, based on its isolation from a normally sterile body site and the symptomatic improvement of the patient after 9 months of TB therapy.[13] This suggests that the novel strain was the causative agent of this patient's disease.

Both the close phylogenetic relationship with species such as *M. szulgai* and *M. kansasii*, which are among the most pathogenic nontuberculous mycobacteria,[13,14] and the presence of an RD1 region with *esat-6* and *cfp-10* genes, which is a virulence factor in *M. tuberculosis*,[15] add to our view that this strain is a human pathogen. On the basis of the data presented here, we describe a novel species to accommodate strain NLA000201958^T, for which we propose the name *Mycobacterium riyadhense* sp. nov.

Description of *Mycobacterium riyadhense* sp. nov.

Mycobacterium riyadhense (ri.ya.dhen'se; N.L. neut. adj., *riyadhense* after Riyadh, capital of the Kingdom of Saudi Arabia and origin of the patient from whom the type strain was isolated).

Slowly growing, nontuberculous mycobacterium that produces rough, white colonies after 28 days of incubation at 36 °C; growth is slower at 25 and 30 °C and no growth occurs at 42 °C. Incorrectly identified as *M. tuberculosis* complex by the GenoType CM assay. Negative for niacin accumulation, heat-stable catalase, β-glucosidase, tellurite reduction, growth on MacConkey agar and tolerance to para-nitrobenzoic acid, hydroxylamine and oleic acid, but positive for nitrate reduction, semi-quantitative catalase, Tween 80 hydrolysis, arylsulfatase and urease activity and tolerance to TCH, thiacetazone and isoniazid. Readily identifiable by its unique rDNA sequences.

The type strain is NLA000201958^T (=CIP 109808^T; =DSM 45176^T), recovered from maxillary sinus lavage fluid.

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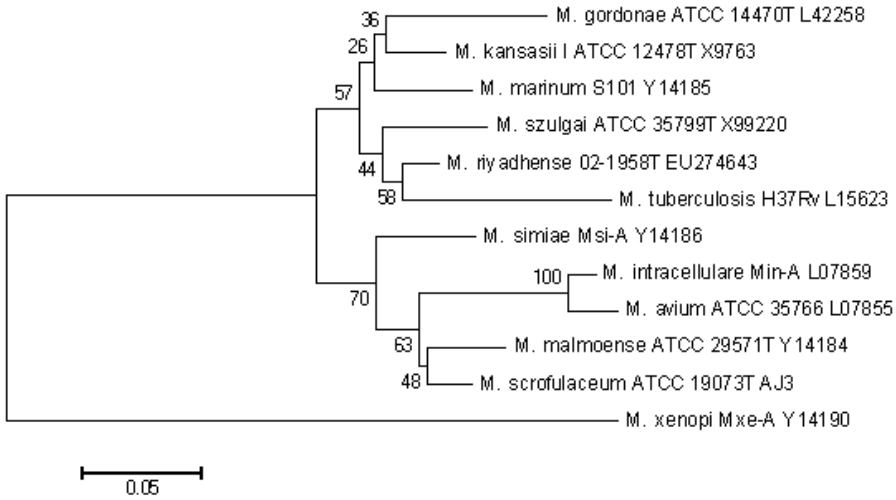
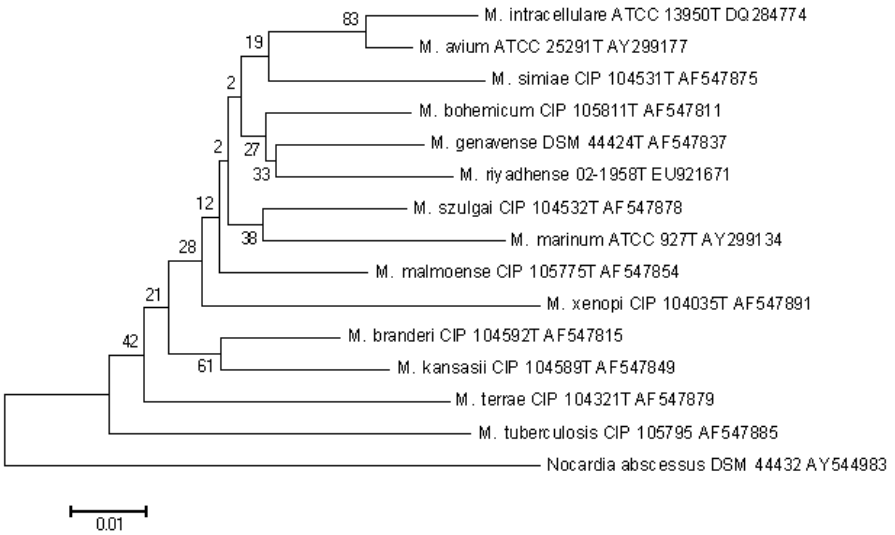


Supplementary Figure S1: Phylogenetic relationship of NLA000201958^T (*Mycobacterium riyadhense* sp. nov.) and related species of *Mycobacterium*, based on ITS sequences. Neighbour-joining tree created and bootstrapped 1000 times with CLUSTAL X[10] and visualized with MEGA 4.0.[11] Bootstrap values are indicated at nodes.



Supplementary Figure S2: Phylogenetic relationship of NLA000201958^T (*Mycobacterium riyadhense* sp. nov.) and related species of *Mycobacterium*, based on *hsp65* gene sequences. Neighbour-joining tree created and bootstrapped 1000 times with CLUSTAL X[10] and visualized with MEGA 4.0.[11] Bootstrap values are indicated at nodes.

Note: The GenBank/EMBL/DDBJ accession numbers for the sequences of the 16S rRNA, 16S-23S ITS, *rpoB*, *hsp65*, *esat-6* and *cfp-10* gene are EU274642, EU274643, EU274644, EU921671, EU552926 and EU552927. Phylogenetic trees based on 16S-23S internal transcribed spacer and *hsp65* sequences of selected mycobacterial species are available as supplementary material in IJSEM Online.

Nontuberculous mycobacteria in Vietnam; more than confounders of culture-based TB diagnosis

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Abstract

Mycobacterium cultures applied during a recent survey in Vietnam yielded nontuberculous mycobacteria in 2.4% of all smear-positive tuberculosis suspects. We identified ten of these nontuberculous mycobacteria as *M. abscessus* (n=8), *M. fortuitum* and *M. porcinum* (both n=1). Based on a retrospective analysis of the ten respective case record forms, three patients might have had NTM disease. The predominance of rapid-growing NTM appears specific to the South East Asia region. Our findings stress the need for identification tools alongside culture facilities. The cost-effectiveness of treatment for these infections in a low-income setting is a major concern.

Introduction

Nontuberculous mycobacteria (NTM) have been isolated from clinical and environmental samples worldwide.[1] Most of the cases of human NTM disease, however, have been reported from countries where the incidence of tuberculosis is low. Their clinical relevance in a setting with a high incidence of tuberculosis remains to be resolved. In both settings, however, NTM can confuse the culture-based diagnosis of tuberculosis.[1,2]

In Vietnam, during a recent drug resistance survey among patients suspected of tuberculosis, *Mycobacterium* culture on Löwenstein-Jensen media was added to the diagnostic tools. Of all 1863 culture positive patients, 45 (2.4%) had a positive culture yielding NTM. We randomly selected ten NTM isolates, cultured in different laboratories, for identification by molecular methods. In addition, we reviewed their case record forms to assess the clinical relevance of the isolated NTM.

Methods

Sputum samples were decontaminated by the NALC-NaOH method and incubated on Löwenstein-Jensen media at 36 °C. All positive cultures were examined for growth characteristics and colony morphology; primary identification as *M. tuberculosis* complex was done using Ziehl-Neelsen staining and the niacin test. From the cultures yielding acid-fast bacilli with a negative niacin test, ten were heat-killed and sent to the Dutch National Mycobacteria Reference Laboratory, for molecular identification. We identified the isolates using the Inno-LiPA MYCOBACTERIA v2 reverse line blot (Innogenetics NV, Gent, Belgium). If no species-specific identification was obtained, we performed sequencing of the 151bp hypervariable region A of the 16S rDNA gene, using previously published methods.[3]

To assess the clinical relevance of the isolates NTM, we applied a simplified version of the American Thoracic Society diagnostic criteria for pulmonary NTM disease,[1] using clinical data retrieved from the case record forms made for the survey.

Table 1: Baseline patient characteristics

Pt.	Sex, Age	Species	Symptoms	Chest radiograph	ATS
1	M, 21	*M. abscessus*	PC	NA	Not met
2	M, 49	*M. abscessus*	PC, WL, NS	NA	Not met
3	M, 58	*M. abscessus*	PC	NA	Not met
4	M, 43	*M. abscessus*	PC	NA	Not met
5	F, 25	*M. abscessus*	PC, WL, NS	NA	Not met
6	F, 49	*M. abscessus*	PC	NA	Not met
7	M, 30	*M. abscessus*	PC	NCL	Met
8	F, 79	*M. porcinum*	PC	NCL	Met
9	M, 49	*M. fortuitum*	PC	NCL	Met
10	M, 29	*M. abscessus*	PC, F	NA	Not met

M/F, male/female; PC, productive cough; WL, weight loss; NS, night sweats; F, fever; NA, no abnormalities; NCL, non-cavitary lesions; ATS, status according to American Thoracic Society diagnostic criteria

Results

We identified the isolates of the ten patients as *M. abscessus* (n=8) and *M. fortuitum* complex (*M. fortuitum* and *M. porcinum*, both n=1). No cross-reactions with the *M. tuberculosis* complex probe of the INNO-LiPA kit, suggestive of mixed *M. tuberculosis* complex and NTM culture, were observed. The baseline clinical characteristics of the patients are detailed in Table 1. All patients were HIV-negative. All of their samples were positive for acid-fast bacilli on direct microscopy. Chest radiographs were made for the surveys, but their results were only reported as I) cavitary lesions, II) non-cavitary lesions or III) no abnormalities. Based on this apparent limitation, we considered both category one and two to be possible signs of mycobacterial disease. Three patients met the ATS diagnostic criteria based on their symptoms, chest radiograph abnormalities and smear and culture positivity for the NTM species.

Discussion

NTM can confuse the diagnosis of tuberculosis in high-incidence settings. A minority of patients in the present study might have even had pulmonary NTM disease. A firm diagnosis of NTM disease, however, should be based on the examination of at least three separate sputum samples and chest radiograph abnormalities that are confirmed to be suggestive of mycobacterial disease.[1] The absence of cavitary lesions in these patients does not preclude a diagnosis of disease due to rapid growing NTM, as these are usually characterized by nodular-bronchiectatic rather than cavitary disease.[1] Detection of acid-fast bacilli during direct microscopy further strengthened the diagnosis of NTM disease. For the purpose of the surveys, these patients were considered unlikely to have TB and received no treatment.

The 45 patients with cultures yielding NTM made up 2.4% of all 1863 culture positives in the survey. This is in line with the classical observation that 1-4% of all patients in high-incidence settings have at least a single culture yielding NTM.[4]

Our small sample suggests that rapid growers may predominate among NTM isolates in Vietnam. This finding has been previously reported from neighboring regions including Thailand, Southern China and Taiwan.[5-7] The isolation of NTM has important implications for tuberculosis control in such high incidence settings. Together with the implementation of culture facilities, there should be (molecular) identification facilities available, especially where liquid culture systems are applied which bar the interpretation of colony morphology. Without identification, patients may receive unwarranted tuberculosis treatment and NTM isolates may be subjected to drug susceptibility testing. Their common resistance to first-line drugs, which is most pronounced in the rapid growing NTM as encountered in this study,[1] may lead to false reports of multi-drug resistant tuberculosis and misguided treatment.

Diagnosing NTM disease brings about the issue of whether or not to treat these infections in a low-income setting. Treatment results vary for the different NTM species and current treatment guidelines advocate the use of expensive drugs over a long period.[1] Both aspects are highly problematic in a low-income setting. *Mycobacterium abscessus*, which was most prevalent among our isolates, is especially problematic due to its natural resistance to most classes of antibiotics.[1]

In conclusion, NTM are cultured from patients suspected of TB in Vietnam and may represent NTM disease, although a survey setting hampers a firm diagnosis. This stresses the need for identification tools alongside culture facilities. In our random sample we found rapid-growing NTM only, which appears specific to the South East Asia region. Diagnosing NTM disease raises of issue of the cost-effectiveness of treatment for these infections in a low-income setting.

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Invasive disease caused by nontuberculous mycobacteria, Tanzania

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Summary

Data on nontuberculous mycobacterial (NTM) disease in sub-Saharan Africa are limited. During 2006-2008, we identified 3 HIV-infected patients in northern Tanzania who had invasive NTM disease; two were infected with "*Mycobacterium sherrisii*" and one with *M. avium* complex sequevar MAC-D. Invasive NTM disease is present in HIV-infected patients in sub-Saharan Africa.

In sub-Saharan Africa, mycobacterial infections are predominantly caused by *Mycobacterium tuberculosis*.[1] In more developed countries, *M. avium* and *M. simiae* are responsible for disseminated disease in HIV-infected persons.[2] To better understand invasive nontuberculous mycobacterial (NTM) infections in HIV-infected persons in sub-Saharan Africa, we studied patients at 2 hospitals in northern Tanzania.

The Study

From July 2006 through August 2008, we collected blood from 723 patients >13 years of age who had axillary temperatures >38°C and who had been admitted to Kilimanjaro Christian Medical Centre and Mawenzi Regional Hospital in Moshi, Tanzania. Standardized clinical information was collected from all patients. For mycobacterial culture, 5 mL from each patient was inoculated into a BacT/ALERT MB bottle and monitored in a BacT/ALERT 3D (bioMérieux, Durham, NC, USA) automated liquid culture instrument. Other tissue samples (not blood) were obtained from patients with suspected invasive mycobacterial disease and incubated on Middlebrook 7H10 and Lowenstein-Jensen media at 36°C. We used AccuProbe MTB and MAC kits (GenProbe, San Diego, CA, USA) to identify members of *M. tuberculosis* complex and *M. avium* complex. NTM were further identified by the INNO-LiPA Mycobacteria v2 reverse line blot (Innogenetics, Gent, Belgium). All assays were used according to the manufacturer's instructions. All reverse line blot identifications were confirmed by performing additional sequencing of the complete 16S rDNA gene, the 16S-23S internal transcribed spacer (ITS), and the 65 kDa heat shock protein (*hsp65*) gene.[3,4]

Of the 723 patients, 30 (4.1%) had mycobacterial bloodstream infections, of which two (9%) were NTM. In one additional patient, NTM were identified in a tissue specimen. We describe the 3 patients with NTM infections.

The first patient was a 49-year-old man with cough and weight loss. His sputum contained acid-fast bacilli, and he simultaneously received a diagnosis of HIV infection with a CD4-positive T-lymphocyte count (CD4 count) of 9 cells/mm^3. Tuberculosis therapy was begun and comprised isoniazid, rifampicin, pyrazinamide, and ethambutol; he was also started on a fixed-dose combination of zidovudine, lamivudine, and abacavir. DNA was extracted from the initial sputum smear taken at the time of presumptive tuberculosis diagnosis according to previously published methods.[5] The GenoType CM/AS reverse line blot assay (Hain Lifesciences, Nehren, Germany) was weakly positive for *M. tuberculosis* complex. The patient's cough resolved, and he completed a 9-month course of tuberculosis therapy. When fever subsequently developed, he was admitted to the hospital; CD4 count was 13 cells/mm^3. Mycobacterial blood culture grew acid-fast bacilli after 12 days of incubation; results of AccuProbe MTB and MAC tests were negative. Heat-killed cells from the positive blood culture were identified as *M. simiae* by the INNO-LiPA reverse-line blot. Sequencing of the full 16S

rDNA gene, ITS, and *hsp65* gene identified the isolate as "*M. sherrisii*." The 16S rDNA and *hsp65* sequences were identical to the *M. sherrisii* American Type Culture Collection (ATCC; Manassas, VA, USA) BAA-832 type strain sequences deposited in the GenBank sequence database under accession nos. AY353699 (16S rDNA) and AY365190 (*hsp65*). The ITS sequence was identical to that of *M. sherrisii* strain FI-95229 (accession no. DQ185132), isolated from sputum of a patient in Italy.[6] The Tanzanian patient was treated with azithromycin, 500 mg/day, and ethambutol, 800 mg/day. His fever abated and he remained well, with 109 CD4 cells/mm^3 as of last follow-up in 2008.

The second patient was a 36-year-old HIV-infected man with a three-month history of fever and weight loss and 31 CD4 cells/mm^3. He had been taking fixed-dose combination stavudine, lamivudine, and nevirapine for five months, but his adherence to therapy was poor. A mycobacterial blood culture grew acid-fast bacilli after 15 days of incubation; AccuProbe MTB and MAC test results were negative. Heat-killed cells from the positive blood culture were identified as *M. simiae* by the INNO-LiPA reverse-line blot and again as *M. sherrisii* by sequencing of the full 16S rDNA gene, ITS, and the *hsp65* gene. The 16S rDNA gene had a single base-pair difference when compared with the *M. sherrisii* ATCC BAA-832 type strain sequence in GenBank. We deposited the new 16S rDNA sequence in GenBank under accession no. EU883389. The *hsp65* sequence was identical to the *M. sherrisii* ATCC BAA-832 strain sequence (accession no. AY365190); the ITS sequence was identical to the *M. sherrisii* strain FI-95229 (accession no. DQ185132) sequence.[6] The patient was treated with azithromycin, 500 mg/day, and ethambutol, 800 mg/day; fever abated. At follow-up in 2008, the patient was continuing treatment with azithromycin and ethambutol but had abdominal pain and hepatosplenomegaly. Abdominal ultrasonography showed retroperitoneal lymphadenopathy. Follow-up mycobacterial blood cultures have been negative.

The third patient was a 36-year-old HIV-infected woman with a 4-month history of bilateral skin lesions affecting the lower extremities (Figure 1) and 206 CD4 cells/mm^3. HIV infection had been diagnosed 18 months earlier; baseline CD4 count was 6 cells/mm^3. She began fixed-dose combination stavudine, lamivudine, and nevirapine soon after her HIV diagnosis. An incisional biopsy from the active margin of a leg lesion showed several foci of dermal necrosis with dense lymphocytic infiltrate and Langhans-type giant cells consistent with granulomatous inflammation (Figure 2). Culture of biopsy material was positive for *M. avium* complex. The isolate reacted only with the *M. avium-intracellulare-scrofulaceum* complex probe of the INNO-LiPA reverse-line blot. The 16S rDNA gene and ITS sequences were identical to the *M. avium* complex ATCC 35770 (Melnick) strain sequences published by Böddinghaus et al.[7] and available in the Ribosomal Differentiation of Microorganisms database (http://rdna.ridom.de). The ITS sequence was also identical to the *M. avium* complex ATCC 35770 strain sequence available in GenBank (ITS sequevar MAC-D, accession

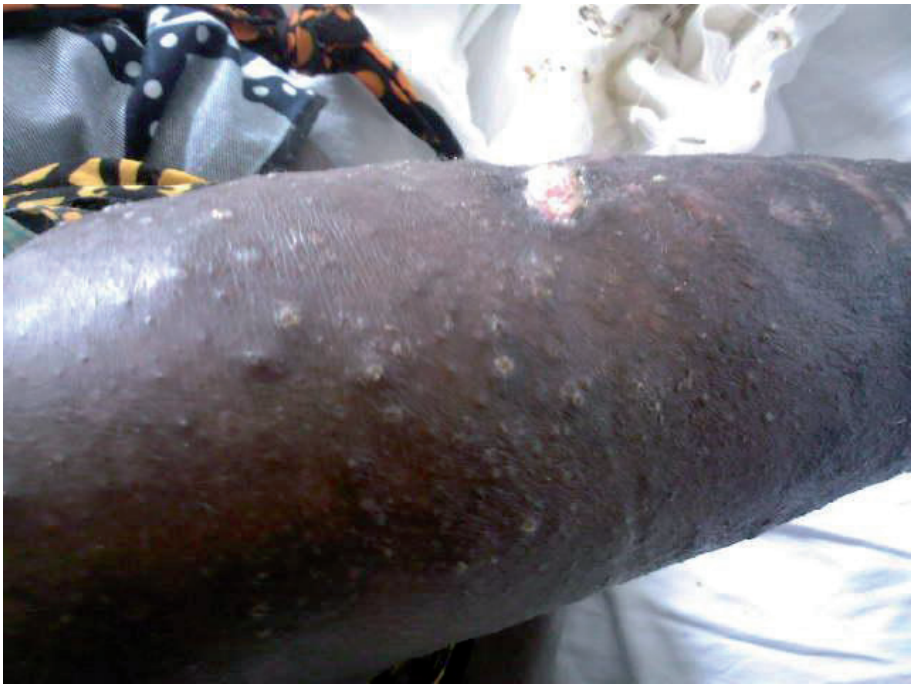

Figure 1: Photograph of skin lesions on right leg of patient 3, taken before treatment

no. L07851). The *hsp65* sequence was identical to the ATCC 35770 sequence (accession no. U85637). Because the full 16S rDNA gene sequence of this strain was not available in GenBank and only a small fragment of *hsp65* was available, we deposited our sequences under accession nos. EU815938 (16S rDNA) and EU935586 (*hsp65*). This patient was treated with azithromycin, 500 mg/day, ethambutol, 800 mg/day, and rifampicin, 600 mg/day. Her lesions abated over the subsequent weeks, and she remained well as of follow-up in 2008.

Conclusions

Improved laboratory techniques enabled us to demonstrate that invasive NTM infections occur in northern Tanzania and include *M. sherrisii* and *M. avium* complex. *Mycobacterium sherrisii* still awaits official recognition.[6] Of *M. sherrisii* infections reported to date,[6,8-12] most have been in HIV-infected patients from Africa.[8-10] Although recommendations for the antimicrobial drug management of these infections have not yet been established, our two patients with *M. sherrisii* disseminated disease responded clinically to the optimization of their antiretroviral therapy regimen and to the combination of ethambutol and azithromycin.

The *M. avium* complex isolated from our third patient is remarkable for its ITS sequevar type. MAC-D has not previously been associated with invasive disease in HIV-infected patients, in whom *M. avium* sequevars, mainly Mav-A

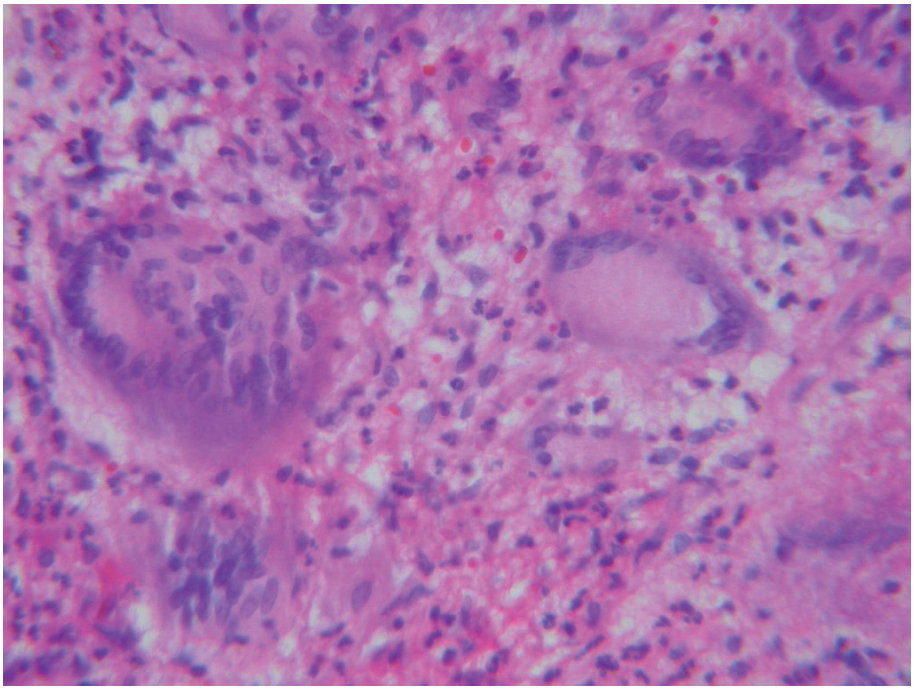

Figure 2: Histopathologic appearance of skin biopsy specimen of patient 3

Dermal necrosis with a dense lymphocytic infiltrate and Langhans-type giant cells, consistent with granulomatous inflammation (hematoxylin and eosin stain, magnification ×40).

and -B, are most common.[12,13] The *M. avium* complex ATCC 35770 reference strain was the first reported strain with a MAC-D ITS. The ATCC 35770 strain, however, was isolated from a sputum sample in a symptomatic patient in the United States.[14] The isolate from our third patient and the ATCC 35770 strain are genetically divergent from other *M. avium* complex members and may represent a separate species within the *M. avium* complex.

Invasive NTM disease in HIV-infected populations in sub-Saharan Africa demands more attention in terms of identification of etiologic agents, clinical relevance, and management. Further insights would be gained if current and future studies on tuberculosis in the region included liquid culture and molecular identification to confirm *M. tuberculosis* infection and establish the epidemiology and clinical relevance of NTM.

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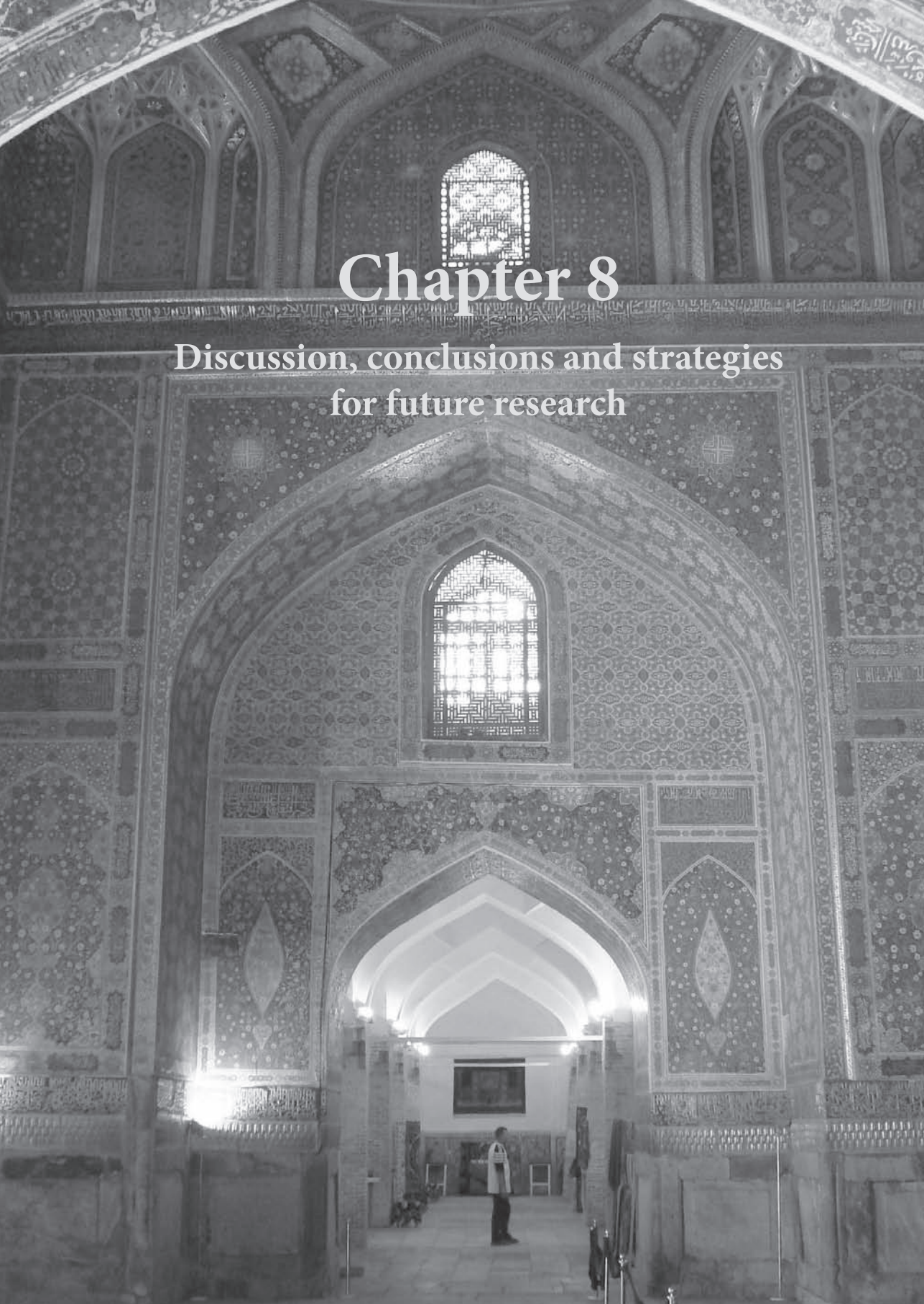

Chapter 8

Discussion, conclusions and strategies for future research

General Discussion

"Een tuingrondsuspensie bij een cavia ingespoten zal ondanks het feit, dat vele honderden bacteriesoorten worden ingespoten op zijn hoogst gasphlegmonen doen ontstaan, een infectie wordt practisch gesproken nimmer op deze wijze opgewekt. Het spreekt dus vanzelf, dat ook de grondmycobacteriën zich als volkomen onschuldige saprophyten zullen gedragen."

["Injection of a potting soil suspension in guinea pigs will, despite injection of hundreds of bacterial species, merely lead to gas phlegmons; [mycobacterial] infections are generally not invoked. Hence, the environmental mycobacteria behave as harmless saprophytes."]
- L.E. Den Dooren De Jong, Dutch bacteriologist, 1939 [1]

Within this thesis, four cardinal issues have been raised. Most important is the observation that the clinical relevance of nontuberculous mycobacteria (NTM) in the Netherlands differs significantly by species. This observation is important as it offers guidance in the treatment decision in individual patients from whom NTM have been isolated. Furthermore, this may serve as a starting point to unravel the interaction between NTM and the human host. Our second observation is that the outcome of treatment for NTM disease is generally disappointing, despite treatment adjustment to *in vitro* susceptibility testing results and clinical trial data. Third, currently used molecular identification tools provide an over-simplified view of the evolutionary divergence among nontuberculous mycobacteria, which may provide part of the reason for the differences in clinical relevance between strains of different species and within species. Lastly, NTM cause disease worldwide, including in settings with a high prevalence of tuberculosis (TB), where identification, drug susceptibility testing and even active drugs may be unavailable.
The rising isolation frequency of NTM in the Netherlands lead to the current studies; during these studies, the species distribution among NTM isolates was found to differ from neighbouring countries. Before addressing the four cardinal issues of this thesis, the isolation frequency and species distribution will be briefly discussed.

Isolation frequency of nontuberculous mycobacteria in the Netherlands

The isolation frequency of NTM has risen during the 2000 to 2008 period in the Netherlands, whereas in the same period the incidence of TB decreased. This rise is not limited to the Netherlands. Many developed countries in Europe, West Asia and North America have recorded rising NTM isolation frequencies.[2] Some authors have rightly claimed that the rising isolation frequency of NTM

reflects simultaneous improvements in laboratory facilities.[3] Microbiological laboratories have benefited from a wide array of novel techniques and materials over the past decade. Highly sensitive automated liquid culture systems have been added to the conventional solid media for *Mycobacterium* culture.[4] Molecular detection and identification assays have been developed that offer rapid inexpensive identification of NTM to laboratories without access to DNA sequencers. These techniques have largely replaced the previous biochemical identification and high-performance liquid chromatography as first line identification methods.[5-7]

There is increasing evidence that the technological advances are not the sole explanation for the rise in NTM isolation.[2,7,8] First, it is important to note that in the Netherlands the percentage of TB cases that could not be confirmed by culture (stable at 33% over the 1999-2005 period) did not decrease, despite introduction of the more sensitive liquid culture systems and molecular detection and identification tools.[9]

Second, the rise in isolation frequency does not affect all species equally; if this rise was based largely on technical improvements, one would expect a uniform rise in isolation of many, if not all, NTM species. In the Netherlands, the rising isolation frequency is largely explained by an increasing isolation frequency of *M. avium* complex (MAC) isolates, mainly *M. avium* in respiratory samples of elderly patients **(Chapter 2.1, 2.9)**. For other species, including *M. simiae*, *M. xenopi*, *M. szulgai* and *M. malmoense*, such an increase was not apparent in the Netherlands **(Chapter 2.2-2.7)**. Other European countries did report rising isolation frequencies for *M. malmoense*.[10]

Third, the increasing isolation frequency of NTM seems to be particularly true for countries where the incidence of TB is in decline.[2,7] This decline in TB incidence suggests that exposure to TB infers cross-protection to NTM disease. This is supported by the fact that countries that halted Bacille Calmette-Guerin (BCG) vaccination subsequently noticed an increase in the incidence of paediatric cervicofacial lymphadenitis caused by NTM.[11,12] Moreover, during decades of decreasing tuberculosis incidence rates, Bleiker and co-workers noted increased skin sensitization to *M. scrofulaceum* and decreasing sensitization to tuberculin in Dutch elementary school children.[13] A similar observation was recently made in the United States, were over the past three decades, the skin sensitization to *M. intracellulare* has increased, in conjunction with an increase in the incidence of *M. intracellulare* disease.[14] Still, pulmonary NTM disease is well known to affect patients with a destroyed lung or upper lobe bronchiectasis caused by prior pulmonary tuberculosis. Thus, several factors other than cross protection by tuberculosis exposure need to be considered responsible for the rise in NTM isolation frequency.

One of the most important factors is probably the ageing population in the Netherlands and many other developed countries. With the increased life expectancy comes an increasing burden of chronic diseases, especially chronic

pulmonary disease. The most recent estimate is that 1.3% of the Dutch population has a formal diagnosis of chronic obstructive pulmonary disease (COPD).[15] In a country of 16.2 million inhabitants, that makes for 210600 COPD patients. Chronic pulmonary disease in itself increases the susceptibility to pulmonary NTM disease. For other chronic diseases, immunosuppressive therapy is applied, which will indirectly lead to increased susceptibility to NTM disease. Such increased susceptibility has been noted specifically in users of anti-tumor necrosis factor-α (TNF) therapy **(Chapter 3)**.[16] These demographic changes have probably also influenced the NTM isolation frequency in the Netherlands **(Chapter 2.1, 2.9)**.

The increased use of showers for personal hygiene may also be a factor in the increasing prevalence of NTM disease.[17] Showers produce fine aerosols of tap water that is known to contain NTM.[18-20] As a result of showering and other environmental contacts, humans are thought to be exposed to 50 to 500 NTM bacilli on a daily basis.[21]

Although the background of the increased clinical NTM isolation may remain controversial, it has obviously led to increased interest in NTM among clinicians.[7] Increasing knowledge of NTM disease in clinicians may, in turn, have lead to more patients being evaluated for and diagnosed with NTM disease. Still, a rise in the incidence of NTM disease has also been noted in populations where the number of patients evaluated remained stable.[22] The increased number of clinical NTM isolates has led to an intense debate on the clinical relevance of this finding. In fact, this debate was the starting point for our studies.

Based on these studies, we now conservatively estimate that NTM are isolated from 800 patients annually in the Netherlands, or 5/100.000 persons per year. In our regional study we found that for one third of all patients with NTM isolates, the NTM could be classified as causative agents of disease **(Chapter 2.1)**. The incidence of disease due to NTM in the Netherlands would then be 1.7/100.000 persons per year.

Species distribution among clinical NTM isolates in the Netherlands

Of all isolates submitted to the Dutch National Mycobacteria Reference Laboratory, part of the National Institute for Public Health and the Environment (RIVM), *Mycobacterium avium* is the most frequently encountered NTM species (23%), followed by *M. gordonae* (15%), *M. kansasii* (8%), *M. intracellulare* (7%) and the *M. fortuitum* complex (5%; see Table 1). Within the Netherlands, this species distribution is likely to differ by region due to varying environmental characteristics. Nonetheless, the species distribution in our clinical study in the Nijmegen-Arnhem region was very much in line with the general distribution of NTM species submitted to the RIVM (Table 1).

The species distribution in the Netherlands is different from that of neighbouring countries, although the paucity of recent, published data precludes in-depth

Table 1: NTM species distribution in clinical samples in various geographic locations

Country	Setting	N	Species distribution (%)									
			MAC	Mav	Min	Mka	Mxe	Mml	Msz	RGM	Mab	Mfc
NL	NRL	4599	39	23	7	8	2	3	1	13	3	5
NL	MC	232	41	28	9	8	2	1	2	11	2	5
England	RL	6668	20	-	-	15	23	2	<1	16	-	8
Scotland	RL	248	33	-	-	4	8	38	0	17	-	-
Belgium	MC	1180	12	-	-	3	35	0	-	-	-	2
Germany	MC	1390	26	-	-	4	13	2	-	-	-	12
France	MC	3200	41	-	-	11	21	1	-	-	-	7
Denmark	NRL	496	40	-	-	1	3	4	<1	7	4	2
Slovenia	NRL	1299	20	12	7	8	29	1	-	16	1	7
Canada	RL	11186	59	-	-	2	26	-	-	13	-	-
USA-NY	SC	505	84	-	-	2	3	0	0	9	3	3
USA	MC	5469	50	-	-	12	1	<1	<1	13	-	10
Korea	SC	794	28	14	14	2	<1	0	4	54	18	27

NL, the Netherlands; USA, United States of America; (N)RL, (National) Reference Laboratory; MC, multi-centre; SC, single centre; MAC, *M. avium* complex; Mav, *M. avium*; Min, *M. intracellulare*; Mka, *M. kansasii*; Mxe, *M. xenopi*, Mml, *M. malmoense*; Msz, *M. szulgai*; RGM, rapid-growing mycobacteria; Mab, *M. abscessus*; Mfc, *M. fortuitum* complex; data are derived from Chapter 2.1 and references 2,8,23-28.

analysis. *Mycobacterium xenopi* has been linked to the English Channel region, based on data from the UK and Belgium.[2,29] Prior studies in South-East England have demonstrated a predominance of *M. xenopi* in that area; this has been linked to the fact that this is a coastal region, with many water birds, which may be a source of *M. xenopi*.[29] A similar species distribution has recently been reported from Slovenia.[25] Despite the Netherlands' long North Sea coast line and its proximity to the English Channel, *M. xenopi* isolation is infrequent. Moreover, *M. xenopi* isolation is not linked to coastal areas of the Netherlands **(Chapter 2.2)**. Based on currently available data, the species distribution in the Netherlands most closely resembles that of Denmark and is remarkably different from that of our direct neighbouring countries Germany and Belgium, as well as from the United Kingdom and North America (Table 1). In North America *M. intracellulare* is the most frequent *M. avium* complex isolate and the most common causative agent of pulmonary NTM disease.[7,30] Conversely, *M. avium* predominates in the Netherlands, both in terms of isolation frequency and clinical relevance (Table 1 & **Chapter 2.1**).

Of the 130 validly published NTM species,[31] approximately 70 have been isolated from clinical samples in the Netherlands **(Chapter 4.1, 5.1)**. It is not known whether the frequency of clinical isolation mirrors the NTM species distribution in the environment. Although the RIVM has data on species distribution in clinical samples, no recent environmental data are available.

Possibly, the human respiratory tract (the most common source of clinical NTM isolates) exerts a selective pressure. Furthermore, it is conceivable that not all environmental NTM can be cultured *in vitro* with the same rate of success. This should be a subject of future studies.

The NTM species distribution in the Netherlands during our study period not only differed from neighbouring countries; it also varied from earlier observations. In the 1960's, *M. kansasii* was reported to be the most frequent causative agent of pulmonary NTM disease in the Netherlands.[32-34] This was different from for example the USA, where at that time the nonchromogenic *M. avium* complex bacteria (or "Battey" bacilli, after the Battey State Hospital, Georgia, USA [35]) were most frequently isolated. It seems that in the three decades between the studies by Manten, van Joost and Selkon, the species distribution in the Netherlands has changed. Here, technical advances and their impact on *Mycobacterium* taxonomy[36,37] as well as environmental and host factors may play an important role. The latter will be covered in the "disease characteristics" section.

Similarly, in the 1960's, the causative agents of paediatric cervicofacial lymphadenitis were mostly *M. scrofulaceum* (21/40), followed by the nonchromogenic "Battey" bacilli. *Mycobacterium scrofulaceum* is now a rarely encountered pathogen and most cases of paediatric cervicofacial lymphadenitis are now caused by *M. avium* **(Chapter 2.1)**.[38] *Mycobacterium malmoense* and *M. haemophilum* are probably the second and third most common NTM causative agents of NTM lymphadenitis in the Netherlands **(Chapter 2.1, 2.6)**.[38,39] This shift in causative agents, away from *M. scrofulaceum,* has been simultaneously noted in the USA.[7] This phenomenon has been attributed to chlorination of tap water, which should select for *M. avium,* as *M. scrofulaceum* is more susceptible to chlorine.[7,21] Studies reviewed by Collins and co-workers, however, did not demonstrate important differences in chlorine susceptibility between NTM species and considered tap water chlorine levels too low to lead to significant reductions in mycobacterial load.[40] Furthermore, in the Netherlands the same species shift was noted in absence of tap water chlorination. Second, after molecular identification tools became more widespread, several scotochromogenic slow growing mycobacteria isolated from children with cervicofacial lymphadenitis have been elevated to separate species status; these include *M. bohemicum*, *M. interjectum*, *M. lentiflavum* and *M. palustre*.[37] *Mycobacterium mantenii* and *M. vulneris*, which we have described, fall into this same category **(Chapter 5.3, 5.4)**. The International Working Group on Mycobacterial Taxonomy also studied several isolates related to, but different from, *M. scrofulaceum*.[41] All these isolates and newly described scotochromogenic slow-growers may have previously been falsely identified as *M. scrofulaceum*.[37]

The clinical relevance of nontuberculous mycobacteria

The first part of our studies focussed on the clinical relevance of NTM isolation. To quantify clinical relevance we assessed the fraction of patients with isolates of a particular species that had disease attributable to bacteria of this NTM species. This approach is novel; previous studies generally described reports of single or a few cases, or were laboratory driven and left the assessment of clinical relevance to the treating physician. In general, most reports focus on cases of true NTM disease and ignore the fraction of the isolates of that same species that could not be associated with clinical disease. So, the literature was not conclusive with regard to the average level of clinical relevance of the described NTM species. This implies that for clinicians faced with a patient from whom an uncommon NTM is isolated, a literature search would probably lead to an overestimation of its clinical relevance due to this publication bias.

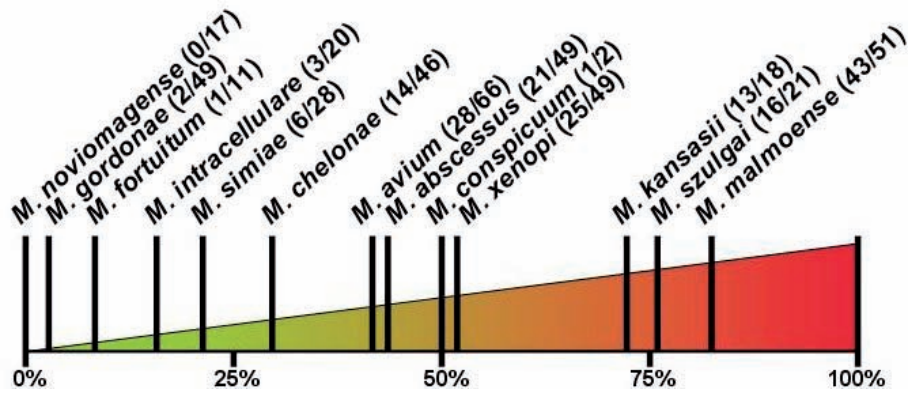

Figure 1: Percentage of patients with NTM disease, per species

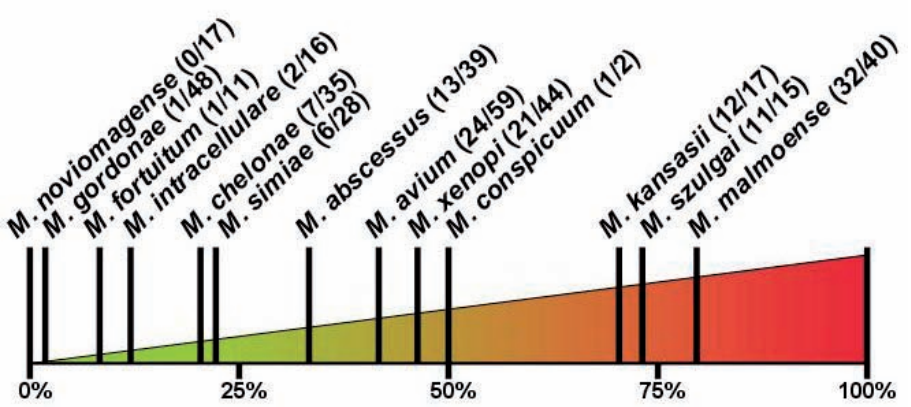

Figure 2: Clinical relevance of pulmonary isolates of NTM species

We used the American Thoracic Society diagnostic criteria for pulmonary NTM disease to assess the relevance of pulmonary isolates. For extrapulmonary isolates, we divided those from the normally sterile sites (e.g. lymph nodes, bone) from those from the non-sterile digestive tract. Whereas the former were considered relevant until proven otherwise (e.g. signs of laboratory contamination), the latter were considered irrelevant, unless accompanied by features suggestive of disseminated NTM disease. We applied these criteria in a regional study, to cover the major species such as *M. avium*, *M. intracellulare* and *M. kansasii*. Later we used these criteria in nation-wide studies focussed on the less frequently isolated *M. xenopi*, *M. simiae*, *M. szulgai*, *M. malmoense*, *M. conspicuum* and the rapid growers *M. chelonae* and *M. abscessus*. Lastly, we used the ATS diagnostic criteria while describing four new species of *Mycobacterium*: *M. noviomagense*, *M. riyadhense*, *M. vulneris* and *M. mantenii*. We have reviewed medical files of 473 patients in the Netherlands with NTM isolates, to conclude that 192 patients (41%) were likely to have true NTM disease **(Chapter 2)**.

The most important observation stemming from our regional and national studies is that the clinical relevance, expressed as the percentage of patients with NTM isolates that met the ATS diagnostic criteria for pulmonary NTM disease or were likely to have extrapulmonary NTM disease, differs by species. These differences can be visualized on a scale (Figure 1), to assist clinicians in their assessment of individual patients from whom an NTM is isolated.
The position on Figure 1 partly reflects the number of cases of extrapulmonary disease due to the respective NTM species because isolation of NTM from extrapulmonary sites is generally indicative of true NTM disease. Species that frequently cause extrapulmonary disease are thus more often clinically relevant than species that are rarely encountered in extrapulmonary samples. The ability to cause extrapulmonary and disseminated disease differs significantly by species. *Mycobacterium avium* is a common causative agent of pulmonary disease, paediatric cervicofacial lymphadenitis and HIV-related disseminated infections. In contrast, *M. malmoense* causes pulmonary disease and lymphadenitis, *M. chelonae* and *M. abscessus* cause pulmonary and focal extrapulmonary disease, but rarely lymphadenitis and *M. simiae* almost exclusively causes pulmonary disease in the Netherlands **(Chapter 2)**. Hence, it is fairer to compare the clinical relevance of pulmonary NTM isolates separately. Based on these data for the various species studied in our regional and nation-wide studies, we have constructed Figure 2.

Whereas *M. avium* is the most frequently isolated NTM in the Netherlands, *M. xenopi*, *M. kansasii*, *M. szulgai* and *M. malmoense* are more often clinically relevant. Although *M. kansasii* and *M. szulgai* are phylogenetically related (and, in fact, most closely related to the *M. tuberculosis* complex **(Chapter 6.2)**), the level of clinical relevance is not higher for these species than for more

Table 2: Clinical relevance of pulmonary NTM isolates in different countries

Country	Year	Setting	N	% clinical relevance by species							
				All	MAC	Mav	Min	Mka	Mab	Mfc	Mxe
NL	99-04	MC	212	25	31	41	13	71	33	9	60
Korea	02-03	SC	794	25	43	34	52	50	45	10	0
USA-NY	00-03	SC	344	24	25	-	-	57	-	-	44
USA	81-83	MC	5469	38	47	-	-	75	-	18	25

NL, the Netherlands; USA, United States of America; NY, New York; MC, multi-centre; SC, single centre; MAC, *M. avium* complex; Mav, *M. avium*; Min, *M. intracellulare*; Mka, *M. kansasii*; Mab, *M. abscessus*; Mfc, *M. fortuitum* complex; Mxe, *M. xenopi*; data are derived from Chapter 2.1 and references 26-28.

distantly related NTM such as *M. malmoense*. This implies that there is no direct correlation between the phylogenetic grouping and clinical relevance. In addition, in The Netherlands 47% of 45 patients from whom *M. xenopi* was cultured from respiratory samples met the American Thoracic Society diagnostic criteria. Contrarily, none of the 17 patients with *M. noviomagense* isolates did, while these two species are phylogenetically closely related **(Chapter 2.2, 5.2)**.

The difference in clinical relevance between NTM species can guide a clinician in his stance towards the symptomatic patient from whom a particular NTM has been isolated. Isolation of *M. kansasii*, *M. malmoense* or *M. szulgai* urges vigilance and intense follow-up, if not the institution of therapy; if one of a series of sputum cultures yields *M. gordonae* or *M. noviomagense*, a more observational stance is probably sufficient, as pulmonary NTM disease is far less likely. Based on small numbers of isolates noted in our regional study we presume that *M. genavense* and *M. celatum* fall into he same category as *M. kansasii*, *M. szulgai* and *M. malmoense*. *Mycobacterium alvei*, *M. palustre*, *M. terrae* and *M. phlei* are likely to be as non-relevant as *M. gordonae* and *M. noviomagense* **(Chapter 2.1, 5.2)**. Still, these findings remain to be confirmed in nationwide studies with larger numbers of patients.

We think that the ATS diagnostic criteria should be changed to accommodate for this difference in clinical relevance. It is conceivable to introduce less strict criteria for the diagnosis of pulmonary *M. kansasii*, *M. malmoense* and *M. szulgai* disease, as their isolation frequently indicates true NTM disease. For *M. kansasii*, the ATS criteria already state that the treatment decision may be guided by a single positive culture, based on its established pathogenic potential.[7] It is important, however, to make note of the subtype of *M. kansasii* isolated, as only one of five subtypes is strongly associated with pulmonary disease.[42,43] Stricter diagnostic criteria for species like *M. chelonae*, *M. abscessus* and *M. simiae* may prevent unwarranted diagnoses of disease. For these three species, we noted several patients who met the ATS diagnostic criteria, while they were unlikely to suffer true NTM disease **(Chapter 2.3, 2.7)**. Figure 2 can

serve as a template to assign sets of different microbiological criteria to groups of species. The clinical and radiological criteria should be strictly maintained. Despite a paucity of available data, we did find striking similarities between the clinical relevance of NTM species observed in our regional study and the few previous studies in other countries; they are visualized in Table 2.

The clinical relevance among MAC isolates observed in this study differs from previous reports from the USA, where *M. intracellulare* was the more frequent respiratory pathogen and clinically more relevant than *M. avium* in non-HIV patients.[7,30] The latter issue was debated in a recent study from another site in the United States, where *M. avium* was still less frequently isolated than *M. intracellulare*, though more frequently clinically relevant.[44] Unfortunately, the nation-wide study performed by O'Brien and co-workers[27] and the recently published study performed by Bodle and co-workers at the Presbyterian Hospital in New York[26] did not distinguish between the MAC species.
Similarly, we found *M. malmoense* to be the most frequently clinically relevant NTM species; 80% of all patients with pulmonary isolates met the ATS criteria. These data roughly match those reported from nearby Scotland and Denmark,[23,24] but differ strongly from the United States. A single study of 60 patients has been performed in the United States, which concluded that true *M. malmoense* disease was likely in just six (10%) of the patients assessed.[45] Differences in clinical relevance of NTM species in various geographical areas suggest variation in host susceptibility or differences in the virulence of the circulating bacteria. Both issues will be discussed in separate paragraphs.

One important limitation associated with our approach is the use of the American Thoracic Society diagnostic criteria. For *M. abscessus*, *M. chelonae* and *M. simiae*, we have noted that patients may meet these criteria while true NTM disease is, in fact, unlikely (Figure 2). Most frequently, these patients responded well to therapies that were highly unlikely to have any impact on an NTM infection. For these three species, the true degree of clinical relevance, hence their position in Figure 2, is likely to be lower than currently estimated **(Chapter 2.3, 2.7, 2.8)**. Another important issue is the revision of the ATS diagnostic criteria in 2007. With more lenient bacteriological criteria, we found that more patients met the revised criteria, compared to the stricter previous criteria published in 1997 **(Chapter 2.8)**.[7,46] Whether this leads to an improved selection of patients for whom therapy is warranted and leads to clinical improvement is uncertain. The authors of the new criteria, however, repeatedly state that meeting these criteria does not, *per se*, necessitate the institution of therapy.[7] This was not emphasized in the former statement. We believe that such comments added for the academic nuance only increase confusion for the clinician **(Chapter 2.8)**.

A second issue which may have influenced our results is the fact that for NTM identification, we have frequently relied on reverse line blot hybridization assays. In later studies we have proven that these tests underestimate the genetic divergence and, albeit infrequently, can lead to false identifications. The identification of *M. riyadhense* as *M. tuberculosis* complex, the identification of *M. sherrisii* isolates as *M. simiae* and the incorrect identification of isolates with unique, novel 16S gene sequences to species level may mean that some of the isolates in our species-specific retrospective studies may not have been that exact species **(Chapter 5.1, 7.2, 7.4)**. The results of our multi-gene sequencing study also revealed isolates of *M. bolletii* and *M. massiliense* among isolates previously designated *M. abscessus*. These species may also be present in the series previously identified as *M. abscessus* and examined in our retrospective study.
Lastly, our studies on clinical relevance were based on retrospective medical file studies. An assessment of clinical relevance based on retrospective data only is hampered by the fact that not all clinical circumstances can be convincingly captured in a retrospective medical file review.

NTM disease characteristics in the Netherlands

In the 1950's, the NTM were first recognized as causative agents of pulmonary disease in the Netherlands. Now, it has become clear that the NTM are causative agents of various types of disease, each within specific populations, though pulmonary NTM disease is most frequently diagnosed. Of all 473 patients with NTM isolates evaluated during our various studies, 407 (86%) had NTM isolated from pulmonary samples; of all 192 cases of NTM disease found during these studies, 138 (72%) were cases of pulmonary NTM disease. Three distinct pulmonary NTM disease types have been distinguished: classical cavitary disease, nodular-bronchiectatic disease and hypersensitivity pneumonitis.[7] From Manten's early observations[32,33] to our current findings, it has been clear that in the Netherlands, the upper lobe cavitary disease type (example in Figure 3) is most frequent **(Chapter 2)**.

The cavitary disease type resembles pulmonary tuberculosis both clinically and radiologically, though its clinical course is usually more prolonged. This disease type primarily affects patients with pre-existing pulmonary disease. Most patients in our studies who suffered pulmonary NTM disease had a history of chronic obstructive pulmonary disease (COPD); the percentage ranged from 83% of patients from with *M. simiae* disease, to 66% for those with *M. malmoense* disease **(Chapter 2)**. These differences suggest that not all NTM equally need a structurally damaged lung to thrive well. Perhaps this explains the differences in species distribution between our current studies and the studies by Manten in the 1960's, when *M. kansasii* dominated.[32] In our era, with the increasing

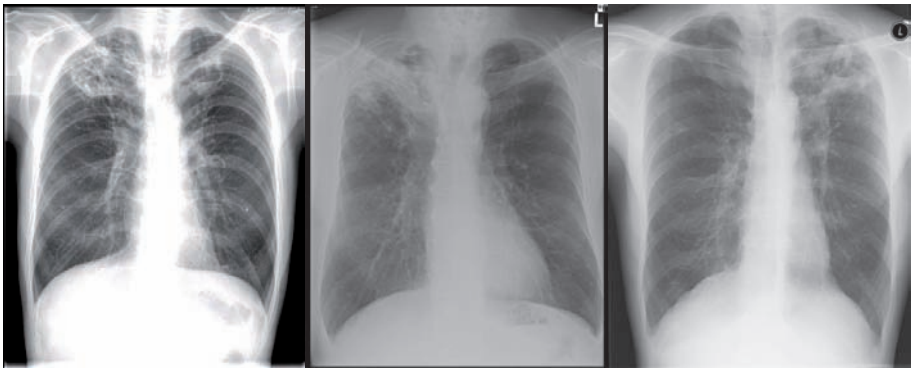

Figure 3: Examples of cavitary lesions caused by *Mycobacterium malmoense* lung disease

incidence of COPD, NTM other than *M. kansasii* succeed in causing pulmonary disease, in the population with pre-existing pulmonary disease.
Many patients had mild to moderate COPD, stage I to II according to the Global initiative for chronic Obstructive Lung Disease (GOLD) criteria.[47] In our regional study, we already noted that those who met the ATS criteria for pulmonary NTM disease were generally younger than those who did not **(Chapter 2.1)**. This suggests that the risk of NTM disease does not increase as COPD progresses. Perhaps NTM disease affects a specific subgroup of COPD patients with an underlying genetic predisposition. This polymorphism, in turn, may have a role in the emergence of COPD, or of a specific COPD phenotype in these patients.
In the United States, nodular-bronchiectatic disease is the most frequent NTM disease type; its radiographic features are shown in Figure 4. This disease primarily affects post-menopausal women, who tend to be taller and leaner than controls and have a distinct body habitus with scoliosis, pectus excavatum and mitral valve prolapse: the "Lady Windermere syndrome".[7,48] It has recently been found that among patients presenting with the Lady Windermere Syndrome, mutations in the Cystic Fibrosis transmembrane conductance regulator (CFTR) gene were significantly more frequently present than in the general population.[49] This disease type is rare in the Netherlands; it is outnumbered 4:1 by cavitary NTM disease **(Chapter 2.1)**. No cases of hypersensitivity pneumonitis were encountered during our retrospective studies. It is not certain whether this represents a reporting bias, or reflects a true absence.
We noted several cases of pulmonary *M. abscessus* disease in patients with cystic fibrosis (CF). CF patients are specifically at risk of developing *M. abscessus* disease. The background of this phenomenon is incompletely understood **(Chapter 2.7)**.[50,51] There are two possible explanations for this predilection. First, the fact that *M. abscessus* bacteria are resistant to most antibiotics classically used to treat CF implies they are progressively selected for by the repeated

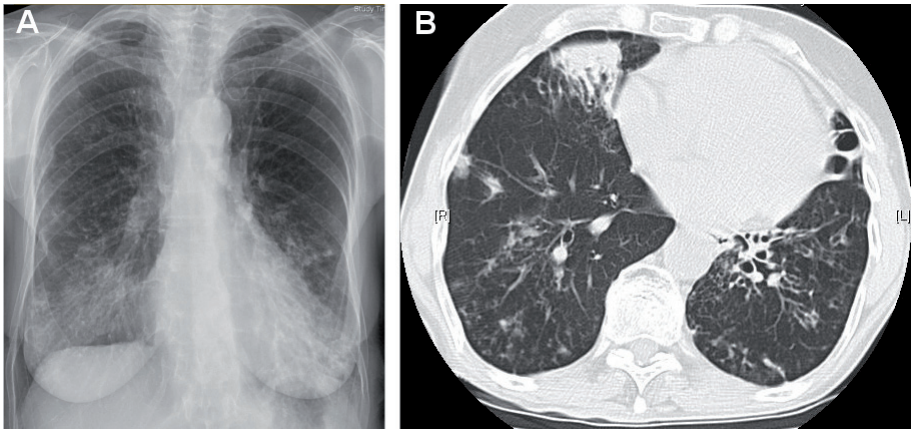

Figure 4: The chest radiograph (A) and corresponding chest CT (B) image of a female patient with pulmonary nodular bronchiectatic *M. intracellulare* disease. Chest radiograph reveals bilateral, predominantly mid to lower lung zone linear and nodular opacities; Chest CT scan reveals peripheral nodular opacities (tree-in-bud pattern), consolidation and bronchiectasis in the right middle lobe, and bronchiectasis in the lingula.
Pictures were kindly provided by Professor Charles L. Daley, National Jewish Health, Denver, USA.

antibiotic treatments, as has been shown for other CF pathogens, including *Stenotrophomonas maltophilia* and *Alcaligenes xylosoxidans*.[52,53] Second, as a result of the dysfunction of the CFTR, CF patients have epithelial lining fluid with elevated sodium chloride (NaCl) concentrations.[54] Interestingly, *M. abscessus* bacteria are characterized in the laboratory by their ability to grow in a saline environment. Ability to grow in 5% NaCl was, in fact, the available method to distinguish *M. abscessus* from related rapid growing NTM prior to the advent and spread of molecular tools for NTM identification.[55]

Little is known of the mechanisms by which the pre-existing pulmonary diseases influence susceptibility to pulmonary NTM disease. It is, for instance, not known what happens directly after acquisition of these bacteria in the lung. Disease caused by NTM is the result of a complex interplay between bacterial virulence factors and host immunity. Interestingly, both sides of the story are currently composed of largely blank pages. In murine models, *M. avium* is able to establish pulmonary infection after intravenous challenge, similar to *M. tuberculosis*.[56-58] *Mycobacterium abscessus* and *M. chelonae*, on the other hand, fail to establish pulmonary infection after intravenous[59] and even intratracheal challenge of Balb/c mice.[60] There is an interesting parallel with an observation in humans. In a series of 200 AIDS patients with documented disseminated *M. avium* complex disease, only 5 patients (2.5%) were found to have concomitant pulmonary disease due to *M. avium* complex.[61] This suggests that NTM have difficulty establishing an infection in healthy lung tissue. In 1959, Emanuel Wolinsky already reported that in a guinea pig model successful pulmonary infection with a variety of NTM was

possible, but only after having induced silicosis in these animals by quartz inhalation.[62] The CFTR mutations in the Lady Windermere syndrome[49] add to the suggestion that patients suffering pulmonary NTM disease without a formally diagnosed pre-existing lung disease, or other risk factors, may not be truly "immunocompetent" or "without predisposing conditions" as has been suggested in many case reports and series. Our clinical findings and the many questions arising from them suggest that more appropriate animal models, mimicking clinical situations including COPD and cystic fibrosis, should be used to investigate the pathophysiology and immunology of pulmonary NTM disease.

Paediatric cervicofacial lymphadenitis is the second most frequent NTM disease entity in the Netherlands; a relatively benign disease which primarily affects previously healthy children below the age of six with no known impairment of immunity. It is characterized clinically by painless swelling of a cervical or submandibular lymph node in the absence of generalized symptoms; other lymph nodes are rarely affected **(Chapter 2.1, 2.6, 5.3, 5.4)**. In the Netherlands, *M. avium* is the most frequent causative agent, followed by *M. malmoense* **(Chapter 2.1, 2.6)**. Surgical excision is the treatment of choice and is more effective than multi-drug treatment.[7,38]

In the Netherlands, 15 to 20 cases of localized skin disease by *M. marinum* are diagnosed annually. This disease is often called the "fish tank granuloma" as most infections nowadays are seen in owners of aquaria with tropical fish; the infection commonly emerges in wounds acquired during cleaning of the aquarium.[7] No cases of *M. ulcerans* skin disease (Buruli Ulcer) were observed in the Netherlands in our study period.

Besides these three frequent NTM disease types, several rare forms of localized NTM disease exist. Bone, skin and soft tissue infections generally arise after trauma, including puncture wounds, surgical wounds and injection sites. Eye infection after laser *in situ* keratomileusis (LASIK) and ear infection in children with tympanostomy tubes are especially notorious;[7] both are frequently caused by rapid growing and highly drug-resistant NTM **(Chapter 2.7, 2.10)**.

In our series of NTM otomastoiditis, we noted very long intervals between the emergence of symptoms and the final diagnosis of NTM disease. We even noted cases of NTM infection in the skin and subcutis, after a dog bite **(Chapter 5.3)** and after cosmetic surgery in the Caribbean **(Chapter 2.7)**. In all these clinical settings, *Mycobacterium* culture may not always be performed and cases may have been missed.

Disseminated NTM disease is very rare and primarily affects patients with impaired immunity. Most cases of disseminated disease have been recorded in patients with advanced HIV disease (CD4 counts <50 cells/ml);[7] this is in agreement with our findings, mainly for *M. szulgai* and *M. avium* **(Chapter 2.1, 2.4)**. Another important HIV-related manifestation of NTM disease is the immune reconstitution syndrome. Here, successful institution or change

of antiretroviral therapy leads to a local or multifocal inflammatory process caused by underlying NTM disease.[63] We have noted two cases of spinal *M. xenopi* disease that we considered to represent immune reconstitution syndromes **(Chapter 2.2)**. Intriguingly, not all NTM pathogens cause HIV-related disseminated disease. This even accounts for bacteria of species that are common causative agents of other NTM disease types. For instance, *M. malmoense*, *M. szulgai*, *M. avium* and *M. abscessus* cause pulmonary disease in similar clinical settings, but only *M. avium* and *M. szulgai* are associated with disseminated infection in patients with AIDS **(Chapter 2)**.

Recently, inherited genetic defects of the interferon-γ/IL-12/IL-23 pathway were discovered, which confer an increased risk of mostly disseminated NTM disease.[64] These genetic defects are rare and we have only observed these in two patients with *M. szulgai* disease **(Chapter 2.4)**. However, only a very minor subset of the patients in whom NTM disease is diagnosed are examined for disorders in these pathways. Infections by other NTM (*M. avium*, *M.gordonae*, *M. peregrinum* and *M. mageritense*) in these patients have been described by van Dissel and co-workers of the Leiden University Medical Centre.[65,66] It is not yet known whether all NTM species can cause disease in these patients with inherited immunological disorders. In their murine model, Ehlers and Richter found that interferon-γ is a crucial determinant in the outcome of infection with most, but not all, nontuberculous mycobacteria tested.[58]

Other patient groups at risk for disseminated NTM disease include those with leukaemia or immunosuppressive drug use. Previously, this mainly concerned patients who received steroid treatment for chronic inflammatory diseases, or patients receiving steroids and other immunosuppressive agents after solid organ transplantation.[7] We have noted several cases of NTM disease attributable to immunosuppressive drug use. We recorded three cases of pulmonary *M. xenopi* disease and one case of *M. szulgai* skin disease in patients receiving steroids for chronic inflammatory diseases. In addition, we observed two cases of disseminated *M. abscessus* skin and joint disease and single cases of pulmonary *M. avium* disease, disseminated *M. malmoense* disease, focal *M. malmoense* skin disease and disseminated *M. chelonae* skin disease, all in patients receiving immunosuppressive drugs for haematological malignancies or solid organ transplantation **(Chapter 2)**.

Recently, the increasing application of the anti-TNF agents for rheumatic and other inflammatory diseases has created a new category of patients at increased risk for NTM disease **(Chapter 3)**.[16] TNF-α has an important role in granuloma formation and maintenance,[67] which are essential in the immunity against mycobacteria. During the early trials with these agents, reactivation of latent *M. tuberculosis* infection was most frequently reported.[68] The introduction of screening measures, first by tuberculin skin test and chest radiograph,[68,69] now often by interferon-γ release assays and chest radiograph **(Chapter 3.2)**, has limited the number of active tuberculosis cases in this patient group. The

number of cases of NTM disease associated with anti-TNF treatment, however, has increased and may now be more frequent than tuberculosis in this patient category;[16] many of these patients have extrapulmonary or disseminated NTM disease. The number of patients who develop NTM disease associated with treatment of their inflammatory disease is likely to increase even further in the future. First, the anti-TNF agents are increasingly used for a wide array of indications, not only in rheumatic disease, but also in sarcoidosis, inflammatory skin disease (e.g. psoriasis) and inflammatory bowel disease (e.g. Crohn's disease). Second, many of the immunosuppressive agents that are currently being developed or tested in rheumatic disease are likely to have a profound impact on the immunity against mycobacteria **(Chapter 3.2)**. Awareness of the possible emergence of NTM disease needs to be induced among future users and prescribers of these drugs. For the recently approved $CD20^+$ B-cell antibody rituximab, the first cases of NTM disease have already been reported.[16,70]

Treatment and outcome of pulmonary disease due to nontuberculous mycobacteria

In 1965, Manten stated that "[effective] chemotherapy for these [NTM] infections is an illusion"; surgical resection was the preferred treatment for NTM disease.[32] Considering the cure rates in the most recent BTS trial, 25-40% after 5 years of follow-up, one may question the progress made since 1965. In our studies, cure rates differed by species. In the regional study we recorded an overall cure rate of 67% for pulmonary NTM disease, though as low as 50% for *M. avium* disease. Higher cure rates were observed in *M. szulgai* (82%) and pulmonary *M. malmoense* (70%) disease **(Chapter 2)**, the latter is in line with the recent BTS trial.[71] Our results are biased since treatment regimens and duration varied widely, our average duration of follow-up was limited and retrospective medical file review is not an optimal method to study treatment outcome.

The sobering cure rates in the recent BTS trial, as well as in our various studies, may partly be explained by host factors, including the pre-existent pulmonary diseases and impairment of immunity. Most deaths in the BTS trial, for example, were due to these conditions rather than NTM disease.[71] Of course, the lack of evidence-based, effective treatment regimens also results from the lack of interest in NTM disease and the low incidence, which hampers the execution of clinical trials.

The introductions of the rifamycins and the newer drug classes, including the fluoroquinolones and macrolides, have not resulted in higher cure rates. Macrolide-based treatment regimens have shown promising results in *M. avium* complex disease in the United States (92% culture conversion)[72] and Japan (84% culture conversion).[7,73] This efficacy could not be reproduced in

Table 3: Common slow- and rapid growing NTM with susceptibility data

Growth rate	Species	Susceptibility to			
		Macrolides	Quinolones	Aminoglycosides	Rifampicin
Slow	*M. avium*	S	R	R	R
Slow	*M. kansasii*	S	S	R	S
Slow	*M. malmoense*	S	R	R	S
Slow	*M. xenopi*	S	S	S	S
Slow	*M. simiae*	R	R	R	R
Rapid	*M. abscessus*	S	R	R	R
Rapid	*M. chelonae*	S	R	R	R
Rapid	*M. fortuitum*	R	S	S	R
Rapid	*M. peregrinum*	S	S	S	R

S, Susceptible; R, resistant

studies in Europe conducted by the British Thoracic Society (BTS), where only 24% of 83 patients with pulmonary *M. avium* complex disease were alive and cured after five years of follow-up.[71] How to explain the apparent controversy surrounding in the efficacy of macrolides in pulmonary *M. avium* complex disease? Three aspects need to be considered.

First, the macrolides have been proven effective mostly in studies performed in the US, where the majority of patients present with nodular-bronchiectatic disease.[7,30,72] This specific disease type may differ in pathogenesis from the cavitary disease that is most common in the Netherlands and other European countries **(Chapter 2)**.[71] The different patient demographics, with a majority of females without a history of pulmonary disease but with CFTR gene mutations,[7,49] support a different pathogenesis. The lack of drug penetration in cavities may be less an issue in nodular-bronchiectatic disease; it is well known that cavitary disease is among the factors associated with poor outcome of intermittent treatment.[74] Possibly, the anti-inflammatory activity of macrolides is an added benefit in nodular-bronchiectatic disease, more so than in cavitary disease. Cavitary and nodular-bronchiectatic pulmonary NTM disease are two separate entities that may need separate treatment regimens to achieve comparable cure rates.

Second, in the United States the term '*M. avium* complex' is commonly used, although most of the isolates are identifiable to the species level and represent *M. intracellulare*.[7,30] We found macrolides to be more active *in vitro* against *M. intracellulare* than against *M. avium* **(Chapter 4.1)**. Hence, in Western Europe, where *M. avium* is isolated more frequently than *M. intracellulare*, one may expect that macrolides are less effective.

Last, death rates were high during treatment and 5 years of follow-up in the BTS trial, due to the extent of pre-existent pulmonary disease in the enrolled patients. The studies in the United States enrolled patients of older age, though their condition was less frequently impaired by chronic pulmonary disease

and follow-up was considerable shorter (19 months); correspondingly, death rates were lower.[7,72]
For the fluoroquinolones, the BTS trial again demonstrated a limited role in treatment of NTM disease.[7] In the light of our *in vitro* results, this comes to no surprise. The new fluoroquinolone moxifloxacin has been shown to be more active than ciprofloxacin against mycobacteria.[7,75] Perhaps there may be a role for moxifloxacin in the treatment of disease due to some NTM species. For *M. avium* disease, however, we expect little efficacy **(Chapter 4.1)**. A combination of fluoroquinolones and macrolides did not result in greater efficacy in the BTS trial.[71] Kohno and co-workers demonstrated a dampening effect of the fluoroquinolones on macrolide activity, both *in vitro* and *in vivo*.[76] For slow growing NTM, a combination of macrolides and fluoroquinolones will not be an effective regimen. Still, it is a regimen that we regularly encountered in our restrospective studies **(Chapter 2)**.

The use of treatment regimens that were not evidence based, or used for too short periods was frequent in all our retrospective studies **(Chapter 2)**. Combined with the frequently observed over- and underdiagnosis, leading to over- and undertreatment of NTM disease, this makes clear that national guidelines for appropriate diagnosis and treatment of NTM disease are urgently needed. Pulmonary NTM infections are most frequent and the Dutch Association of Physicians in Pulmonary Diseases and Tuberculosis (Nederlandse Vereniging van Artsen voor Longziekten en Tuberculose; NVALT) is the most likely candidate to supervise the establishment and dissemination of such guidelines. Based on our retrospective analyses of clinical NTM disease and *in vitro* drug susceptibility, we believe the advice based on the results of the recent BTS trial can be extrapolated to the Netherlands. The rifampicin and ethambutol regimen should be used as an initial basic regimen for disease due to slow-growing NTM (for frequently isolated slow- and rapid growing NTM, see Table 3). Macrolides may be added if the patient does not improve, or in case of a relapse. Future studies should address their place in the initial regimen in case of nodular-bronchiectatic disease.
The additional value of aminoglycosides, advocated by the ATS for the first months of treatment of cavitary *M. avium* complex disease,[7] remains to be established in European studies. For the rapid growers, a combination of a macrolide, an aminoglycoside and one or two companion drugs selected on basis of *in vitro* drug susceptibility patterns, as proposed by the ATS, is the preferred regimen.[7] For most NTM species, however, no controlled trials have been done and little supportive evidence to facilitate guidelines exists.

The clinical utility of drug susceptibility testing (DST) of NTM isolates remains an open question; within the American Thoracic Society guidelines, DST is considered most beneficial in case of delayed culture conversion or failure of

therapy; baseline testing is only advised for *M. avium* complex isolates, against the macrolides and for *M. kansasii*, against rifampicin.[7] The utility of DST in case of relapse or failure is increased if a baseline test is available, to discern acquired resistance. Hence, we believe baseline testing, i.e. DST prior to the institution of therapy, is appropriate for all NTM that are considered clinically relevant. Here, again, our quantification of clinical relevance of the various species (Figures 1 and 2) may prove useful.

For the first-line anti-tuberculosis drugs (isoniazid, rifampicin, ethambutol, streptomycin), discrepancies between *in vitro* activity and *in vivo* treatment outcome complicate the interpretation of the DST result **(Chapter 4.1)**.[71,77] Various factors may be involved in these discrepancies. First, Banks and co-workers noted substantial synergism between rifampicin and ethambutol, which may partially explain good treatment outcome in cases of *M. avium* disease caused by bacteria resistant to both rifampicin and ethambutol. Similarly, synergism between streptomycin and rifampicin was recorded.[78] Perhaps a combined rifampicin/ethambutol susceptibility test is clinically more valuable than testing separate drugs, to which resistance is common **(Chapter 4.1)**.[78] Second, the drug concentrations within NTM disease lesions and macrophages, thus the actual exposure of the NTM to antimycobacterial drugs, may be higher than the concentration used in *in vitro* DST. Moreover, cut-off points for *in vitro* resistance of NTM are usually extrapolated from those of *M. tuberculosis* and may not be representative.

Here, pharmacokinetic studies may provide useful data to reconsider the currently used breakpoint concentrations. It has been observed that patients with pulmonary *M. avium* complex disease achieve higher serum concentrations of rifampicin than tuberculosis patients.[79] Similar studies in patients with cavitary NTM disease are warranted in the Netherlands, preferably correlating the concentrations to bacteriological and clinical improvement and treatment outcome in a pharmacodynamic study. To date, no such studies have been performed in NTM disease. Preferably, serum concentrations, the tissue diffusion and intracellular drug concentration within macrophages should be investigated. These are likely to increase our understanding of the discrepancies between *in vitro* activity and *in vivo* treatment outcome.

The second line drugs (prothionamide, cycloserine, clofazimine) are highly toxic and their efficacy has not been proven.[80,81] As a result, there is little reason to include them in standard DST schemes, especially as part of the isolates will not be considered clinically relevant and the respective patients will not be treated. For now, mainly for the slow-growing NTM, it may suffice to test macrolides, aminoglycosides and the newer drugs with established therapeutic potential (moxifloxacin, linezolid, tigecycline). For the rapid growers, where drug resistance is a grave issue, this set should be expanded with carbapenems and cefoxitin.[82]

The optimal DST method also remains subject of debate. The Clinical and Laboratory Standards Institute (formerly National Committee for Clinical Laboratory Standards) now advocates the use of broth microdilution methods, especially for the rapidly growing NTM. For slow growers, agar dilution and broth microdilution methods are considered appropriate.[82] In the Netherlands, we use an agar dilution method for all NTM. This method was historically selected as it was the method of choice for *M. tuberculosis* DST, for both first and second-line drugs.[83,84] This may partly explain the differences in drug susceptibility patterns between our study **(Chapter 4.1)** and previously published studies applying the broth microdilution techniques. The level of resistance to aminoglycosides (see Table 3) exceeds that observed in previous studies.[7,82,85] Especially for the rapid growing NTM, this may have an undesirable impact on treatment regimens.

For *M. tuberculosis* complex bacteria, the RIVM now performs DST in the MGIT automated liquid culture system. The abolition of the agar dilution method for *M. tuberculosis* complex has led to renewed debate on the platform of choice for NTM. Currently, the CLSI recommended broth microdilution method is being tested at the RIVM.

To optimise treatment of NTM disease, newer and more active drugs are still strongly needed. Preferably, the *in vitro* activity of these drugs should be concordant with their clinical activity and thus treatment outcome. Within this thesis we have assessed the potential of the antipsychotic drug thioridazine. Strictly, thioridazine is not a new drug, as it has been used extensively to treat schizophrenia for several decades.[86] Its antimycobacterial activity had not been fully explored, though. We found promising activity against *M. tuberculosis*, even against multi- and extensively drug-resistant strains; unfortunately, its activity against NTM was limited **(Chapter 4.2)**. Although thioridazine is concentrated within macrophages,[86] where the NTM reside during (early) infection **(Chapter 6.3)**, this drug is unlikely to have any additional value in the treatment of NTM disease **(Chapter 4.2)**.[87]

Combined chemotherapy and surgery may be the most effective intervention in a select category of patients that do not respond favourably to the initial therapy regimen; therapy-resistant cavitary disease, preferably of a single lobe of one lung is a good indication for surgery in operable patients.[88,89] Pneumonectomy for those with an infected destroyed lung may be feasible, although if there are lesions in the other lung, they should be minimal. Cavitary lesions in the other lung are a contra-indication for pneumonectomy.[90] Although it requires keen timing, eligible patients and centres with specialized surgeons, pulmonary physicians and good follow-up facilities, we believe that increased application of surgery for pulmonary NTM disease can greatly improve outcome **(Chapter 4.3)**.

Genetic divergence: from gene sequence to clinical relevance

Our finding that the clinical relevance of NTM differs significantly by species is an indication that bacterial factors are associated with clinical relevance. Very little is known of bacterial virulence factors and their mode of action in NTM. In *M. avium*, two genes associated with virulence have been identified. In 1998 a conserved coding sequence for the macrophage-induced gene *mig* was found to be associated with increased virulence of *M. avium* and human disease,[91] although this association could not be reproduced in a recently published study.[92] Recently, a PPE gene related to Rv1787 of *M. tuberculosis* was shown to promote survival and growth of *M. avium* within human macrophages and in a murine disease model.[93] Since NTM are environmental bacteria and humans are likely to be an accidental host, one may wonder whether something like a virulence factor actually exists. Is a mechanism that allows NTM to survive in the hostile environment of the human lung not merely a fortunate result of adaptation to varying environmental niches? This broad adaptation is well represented by the size of the genome; the generalists *M. smegmatis* (7.0 Mb) and *M. marinum* (6.6 Mb) have larger genomes than specialist, host-adapted bacteria such as *M. leprae*, *M. tuberculosis* and *M. avium paratuberculosis* (3.8, 4.4 and 4.8Mb respectively), which have adapted to a limited host range by reductive genomics and lateral gene transfer.[94] Genomes of intracellular bacteria tend to lose genes for metabolic diversity, needed for survival in diverse and potentially nutrient-poor environments, but retain those encoding transcription, translation and other basic processes that are important regardless of ecological niche.[95,96]

We have explored the bacterial factor associated with clinical relevance, by two separate approaches. At the macro level, we have explored differences in virulence between species. We have looked into the presence of the Region of Difference 1 (RD1) and its role as a virulence factor in NTM. The RD1 is an important virulence factor for *M. tuberculosis*.[97] It is striking, in this respect, that most NTM species found to harbour an RD1 element in their genome (*M. kansasii*, *M. szulgai*, *M. marinum*, *M. riyadhense*) are clinically highly relevant (Figure 1 & 2) **(Chapter 2.1, 2.4, 6.2, 7.2)**. On the other hand, this element is absent in other clinically relevant species, most notably *M. malmoense* and *M. avium* **(Chapter 2.1, 2.6, 6.2)**. Moreover, the RD1 genes are thought to facilitate the translocation of mycobacteria from the phagolysosome to the cytosol of macrophages.[98] Translocation to the cytosol could not be convincingly demonstrated for *M. szulgai* in our THP-1 cell infection model, despite presence of an RD1-like element **(Chapter 6.3)**. Similarly, in *M. smegmatis*, the RD1 genes are thought to be involved in bacterial conjugation, rather than translocation from the phagolysosome to the cytosol of macrophages.[99,100] The genetic differences between the *M. tuberculosis* complex and NTM in genes associated with virulence such as the RD1 or their expression and product secretion may explain the generally lower virulence of NTM **(Chapter 6.2,**

6.3). For now, presence of an RD1 remains merely a phylogenetic character **(Chapter 6.2)**.

At the micro level, we have explored intraspecies genetic diversity within semi-conserved housekeeping genes, i.e. genes involved in important cellular processes whose disruption by genetic polymorphism would lead to loss of bacterial viability. Subdividing NTM species, based on variance in housekeeping genes, may not be merely an academic or taxonomic exercise. Different subgroups may show different levels of clinical relevance or even different types of disease. *Mycobacterium kansasii* is a good example; although five subtypes are known, only two cause disease in humans. One causes mainly pulmonary disease (type I), whereas the other is a causative agent of disseminated disease in immunocompromised patients (type II). The remaining types are considered non-pathogenic environmental bacteria.[42,43] This intraspecies divergence was already noted by Coster in his animal inoculation experiments in the 1950s; not all Syrian gold hamsters inoculated with similar loads of various "yellow bacillus" (the contemporary name for *M. kansasii*) isolates developed disease, suggesting that more and less pathogenic strains existed.[101] In our retrospective study on *M. xenopi* we noted a similar phenomenon. Strains of the *M. xenopi* II 16S sequence type were more often clinically relevant than the ones of type I **(Chapter 2.2)**. In our multi-gene sequencing study, we noted interesting levels of intraspecies divergence in all species tested. Interestingly, the divergence followed a mosaic rather than stochastic pattern. Lateral gene transfer as a driving force in bacterial evolution is not a far-fetched option for NTM. Their environmental habitat provides for intense contact with other bacterial species and genera. Nevertheless, the extent of divergence was such that it was unlikely to result from lateral gene transfer **(Chapter 6.1)**. A long evolutionary development through stochastic processes is more likely.

We were not able to discern a *M. xenopi* or *M. malmoense* subgrouping significantly associated with clinical disease; our study served as an exploration, rather than proof of concept, as the number of isolates was quite low **(Chapter 6.1)**. If the intraspecies genetic divergence within house-keeping genes can be extrapolated to virulence-associated genes, however, intraspecies genetic divergence may still influence clinical relevance. Some isolates of a certain species may or may not cause disease based on variance within genes (and their products) essential in the disease process.

Overall, the knowledge on virulence factors in NTM lags significantly behind in comparison to other environmental opportunistic pathogens. For instance, a variety of mechanism has been described for *Pseudomonas aeruginosa*, an environmental pathogen that affects categories of patients also at risk for NTM disease. *Pseudomonas aeruginosa* is known to create biofilms in which they are less susceptible to antimicrobials and these bacteria can switch to a mucoid phenotype and even a hypermutator state, associated with immune evasion and increased virulence. By quorum sensing, a system by which bacteria sense their

cumulative numbers and change their behaviour accordingly, the *Pseudomonas* bacteria can assess their numerical presence and time their phenotypical and genotypical change.[53,102] A similar change in colony morphology, associated with increased virulence and drug resistance, has recently been observed for *M. abscessus*,[103] although it is not known whether this change reflects a change in behaviour such as biofilm formation. Other fascinating assets, such as quorum sensing, have not been studied in NTM. Conjugation has been described as a function of ESAT-6 and CFP-10 secretion in *M. smegmatis*.[99,100] This is the closest to quorum sensing known for NTM. The genetic divergence among NTM makes that these kinds of virulence factors are unlikely to be distributed throughout the genus, as we have noted for the RD1 **(Chapter 6.2)**. A lot is still to be learned on the mechanisms involved in the actual establishment of NTM infections. Furthermore, it is unknown whether these are uniform in all NTM pathogenic to humans.

The genetic divergence among NTM questions our currently used identification tools. Perhaps replacement of the 16S rDNA gene by the *rpoB* gene sequence as the molecular target for identification to species level among rapid growing NTM is an improvement, because this gene offers a higher level of discrimination.[104] Instead of choosing a more variable target, it may be worthwhile to switch to a system that includes sequencing of multiple targets.[105] As we have noted in our multi-gene sequencing study, this leads to distinction of particular subtypes within NTM species. These subtypes may have clinical correlates **(Chapter 6.1)**; systematic identification of NTM in a multi-gene sequencing scheme will shed light on these correlates. Here, the genetic divergence among NTM calls for new, more exact species definitions. Ultimately, whole genome sequencing will provide the best identification method and will reveal the true extent of intra- and inter-species genetic divergence among NTM, although it is for technical and financial reasons not yet within reach for this purpose.

Whole genome sequences of clinical isolates of the major NTM species, both clinically relevant and non-relevant, can be used to build the ultimate phylogenetic tree of the genus *Mycobacterium*. This tree can be appended with clinical data to allow systematic studies of virulence associated genes. Currently, however, the vast majority of mycobacteria subjected to whole genome sequencing are *M. tuberculosis* complex members.[106]

Given the fact that the clinical relevance of NTM differs by species, the description of new species can be clinically meaningful. A more distinctive taxonomy is frequently considered a nuisance by clinicians. The ongoing use of terms like '*M. avium* complex' is testimony to this sentiment.[107] Recognition of species of particular pathogenicity or causative agents of a particular disease type and their subsequent description as novel species may be of great help in clinical practice and can serve to unravel bacterial virulence factors. We have elevated the group of *M. avium* complex isolates characterised by the MAC-Q 16S-23S internal transcribed spacer sequevar to species status as they

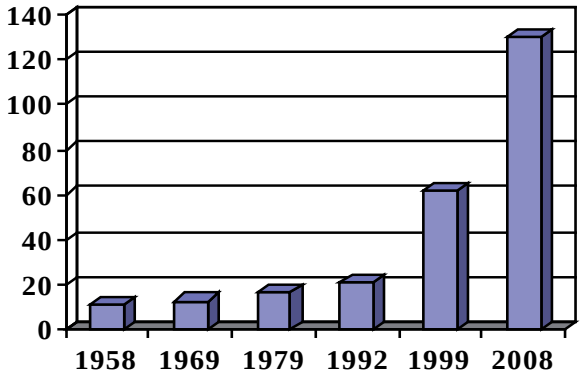

Figure 5:
The number of validly published *Mycobacterium* species over time

Numbers were obtained from references 31,34,35,37,108,109.

were causative agents of extrapulmonary disease. If this is confirmed in larger case series, whole genome sequencing and comparison with *M. avium* and *M. intracellulare* may help to understand the aetiology of NTM disease and the apparent tropism of *M. vulneris* **(Chapter 5.3)**.[106] Alternatively, distinction of *M. noviomagense* as a novel species, genetically related to *M. xenopi*, is clinically meaningful as pulmonary *M. noviomagense* isolates were clinically not relevant, which is very different from the frequently clinically relevant *M. xenopi* (Figure 2) **(Chapter 2.2, 5.2)**. Future comparative studies can reveal the causative mechanisms behind these clinical observations. Integration of clinical data in new species descriptions is helpful to clinicians to determine their stance towards patients with a pulmonary isolate of *M. noviomagense*, *M. mantenii* or other novel species.
For NTM, it may be important to use clinical utility as a criterion for description of novel species. We agree with Telenti, who has proposed that "clinical meaningfulness should be the key to taxonomic precision".[107] The number of validly published NTM species has exploded after the introduction and spread of molecular identification tools in the early 1990's (Figure 5).

In our re-analysis of previously unidentifiable NTM isolates, we recorded 53 groups of isolates with novel 16S gene sequences, of which one third (n=18) could be assigned to recently published species. Still, that leaves 35 groups of isolates that may represent novel species; some isolates were from extrapulmonary sources and thus likely causative agents of human disease. Hence, even if clinical meaningfulness is a decisive factor to describe new species, the number of NTM species is likely to reach 200 by the year 2010 (Figure 5). Is such a large number of NTM species feasible in daily clinical practice? Based on the whole genome sequences appended with clinical data, a grouping of NTM into groups based on virulence factors, rather than specific species names, may be a worthwhile future strategy: from gene sequences to clinical relevance **(Chapter 5.1, 6.1)**.[106]

Clinical relevance of NTM isolation in other geographical regions

The number of countries that have reported NTM isolation or disease has increased. Marked regional differences in NTM species distribution exist. The species that seemed to be dominant in Oman are slightly different from those found in the Netherlands **(Chapter 7.1)**. In a small study in Vietnam, however, we found a very different species distribution, marked by predominance of rapid growers similar to adjacent regions of China, Taiwan and Thailand **(Chapter 7.2)**. Such differences are clinically important, as treatment can vary for different species.

In areas with a high prevalence of tuberculosis (TB), it is often assumed that all acid-fast bacilli visualized by Ziehl-Neelsen staining of clinical samples or *Mycobacterium* cultures are *M. tuberculosis* complex bacteria. This is not always the case, though, and this has an impact on clinical care and surveillance of TB.[110] The clinical relevance of NTM isolation from respiratory samples in areas with a high prevalence of TB remains uncertain. In most of these areas, physicians already struggle to cope with the high number of TB patients, leaving little attention to the possibility of NTM disease. In many such regions, culture and identification facilities are lacking, hampering even the diagnosis of TB and rendering distinction between TB and NTM disease impossible.

In many regions of Africa the high TB prevalence is further complicated by a high burden of HIV infection. For patients with HIV infection, distinguishing NTM disease from TB is important, as treatment of NTM disease is different from that of TB **(Chapter 7.4)**. Still, the same lack of diagnostic tools bars diagnosing NTM disease. Several studies found NTM in clinical samples in Africa, although few have examined their clinical relevance.[111] After the onset of the HIV epidemic, a few studies have reported cases of disseminated NTM disease.[112,113] A recent study in Zambia clearly demonstrated extrapulmonary NTM disease, mostly in HIV-infected patients.[114] Our work in Tanzania demonstrated that disseminated NTM disease is also prevalent there **(Chapter 7.4)**, though the causative agents are different from the Netherlands and the United States, where *M. avium*, *M. kansasii* and *M. xenopi* are important **(Chapter 2.1, 2.2)**.[115,116]

The NTM species distribution differs by region and some NTM species are restricted to particular areas and not found elsewhere. The misidentification of *M. sherrisii* isolates as *M. simiae* in our Tanzanian study **(Chapter 7.4)** showed that currently available molecular identification tools, many of which are designed to identify NTM in Europe and the USA, may be of limited use in other parts of the world, where different NTM species exist. In addition, the data on clinical relevance obtained in our studies in the Netherlands can probably not be extrapolated to African or Asian countries. Similarly, we have noted that results from studies in the United States proved irreproducible in the Netherlands **(Chapter 2.1)**. Therefore, each region should perform its own investigations into the NTM species present and their clinical relevance.

Conclusions and strategies for future research

The clinical relevance of NTM differs by species. This provides guidance to clinical care. This guidance should be further improved by systematic study of additional species and re-evaluation of the already studied NTM species once their case volume has increased. Disease due to NTM results from a complex and incompletely understood interplay of host and pathogen factors. Several host factors, including advanced HIV infection, CFTR gene mutations and interferon-γ or interleukin-12 receptor deficiencies, have been shown to increase susceptibility to NTM disease.[7,49,64,65] Bacterial virulence factors remain largely unknown. The role of ESAT-6 and CFP-10 as virulence factors in *M. tuberculosis* complex bacteria does not hold true for *M. szulgai*. The translocation by *M. tuberculosis* and *M. marinum* from the phagosomes to the cytosol in macrophages could not be convincingly demonstrated for *M. szulgai* **(Chapter 6.2, 6.3)**. Whether this results from a lack of transcription or secretion, or whether these genes and their products have very different functions in *M. szulgai* remains to be investigated. Similar genes are also present in *M. kansasii* and *M. riyadhense*, but the functions of their products in these species remains to be investigated.

Despite the findings for ESAT-6 and CFP-10, it may prove worthwhile to perform whole genome sequencing of strains of the clinically relevant species (i.e. *M. kansasii*, *M. szulgai*, *M. malmoense*), most of which are phylogenetically related to the *M. tuberculosis* complex **(Chapter 6.2)**, to assess the presence and absence of other virulence factors and their secretion mechanisms. The already sequenced *M. tuberculosis*, *M. bovis* and *M. bovis* BCG strains can serve as a template for these analyses. Interestingly, whole genome sequencing of NTM is performed now, though the choice of species has not been based on clinical relevance. Currently, sequences are available for *M. avium hominissuis* 104, *M. avium paratuberculosis* K10, *M. marinum*, *M. ulcerans*, *M. vanbaalenii* and *M. smegmatis*. Whole genome sequences of *M. abscessus* and *M. kansasii* are currently determined.[106]

We do not know whether the clinical isolates mirror the NTM species distribution in the environment in the Netherlands. Perhaps, the human respiratory tract (the most common source of clinical NTM isolates) exerts a selective pressure. This would be important for the concept of clinical relevance and mycobacterial virulence. Sampling of natural and man-made environments, accompanied by molecular identification and preferably typing of encountered NTM should be performed in association with a prospective clinical study within a confined region.

We have used the degree of clinical relevance in humans as a measure of pathogenicity. Still, this concept has not been sufficiently validated. Animal infection experiments with isolates of various species and with relevant and non-relevant isolates of the same species may shed light on this issue. Such studies could reveal the relative importance of host and pathogen factors in

NTM disease. Simultaneously, this would offer the opportunity to study the consistency of the immunological reaction to various NTM species. The obvious differences in clinical relevance of *M. malmoense* and *M. intracellulare* between the United States and the Netherlands could also be studied by murine infection with clinical isolates from both countries. The choice of animals and infection route is very important and it may even be necessary to invoke conditions that predispose to NTM disease (e.g. silicosis, COPD, CF), similar to early guinea pig studies with silicosis induction to facilitate pulmonary *M. kansasii* infection.[62]

Outcome of treatment for NTM disease is often disappointing; the success with macrolide-based regimens observed in the United States[72] is not observed in the Netherlands **(Chapter 2.1)**, or in the recent BTS study.[71] More trials are needed to address this issue. These must be multi-country trials and need to explore the additional effect of moxifloxacin, as well as novel compounds and combinations. Meanwhile, we recommend that regimens that proved most effective in previous trials should be increasingly used in the Netherlands. In our retrospective surveys we noted a widespread use of inferior regimens of too short duration. This is an unwanted situation in a country where access to up-to-date medical literature is easy. In addition, we noted both over- and undertreatment of NTM disease. This suggests that the use of diagnostic criteria, available from both the ATS and BTS,[7,117] is not commonplace in the Netherlands. The limited use of available guidelines for diagnosis and treatment of NTM disease makes clear that national guidelines for appropriate diagnosis and treatment of NTM disease are needed. We believe that separate diagnostic guidelines are needed for pulmonary disease due to species of different clinical relevance. Strict criteria should be implemented for species generally of low clinical relevance (*M. gordonae*, *M. noviomagense*, *M. chelonae*) and more lenient criteria for the highly clinically relevant species (*M. kansasii*, *M. szulgai*, *M. malmoense*) **(Chapter 2)**. Moreover, increased consultation and referral of patients to the two tuberculosis reference hospitals available in the Netherlands (Dekkerswald and Beatrixoord) would be beneficial. These centers have extensive knowledge and experience in handling NTM disease. Dekkerswald also offers surgery for pulmonary NTM disease. Surgery proved effective for patients who did not respond to optimal chemotherapy regimens in our pilot study; it may help to improve the cure rates, even though it is suitable for a select category of patients only **(Chapter 4.3)**.

We found substantial genetic divergence within NTM species. This may help to explain the bacterial side of the principle of clinical relevance; particular subtypes may be associated with clinical relevance, as we noted for *M. xenopi* **(Chapter 2.2)**. The differences in clinical relevance of *M. intracellulare* and *M. malmoense* between the Netherlands and the United States are an interesting starting point for a multi-gene sequencing study of representative isolates of both species from both countries.

It was difficult to obtain national coverage for our clinical relevance surveys; individual laboratories had to be contacted to obtain insight in their databases. NTM disease is not a reportable condition in the Netherlands and there is no obligation to send isolated NTM to the national reference laboratory (RIVM). Although obligatory strain referral may be impossible to arrange and superfluous, a laboratory reporting system would be of great benefit to monitor the NTM isolation in the Netherlands. We hope that our results will lead to such a reporting system and that this will encourage and facilitate future research.

We have been able to lift the carpet and demonstrate that NTM isolation is a relevant clinical and laboratory issue in the Netherlands and other countries, including countries with a high prevalence of tuberculosis. We hope to have encouraged researchers in many countries to survey the local NTM isolation frequency and the degree of clinical relevance. Such surveys will enable countries to develop their own diagnosis and treatment guidelines, using the ATS diagnostic criteria and both ATS and BTS treatment guidelines as a backbone. It is apparent that the American or British situation and its resulting guidelines[7,117] can not be directly extrapolated to other countries, as acknowledged in the ATS document.[7]

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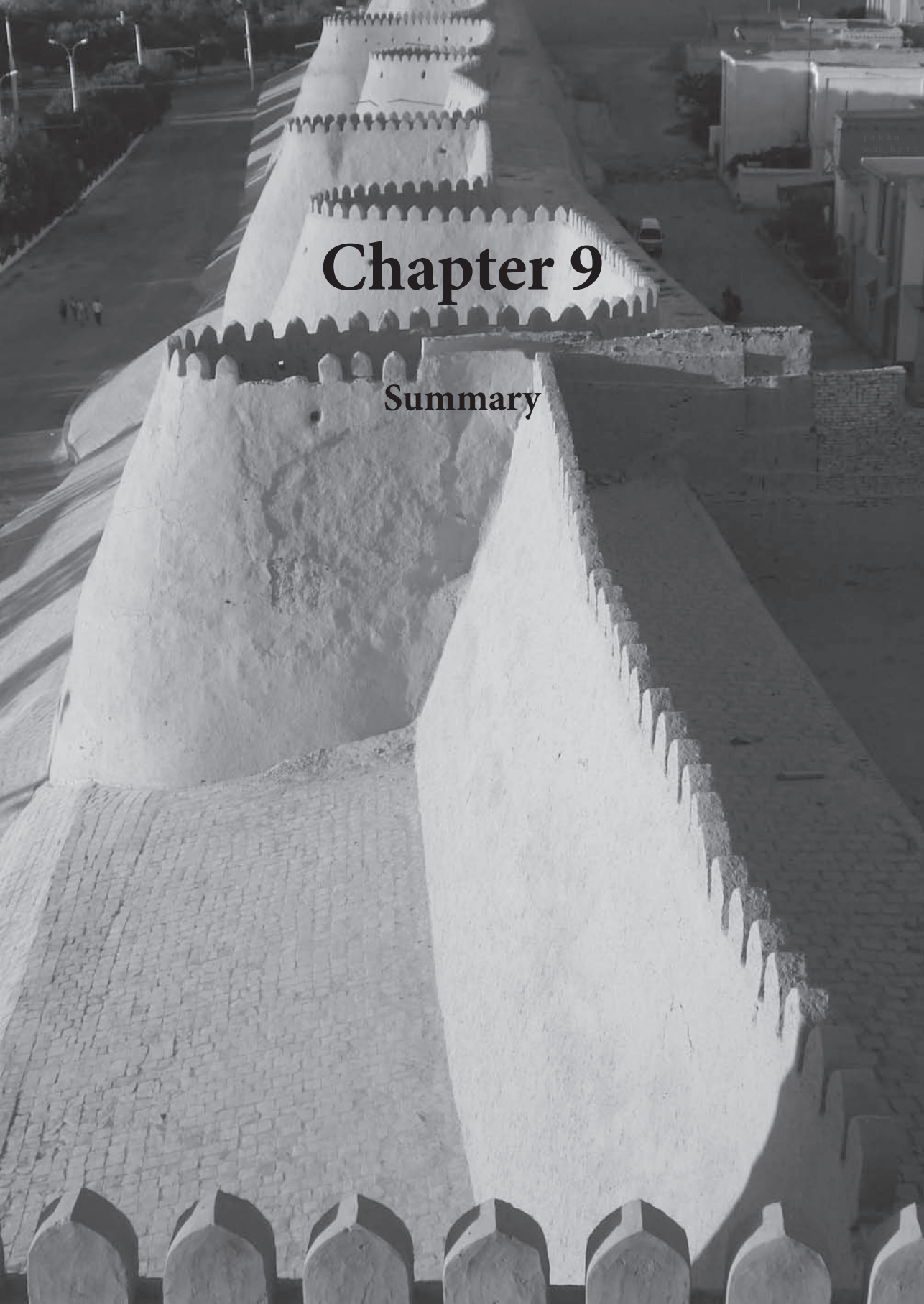


Chapter 9

Summary

This thesis summarizes joint studies on the clinical relevance of nontuberculous mycobacteria, by the University Lung Centre Dekkerswald (part of the Radboud University Nijmegen Medical Center) and the National Institute for Public Health and the Environment.

In **Chapter 1** we present a general introduction on nontuberculous mycobacterial disease, including a historical perspective. Our starting point is that the genus *Mycobacterium* consists of three main groupings: I) the *Mycobacterium tuberculosis* complex, II) *Mycobacterium leprae* and III) the nontuberculous mycobacteria. While clinical isolation of nontuberculous mycobacteria (NTM) has become increasingly frequent in recent years, their clinical relevance was largely unknown.
Whereas the *M. tuberculosis* complex and *M. leprae* bacteria are obligate pathogens for humans and other animals, the NTM, which consist of over 100 validly published species, are mostly opportunistic environmental pathogens mainly affecting patients with impaired local or systemic immunity. Their omnipresence in the environment implies that if NTM are cultured from non-sterile clinical samples, e.g. those from the respiratory and digestive tract, this does not indicate NTM infection *per se*; contamination or pseudo-infection should be ruled out. To accommodate for this, the American Thoracic Society (ATS) has developed diagnostic criteria for NTM lung disease, consisting of clinical and microbiological criteria.

In **Chapter 2**, we have quantified the clinical relevance of isolation of various NTM species. To determine the clinical relevance, we retrospectively assessed the percentage of patients that met the ATS diagnostic criteria for pulmonary NTM disease, as well as the frequency and clinical features of extrapulmonary disease, per species. In a regional study of all clinical NTM isolates we found that 25% of all NTM isolated from respiratory tract samples were causative agents of pulmonary NTM disease. Taking both pulmonary and extrapulmonary isolates into account, 33% of all clinical NTM isolates were causative agents of true NTM disease. In the regional and subsequent national studies we found that this percentage differs significantly by species, from less than 5% (for *M. noviomagense*, *M. gordonae*), to well over 70% (*M. kansasii*, *M. szulgai* and *M. malmoense*). This finding has important implications for clinical practice. The identification of cultured NTM can assist the clinician in determining his stance towards the individual patient. We suggest that future ATS diagnostic criteria should accommodate for these differences in clinical relevance. For species of limited clinical relevance, the criteria should be stricter to prevent unwarranted diagnosis and treatment of NTM disease. Conversely, for highly relevant species, more lenient criteria can reduce diagnostic delay and undertreatment.
From the retrospective studies we learned that the diagnosis and treatment of NTM disease in the Netherlands can be improved. The use of available

diagnostic criteria and treatment guidelines seemed limited; we recorded significant over- and underdiagnosis, leading to over- and undertreatment of NTM disease. Moreover, the use of treatment regimens that were not evidence based, or of too short duration was frequent. Possibly as a result, cure rates for pulmonary NTM disease were suboptimal. In our regional study, we recorded an overall cure rate of 67%. Cure rates also differed by species and ranged from 50% (for *M. avium*) to 82% (*M. szulgai*). These cure rates are comparable to those of multi- or extensively drug-resistant tuberculosis and emphasize the strong need for new anti-mycobacterial drugs and adjunctive therapies.
From our retrospective studies, a rather homogeneous patient population emerged, characterized by a predominance of males, an average age of 60 years and pre-existing pulmonary disease. Cavitary disease was the most common pulmonary NTM disease type in these patients. Pre-existing pulmonary disease and cavitary NTM disease are both major factors negatively influencing treatment outcome. The increasing isolation frequency of NTM in the Netherlands was largely due to a rise in *M. avium* isolates from pulmonary samples exactly in this patient category.
We consider the increased frequency of clinical NTM isolation to be mainly a result of the ageing of the Dutch population, with an increasing prevalence of chronic (pulmonary) diseases and immunosuppressive treatment for these diseases, as well as a decline in cross-protection from exposure to tuberculosis. Improvements in laboratory techniques and the increased use of showers for personal hygiene may have also had an impact.

Within **Chapter 3**, we focused on an emerging category of patients at risk for NTM disease, those with rheumatic or other immune-mediated inflammatory disease treated with antibodies against cytokines or receptors with pivotal roles in inflammatory pathways. These include the anti-tumor necrosis factor-α (anti-TNF) agents and are collectively referred to as 'biologicals'. The first clinical trials using anti-TNF agents revealed an increased risk of serious infections, including reactivation of latent tuberculosis. We described a case of pulmonary *M. szulgai* disease associated with anti-TNF therapy and reviewed the impact of the various biologicals on immunity against mycobacteria. NTM disease has already been recorded in patients undergoing anti-TNF, anti-IL-1 and $CD20^+$ B-cell antibody treatment for rheumatic disease. Among the drug classes currently in development for anti-rheumatic therapy, the IL-17, IL-23 and JAK/STAT-signaling modifying agents and metalloproteinases are also likely to confer an increased risk of mycobacterial infection. As the biologicals and the indications for their use diversify, associated cases of NTM disease are likely to become more frequent. All patients receiving biologicals should be screened for latent tuberculosis infection and carefully monitored for mycobacterial disease.

With the clinical relevance of NTM established and populations at risk identified, it is sensible to shift the focus to treatment of NTM disease. In Chapter 2, we found the outcome of treatment was often poor. Therefore, in **Chapter 4**, we looked into potential improvements of therapy for NTM disease. We studied the role of drug susceptibility testing, a potential novel drug and adjunctive surgery.

Drug susceptibility testing demonstrated that clarithromycin and rifabutin are *in vitro* most active against NTM. The utility of testing other first and second line anti-tuberculosis drugs is limited. For the first line drugs (isoniazid, rifampicin and ethambutol) there are discrepancies between *in vitro* activity and *in vivo* outcome of treatment; the activity of the second line drugs (clofazimine, cycloserine, prothionamide) is not supported by clinical data. To improve therapy guidance, these should be substituted for drugs with proven clinical efficacy, correlating with *in vitro* susceptibility, especially for rapid growing NTM. Baseline testing, i.e. drug susceptibility testing prior to the institution of therapy, is appropriate for all clinically relevant NTM isolates; follow-up testing is warranted in case of therapy failure or relapse.

Considering the urgent need for new antimycobacterial drugs, we tested the activity of thioridazine, a widely available and out-of-patent antipsychotic drug with antimicrobial properties. We measured potent activity against *M. tuberculosis*; the activity against NTM was up to 8 times lower. This seems to preclude a role for thioridazine in treatment of NTM disease.

In case of failure of conventional drug treatment for pulmonary NTM disease, adjunctive surgical resection of the affected parts of the lungs has been advised. We reviewed the outcomes of eight patients who underwent surgery for NTM lung disease in Dekkerswald. Surgery resulted in long-term culture conversion in all but one patient. The indications and timing of surgery were critical factors; these were insufficiently addressed in available literature. We conclude that benefits of surgery should be considered for every individual patient in whom NTM lung disease is diagnosed and re-evaluated after six months of treatment. Where possible, surgery should be pursued and timely conducted. To assist pulmonary physicians, we have proposed indications for surgery.

Clinical relevance and optimal treatment regimens have been defined for well-established NTM species. In **Chapter 5**, we explored the heterogeneity among NTM and concluded that at least 4% of all NTM isolates submitted to the national reference laboratory can not be assigned to validly published species. Strains related to, but different from, the *M. avium* complex and *M. fortuitum* complex members were especially prevalent.

Some groupings of previously unidentifiable mycobacteria stood out as they were frequently isolated, or isolated from extrapulmonary samples and were thus probably associated with clinical NTM disease. Four of these were studied in-depth and could be elevated to separate species status, with

an assessment of their clinical relevance. We discerned the clinically non-relevant *M. noviomagense* from the closely related and frequently clinically relevant *M. xenopi*, described *M. vulneris* and (in Chapter 7) *M. riyadhense* as causative agents of extrapulmonary NTM disease and found that *M. mantenii* is a causative agent of pediatric cervicofacial lymphadenitis but not relevant in pulmonary samples. More discriminative mycobacterial taxonomy can be of clinical utility; in fact, we conclude that clinical meaningfulness should be the key to taxonomic precision in NTM.

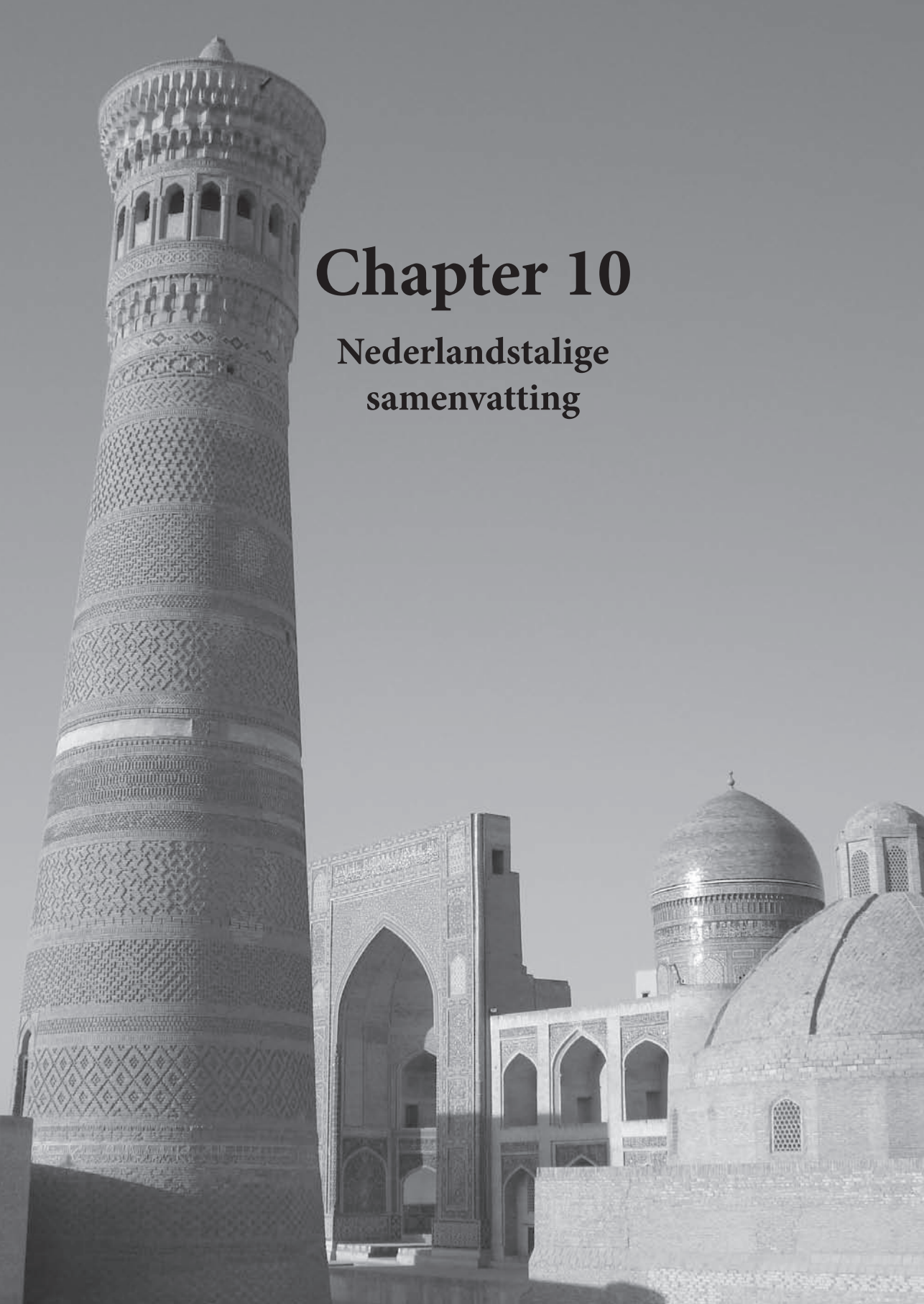
In **Chapter 6** we zoomed further in, on the genetic divergence within NTM species. Infectious diseases result from complex interplay of host and pathogen factors; genetic divergence within NTM could provide important insights in the bacterial factors in the issue of clinical relevance. Particular subgroups within species may be clinically more relevant than others. By sequencing multiple house-keeping genes in various NTM species, we were able to reveal, for the first time, the extensive genetic divergence within NTM species. Still, we could not correlate subgroups based on variation in any of the house-keeping genes with clinical disease. The next approach was to look at a specific virulence factor, a gene whose product is essential in the pathogenesis of NTM disease. We therefore explored the presence and role of the *esat-6* and *cfp-10* genes, which have central roles in virulence of *M. tuberculosis*. The proteins encoded by these genes allow *M. tuberculosis* to escape from the phagolysosome, where it should be neutralized, to the cytosol in human macrophages. We found similar genes in *M. szulgai*, *M. kansasii*, *M. riyadhense* and *M. marinum*, species that are highly clinically relevant and phylogenetically related to the *M. tuberculosis* complex. In *M. szulgai*, we tested the function of these genes in an *in vitro* macrophage infection. Here, however, we recorded by cryo-immunogold electron microscopy that presence of *esat-6* and *cfp-10*-like genes does not allow *M. szulgai* to escape from the phagolysosome to the cytosol in macrophages. The actual pathogenesis of NTM infection remains to be established.

Finally, in **Chapter 7**, we have looked across our borders to assess the occurrence and clinical relevance of NTM isolation in different settings in very different geographical regions. In Oman, we encountered a situation similar to the Netherlands, with similar species causing similar pulmonary NTM disease types. In nearby Saudi Arabia, we identified a novel NTM species causing a soft tissue infection after blunt trauma. In Tanzania, we identified NTM as causative agents of HIV-related disseminated disease. In Vietnam, we identified a random sample of NTM isolates in a tuberculosis drug susceptibility survey, a setting that hampers assessment of the clinical relevance of the isolates. In the Tanzanian and Vietnamese setting we noted a predominance of NTM that are rare or absent in the Netherlands. Hence, we conclude that identification

methods intended for use in the European or North American setting may not function optimally in other geographical regions, where different distributions of NTM species may be present. Although incidence and disease manifestations likely differ by setting, we concluded that NTM are present and cause disease worldwide, including in settings with a high prevalence of tuberculosis.

We have demonstrated that NTM isolation is a relevant clinical and laboratory issue in the Netherlands and other countries, including countries with a high prevalence of tuberculosis. In **Chapter 8**, the combined results of our studies are discussed within their scientific context. We conclude that future research on NTM should focus on three subjects. First and perhaps foremost, the pathogenesis of NTM infections is largely unknown. Prospective clinical studies should address host factors influencing susceptibility to NTM disease; whole genome sequencing of clinically relevant NTM as well as *in vitro* and *in vivo* infection experiments can help to reveal bacterial virulence factors. Second, improvement of treatment outcome is urgently needed. Pharmacokinetic studies are warranted to optimize therapy with currently available drugs. New antimicrobials should be tested for their activity against NTM and this specifically includes new antituberculosis drugs currently under development. Simultaneously, existing diagnostic and therapeutic guidelines should be disseminated and expert consultation should be propagated among respiratory and infectious diseases specialists. Third, the environmental sources of NTM infection remain uncertain and deserve further investigation. Water and soil samples have yielded NTM and some of these could be linked to human disease. Still, we do not know where these NTM actually reside and multiply.

Last, it is difficult to advocate increased attention for NTM disease when its true incidence, prevalence and the burden of disease remain to be quantified. As a start, we propose an obligatory, although not necessarily real-time, laboratory reporting system for the Netherlands. Combined with clinical data this will reveal the true burden of NTM disease in the Netherlands.



Chapter 10

Nederlandstalige samenvatting

Dit proefschrift omvat het eerste deel van een gezamenlijk onderzoek van het Universitair Longcentrum Dekkerswald (onderdeel van de vakgroep Longziekten van het UMC St. Radboud te Nijmegen) en de afdeling Mycobacteriën van het Rijksinstituut voor Volksgezondheid en Milieu te Bilthoven, naar de klinische relevantie van nontuberculeuze mycobacteriën.

In **Hoofdstuk 1** introduceren wij de stand van de wetenschap aangaande ziekte door nontuberculeuze mycobacteriën voorafgaand aan dit onderzoek, inclusief een historisch overzicht. In het kort omvat het geslacht *Mycobacterium* drie hoofdgroepen: I) het *Mycobacterium tuberculosis* complex, II) *Mycobacterium leprae* en III) de nontuberculeuze mycobacteriën (NTM). Hoewel in Nederland het aantal klinische monsters waaruit NTM worden gekweekt is gestegen gedurende het afgelopen decennium is er nog weinig kennis omtrent de klinische relevantie hiervan.
Waar het *M. tuberculosis* complex en *M. leprae* obligate pathogenen zijn voor de mens en dier zijn de NTM, waarvan al meer dan 100 verschillende species zijn beschreven, doorgaans opportunistische pathogenen die vanuit milieubronnen infecties veroorzaken bij patiënten met verlaagde lokale of systemische immuniteit. Door hun veelvuldig voorkomen in ons milieu betekent het kweken van NTM vanuit een niet-steriel monster, bijvoorbeeld een monster uit de luchtwegen of het maag-darmstelsel, niet per definitie dat er sprake is van een infectie; contaminatie of pseudo-infectie dienen te worden uitgesloten. Om het maken van dit onderscheid te ondersteunen heeft de American Thoracic Society (ATS) criteria opgesteld voor het diagnosticeren van longinfecties door NTM, bestaande uit klinische en microbiologische criteria.

In **Hoofdstuk 2** hebben wij de klinische relevantie van het isoleren van verscheidende NTM species gekwantificeerd. Hiertoe bepaalden wij per species retrospectief het percentage patiënten dat voldeed aan de ATS criteria voor NTM longinfecties, alsmede de frequentie en klinische uitingsvormen van extrapulmonale NTM infecties. In een regionaal onderzoek dat alle klinisch geïsoleerde NTM omvatte oordeelden wij dat 25% van alle uit longmaterialen gekweekte NTM veroorzakers waren van daadwerkelijke NTM longinfecties. Wanneer we ook de extrapulmonale monsters meenamen, konden we stellen dat 33% van alle klinische geïsoleerde NTM verwekkers waren van ware NTM infecties. In de regionale en hierop volgende nationale studies stelden wij vast dat dit percentage significant verschilt tussen de verschillende NTM species, variërend van minder dan 5% (*M. noviomagense*, *M. gordonae*) tot boven de 70% (*M. kansasii*, *M. szulgai* en *M. malmoense*). Deze bevinding heeft een belangrijke klinische weerklank. De identiteit van de NTM in een klinisch monster kan de clinicus ondersteunen in het bepalen van zijn grondhouding ten aanzien van een kweekpositieve patiënt. Wij suggereerden

dat toekomstige ATS criteria rekening moeten houden met deze verschillen in klinische relevantie tussen de verschillende NTM species. Voor de minder relevante species kunnen striktere diagnostische criteria onterechte diagnoses en behandelingen voorkomen. Voor de zeer relevante species daarentegen kunnen soepeler criteria het stellen van de definitieve diagnose bespoedigen.
Uit onze retrospectieve studies leerden wij ook dat de diagnostiek en behandeling van NTM infecties in Nederland verbetering behoeven. Het gebruik van bestaande richtlijnen voor diagnostiek en behandeling is geen gemeengoed; wij bemerkten zowel over- als onderdiagnostiek, leidend tot over- en onderbehandeling van NTM infecties. Daarbij viel het veelvuldig instellen van niet bewezen effectieve behandelingen, of behandelingen van te korte duur, op. Mogelijk als gevolg hiervan waren de uitkomsten van therapie voor NTM longinfecties vaak teleurstellend. In onze regionale studie zagen wij bij 67% van de behandelde patiënten een gunstige uitkomst. De uitkomst van therapie verschilde echter ook per species en varieerde van 50% (*M. avium*) tot 82% (*M. szulgai*) gunstige uitkomst. Deze uitkomsten zijn vergelijkbaar met die van multi- of extensief drugresistente tuberculose en benadrukken de sterke behoefte aan nieuwe antimycobacteriële middelen en ondersteunende therapieën.
De homogeniciteit binnen de patiëntenpopulaties van onze verschillende retrospectieve studies was opvallend; de populaties bestonden voornamelijk uit mannen, met een gemiddelde leeftijd van 60 jaar en pre-existente (chronische) longziekten. Holtevormende NTM longinfectie was de meest voorkomende vorm van NTM ziekte in deze patiënten. Zowel pre-existente longziekten als de holtevormende NTM longinfecties vergroten het risico op het falen van therapie. De toename in het aantal NTM isolaten in Nederland wordt ook grotendeels veroorzaakt door een toename in het aantal *M. avium* isolaten uit longmaterialen exact in deze patiëntcategorie.
We beschouwen deze toename in het aantal klinische NTM isolaten voornamelijk als een gevolg van de vergrijzing van de Nederlandse bevolking, met een toename van chronische (long)ziekten en immunosuppressieve behandelingen hiervan, maar ook van afnemende kruisbescherming door blootstelling aan tuberculose. Daarnaast hebben ook verbeteringen in de laboratoriumdiagnostiek en het toenemende gebruik van douches voor persoonlijke hygiëne een mogelijke invloed gehad.

In **Hoofdstuk 3** hebben we extra aandacht besteed aan een nieuw opkomende groep patiënten met een verhoogd risico op NTM infecties, te weten patiënten met reumatische of andere immuun-gemedieerde inflammatoire ziekten die worden behandeld met antilichamen tegen cytokines of receptoren met belangrijke functies in het inflammatoir proces. De middelen omvatten o.a. de tumor necrosis factor-α (TNF-α) blokkers en worden in de Engelstalige literatuur vaak aangeduid als "biologicals". De eerste klinische studies

met TNF-α blokkers toonden al een verhoogd risico op ernstige infecties, waaronder reactivaties van latente tuberculose. Wij beschreven een patiënt met een pulmonale *M. szulgai* infectie tijdens gebruik van TNF-α blokkers en schreven een overzichtsartikel over de impact van de verschillende "biologicals" op de afweer tegen mycobacteriën. Inmiddels zijn NTM infecties beschreven zowel bij patiënten die TNF-α blokkers gebruikten, als bij patiënten die anti-IL-1 en CD20+ B-cel antilichamen gebruikten voor reumatische aandoeningen. Onder de verschillende klassen antireumatische middelen die momenteel worden ontwikkeld mag van de IL-17 en IL-23 blokkers, de JAK/STAT signaaltransductie-modificerende middelen en de metalloproteïnases worden verwacht dat zij de afweer tegen mycobacteriën negatief beïnvloeden. Aangezien het aantal "biologicals" en de indicaties voor hun gebruik snel uitbreiden is een toename in het aantal geassocieerde NTM infecties te verwachten. Alle patiënten die deze middelen gebruiken moeten worden gescreend op latente tuberculose en nauwlettend worden gecontroleerd op het optreden van mycobacteriële infecties.

Na het vaststellen van de klinische relevantie van NTM en het identificeren van risicopopulaties is het logisch de aandacht te verleggen naar de behandeling van NTM infecties. In Hoofdstuk 2 vonden we al dat de uitkomst van therapie vaak teleurstellend was. Daarom zijn we in **Hoofdstuk 4** op zoek gegaan naar mogelijkheden om de uitkomst van therapie van NTM infecties te verbeteren. Hiertoe bestudeerden wij de rol van resistentiebepalingen, een mogelijk nieuw geneesmiddel voor mycobacteriële infecties en de waarde van chirurgie als toevoeging aan medicinale therapie.
Uit de door ons verzamelde resistentiedata kwam naar voren dat clarithromycine en rifabutin *in vitro* het meest actief zijn tegen NTM. Het nut van resistentiebepalingen tegen andere eerste- en tweedelijns antituberculose middelen is beperkt. Bij de eerstelijns middelen (isoniazide, rifampicine en ethambutol) zijn er discrepanties tussen de *in vitro* activiteit en de *in vivo* uitkomst van therapie; de activiteit van tweedelijnsmiddelen (clofazimine, cycloserine en prothionamide) wordt niet ondersteund door klinische gegevens. Om therapieadvies en begeleiding te verbeteren dient het testen van deze middelen te worden vervangen door resistentiebepalingen tegen middelen met een bewezen klinische effectiviteit, bij voorkeur overeenstemmend met de *in vitro* activiteit. Dit gaat in het bijzonder op voor de snel groeiende NTM waar resistentie een groot probleem is. Het bepalen van uitgangswaarden qua resistentie, voorafgaand aan therapie, is wenselijk voor alle klinische relevante NTM isolaten. Vervolgtesten zijn geïndiceerd bij falen van therapie of terugkeer van ziekte na therapie.
Gezien de urgente behoefte aan nieuwe antimycobacteriële middelen, testten wij de activiteit van thioridazine, een overal beschikbaar patentloos antipsychoticum met antimicrobiële eigenschappen. Wij maten een

veelbelovende activiteit tegen *M. tuberculosis*; de activiteit tegen NTM was echter tot wel 8 maal lager. Thioridazine lijkt dan ook geen rol van betekenis te kunnen spelen in het verbeteren van de behandeling van NTM infecties.
Wanneer medicinale therapie voor NTM infecties faalt, wordt chirurgische verwijdering van de aangedane longdelen als laatste redmiddel aangeraden. Wij bestudeerden de uitkomsten van dergelijke chirurgie bij acht patiënten die werden geopereerd voor NTM longinfecties in Dekkerswald. Chirurgie resulteerde in een langdurige omslag naar kweeknegativiteit in zeven van de acht patiënten die werden geopereerd. De indicatiestelling en timing van chirurgie bleken hierbij van doorslaggevend belang; juist deze zaken komen in de bestaande literatuur nauwelijks aan bod. Wij concluderen dat de toegevoegde waarde van chirurgie overwogen dient te worden bij iedere patiënt bij wie een NTM longinfectie wordt vastgesteld en dat dit opnieuw geëvalueerd dient te worden na zes maanden medicinale behandeling. Waar mogelijk dient chirurgische behandeling te worden nagestreefd en tijdig uitgevoerd. Ter ondersteuning van longartsen hebben wij indicaties voor chirurgie opgesteld.

De klinische relevantie en optimale therapie is vastgesteld voor beschreven NTM species. In **Hoofdstuk 5** verkenden wij de heterogeniciteit onder NTM en concludeerden dat ten minste 4% van alle NTM isolaten ingezonden naar het nationale referentie laboratorium niet kon worden toegewezen aan een officieel beschreven species. In het bijzonder ging het hier om stammen verwant aan, maar niet behorend tot de leden van, het *M. avium* complex en *M. fortuitum* complex.
Enkele groepen van deze eerder niet identificeerbare mycobacteriën vielen op doordat zij frequent werden geïsoleerd, of omdat zij geïsoleerd werden uit normaal gesproken steriele materialen en dus zeer waarschijnlijk veroorzakers van NTM infecties waren. Vier van deze groepen werden intensief onderzocht en konden beschreven en erkend worden als nieuwe species, met een inschatting van hun klinische relevantie. Zo onderscheidden wij het klinisch niet relevante species *M. noviomagense* van het sterk verwante en wel frequent klinisch relevante species *M. xenopi*. Ook beschreven wij *M. vulneris* en (in Hoofdstuk 7) *M. riyadhense* als verwekkers van extrapulmonale NTM infecties en identificeerden wij *M. mantenii* als verwekker van cervicofaciale lymfadenitis bij kinderen, terwijl dit nieuwe species in longmaterialen klinisch niet relevant bleek. Een beter onderscheidende taxonomie binnen het genus *Mycobacterium* kan dus klinisch betekenisvol zijn; wij concluderen dan ook dat deze klinische betekenis een voorwaarde zou moeten zijn voor taxonomische precisie binnen de NTM.

In **Hoofdstuk 6** zoomen we verder in, op de genetische divergentie binnen NTM species. Infectieziekten zijn het gevolg van een compelx samenspel van gastheer en pathogeenfactoren; genetische divergentie binnen NTM species

zou dus inzicht kunnen verschaffen in de bacteriële factoren verantwoordelijk voor klinische relevantie. Bepaalde subgroepen binnen species kunnen klinisch relevanter zijn dan andere. Door het sequencen van meerdere huishoudgenen in verscheidene NTM species konden wij, voor het eerst, de enorme genetische divergentie binnen deze species aantonen. Ondanks deze divergentie konden wij geen associaties vinden tussen subgroepen op basis van variatie in de huishoudgenen en klinische ziektevormen. De volgende stap bestond uit het bestuderen van een specifieke virulentiefactor, dat wil zeggen een gen waarvan het product een cruciale rol speelt in de pathogenese van NTM infecties. Hiertoe verkenden wij de aanwezigheid en functie van *esat-6* en *cfp-10* genen, die cruciale rollen spelen in de virulentie van *M. tuberculosis*. De eiwitten waarvoor deze twee genen coderen maken het *M. tuberculosis* mogelijk om te ontsnappen uit het fagolysosoom, waar de bacterie onschadelijk gemaakt dient te worden, naar het cytosol van humane macrofagen. Wij vonden sterk gelijkende genen in *M. szulgai*, *M. kansasii*, *M. riyadhense* en *M. marinum*, stuk voor stuk klinisch zeer relevante species en fylogenetisch verwant aan het *M. tuberculosis* complex. Middels een *in vitro* macrofaaginfectie testten wij de functie van deze genen in *M. szulgai*. Hier zagen wij, met behulp van cryo-immunogold elektronenmicroscopie, dat de aanwezigheid van *esat-6* en *cfp-10* genen het voor *M. szulgai* niet mogelijk maakt te ontsnappen uit het fagolysosoom naar het cytosol van macrofagen. De pathogenese van NTM infecties blijft dus vooralsnog onbekend.

Tenslotte hebben we, in **Hoofdstuk 7**, een blik over onze landsgrenzen geworpen om de klinische relevantie van NTM in zeer verschillende geografische gebieden in te schatten. In Oman troffen we een situatie aan met sterke gelijkenissen met de Nederlandse situatie, zowel qua aangetroffen species als qua manifestaties van NTM longinfecties. In Saoedi Arabië identificeerden wij een nieuw species dat een weke delen infectie veroorzaakte na stomp trauma. In Tanzania toonden we aan dat NTM verwekkers zijn van HIV-gerelateerde gedissemineerde infecties. In Vietnam identificeerden wij een groep willekeurig gekozen NTM die waren aangetroffen gedurende een surveillance studie naar drugresistentie onder *M. tuberculosis* complex bacteriën. Helaas bood deze setting weinig kansen om de klinische relevantie van deze isolaten goed in te schatten. Zowel in Tanzania als in Vietnam bemerkten wij dat species die in Nederland zeldzaam of afwezig zijn daar de overhand hadden. Hierop concluderen wij dat de identificatiemethoden die ontwikkeld zijn voor gebruik in de Europese of Noord-Amerikaanse setting minder kunnen functioneren in andere regio's ware andere NTM species aanwezig kunnen zijn. Hoewel de incidentie en manifestaties van NTM infecties waarschijnlijk verschillen per setting, kunnen wij concluderen dat NTM wereldwijd aanwezig zijn en ziekte veroorzaken, ook in settings met een hoge prevalentie van tuberculose.

Wij hebben aangetoond dat NTM een relevant klinisch en laboratoriumtechnisch probleem vormen, zowel in Nederland als daarbuiten. Dit geldt ook voor landen met een hoge prevalentie van tuberculose. In **Hoofdstuk 8** bediscussiëren wij de bevindingen uit onze verschillende studies binnen hun wetenschappelijke context. Wij concluderen hieruit dat het onderzoek naar NTM zich toe dient te gaan spitsen op drie hoofdthema's. Ten eerste blijft de pathogenese van NTM infecties goeddeels onbekend. Prospectieve klinische studies dienen gastheerfactoren te onderzoeken die de gevoeligheid voor NTM infecties beïnvloeden. Aan de kant van de pathogenen is het raadzaam om van enkele klinisch relevante NTM het volledige genoom te laten sequencen en *in vitro* en *in vivo* infectie-experimenten uit te voeren, teneinde meer inzicht te verwerven in virulentiefactoren. De tweede peiler dient de verbetering van de uitkomst van therapie te zijn. Farmacokinetische studies zijn van belang om de huidige therapie te optimaliseren. Daarnaast dienen nieuwe antibiotica ook te worden getest op hun activiteit tegen NTM en dit behelst in het bijzonder de nieuwe antituberculose middelen die op dit moment in ontwikkeling zijn. Gelijktijdig dienen de bestaande richtlijnen voor diagnostiek en behandeling verder te worden verspreid en dient het consulteren van experts gepropageerd te worden onder longartsen en infectiologen. Het derde belangrijke thema is het bestuderen van milieubronnen van NTM. Uit water- en grondmonsters worden veelvuldig NTM geïsoleerd en sporadisch zijn deze ook al gekoppeld aan ziektegevallen bij de mens. Toch weten we nog onvoldoende over waar deze bacteriën zich bevinden en waar zij zich daadwerkelijk kunnen vermenigvuldigen.

Tot besluit is het zeer lastig om meer aandacht voor de NTM infecties te genereren wanneer basale zaken als incidentie, prevalentie en ziektelast nog niet gekwantificeerd zijn. Als startpunt stellen wij voor om in Nederland een meldingsplicht, zij het niet real-time, in te stellen voor laboratoria die NTM aantreffen in klinische monsters. In combinatie met klinische gegevens zal dit de ware ziektelast door NTM in Nederland inzichtelijk maken.

Dankwoord

dank *de; m* (betuiging van) goede gezindheid tegenover iemand vanwege een geschenk, bewezen dienst enz.

be·dan·ken *-dankte, h -dankt*
1 dank betuigen: *iemand voor iets ~*
2 ontslag nemen of geven: *~ als lid*
3 afwijzen: *daar bedank ik voor*

Bedankt. Zeven letters. Het is een kort woord, een klein gebaar, maar toch van zo'n groot belang. Er zijn vele mensen die in de loop van dit promotietraject van mij een oprecht "bedankt" hebben moeten horen; ik hoop dat ik het jullie allemaal eens gezegd heb.

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Richard, ik moet beginnen je te bedanken voor het feit dat je me vers uit de co-schappen een AGNIO plaats met onderzoekstijd hebt aangeboden; daar is dit alles immers uit voortgekomen. Zodra het promotietraject vorm kreeg, heb je daar alle ruimte voor gecreëerd. Bedankt voor je supersnelle commentaren op de stukken, zelfs vanaf je vakantieadres, je heldere visie op de onderzoeksstrategie, maar zeker ook voor het stroomlijnen van het laatste, formele, deel van dit mooie traject.
Martin, tijdens het tuberculose onderwijs voorafgaand aan mijn co-schappen in Tanzania heb je mijn laatste twijfels weggenomen: ik moest iets gaan doen met mycobacteriele infecties. De wetenschappelijke stage die je me bij terugkomst aanbood bleek een opmaat voor vier prachtige jaren, eerst vooral in de kliniek op Dekkerswald, later meer vanuit het RIVM. Onze besprekingen op de vrijdagmiddag hebben de inhoud en vorm van dit proefschrift bepaald, maar verliepen zo ontspannen dat dat achteraf vaak als bijzaak aanvoelde. De humor en je eeuwige positiviteit zijn gelukkig wel van mens op mens overdraagbaar! Wanneer gaan we naar de Wakhan corridor?
Dick, het is alweer vier jaar geleden dat we elkaar voor het eerst ontmoetten en toch leer je me iedere dag nog zoveel nieuws. Bedankt voor de grote mate van vrijheid die ik heb gekregen en voor je bijdragen, op alle mogelijke manieren, aan dit proefschrift; ze waren van onschatbare waarde. Zowel je loopbaan als je werkwijze zijn een doorlopende bron van inspiratie voor me. Het is dan ook heerlijk om te weten dat ik nog meer dan een jaar in je fantastische team mag meedraaien, met hopelijk een mooi feest ergens in dat jaar. Als alles goed gaat ben je ook op je nieuwe post in Nijmegen nog niet van me af!

De overige leden van het Research Atypische Mycobacteriën (RAM)-team hebben een enorm belangrijke bijdrage aan dit proefschrift geleverd, op vele manieren.
Wiel de Lange, als ik teruglees door dit proefschrift zie ik veel terug van de vele namiddagen die we op je werkkamer achter de PC hebben doorgebracht. Alle casuïstiek die je, als consulent op NTM gebied, met me hebt gedeeld hield tijdens het rondreizen, de statusinzages en het labwerk het belang van al dit onderzoek altijd op mijn netvlies... maar gelukkig konden we het ook gewoon over drummen hebben.
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Wouter Hoefsloot, Mister *M. malmoense*, al vanaf het begin zijn we samen aan het werk aan de NTMs. Bedankt voor de fantastische samenwerking, of het nu was op de longrevalidatie of tijdens statusinzages voor de regio-studie. Dankzij de vele gedeelde interesses zijn onze etentjes altijd gezellig en van iets langere duur dan gepland. Heel erg bedankt dat je paranimf wilde zijn!

Dit proefschrift is het resultaat van de samenwerking tussen twee fantastische clubs. Graag bedank ik iedereen binnen het Universitair Longcentrum Dekkerswald en de afdeling Longziekten van het UMC St. Radboud te Nijmegen. De twee jaar van klinisch werk gecombineerd met onderzoek waren een prachtige tijd. De destijds actieve longartsen Anton Cox, Erik van der Heijden, Johan Molema, Jolanda van Haren-Willems, Miep van der Drift, Monique Reijers, Olga Schuurbiers, Tewe Verhage en Yvonne Heijdra ben ik veel dank verschuldigd. Ik heb ontzettend veel van jullie mogen leren, van polysomnografie tot pleurapunctie en daar heb ik nog steeds veel profijt van! Ook bedank ik alle arts-assistenten met wie ik heb samengewerkt voor de leuke tijd en alle collegialiteit; ik besef me maar al te goed dat jullie nog een stap harder moesten lopen tijdens mijn onderzoeksdagen…
Saar van Nederveen-Bendien verdient, als RAM-team lid van het eerste uur, een extra woord van dank. Saar, bedankt voor al je bijdragen aan de verschillende stukken, in het bijzonder ons regio onderzoek; veel succes in Den Haag!

Vanuit de kliniek verhuisde ik naar de afdeling Mycobacteriën van het RIVM. Hoewel de overstap bijna niet groter kon zijn, werd ik vanaf dag één volledig in jullie hechte club opgenomen en mocht ik overal aan meedoen en denken. Anne-Marie van den Brandt, Arnold Herrewegh, Arnout Mulder, Jessica de Beer, Kristin Kremer, Mimount Enaimi, Miranda Kamst, Rina de Zwaan, Tridia van der Laan, zonder jullie was dit boekje er nooit geweest; heel erg bedankt, voor alles!

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Dit proefschrift was onmogelijk geweest zonder de belangeloze medewerking van alle longartsen, infectiologen, oogartsen, KNO-artsen, huisartsen en neurologen die NTM "vingen" bij hun patiënten en de artsen-microbioloog die ze ook echt aan wisten te tonen en de schakel naar de behandelend artsen vormden. Hartelijk dank voor de altijd vriendelijke ontvangst en de mogelijkheid te leren van jullie ervaringen met NTM infecties.
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Chapter Seven exists because of the great collaborations with equally great people from diverse parts of the world. A big word of thanks goes out to Sahal al-Hajoj and Fahad Al-Rabiah (Saudi Arabia), Sara Al-Mahruqi and Suleiman Al-Busaidy (Oman), John Crump (USA/Tanzania) and Nguyen Van Hung (Vietnam).
Enrico Tortoli, thanks for the introduction into the fascinating world of mycobacterial taxonomy and the great and ongoing collaboration; I will make sure there will be a *Mycobacterium tortolii* one day, that's a promise.
I could devote a full chapter to thanking the European Society for Mycobacteriology and all its members but prefer to do so personally during the next meeting. The annual meetings are fantastic in many ways. See you all in Slovenia, 2010!

For a lot of what I have learnt on NTM disease, I am indebted to my fantastic North American colleagues David Griffith, Richard Wallace Jr., Timothy Aksamit, Kevin Winthrop, Theodore Marras, Kenneth Olivier, Gwen Huitt and of course Michael Iseman and Charles Daley. Our annual get-togethers at the American Thoracic Society conference were a wonderful concoction of fun and science. It was an immense honor to contribute to the postgraduate course on NTM disease. Professor Charles Daley, dear Chuck, thank you so much for your willingness to participate in the Doctoral Thesis Committee. I already look forward to join your great team for a while next year.

Joe Falkinham III, we have only met once (yet), but an hour of talking to you saves a year in the laboratory! Thanks for sharing your experience and the knowledge of the works of researchers that predate even my grandparents.

Graag wil ik de Nederlandse leden van de manuscriptcommissie, Prof. Dr. J.W.M. van der Meer en Prof. Dr. P.E. Verweij bedanken voor de beoordeling van dit proefschrift.

De directie van het RIVM/CIb wil ik bedanken voor de mogelijkheid die ik heb gekregen om in het instituut de laatste fase van mijn promotietraject in te vullen. Voorlopig blijf ik nog even, want er is nog meer te doen.

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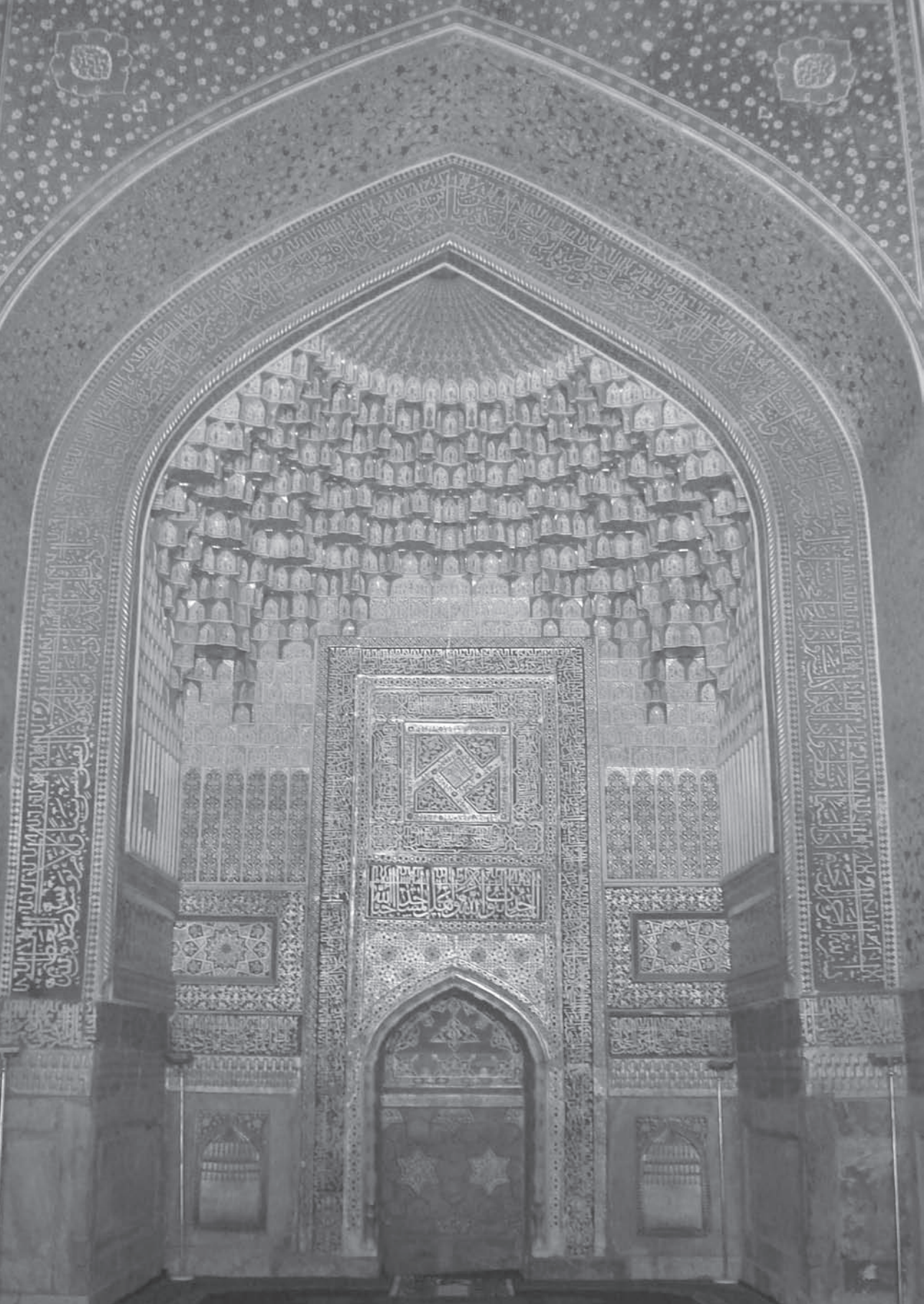

Biography

Jakobus (Jakko) van Ingen was born on June 14th, 1979 in the village of Opheusden, the Netherlands, as the youngest in a family of three children. After attending the "Jan Ligthart" elementary school in Opheusden, he attended secondary school in the nearby city of Wageningen, at what was then called the "Wagenings Lyceum". In a period of nationwide secondary school reforms, this school changed names to "Het Wagenings" and eventually "Pantarijn".

He started a study in Medicine at the Catholic University Nijmegen (currently "Radboud University Nijmegen"). After a one-year journey through South-East Asia, he started his internships in the year 2002. Because of a growing interest in tuberculosis, he did his final internships in the Rubya District Designated Hospital in Rubya, in North-West Tanzania. There he focused on patient management in the Isolation Ward for tuberculosis and leprosy patients, quality management in the hospital laboratory and the use of voluntary counseling and testing for HIV.

After his internships, a three-month research program was the final stage before graduating as a medical doctor. For his research program, he joined Prof. Dr. Richard Dekhuijzen, Dr. Martin Boeree and Dr. Dick van Soolingen to perform a pilot study on the clinical relevance of nontuberculous mycobacteria. This program was to lead to the formal description of *Mycobacterium noviomagense* two years later. After successfully completing this research program in April 2005, he started as an medical doctor at the University Lung Centre Dekkerswald, a former tuberculosis sanatorium, which now hosts one of the Netherlands' two specialized hospitals for tuberculosis treatment. Here, he combined clinical work as a medical doctor with research, supervised by Martin Boeree and Dick van Soolingen. The research entailed a nationwide study on the clinical relevance of *M. xenopi* isolation and a survey of the clinical relevance of nontuberculous mycobacteria in the Nijmegen-Arnhem region.

After one year, the amount of time devoted to research was increased and plans of setting up a PhD program formalized. In 2007, after two years at Dekkerswald, he moved to the National Mycobacteria Reference Laboratory, headed by Dr. Dick van Soolingen and part of the National Institute for Public Health and the Environment, to finalize his PhD thesis on the nontuberculous mycobacteria and test a novel bacterial typing method for nationwide molecular epidemiological surveillance of tuberculosis.

The work on the nontuberculous mycobacteria has already led to twenty-two accepted articles in international peer-reviewed journals and over twenty presentations at conferences in nine different countries. For this work he was awarded the 2008 Nijmegen University Center for Infectious diseases Young Talent Award as wells as the 2008 Young Researcher Award of the Center for Infectious Diseases Control of the Dutch National Institute for Public Health and the Environment.

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