Clinical relevance of non-tuberculous mycobacteria in Zambia

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Contents

Chapter 1

Introduction and outline of the thesis

This thesis describes a series of studies on the clinical relevance of the isolation of non-tuberculous mycobacteria in Zambia. In this introduction an overview is provided of various aspects of mycobacteria described in the literature. Thereafter, the study sites in Zambia where the study was performed are presented. In the last part of this chapter the aims and the outline of the study are detailed.

INTRODUCTION TO MYCOBACTERIA

Mycobacteria are Gram-positive, aerobic bacteria belonging to the family Mycobacteriaceae and one of several mycolic-acid-containing genera within the order Actinomycetales (1,2). The family Mycobacteriaceae comprises only one genus; *Mycobacterium*, a name coined by Lehman and Neuman in 1896 (3).

Bacteria of species within the genus *Mycobacterium* exhibit acid–alcohol fastness, contain genomic DNA with a high G–C content (molecular GC content of 61–71%), and have similar mycolic-acid structures in their cell wall (2).

The non-tuberculous mycobacteria (4) are *Mycobacterium* species different from those belonging to the *Mycobacterium tuberculosis* complex (e.g., *M. tuberculosis, M. bovis, M. africanum, and M. microti*) and *M. leprae*. NTM can be subdivided in many different species with varying rates of evolutionary divergence, variable biochemical characteristics, clinical presentation, clinical relevance and susceptibility to anti-mycobacterial agents. NTM are generally free-living organisms that are ubiquitous in the environment.

These organisms are traditionally designated 'atypical mycobacteria' and this term was introduced by Pinner in 1935 (5). Atypical mycobacteria, have also been named 'anonymous-', 'non-tuberculous-', 'environmental-', 'saprophytic-', or 'opportunistic' mycobacteria, and 'mycobacteria other than tubercle bacilli'. However, these 'atypical' mycobacteria are not merely, as the name seems to imply, variants of *M. tuberculosis*- the 'typical' mycobacteria. In contrast, the atypical mycobacteria constitute an evolutionary divergent group of bacteria with a wide variability in biological features and in ability to cause disease in the humans and animals. Thus, atypical mycobacteria are not 'unusual' *M. tuberculosis* strains. The name 'saprophytes' seems to be incorrect, since it is not justified terming a micro-organism 'non-pathogenic' for man by means of characteristics such as the production of pigment, the rate of growth or the non-virulence in a guinea pig as was done early in the last century. During that time teaching held that a non-tuberculous acid-fast organism that did not produce disease in the guinea pig was a saprophyte. Moreover, a significant proportion of the atypical *Mycobacterium* species is able to cause clinical disease in man similar to what is caused by *M. tuberculosis* complex bacteria. This implies none of these terms have been universally accepted, but the designation 'non-

tuberculous mycobacteria' (NTM) seems to be the most accepted term at this moment in the international context. This designation has been endorsed by the American Thoracic Society (ATS) in their 1990 statement on the Diagnosis and Treatment of disease caused by NTM (6). The diseases caused by NTM have been termed 'mycobacteriosis' and 'opportunistic mycobacterial diseases'.

DISCOVERY AND CLASSIFICATION OF MYCOBACTERIA

Robert Koch discovered the tubercle bacillus in 1882 (7). Shortly thereafter, various mycobacteria other than *M. tuberculosis* were described. A report by Olmacher (8) in 1901 described an acid-fast organism isolated from the sputum of a young woman who was clinically resembling pulmonary tuberculosis. Others reported the isolation of acid-fast bacilli from the sputum of patients with both normal and abnormal chest X-rays (9). But because these micro-organisms lacked pathogenicity in the guinea pig model, there was reluctance to accept them as pathogenic for human beings. The recognition of these organisms as pathogenic to man is relatively recent, with series of patients suffering from chronic pulmonary disease being reported in the literature since the middle 1950s (10-12). The earliest understanding that these organisms, known initially as the *yellow bacillus* (*M. kansasii*) or the *Battery bacillus* (*M. avium* complex; after the Battery Sanatorium in Georgia where it was first recognized), can act as true pathogens took significant time. Early scientist and clinicians such as Ernest Runyon (associated with the Runyon classification) (12), William Shaffer (introduction of serotyping of *M. avium-intracellulare-scrofulaceum*), John Chapman, Emanuel Wolinsky (the major treatise on these organisms as pathogens) (13), and Chai Ahn (diagnostic criteria and drug trials) (14) were major contributors to the effort that resulted in the attention these organisms nowadays receive.

In 1954, Timpe and Runyon (12) argued that the 'mycobacteria other than *M. tuberculosis*' cause disease in humans, and, subsequently, Runyon developed the first classification of these organisms. The NTM were classified into four groups on the basis of growth rates, colony morphology, and pigmentation in the presence and absence of light (15). Though now outdated, this classification allowed microbiology laboratories to more readily identify individual species of NTM, resulting in clearer characterization of distinct diseases or syndromes associated with these organisms.

The identification of mycobacteria was traditionally based on biochemical features and growth characteristics. Because mycobacteria generally multiply at a relatively slow pace, this process involved weeks of incubation.

With the availability of 16S ribosomal DNA sequencing, high-performance liquid chromatography (HPLC), and 65 KD gene polymerase chain reaction-restriction fragment length polymorphism analysis (PRA), the number of new species of NTM has risen dramatically in recent years with the naming of species such as *M. genavense*, *M. interjectum*, *M. triplex*, *M. celatum*, and *M. lentiflavum*. At present over a 100 species are recognized in the genus *Mycobacterium* (13,16).

Within the genus *Mycobacterium*, four groups of human pathogens can be delineated on the basis of genetic, microbiological, clinical, and epidemiologic characteristics:

1. The *M. tuberculosis* complex (*M. tuberculosis*, *M. bovis*, *M. bovis* BCG, *M. africanum*, *M. canettii*, *M. pinnipedii* and *M. microti*).
2. *M. leprae.*
3. Slowly growing NTM (photochromogens, scotochromogens and nonchromogens or Runyon group I, II and III, respectively).
4. Rapidly growing mycobacteria (Runyon group IV).

Worldwide, the most common NTM species causing human disease are the slowly growing mycobacteria of the *M. avium* complex (MAC), and *M. kansasii.* Less commonly encountered human pathogens are the slowly growing bacteria of the species *M. marinum*, *M. xenopi, M. simiae, M. malmoense*, and *M. ulcerans*, and the rapidly growing bacteria of the species *M. abscessus, M. fortuitum*, and *M. chelonae*. Certain relatively common laboratory isolates, such as *M. gordonae*, are important to be known by clinicians because they are almost invariably contaminants and not true pathogens.

Traditional methods for speciation of mycobacterial isolates are based upon growth characteristics and biochemical test results, requiring 4-6 weeks for the whole identification procedure. Furthermore, the reproducibility of this approach was limited. Another disadvantage was the inflexibility of this procedure in discovering new species and subspecies. Test results that did not fit the established schematics of particular (sub-) species were seen as exceptional and irreproducible, and it was difficult to link such observations to mycobacterial groups, not previously recognised.

In the 1980s, the first molecular tests were introduced to distinguish between *M. tuberculosis* complex bacteria, and the NTM (Gen-probe, San Diego, California, USA). Also specific nucleic acid probes tests became available for recognition of the most commonly encountered clinical isolates of NTM, including *M. kansasii*, the *M. avium* complex, and *M. gordonae*.

In the 1990s, 16S DNA sequencing was introduced and the identification of mycobacteria not only became less time consuming, but also much more robust. Phenotypic features tend to change and do not always respect the limits of man-made classifications. Genetic information by its nature allows accurate phylogenetic grouping and this yields

an improved insight in the clinical relevance of certain species and sub-species of mycobacteria. However, routine molecular identification of mycobacteria is currently mainly based on single genetic traits (mostly 16S and the 16S-23S spacer sequences) (17) and the list of new species and sub-species is growing rapidly. Until now (01 Agust 2007), 127 *Mycobacterium* species have been described (http://www.bacterio.cict.fr/m/mycobacterium.html).

It is expected that in the near future the insight in the phylogeny of mycobacteria will grow significantly. Also many currently recognized species may be further divided in two or more new species and sub-species. Particular sub-species may have more clinical relevance than other sub-species. For instance, on the basis of DNA polymorphism in the gene coding for the 16S-23S rRNA internal transcribed spacer, the species *M. kansasii* was recently divided in several sub-groupings (18). *M. kansasii* sub-group I is often associated with clinical disease, whereas the other subgroups are more likely to represent environmental bacteria. It is, therefore, expected that with the increase in genetic information available on NTM, our knowledge on the clinical significance of NTM will improve significantly.

ENVIRONMENTAL NICHES, COLONIZATION AND INFECTION

NTM are ubiquitous in the environment and can colonize or infect people and animals. In contrast to *M. tuberculosis* and *M. leprae*, species that affect only mammals, NTM form an integral part of the natural environment and may also prevail in certain man-made environments, thereby exposing susceptible individuals leading to colonization and infection. NTM act like saprophytes, commensals, and symbionts and are common inhabitants of a wide variety of environmental reservoirs throughout the world, including natural and municipal water, soil, aerosols, protozoans, domestic- and wild animals, milk- and food products. NTM may be abundant in certain natural surroundings or niches, where climatological factors are advantageous for their growth. There are geographical variations in the distribution of specific NTM organisms (19), most likely reflecting regional differences in the mycobacterial habitats.

However, only a very small proportion of all human-mycobacteria interactions progress to outright mycobacterial infection. Such progression is much more common in immune-compromised patients, especially those with AIDS (20). The relatively low incidence of infection due to NTM, despite high levels of exposure, suggests that NTM generally have a low pathogenicity for man (21,22).

The ubiquitous distribution of NTM contributes to the difficulties in interpreting positive culture results. Most NTM can inhabit body surfaces or secretions without causing disease. Thus, the mere isolation of NTM from respiratory specimens in itself may

indicate harmless colonization, but also infection. Furthermore, NTM can potentially contaminate smears and cultures during the acquisition, transportation and laboratory processing of specimens, which may lead to an incorrect diagnosis of tuberculous disease. The clinical manifestations of the infections vary among the NTM species, and distinguishing between colonization or contamination and true infection is complicated.

In this thesis, the term 'colonization' is used to refer to the repeated recovery of viable NTM organisms from properly collected clinical specimens, in the absence of clinical manifestations, tissue invasion or other sign of damage to the host. The NTM multiplicate on a body surface without evoking an immune response.

The term 'infection' will be reserved for disease states in which there are signs or symptoms suggesting a pathologic process. NTM have invaded the body tissues.

The term 'disease' will be used to indicate the clinical manifestation of damage that results from the host-NTM interaction. This is the case in a symptomatic patient who does meet the diagnostic criteria in the guidelines formulated by the American Thoracic Society (ATS) or British Thoracic Society (BTS), which includes pathology on the chest radiograph or chest high-resolution computed tomography (HRCT) scan, repeated sputum samples, bronchial wash or lung biopsy positive for NTM and exclusion of other disorders (23,24).

A 'pseudo-infection' is defined as a positive culture result from a patient without evidence of true infection or colonization, which is typically caused by contamination during specimen handling (25).

These terms (colonization, infection and disease) are used to describe the host-microbe relationship and have been in use for nearly a century. Over the years the concepts of host-pathogen interaction changed and the definitions for these terms were revised. In the literature the definitions of these term are often not clarified. In several publications the difference between rates of colonization and rates of infection or disease are not evident. This may lead to confusion and may overestimate the clinical importance of NTM.

EPIDEMIOLOGY OF NTM INFECTION

Although reports listing the significance of NTM differ in various geographic parts of the world, there does seem to be a definite geographic distribution for some organisms. In the United States, NTM lung disease is most commonly attributable to *M. avium* complex, with *M. kansasii* being second (26). In the United Kingdom, *M. kansasii* is the pathogen most commonly associated with NTM lung disease in England and Wales, while *M. malmoense* is the most commonly encountered NTM in Scotland. *M. xenopi* predominates in Southeast England (23). In Japan, the most common cause of NTM pulmonary disease

is *M. avium* complex, followed by *M. kansasii* (27). The distribution of NTM and the incidence of disease caused by them is not yet fully known in most parts of the world.

The reported rates of NTM colonization and infection are likely to be underestimates, with the former probably less accurate than the latter, given that people without significant symptoms are not likely to have intensive diagnostic work-up for possible NTM infection. The lack of systematic reporting of NTM infection in most nations limits the ability to derive accurate estimates of incidence and prevalence of colonization and infection. Nevertheless, there is sufficient data to conclude that NTM disease rates vary widely depending on the population studied and its geographic location. NTM disease is clearly a major problem in certain groups, including patients with underlying lung disease and also in individuals with impaired immunity. North American rates of colonization and infection have been reported to range from approximately 1-15 per 100,000 and 0.1-2 per 100,000, respectively. Generally, similar rates have been reported in European studies, with the exception of extremely high rates in an area of the Czech Republic where mining is the dominant industry (28). These studies have also shown marked geographic variability in prevalence. Rates in Japan and Australia were similar to those reported in Europe and North America.

On the whole, the impression seems to be that NTM infections in humans are on the increase. However, most data reporting high rates of infection with NTM come from Northern European countries or the United States. In African countries, despite the fact that environmental exposure to NTM is very high in that part of the world, infections with NTM seem to be very rare, even among patients with AIDS (29). Though, not much data is available to date regarding NTM infection in Africa.

Europe

The NTM isolation rates in Finland between 1991 and 1993 were published in a brief report (30). The mean annual isolation rate was 6.6 per 100,000 and 55% of the isolates were MAC, 20% *M. chelonae* and *M. fortuitum*, 15% *M. gordonae* and 10% *M. malmoense*. Numerous reports on the epidemiology of NTM in the United Kingdom have been published. A report from Wales presents data from the period of 1953 to 1978 (31). Only cases with NTM infection are reported, but the definition of NTM infection was not explicitly stated in that paper. A greater than twofold increase in rates of disease over a decade was found, from 0,2 to 0,5 per 100,000 in the periods 1953-1957 to 1963-1967. *M. kansasii* was the most common cause of disease (67%), followed by MAC (28%) and *M. xenopi* (4%). Data from Scotland suggest high rates of infection with *M. malmoense* (32,33). A study on the period 1990 and 1993 reports an incidence rate of 1.3 per 100,000 for *M. malmoense* pulmonary disease in the Lothian region (33). Rates of infection for the

nation as a whole and Lothian in particular are also presented, reporting *M. malmoense* as the most common isolate encountered, followed by MAC.

A report from Switzerland assessed the frequency of clinical disease in all 513 HIV-negative patients from whom NTM were isolated at the University of Zürich between 1983 and 1988 (34). Thirty-four patients (6.7 percent) were thought to have clinical infections: twenty-one had pulmonary disease, ten had soft tissue disease, and one suffered from disseminated disease. The most common isolates were MAC in 16 patients and *M. kansasii* in nine patients. *M. terrae*, *M. fortuitum*, *M. marinum*, and *M. malmoense* were also found.

A study in France of the period between 1991 and 1995, limited to the prevalence of *M. kansasii* in a Paris suburb with a high prevalence of HIV, reported rates of infection and disease of 1.1 and 0.5 per 100,000, respectively (35). Several studies from the Czech Republic have reviewed the experience with *M. kansasii* isolation in the Karvina district of North Moravia, where mining is a major industry (28,36,37). Over a 12 year period ending in 1979, one study reported a greater than 10-fold increase in rates of both colonization and infection, determined to be 17 and 12 per 100,000, respectively, with a simultaneous decrease in the rate of tuberculosis from 30 to 20 per 100,000 (28).

The Netherlands

In the period 2002 through 2006 the number of NTM isolates sent to the National institute for Public Health and Environment (RIVM) amounted 407, 437, 571, 584 and 669 yearly. The underlying reasons for this apparent increase in the number of NTM isolations are currently unknown. It could be due to increased attention to possible NTM infection. However, in the same period the number of newly diagnosed, culture positive tuberculosis cases in this period decreased from 1098 in the year 2002 to 790 in the year 2006. This suggests that there is an inverse correlation between the trends of tuberculosis and NTM infection.

M. kansasii infection rates were reported from The Netherlands for 1-year period over 1978-1979 (38). The data suggest rates of colonization and infection of 0.2 and 0.1 per 100,000, respectively. The rate of all NTM infections was 2.9 per 100,000. Most isolates were from the mining province of Limburg, and the authors speculate that this reflects a particular susceptibility of miners, probably due to underlying lung disease that is prevalent among them.

Northern America

A number of useful studies on isolation of NTM have been performed in North America (39-41). A Centers for Disease Control (CDC) laboratory survey of NTM isolates from 42 of 54 mycobacterial laboratories across the United States was performed in 1979 (42). Data were reported as total numbers of isolates rather than on patient denominators.

Thirty-two percent of the mycobacterial isolates were NTM, comprising a sample of 7,764 positive specimens. Of NTM isolates, 58% were MAC, 15% *M. fortuitum*, 10% *M. kansasii*, and 9% *M. scrofulaceum*. MAC was most common in all regions, followed by *M. fortuitum* and *M. kansasii* with exception of the Midwest where isolates of *M. kansasii* outnumbered those of *M. fortuitum*. National rates of isolation were 2.5, 0.7 and 0.5 per 100,000 for MAC, *M. fortuitum*, and *M. kansasii*, respectively. The highest rates of MAC were observed in South Eastern Atlantic states and several states along the U.S.-Canadian border. *M. kansasii* is most commonly found in some Midwestern and several Southern states. These data were updated and expanded upon 1980, again including only the total number of positive isolates rather than patients (39). A similar proportion of mycobacterial isolates were NTM (35%). Isolation rates and the distribution of the species were generally similar as reported previously.

A Canadian study on positive NTM isolates in the province of Manitoba calculated rates based on the number of patients with positive cultures from 1988 to 1990 (40). Given that 438 patients had positive cultures, and the population comprised 1.5 million individuals, the mean annual rate of infection over the two-year study period was an astonishing 14.6 per 100,000. This is much higher than in the bordering state of Minnesota, based on data reported 10 years previously by Good (39). Other studies (43,44) did not find similarly high rates in British Columbia and reported levels of 2-4 per 100,000.

In a survey of 26 states and 22 city health departments in the United States, recording laboratory and clinical information on patients with NTM positive specimens between 1982 en 1983, MAC was found the most common NTM causing disease (26). Overall colonization and infection rates were 4.5 and 1.8 per 100,000, and more than 80% were cultured from pulmonary sites.

Studies reviewing both microbiologic and clinical data on the rates of NTM disease in North America span nearly four decades (26,43-47). A study of all cases of mycobacterial disease from the province of Newfoundland between 1957 and 1960 reported that two percent were caused by NTM (46). Two thirds of NTM cases were pulmonary. The average annual rate of NTM disease was 2.0 per 100,000 overall and 1.3 for pulmonary disease.

Data on all mycobacterial isolates from the province of British Columbia from 1960-1981 were reported in two publications (43,44). The annual incidence rate of disease per 100,000 rose from 0.08 in 1960 to 0.60 in 1980. MAC was the most commonly isolated species, followed by rapid growers, *M. xenopi*, and *M. kansasii* (44). These two reports present a thorough analysis and suggest a steadily increasing rate of NTM infection and disease over more than two decades.

Studies from the United States of America have shown tremendous geographic variability. Rates of colonization and infection for all NTM have generally been reported at approximately four and two per 100,000, respectively. About 50-60% of cases was caused

by MAC, 20% by *M. kansasii* and approximately 10% by rapid growers. *M. kansasii* has consistently been reported to cause disease in the majority of cases with a positive isolate. Finally, of the few reports that have presented data on disease rates over time, most (43,44,48) have found a significant increase.

A recurring observation is the increase in rates of infection and disease in time. The reason for this increase is unclear but may be caused by several factors. The possibility exists that the apparent increase is either spurious or less significant than studies would suggest. Changes in clinician awareness leading to increased investigations, or laboratory methods leading to isolation and identification of previously unnoticed organisms, could play a role in this trend, and studies have been published that support (49) and refute (50) this argument. A true increase could be related to the host, the pathogen, or some interaction between the two.

Host changes leading to increased susceptibility could play an important role, with increased numbers of patients with inadequate defenses from diseases such as HIV infection, malignancy, or simply advanced age (50). An increase in susceptibility could also relate to the decrease in infection with two other mycobacteria. It has been speculated that infection with *M. tuberculosis* (47) and Bacillus Calmette-Guerin (BCG) (51,52) may provide cross-immunity protecting against NTM infection. Many investigations have observed decreasing rates of *M. tuberculosis* concomitant with the increases in NTM. In addition, studies from Sweden (51) and the Czech Republic (52) have found that children who were not vaccinated with BCG had a far higher rate of extra-pulmonary NTM infection.

Potential changes in the pathogens include increases in NTM virulence, and it has been argued that this should be considered as a possible contributing factor (53). Finally, an interaction between the host and pathogen could involve a major increase in pathogen exposure or potential inoculum size. This may be occurring secondary to the increase in popularity of showering as a form of bathing (54), a habit that greatly increases respiratory exposure to water contaminants. Furthermore, disinfection of drinking water with chlorine and disinfection attempts in medical and industrial settings may likewise select for mycobacteria (55).

The heterogeneity of study methods in identifying cases and the lack of a uniformly applied definition of disease makes it difficult to compare rates between studies. Moreover, the lack of systematic reporting of NTM infection in most nations limits the ability to derive accurate estimates of infection and disease. Regardless, there are more than adequate data to conclude that NTM disease rates vary widely depending on population and geographic location. NTM disease is clearly a major problem in certain groups, including patients with underlying lung disease and also in individuals with impaired immunity.

The rates of NTM infection and disease are increasing, so the problem will likely continue to grow and become a far more important issue than current rates suggest.

Africa

The only available population-based studies in the continent of Africa have been conducted in South Africa and they report extremely high rates of infection. A number of studies on the epidemiology of NTM have been performed in South Africa. These are generally limited to select populations. For an overview of the literature on prevalence/incidence rates of colonization/infection/disease of NTM in Africa see Table 1. The results of mycobacterial studies of sputum cultures from large random samples of South African native people were reported in two studies (56,57). The sputum from 1,196 Zulus in Natal, South Africa, was obtained and examined for the presence of mycobacteria (56). No clinical information was collected. Sputum was obtained either spontaneously or induced by mechanical irritation of the epiglottis. Nine samples grew *M. tuberculosis* and 17 grew NTM, translating into prevalence rates for *M. tuberculosis* of 750 per 100,000 and prevalence rates of colonization/infection for NTM of 1,400 per 100,000. In the other study aerosol-induced sputum from 2,230 Xhosa people in the Transkei region was obtained (57). Ninety specimens grew *M. tuberculosis* and 150 grew NTM, yielding prevalence rates for *M. tuberculosis* of 4,300 and prevalence rates of colonization/infection for NTM of 6,700 per 100,000. According to the authors there was no evidence that the isolated NTM presented with any health threat. Moreover, 79% of the NTM cultured were manifested as 1-colony isolates. Therefore, the 150 NTM represented probably colonization.

Both studies utilized population-based methods to determine the prevalence of pulmonary mycobacterial infection. However, the selected population had an extremely high rate of tuberculosis, probably resulting in bronchiectasis and leading to NTM infection. It is doubtful whether these prevalence rates can be generalized to other populations.

Two South African NTM studies focussed on a population of gold miners (58,59). The records of a cohort of HIV-negative gold miners, investigated for suspected pulmonary mycobacterial disease between 1993 and 1996, were reviewed (59). Annual rates of NTM infection/disease were found to be 101 per 100,000 with the two most common organisms causing 66 (*M. kansasii*) and 12 (*M. scrofulaceum*) per 100,000, respectively. Interestingly, MAC made up for only 6% of all isolates and the rate of MAC disease was not reported. The same group consequently presented data on another cohort of gold miners, this time including HIV-positive patients (58). Rates of NTM disease were somewhat lower than in the earlier report (37 *M. kansasii* and 8.8 *M. scrofulaceum* per 100,000, respectively).

In Ethiopia the prevalence of pulmonary tuberculosis was assessed in out patients in Addis Abada (60). Of 509 consecutive outpatients evaluated on the basis of a clinical suspicion of pulmonary tuberculosis 168 (33%) were culture positive for *M. tuberculosis*. In this population were two patients with isolates classified as *M. avium* complex.

Table 1: Overview of literature on prevalence/incidence rates of NTM colonization/infection/disease in Africa.

Setting	Study	Year	Size	Specimen	Smear pos	Culture results		Clinical information	Incidence/prevalence/proportion of colonization, infection or disease NTM	Ref
						M. tuberculosis	NTM			
South Africa, Natal	random sample population	1974	1,196 (on 1136 microscopy performed,1149 culture)	sputum spontaneously or induced	15 (9 *M. tuberculosis* in culture)	9	17	X-ray, PPD	prevalence of colonization/infection: 1,400 per 100,000	56*
South Africa, Transkei	random sample rural population	1977	2,230	aerosol induced sputum	47 (36 *M. tuberculosis* and 6 NTM in culture)	90	150	X-ray, PPD	prevalence of colonization/infection: 6,700 per 100,000	57*
South Africa, Free State province	Gold miners (HIV-pos and neg patients) with NTM in sputum	1996-1997	118	sputum spontaneously	69	-	118 (32 met the ATS case definition for NTM disease)	ATS criteria	incidence of infection/disease: 174 per 100,000 incidence of disease: 54 per 100.000 (*M. kansasii* 37 per 100,000 and *M. scrofulaceum* 8.8 per 100,000)	58*
South Africa, Welkom	cohort of HIV-neg gold miners with *M. kansasii* or *M. scrofulaceum* in sputum	1993-1996	202 with *M. kansasii*, 41 with *M. scrofulaceum*	sputum spontaneously	164 with *M. kansasii* and 35 with *M. scrofulaceum* in culture	-	297 NTM: 202 *M. kansasii*, 41 *M. scrofulaceum*, 18 *M. avium-intracellulare* complex, 36 nonpathogenic NTM species	clinical signs/symptoms and radiological features	incidence infection/disease: 101 per 100,000 for most common NTM or 125 per 100,000 for all NTM incidence of infection/disease: 78 per 100,000 (*M. kansasii* 66 per 100,000 and *M. scrofulaceum* 12 per 100,000)	59*
Ethiopia, Addis Ababa	out patients with clinical suspicion of pulmonary tuberculosis	1996	509	sputum spontaneously	91 with *M. tuberculosis* in culture	168	2 *M. avium* complex (one smear positive)	clinical signs/symptoms and radiological features	proportion patients colonized/infected: 0.4% (2/509)	60*
Kenya	rural and urban population adults who presented with acute pneumonia	1994-1996	281 clinical episodes of acute pneumonia (272 patients)	sputum or percutaneous transthoracic lung aspiration	23 (of which 3 remained culture neg) of the 53 patients with pos mycobacterial cultures	26	11 (*M. fortuitum/M. chelonae* (3), *M. szulgai* (2), *M. kansasii* (2), *M. terrae* (1), other NTM (3)) (3 cultures lost in transit, 13 too contaminated for speciation)	clinical signs/symptoms and radiological features	proportion patients colonized/infected: 3-6% (11/281)	61*

Setting	Study	Year	Size	Specimen	Smear pos	Culture results		Clinical information	Incidence/prevalence/proportion of colonization, infection or disease NTM	Ref
						M. tuberculosis	NTM			
Kenya, Nairobi	cohort study of HIV-pos and HIV-neg patients with suspected tuberculosis	1989-1990	355	mainly sputum	342 sputum results: 288 smear pos	320	no NTM	clinical signs/symptoms and radiological features	no NTM	62*
Nigeria, Lagos	random sample of patients suspected for pulmonary tuberculosis	1983	668 (of the 2,784 patients suspected for tuberculosis)	repeated sputum samples	not mentioned (of 102 mycobacterial isolates)	87 (and 4 *M. bovis*)	11 (6 *M. avium*, 4 *M. kansasii*, 1 *M. fortuitum*)	clinical signs/ symptoms, chest X-ray, PPD	proportion patients with disease: 2% (11/668)	68*
Zambia, Lusaka	patients suspected for tuberculosis at the University Teaching Hospital	1989	249	sputum, pleural fluid, lymph node, pericardial fluid, lung biopsy	101	137	no NTM	clinical signs/ symptoms, chest X-ray, PPD	no NTM	69*
Zambia	Survey for tuberculin testing: people coughing ≥ 2 wks	1980?	411 reported a chronic cough: 254 sputum samples	sputum	not mentioned	8	3 NTM (species not mentioned).	clinical signs	proportion patients colonized/ infected: 1% (3/254)	70*
Rwanda, Kigali	HIV-1 infected adults with pulmonary disease of undetermined aetiology	1990	111	bronchoalveolar lavage (BAL) and transbronchial biopsy (TBB).	only patients with smear negative sputum for AFB were included	12 (and in 9 diagnosis by histopathologic examination and in 4 AFB positive but culture negative)	no NTM	history, physical examination, chest X-ray, no reaction on antibiotics, sputum negative for AFB, blood culture negative for bacteria	no NTM	9**
Côte d'Ivoire, Abidjan	prospective study: HIV-infected adults with unexplained fever and fewer than 15% of CD4 cells	1996-2002	721	blood and sputum, urine, faeces, pleural fluid	not mentioned	not mentioned	37 NTM identified as *M. intracellulare* and *M. avium*	Follow-up of patients through standardized procedures (visit social worker, examination by physician, open access to study clinic)	incidence of NTM disease 1.8/100 person-years overall and 12.2/100 person-years in patients with CD4 cell counts < 100 cell/mm^3	12**

Setting	Study	Year	Size	Specimen	Smear pos	Culture results		Clinical information	Incidence/prevalence/proportion of colonization, infection or disease NTM	Ref
						M. tuberculosis	NTM			
Kenya, Nairobi (and United States, Finland, Trinidad)	HIV-infected persons, CD4 counts < 200 x 10^6/l	1991-1994	566	blood / sterile specimen	not mentioned	21	56 positive for MAC	risk interview, skin tests with purified protein derivative (PPD) and *M. avium* sensitin.	prevalence rate of disseminated MAC were 2.4-2.6% in Kenya and Trinidad (compared to 10.5-21.6% in United States and Finland)	24**
Kenya, Nairobi	symptomatic adults with advanced HIV infection	1992	48	blood	not mentioned	11	3 positive for *M. avium*	chronic history of diarrhea and/or fever and/or cough, clinically wasted	minimum rate for HIV-associated disseminated *M. avium* infection in patients admitted to the hospital in Nairobi approximately 1%.	27**
Côte d'Ivoire, Abidjan	HIV-pos deaths autopsied	1991	294	autopsy	92 of the 94 cases AFB histological	TB diagnosed in 106 (In 16 cases samples, tissue cultures with *M. tuberculosis*)	Histology indicative of NTM in 7 deaths (no tissue cultures with NTM in 16 cases sampled)	died adults admitted to medical or neurology ward or brought in dead to mortuary	none of the NTM was the prime cause of death	37**
Uganda, Kampala	patients with AIDS (blood cultures) patients HIV pos and neg (clinical isolates)	1990	45 patients with blood cultures 165 patients with clinical isolates (89 HIV pos, 53 HIV neg, 23 unknown HIV)	blood and clinical specimens	not mentioned	blood: 4 clinical isolates: 133 (additional 25 strains niacin pos, not further identified)	blood: no *M. avium* (in former study also no *M. avium* in 50 blood cultures) clinical isolates: 6 *M. fortuitum*, 1 *M. flavescens*	-	none of the NTM were thought to be clinically significant	41**
South Africa, Johannesburg	prospective study: hospitalised HIV-pos black patients with CD4 counts < 100 cells/mm^3	1998	100	blood and sputum, bone marrow, lymph node, pleural fluid, CSF	not mentioned	54	10 disseminated MAC (of whom 3 co-infected with *M. tuberculosis*)	detailed history, physical examination	point prevalence of disseminated *M. avium* complex infection was 10%	47**

Definition of abbreviations: ref = reference, neg = negative, pos = positive.

* Reference in chapter 1: Introduction and outline of the thesis.

** Reference in chapter 11: Summary and General discussion.

Kenyan adults who presented with acute radiologically-confirmed pneumonia were studied to define the aetiology and outcome of acute pneumonia in a rural and urban population (61). In total 281 clinical episodes of acute pneumonia were examined. Acid-fast bacilli were observed in sputum smears from 23 patients, of which three remained culture negative. Mycobacterial cultures were positive in 53 patients (17 from lung aspirates, 47 from sputum) and a *Nocardia* species was isolated from one patient. *M. tuberculosis* was found in 26 cultures, and 11 were identified as NTM (*M. fortuitum/M. chelonae* (3 cultures), *M. szulgai* (2), *M. kansasii* (2), *M. terrae* (1), and other NTM (3). Three cultures were lost in transit, and 13 were too contaminated for further speciation. In a cohort study of 355 HIV-positive and HIV-negative patients with suspected tuberculosis in Kenya no NTM were found in specimens (mainly sputum) taken at presentation (62).

A number of studies have reported that in sub-Saharan Africa *M. tuberculosis* is a frequent cause of bloodstream infection (63-65) and that *M. avium* complex is an uncommon cause of disseminated infection among HIV-1 infected persons (29,63,64,66,67).

In Malawi the aetiology of 70 patients, out of 233 patients, with a bloodstream infection was determined (64). Of the isolated pathogens 21 (28%) were belonging to *M. tuberculosis* and one to *M. bovis*. In addition, three NTM were found: one each of *M. avium* complex, *M. simiae*, and species belonging to the SAV group (*simiae-avium* group mycobacteria, i.e. organisms that resemble *M. avium* complex by conventional biochemical tests but that have HPLC profiles that are more consistent with *M. simiae*).

In Nigeria a total of 2,784 cases with persistent symptoms of lower respiratory tract infections were seen in chest clinics in Lagos (68). Of these, 668 were randomly selected and screened for pulmonary tuberculosis. Repeated sputum samples were cultured and 102 mycobacterial isolates were obtained. Of these isolates, 87 (85%) were identified as *M. tuberculosis*, four (4%) as *M. bovis* and 11 (11%) as NTM. Among these NTM, six were classified as *M. avium*, four as *M. kansasii* and one as *M. fortuitum*.

In Zambia, 249 patients suspected for tuberculosis were recruited to a cohort study to investigate the interaction between tuberculosis and HIV (69). Sputum, pleural fluid or tissue were examined. *M. tuberculosis* was cultured in 57% (39/67) of HIV negative cases and 54% (98/182) of HIV positive cases. No NTM were isolated.

In a survey for tuberculin testing in Zambia all people were asked if they had a cough more than two weeks duration. If so, sputum was collected from all people over nine years old (70). Of 1,692 people, 411 reported a chronic cough and 254 sputum samples were obtained. Of these sputum samples 8 *M. tuberculosis* and 3 NTM were isolated (species not mentioned).

TRANSMISSION AND PREDISPOSING FACTORS

The biological variation among NTM is large when considering the ability to cause clinical disease in man and to affect various target organs or tissues.

In contrast to *M. tuberculosis* and *M. leprae* that affect only mammals, NTM form an integral part of the natural environment and may also prevail in certain man made environments, such as hot water tanks and tap water, thereby infecting and causing disease in vulnerable individuals. Transmission of infection occurs by inhalation or ingestion of aerosolized organisms from soil, dust and water spray and less frequently from instrumentation such as bronchoscopy or by inoculation. Damaged skin tissues favour infections by particular NTM species including *M. abscessus* (71). Direct transmission from animals is not important for human infections (72), and human-to-human transmission has not been demonstrated so far. Therefore, patients with disease are not generally isolated. Disease in humans almost certainly arises as a result of contact with bacilli in the environment. Investigation is continuing into the exact mechanisms of transmission of NTM disease from the environment to man.

Certain types of conditions including congenital- or acquired immune deficiency of T-cells or macrophages, cystic fibrosis (CF) or bronchiectasis, tend to facilitate NTM infection. Trauma that is often associated with introduction of foreign material such as wood splinters or sand particles, unsterile injections, or surgically implanted cosmetic or medical devices in tissue, may promote disease with different NTM (73-79). The course of infection depends on the characteristics of the NTM species, the presence of predisposing host factors and the clinical setting (80,81).

Risk factors may be divided, in decreasing order of importance, into coexisting medical conditions, living and work environment, and patient demographics.

Coexisting medical conditions likely provide the most powerful risk factor for NTM infection. These risk factors may be divided into impairment of local pulmonary defences and generalized immune defects, with examples including CF and HIV, respectively. Preexisting lung disease, including silicosis and other pneumoconiosis (31,36,59), chronic obstructive pulmonary disease (32,35,36), bronchiectasis (35,36), and other structural changes in the anatomy of the lung consistent with previous tuberculosis (59) have been identified as important risk factors. Alcohol abuse (54), diabetes mellitus, malignancy (32), and smoking (54) also have been associated with NTM infection.

Living and work environment has consistently been identified as an important risk factor for NTM colonization and infection. Studies covering large geographic areas have generally found an increased risk of NTM colonization in people living in warmer regions (82).

Living in urban versus rural settings has been associated with altered rates and patterns of NTM colonization in several studies (26,44,45,47). The most commonly cited environmental risk factor for NTM is the work environment, specifically mining, other heavy industries such as smelting. Also, residence in areas where these industries dominate may be a risk factor (38,58,59).

Certain demographic features have been identified as risk factors for NTM infection, including age, sex, or combination of the two. Increasing age has almost universally been identified as a risk factor for NTM (43,82) as well as male sex (43,45,47). However, exposure to NTM in the work environment during cumulative years could be a confounding factor in that respect. Studies have also documented observations regarding the patient profiles of 'elderly females' and 'middle-aged males' being at increased risk for MAC (26,47) and *M. kansasii* (37).

Immune competent patients infected by NTM can be stratified into two groups: the first comprises patients, usually men of more than 50 years old, primarily white with pre-existing lung disease such as chronic airways disease, fibrocavitary diseases and bronchiectasis and often alcoholics and/or smokers (14,83,84). A more recently recognized group of patients comprises predominantly elderly (over age 50) non-smoking women with no pre-existing pulmonary disease and who have interstitial patterns on chest radiography (50,85,86).

CLINICAL MANIFESTATIONS OF INFECTIONS DUE TO NTM

In broad terms, the following main categories of clinical symptoms caused by NTM can be distinguished: pulmonary disease, superficial lymphadenitis, skin and soft tissue infection, and disseminated disease. Pulmonary disease accounts for up to 90% of all cases.

Furthermore, NTM are occasionally involved in nosocomial infections and pseudo-outbreaks (73,74) Unfortunately, official figures on infections due to NTM cannot be given because they are mostly not incorporated in surveillance programs.

In The Netherlands one focal centre performs the secondary laboratory diagnosis of tuberculosis and mycobacterioses; the RIVM. At the RIVM in the year 2005 nearly 600 isolates of NTM were received, and the actual number of NTM isolates in this country may be much higher. In most of the concerned cases there was a clinical ground underlying the decision to culture mycobacteria. The magnitude of clinical relevance of isolation of particular NTM is currently under investigation at the RIVM, in collaboration with University lung centre Dekkerswald in Nijmegen.

NTM infections of the lung often occur in the context of pre-existing lung disease, especially chronic obstructive pulmonary disease (COPD), bronchiectasis, pneumoconiosis, cystic fibrosis, and a history of tuberculosis (87-89). As a result, the clinical manifestations of NTM-associated lung disease are often similar to those of the underlying disease and are also present in patients with NTM lung disease who do not have pre-existing pulmonary disease. Progressive pulmonary disease is primarily caused by *M. avium* complex (MAC) and *M. kansasii*. Other species which cause lung disease include *M. abscessus*, *M. fortuitum*, *M. xenopi*, *M. malmoense*, *M. szulgai*, *M. simiae*, and *M. asiaticum* (89).

Differences in geographic distribution play a prominent role in the epidemiology of NTM pulmonary disease. *M. xenopi* is relatively more common in Europe, Great Britain, and Canada, while *M. malmoense* is relatively more common in Scandinavia and Northern Europe (13,90).

Cervical lymphadenitis, especially in children younger than 5 years of age, is caused mostly by MAC and *M. scrofulaceum* in the United States, and, in Northern Europe mostly by *M. malmoense*. However, the most common cause of this infection in Europe is *M. tuberculosis*.

Skin and soft tissue infection usually occurs from percutaneous inoculation (e.g.; trauma or surgery) and are caused primarily by *M. marinum* and *M. ulcerans* and the rapid growers *M. chelonae* and *M. fortuitum*. Chronic granulomatous infections of bursae, joints, tendon sheaths and bones are seen after direct inoculation of NTM through accidental trauma, surgical incisions, puncture wounds or injections.

Disseminated infections of NTM are rare and most commonly caused by *M. chelonae*, *M. abscessus* and *M. fortuitum*. A review of the literature from the early 1990s identified worldwide 54 cases over 14 years (91). The diseases occurred most commonly in patients who had profound immuno-suppression and presented as multiple subcutaneous nodules (pseudo erythema nodosum) or abscesses that drained spontaneously (92). The majority of disease due to NTM in HIV-infected patients is caused by *M. avium* complex (MAC). In these patients usually disseminated disease is reported, although localized forms of MAC infection are currently being reported with the widespread use of more effective antiretroviral therapies.

THE LABORATORY DIAGNOSIS OF NTM

Microscopy

Microscopic examination of sputum smears after staining, culture on specific (liquid and solid) media, and molecular detection are nowadays the cornerstones of the laboratory

diagnosis of mycobacterioses, including tuberculosis. Usually, in poor resource settings, microscopy is still the only tool available for the diagnosis of tuberculosis. All mycobacteria share the characteristic of 'acid-fastness', i.e.; after staining with carbol-fuchsin or auramine-rhodamine, they do not decolorize with acidified alcohol. Thus, the common term acid-fast bacilli (AFB) is essentially synonymous with mycobacteria. Nocardia, the main exception, is weakly or variably acid-fast.

Specimens may be stained with the Ziehl-Neelsen stain or one of its modifications, such as the Kinyoun stain, and examined by routine light microscopy. However, microscopy is relatively insensitive, since at least 10,000 organisms per millilitre of sputum are required for smear positivity (93). Thus, other procedures are often performed in order to increase the sensitivity of direct microscopy of clinical specimens. Most laboratories in the Western world use a fluorochrome stain such as auramine-O or auramine-rhodamine and examine specimens by fluorescence microscopy. In order to increase the sensitivity, it is also recommended to use *N*-acetyl L-cysteine (NALC) for liquefaction of the sputum together with concentration. Furthermore, liquid specimens may be centrifuged first before the sediment is stained.

Culture

Confirmation of the presence of mycobacteria in clinical specimens has traditionally required culture, because of the relative insensitivity of direct microscopy. In general, clinical specimens that are normally sterile, such as blood, cerebrospinal fluid, or serous fluids, can be inoculated directly onto media. In contrast, non-sterile specimens, such as sputum or pus, must first be chemically decontaminated, in order to eliminate common bacteria and fungi that would overgrow the culture. However, decontamination procedures inhibit the growth of mycobacteria as well. Clinical specimens for mycobacterial cultures should be inoculated onto one or more solid media (e.g.; Löwenstein-Jensen or Middlebrook 7H11 media) and into a liquid medium such as BACTEC 12B broth or MGIT broth (both manufactured by Becton Dickinson). Growth of visible colonies on solid media typically requires three to eight weeks. Primary cultures in liquid media, such as in the MGIT system, usually produce results within 10 to 14 days. However, this method is not hundred percent sensitive; as a result, this culture method supplements but not replaces traditional solid media.

Molecular methods

Traditional methods of speciating mycobacterial isolates were based upon growth characteristics on solid media and subsequent biochemical tests, requiring additional weeks for subcultures. These time-consuming methods are being replaced with more rapid techniques. Commercial DNA probes (AMPLICOR nucleic acid amplification test (AccuProbe) (Roche Diagnostic Systems, Inc., Branchburg, New Jersey) and Gen-

Probe Amplified *Mycobacterium tuberculosis* Direct Test (MTD) (Gen-Probe, San Diego, California) have been available for some time for identification of clinically important mycobacterial species, including *M. tuberculosis* complex, *M. avium*, *M. intracellulare*, *M. avium* complex, *M. kansasii*, and *M. gordonae*. The tests are based on species-specific DNA probes that hybridize with ribosomal RNA released from bacteria. Other methods (INNO-LiPA Mycobacteria; Innogenetics, Ghent, Belgium and GenoType Mycobacterium CM/AS, Hain Lifescience GmbH, Nehren, Germany) are based on reverse hybridization, in which the mycobacterial 16S-23S internal transcribed spacer region or the 23S gene region are amplified by polymerase chain reaction (PCR), and amplicons are subsequently hybridized with probes for several mycobacterial species on paper strips.

Polymerase chain reaction (PCR)-based sequencing consists of amplification of mycobacterial DNA with genus-specific primers and sequencing of the amplicons. The micro-organism is identified by comparison of the nucleotide sequence with reference sequences. The target most commonly used is the gene coding for the 16S ribosomal RNA. Several other target genes have been characterized for this purpose (genes coding for the 32 kDa protein (95), the 65 kDa heat shock protein (96), and the 16S-23S ribosomal RNA internal transcribed spacer (94)).

DIAGNOSIS OF NTM DISEASE

The diagnosis of NTM disease is highly complicated. Prior to the 1950s, it was generally accepted that NTM do not cause human disease. Unlike *M. tuberculosis*, NTM were found of low virulence in the guinea pig model; as a result, when they were found in sputum of humans, it was difficult to know whether their presence represented disease or simply colonization. Finding any *M. tuberculosis* in sputum is regarded as abnormal and indicative of disease, but such is not the case with NTM. Healthy people may expectorate NTM without having evidence of this disease. The isolation of a few colonies of NTM from the sputum of a healthy person may have no clinical significance. If these organisms are isolated from a person with an abnormal chest X-ray film, further evaluation is necessary.

When NTM are isolated from a usually sterile site (e.g., blood, bone marrow, lymph nodes, synovial fluid), the diagnosis of true disease is generally straight forward. However, when NTM are isolated from non-sterile sites, such as sputum or broncho-alveolar lavage (BAL) fluid, the diagnosis is less definitive, especially when the colony numbers are low or NTM are isolated from only one cultured specimen. A diagnosis of infection in such a case depends upon other clinical findings and the presence or absence of other pathogens. Organisms that in immune competent persons are considered 'commensals',

can be opportunistic pathogens in patients with advanced HIV disease and immunodeficiency (97).

The criteria set in a Statement by the American Thoracic Society (ATS) in 1997 and these formulated by the British Thoracic Society (BTS) serve as a useful guide to the diagnosis of NTM disease in patients with or without HIV co-infection (23,89). The guidelines of the ATS have been recently revised (February 2007) (24). The diagnostic criteria for NTM pulmonary infections include both imaging studies consistent with pulmonary disease and recurrent isolation of mycobacteria from sputum or bronchial wash in a symptomatic patient.

Diagnostic criteria for NTM lung disease

In 1997, the American Thoracic Society (ATS) issued a revised set of diagnostic criteria for NTM lung disease and recently (February 2007) the third statement was published (24,89). In 2000, the British Thoracic Society (BTS) also published its guidelines for the management of NTM lung disease (23). According to the BTS guidelines, NTM lung disease is indicated when positive cultures develop from specimens of sputum obtained at least seven days apart (positive culture twice) in a patient whose chest radiograph suggests mycobacterial infection and who may or may not manifest symptoms or signs.

The ATS guidelines of 1997 included diagnostic criteria that are more strict than those of the BTS statement. In this thesis, the ATS criteria of 1997 were applied. In symptomatic patients with infiltrate, nodular, or cavitary disease, or a high-resolution CT scan showing multi-focal bronchiectasis and/or multiple small nodules, the following apply:

A. If three sputum/bronchial wash results are available from the previous 12 months:
    1. three positive cultures with negative AFB smears results.

    or

    2. two positive cultures and one positive AFB smear.
B. If only one bronchial wash is available:
    1. positive culture with a 2+ to 4+ AFB smear or a 2+ to 4+ growth on solid media.
C. If sputum/bronchial wash evaluation are non-diagnostic or another disease cannot be excluded:
    1. trans-bronchial or lung biopsy yielding a NTM.

    or

    2. biopsy showing mycobacterial histo-pathologic features and one or more sputa or bronchial washings are positive for an NTM even if only in low numbers.

    or any growth from usually sterile extra-pulmonary site

The new ATS guidelines (2007) include diagnostic criteria that are more lenient than the former statement (24). The clinical criteria include a symptomatic patient with

pulmonary symptoms, nodular or cavitary opacities on chest radiograph, or an HRCT scan that shows multifocal bronchiectasis with multiple small nodules. In addition, the microbiologic criteria comprise positive culture results from at least two separate sputum samples, or positive culture results from at least one bronchial wash or lavage, or lung biopsy with mycobacterial histopathologic features (granulomatous inflammation or AFB) and positive culture for NTM, or lung biopsy showing mycobacterial histopathologic features (granulomatous inflammation or AFB) and one or more sputum or bronchial washings that are culture positive for NTM.

Noteworthy is the exclusion of positive smear(s) of sputum or bronchial wash and the exclusion of extra-pulmonary NTM isolates from the diagnostic criteria. Furthermore, expert consultation should be obtained when NTM are recovered that are either infrequently encountered or that usually represent environmental contamination. 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.

Signs and symptoms of pulmonary disease caused by NTM are variable and non-specific. NTM frequently occurs in the context of underlying lung disease, which can also cause symptoms. As a result, the distinction between transient infection or contamination and true infection is often difficult. Most patients present with cough that becomes productive as the disease progresses. Fatigue, weight loss, malaise, fever, dyspnoea, haemoptysis, and chest discomfort may also occur as the disease progresses. In most patients, the symptoms have been present for months to years and attributed to chronic bronchitis or bronchiectasis.

The radiographic findings of NTM lung disease are variable, depending in part upon the species. Findings consistent with NTM pulmonary infection on chest radiograph or high resolution computed tomography scan include infiltrates (usually nodular or reticulo-nodular), cavities, multi-focal bronchiectasis, and/or multiple small nodules.

Only for a limited number of NTM infections a skin test is commercially available. Cross-reactivity between purified protein derivatives from various NTM species has limited skin testing with purified protein derivative (PPD) to epidemiologic and descriptive studies (98).(99)

Given the lack of diagnostic specificity of chest radiography and skin testing, the diagnosis of NTM lung disease requires microbiologic confirmation. The diagnostic evaluation should consist of smear and culture of at least three sputum specimens obtained in the morning. If the aetiology of the patient's symptoms remains uncertain and the infiltrate persists, bronchoscopy with broncho-alveolar lavage (BAL) should be performed.

THE STUDY SITE; ZAMBIA

Zambia, officially the Republic of Zambia, is a landlocked country in Southern Africa. It borders the Democratic Republic of the Congo to the north, Tanzania on the North-East, Malawi on the East, Mozambique, Zimbabwe, Botswana, and Namibia to the South, and Angola on the West. The former name of this country was Northern Rhodesia, nowadays the country is named after the Zambezi river.

Zambia has a tropical climate and consists mainly of high plateau with some hills and mountains. At 752,614 square km it is the 39th-largest country in the world. Zambia is drained by two major river basins: the Zambezi River basin in the South; and the Congo River basin, in the North. Of the two basins, the part of Zambia drained by the Zambezi River basin is about three-quarters of the country's total area.

Zambia is divided into nine provinces, each administered by an appointed deputy minister who essentially performs the duties of a governor. The provinces are divided into 72 districts. The provinces are: Central, Copperbelt, Eastern, Luapula, Lusaka (home of the capital city, Lusaka), Northern, North-Western, Southern, and Western Province. Within

Table 2: Statistics Zambia 2004.

General information	
Total population	11,668,000
GDP per capita (Intl $)*	1,013
Life expectancy at birth m/f (years)	40.0/40.0
Healthy life expectancy at birth m/f (years)	34.8/35.0
Adult mortality m/f (per 1000)	683/656
Total health expenditure per capita (Intl $)*	51
Total health expenditure as % of GDP	5.4

* Intl $ (International dollar): a common currency unit that takes into account differences in the relative purchasing power of various currencies.

Table 3: Health indicators in Zambia.

Health Indicator	Estimated figures
Life expectancy at birth	38.1 (2004)
Infant mortality rate (per 1000 live births)	102 (2004)
Under-5 mortality rate (per 1000 live births)	182 (2004)
Total fertility rate	5.5 (2004)
Maternal mortality rate (per 100,000 live births)	750 (2006)
Crude death rate (deaths per 1000 population)	19.9 (2006)
Crude birth rate (births per 1000 population)	41 (2006)

Figure 1: Map Africa and Zambia.

each district there are administrative subdivisions known as Traditional Authorities, which are presided by chiefs. The smallest administrative unit is the village.

Over 70% of Zambians live in poverty. Per capita annual income is currently about $395 and places the country among the world's poorest nations. Zambia is also one of Sub-Saharan most highly urbanized countries. Almost one-half of the country's 10 million people are concentrated in a few urban zones strung along the major transportation corridors, while rural areas are underpopulated. Unemployment and underemployment are serious problems.

Zambia's population is comprised of about 72 Bantu-speaking ethnic groups but almost 90% of Zambians belong to the nine main ethnolinguistic groups, which are the Bemba, Nyanja-Chewa, Tonga, Tumbuka- spoken in the Eastern Province and Eastern part Northern Province, Lunda, Luvale, Kaonde, Nkoya and Lozi. Each ethnic group is concentrated in a particular geographic region of the country and many groups are very small and not as well known. The official language is English. Most Zambians are subsistence farmers. The predominant religion is Christianity which is also the official national religion. Islam also has a visible presence, especially in urban settings. Expatriates, mostly British (about 15,000) or South African, live mainly in Lusaka and in the Copperbelt in Northern Zambia, where they are employed in mines and related activities. Zambia also has a small, but economically important Asian population, most of whom are Indians. There is low literacy rate: 68% in adults in 2004. Secondary school enrolment is low with 25.8% of the children.

Almost 50 percent of the population consists of children under the age of 15 and a further 20 to 25 percent are women of childbearing age (world resources institute). The World Health Organization reports that the life expectancy at birth in Zambia is just 40 years

and the under-five child mortality rate is approximately 180 per 1,000. The impact of inadequate health care facilities on this group, as well as the population as a whole, is compounded by poverty, disease and malnutrition. The effects of the HIV/AIDS epidemic are devastating, with an estimated 15 to 20 percent of adult Zambians being infected, about 900,000 living with AIDS and over 550,000 AIDS orphans, the great majority of who are not infected.

The arrival of the HIV/AIDS pandemic has caused a re-emergence of tuberculosis throughout Southern Africa, where the average tuberculosis case rate between 1964 and 1984 remained constant at 100 per 100,000 people. Since the advent of AIDS, TB case rates in Zambia increased nearly fivefold to over 500 per 100,000 people in 1996. There are now in excess of 78,000 new tuberculosis cases reported every year with an estimated incidence of 650 per 100,000 people. Of the adult people (15-49 years) with tuberculosis, 54% is HIV positive.

HOSPITALS IN ZAMBIA WHERE THE STUDY WAS PERFORMED

St. Francis Hospital

St. Francis Hospital is a large, rural hospital serving a local population of over 200,000 within a 60 km radius and receiving specialist referrals from all over Eastern Province (about 1.5 million people). It is located in the district of Katete in Zambia's Eastern Province. St. Francis Hospital was started in 1948 by an Anglican Missionary. It first consisted of wards and staff accommodation built of wooden poles, mud walls and thatched roofs. The hospital rapidly expanded and became a teaching hospital for surgeons and midwives. In 1987, the Roman Catholic Church in Zambia joined the Anglicans in running the hospital. The largest mission hospital in Zambia is now fully integrated into the Zambian Health Service and partly funded by the Zambian Government, but also receives funding via the Anglican and Catholic Churches and from overseas support groups in The Netherlands and the U.K. St. Francis hospital has a predominantly Zambian staff, but also uses on volunteers from overseas to compensate for the national shortage of clinical staff. It is about 500 km from the capital of Lusaka. This is a five or six hour journey on the 'luxury' coaches. The hospital contains about 360 beds. However, this total is never a limit to the number of in-patients in the hospital, as space on the floor can always be found and it is a common occurrence for patients to be found sleeping on the floors of the hospital wards. St. Francis Hospital also runs two nursing schools and a network of 14 rural clinics which serve a population of 157,000. In addition, the hospital supports over 6,000 AIDS orphans living in proximity to the hospital in a centre for voluntary HIV/

AIDS testing and counselling. Furthermore, it provides community education on HIV/AIDS prevention and in home support to those individuals living with HIV/AIDS.

Yeta district Hospital

Yeta district Hospital in Sesheke in the Western Province of Zambia is a first level hospital with 101 beds. The hospital is owned by the government.

Our lady's Hospital

Our lady's Hospital in Chilonga is a 182 bed second level hospital, which means it serves as a referral hospital for the Northern Province. A school of nursing and midwifery is attached to the hospital, and it participates in the training of clinical officers.

AIMS AND OUTLINE OF THIS THESIS

Tuberculosis remains a problem of large dimension in Africa and *Mycobacterium tuberculosis* is the most important causative agent of this infectious disease. However, it is known that in developed countries also NTM play a significant role in the aetiology of tuberculosis-like syndromes, especially in immune-compromised patients. In Africa, where HIV-infection is highly prevalent, the contribution of NTM to the problem of tuberculosis has so far been examined on a very limited scale.

From a hospital in Sesheke, Zambia a request was received at the laboratory of medical microbiology, Medical Centre Rijnmond-Zuid in Rotterdam to support a research project on the diagnosis of tuberculosis. In this African hospital, many patients were clinically suspected for tuberculosis but had a negative Ziehl-Neelsen staining of sputum. During a tuberculosis surveillance study, we regularly isolated NTM from various clinical specimens of Zambian patients with pulmonary syndromes. The clinical significance of a part of these NTM was questionable and the present study aims to resolve this enigma.

Patients who were admitted to different hospitals with chronic complaints during various time periods from 2001 to 2004, were included in the study. Most of the study was performed in St. Francis Hospital in Katete (Eastern province). Other hospitals involved were Yeta District Hospital in Sesheke (Western province) and Our Lady's Hospital in Chilonga (Northern province). Because mycobacterial infections can affect all parts of the body, we decided to include all patients over 15 years of age who with signs and symptoms in any part of the body for more than two weeks.

The clinical relevance of the isolation of NTM in patients with a positive or negative HIV status in Zambia is evaluated. The role of NTM in human disease in Africa may be underestimated and is examined in more detail.

Part I: Diagnosis of tuberculosis

In the study described in **Chapter 2**, we compare the effect of two decontamination procedures on the sensitivity to detect *M. tuberculosis* complex and NTM in sputum samples, as culture is considered the gold standard for the diagnosis of tuberculosis. Most clinical sputum samples contain a variety of micro-organisms that can easily overgrow *M. tuberculosis*. Decontamination of these samples is therefore crucial in preventing contamination of a *Mycobacterium* culture. However, the recovery of mycobacteria is negatively influenced by decontamination.

Because usually only microscopic examination is available to confirm the diagnosis of tuberculosis in Africa, the involvement of acid-fast NTM in tuberculosis-like syndromes might result in the misdiagnosis of tuberculosis. A new promising diagnostic tool for the rapid identification of mycobacteria is Raman spectroscopy. Another recent development. the 'electronic nose' also shows potential for the direct identification of mycobacteria in clinical samples. These new methods are evaluated in **Chapter 3** and **Chapter 4**, respectively.

Part II: Clinical relevance of NTM

In the first pilot study described in **Chapter 5**, the role of NTM in the development of tuberculosis-like disease in Sesheke and the confusion that this may cause in the diagnosis of tuberculosis by microscopy, is evaluated. In the subsequent pilot study described in **Chapter 6** the possible role of NTM in tuberculosis-like diseases in various geographic locations, in addition to Sesheke, was compared.

Eight patients from whom NTM was cultured from normally sterile body sites were described in detail in **Chapter 7**. In order to exclude the possibility of a laboratory cross-contamination, a novel amplified fragment length polymorphism (AFLP) DNA typing method for a frequently found NTM was applied. Only a limited number of studies on the epidemiology of NTM has been performed in Africa. In the study described in **Chapter 8** the frequency and clinical relevance of NTM in the population living in the area around the hospital was compared with a patient population admitted to the hospital. In **Chapter 9**, the accuracy of clinical diagnosis of tuberculosis in Zambia in the era of increasing HIV prevalence is summarized.

Studies have indicated that host genetic factors are major determinants of susceptibility to mycobacterial infection in humans. Not much is known on possible racial differences involved in the susceptibility to infections diseases like tuberculosis. It is likely that susceptibility to most micro-organisms is determined by a large number of polymorphic

genes, and identification of these markers will provide insights into this mechanism. The DNA polymorphism of genes that determine susceptibility to infection with mycobacteria, in Zambian patients, is described in **Chapter 10**.

Finally, in **Chapter 11** the results of the studies described in Chapter 2 to 10 are summarized and discussed. Questions that could be the basis for future research are put forward.

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Chapter 2

Comparison of NaOH-*N*-acetyl cysteine and sulfuric acid decontamination methods for recovery of mycobacteria from clinical specimens

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ABSTRACT

We compared the NaOH-*N*-acetyl cysteine (NaOH-NALC) and the sulfuric acid decontamination procedure in the detection of mycobacteria using the Mycobacteria Growth Indicator Tube (MGIT). In total 219 sputum specimens were collected from 142 Zambian patients and subjected to mycobacterial culture. One half of the specimen was decontaminated with NaOH-NALC and the other half was decontaminated with sulfuric acid. From the 438 samples a total of 261 (60%) cultures yielded growth of mycobacteria, consisting of 22 different species. The sulfuric acid method was more successful than the NaOH-NALC method in recovering mycobacteria in MGITs (146 versus 115 respectively, p=0.001). Of the 146 positive mycobacterial cultures recovered after sulfuric acid decontamination 28 were *Mycobacterium tuberculosis*, 84 nontuberculous mycobacteria (NTM) and 34 acid fast bacterial isolates which could not be identified to the species level. The 115 mycobacteria recovered by the NaOH-NALC method consisted of 34 *M. tuberculosis* strains, 55 NTM and 26 acid fast bacteria that could not be identified. The most frequently isolated NTM were *Mycobacterium lentiflavum* and *Mycobacterium intracellulare*. Comparing the two decontamination methods the recovery of NTM in the sulfuric acid group was significant higher than in the NaOH-NALC group (p=0.001). In contrast, no significant difference was found for the recovery of *M. tuberculosis*. These results show that the decontamination method used affects the recovery of nontuberculous mycobacteria in particular.

INTRODUCTION

Tuberculosis continues to be a tremendous public health problem in both developing and industrial countries. *Mycobacterium tuberculosis* complex strains are still responsible for the majority of *Mycobacterium* infections worldwide (5). However, there has been an increase in infections caused by nontuberculous mycobacteria (NTM) mainly due to the AIDS pandemic (12). Culture is considered the gold standard for the detection of *M. tuberculosis*, but most clinical sputum samples contain a variety of microorganisms that may overgrow *M. tuberculosis*. Decontamination of these samples is therefore crucial in preventing contamination of the mycobacterial culture. However, also the recovery of mycobacteria is negatively influenced by decontamination (13,15). The decontamination method applying sulfuric acid was used in our laboratory in the former days and NaOH-*N*-acetyl cysteine (NaOH-NALC) is currently most widely used. It is unclear which method provides maximum decontamination while maintaining the viability of *M. tuberculosis* complex and which species of mycobacteria are more sensitive to a specific decontamination method (9,10). The isolation of *M. tuberculosis* complex and/or NTM has epidemiological implications and is also relevant for the management of patients with regard to appropriate treatment, isolation and contact tracing. Antibiotic treatment may vary according to the species encountered, and certain species may require no antibiotic therapy at all.

These considerations have prompted us to compare the effect of these two decontamination procedures on the sensitivity of the detection of *M. tuberculosis* complex and NTM in sputum samples. The study was carried out in two rural hospitals in Zambia where the HIV prevalence was approximately 20%.

MATERIALS AND METHODS

From March to June 2001, 219 sputum specimens were collected from 142 chronically ill patients and cultured for mycobacteria in Zambia. After informed consent each patient admitted on the medical ward in the hospital and who was coughing for more than two weeks was included in the study. During three consecutive days morning sputum was collected from these patients and two separate specimens were cultured for mycobacteria. The third morning sputum specimen was stored at −20°C. About 5% of the patients were not able to produce sputum and about 35% of the patients could not submit a second sputum sample because they did not have a productive cough anymore, discharged themselves or died. Before decontamination, the 219 specimens were divided in two samples. The specimen was macroscopically divided and the (muco)purulent parts were separated equally. The specimen was assessed microscopically according to the criteria from the ASM-manual for clinical microbiological procedures, i.e. basically on the basis of the

number of squamous epithelial cells and leukocytes (11). One half of the specimen was decontaminated with NaOH-NALC and the other half was decontaminated with sulfuric acid. All specimens were collected, decontaminated by NaOH-NALC and sulfuric acid, inoculated and incubated in Zambia. After transport of all tubes the work-up of the positive cultures was done in The Netherlands. Each specimen of the patient was collected in a container that was never used before and was imported from The Netherlands. The stock of decontamination fluid was sterilised twice a week and each day a fresh tube or bottle was opened.

Decontamination with NaOH-N-acetyl cysteine (13)

For the NaOH-NALC procedure 2.5 ml of the sputum specimen was put in a 15 ml glass centrifuge tube with a screw cap. An equal volume of NaOH-NALC decontamination solution (6.8% NaOH, 2.9% sodium citrate, 0.5% NALC, final concentration of NaOH: 1.7%) was added. After vortexing the specimens was left at room temperature for 15 min. Subsequently 5 ml of sterile 0.067 M phosphate buffer (pH 6.8) was added and the mixture was centrifuged at 3660×*g* for 15 min. The supernatant was discarded and the sediment was inoculated in a Mycobacteria Growth Indicator Tube (MGIT; Becton Dickinson Microbiology Systems, Cockeysville, MD).

Decontamination with sulfuric acid (15)

For the sulfuric acid (6.0%) decontamination, 2.5 ml of the specimen was put in a 15 ml glass centrifuge tube with a screw cap and an equal volume of sulfuric acid (final concentration of sulfuric acid: 3.0%) was added. After vortexing the specimen was left at room temperature for 15 min for decontamination. Then, 5 ml sterile normal saline was added and the mixture was vortex-mixed again. After centrifugation for 15 min at 3660×*g*, the supernatant was discarded and the sediment was suspended in 10 ml of normal saline. After another centrifugation for 15 min at 3660×*g*, the supernatant was discarded and the sediment was inoculated in an MGIT.

Media, incubation, reading, identification

Half a ml of the decontaminated specimens was inoculated into an MGIT with PANTA (polymyxin B, amphotericin B, nalidixic acid, trimethoprim, and azlocillin) according to the protocol of the manufacturer. The inoculated tubes were incubated for at least 8 weeks at 37°C in Zambia or in The Netherlands. The tubes were incubated between 2 and 12 weeks in Zambia and the time of incubation was extended for at least 8 weeks in The Netherlands. They were read macroscopically every week and smears stained by Ziehl-Neelsen were made from all tubes in The Netherlands. Mycobacteria were identified by the Accuprobe (bioMérieux) or 16S rDNA sequencing (8). From 60 samples, identifica-

tion of the acid fast bacteria (AFB) was not possible due to contamination or due to loss of viability of the bacteria during the process of culture, transport, and/or identification.

Data analysis

Data were analysed with SPSS 11.0. The McNemar test was used to compare paired dichotomy variables and a *P*-value <0.05 was considered significant.

RESULTS

From 142 patients 219 sputum specimens were collected. These specimens were divided in two parts (in total 438 samples) that were decontaminated with NaOH-NALC or sulfuric acid. At least 70% of the sputum specimens had a quality representative for the lower respiratory tract (Quality score≥1). Of the 142 patients 37 had a sputum culture positive for *M. tuberculosis* and from these patients 18 had also a positive smear for AFB made from these samples.

From the 438 samples an overall number of 261 (60%) cultures yielded growth of mycobacteria, representative of 22 different species (Table 1). *M. tuberculosis* was most commonly recovered (*n*=62), followed by *Mycobacterium lentiflavum* (*n*=33) and *Mycobacterium intracellulare* (*n*=31). From 60 cultures positive with acid fast bacteria (AFB) no identification was possible (26 were found in the NaOH-NALC decontamination method and 34 in the sulfuric acid method).

Table 1: Culture results after decontamination with NaOH-NALC or sulfuric acid of split specimens.

Sulfuric acid	NaOH-NALC						
	Negative	*M. tuberculosis*	*M. lentiflavum*	*M. intracellulare*	NTM[a]	AFB no id[b]	Total[c]
Negative	48	3	3	2	8	9	73
M. tuberculosis	5	14	0	1	5	3	28
M. lentiflavum	12	3	3	3	1	4	26
M. intracellulare	3	1	0	2	3	4	13
NTM[a]	19	5	1	6	11	3	45
AFB no id[b]	17	8	0	3	3	3	34
Total[d]	104	34	7	17	31	26	438

The numbers indicate the culture results of half of the specimen after decontamination with one of the two methods. Reading as a 2×2 table.

[a]NTM consisted of *M. mucogenicum* (*n*=7), *M. fortuitum* (*n*=5), *M. chelonae* (*n*=5), *M. terrae* (*n*=3), *M. goodii* (*n*=2), *M. gordonae* (*n*=1), *M. asiaticum* (*n*=1), *M. triplex* (*n*=1), *M. rhodesia* (*n*=1), *M. avium* (*n*=1), *M. obuense* (*n*=1) and 8 different unidentified *Mycobacterium* species (*n*=48).

[b]From 60 positive cultures with acid fast bacteria (AFB) no identification (id) was possible.

[c]Recovering of mycobacteria by Sulfuric Acid method: 28+26+13+45+34=146.

[d]Recovering of mycobacteria by Nalc-NaOH method: 34+7+17+31+26=115.

In many sputum specimens two different mycobacteria species were isolated (Table 1). For instance, in the samples decontaminated with NaOH-NALC 34 *M. tuberculosis* strains were identified while in the other part of the same specimen decontaminated with sulfuric acid in 14 samples *M. tuberculosis* was also identified but in 3 samples no mycobacteria were found, in 3 samples *M. lentiflavum* and in 1 sample *M. intracellulare* was isolated, in 5 samples other NTM were found and in 8 samples the identification of the AFB was not possible.

Of the 261 isolates, 25 were detected exclusively by the NaOH-NALC method (the MGIT of the sulfuric acid method was negative) versus 56 by the sulfuric acid method (the MGIT of the NaOH-NALC method remained negative). The 25 mycobacteria detected only by the NaOH-NALC method consisted of 3 strains of *M. tuberculosis*, 13 NTM and 9 AFB with no identification. Of the 56 mycobacteria detected only in the sulfuric acid method 5 strains belonged to *M. tuberculosis*, 34 to NTM and 17 AFB with no identification.

The sulfuric acid method was more successful than the NaOH-NALC method in recovering mycobacteria via MGITs (146 and 115 respectively, p=0.001). Of the 146 mycobacteria (recovered by sulfuric acid method) 28 belonged to *M. tuberculosis*, 84 to NTM and 34 AFB with no identification. The 115 mycobacteria recovered by NaOH-NALC method consisted of 34 strains of *M. tuberculosis*, 55 NTM and 26 AFB with no identification. Comparing the samples decontaminated with NaOH-NALC and those decontaminated with sulfuric acid no significant difference was found in recovering *M. tuberculosis* (p=0.39). However, the sulfuric acid method appeared a significantly better decontamination method than the NaOH-NALC method for recovering NTM (p=0.001).

The 146 positive mycobacterial cultures detected by the sulfuric acid method consisted mostly of *M. tuberculosis* (28), *M. lentiflavum* (26) and *M. intracellulare* (13). The 115 mycobacteria isolated after decontamination with NaOH-NALC were mainly identified as *M. tuberculosis* (34), *M. intracellulare* (17), and *M. lentiflavum* (7). Of the 62 *M. tuberculosis* isolates 14 were detected by the NaOH-NALC method as well as by the sulfuric acid method. 14 *M. tuberculosis* isolates where detected exclusively by the sulfuric acid method and 20 only by the NaOH-NALC method.

DISCUSSION

In the present study we found a high rate of positive cultures for mycobacteria. Of the 261 isolates positive for mycobacteria 62 (24%) were identified as *M. tuberculosis* and 139 (53%) were identified as NTM. Significantly more NTM were recovered by the sulfuric acid decontamination method than by the NaOH-NALC method (84 versus 55 respectively). There was no significant difference between the sulfuric acid decontamination

method and the NaOH-NALC method for the detection of *M. tuberculosis* (28 versus 34 respectively).

In the study of Burdz et al. no clearly superior method between NaOH-NALC, modified Petroffs' method or the Yamane procedure was found in affecting the viability of *M. tuberculosis* H37Ra (2). A limited number of earlier studies showed differences between the recovery of mycobacteria by different decontamination methods. Salfinger and Kafader compared NaOH-NALC with sodium dodecyl sulfate (SDS)-sodium hydroxide for the detection of mycobacteria by BACTEC Middlebrook 7H12 medium and Löwenstein-Jensen slants (17). They concluded that SDS-NaOH was superior to NaOH-NALC as a pre-treatment method. On the other hand, in the study of Pfyffer et al. clinical specimens were processed with SDS and NaOH-NALC using the MGIT (16). Specimens pre-treated with SDS resulted in both poor recovery and delayed mean time to detection of AFB. In both studies the number of NTM strains isolated were much less than in our study.

The collection and processing of specimens was done in a way to avoid cross contamination. All disposables needed were imported from The Netherlands and for sterilisation the autoclave of the hospital was used. The restriction fragment length polymorphism (RFLP) patterns of the isolated *M. tuberculosis* strains were almost all different (data not shown) virtually excluding the possibility of a cross contamination in the laboratory in Zambia. Furthermore, to assure that the isolated NTM were not contaminants introduced in the laboratory, sputum specimens (stored at −20°C) of 20 patients were checked and confirmed positive for mycobacteria by amplified rDNA restriction analysis (4). In addition AFLP DNA typing was performed from 10 *M. lentiflavum* strains that showed that different strains were isolated and therefore a laboratory contamination could be considered unlikely (1). Although not all *M. lentiflavum* isolates have clinical significance the first case of human disease was a patient with spondylodiscitis who markedly improved on multiple antituberculous agents (18). Other clinical cases were reported later: four cases of cervical lymphadenitis, a case of cavitary pulmonary disease, one disseminated infection in a woman undergoing steroid therapy, a disseminated infection and hepatic abscess in an AIDS patient (3,6,7,14,19).

Both decontamination methods eliminate most of the contaminating bacteria in the sputum specimen but also reduce the viability of the mycobacteria in the specimen. The alkaline or acid environment is toxic not only to contaminating organisms but to mycobacteria as well. In the present study a complete shift is observed in the ratio of *M. intracellulare* and *M. lentiflavum*. In the NaOH-NALC method the ratio is 17/7 and in the sulfuric acid method the ratio is 13/26. The cell viability of different species of mycobacteria is probably not the same for various decontamination methods. The effect of genetic variation of mycobacteria on the sensitivity to the decontamination process is probably another reason (20).

In the present study NTM were apparently more susceptible to killing by NaOH. The final concentrations for NaOH (1.7%) and sulfuric acid (3.0%) employed in this study were higher than the recommended concentrations (1.0% and 2.0% respectively). These concentrations were according to protocols used in The Netherlands because of a higher contamination rate of the sputum. This might be another reason for the different percentage of recovery of NTM between NaOH-NALC and sulfuric acid decontamination.

The type of decontamination method applied definitely influences the recovery of mycobacteria, NTM in particular. This has clear consequences for the quality of both microbiological diagnosis and treatment of patients. More research is needed for the evaluation of the clinical relevance of the various NTM isolated.

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Chapter 3

Rapid identification of mycobacteria by Raman spectroscopy

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ABSTRACT

A number of rapid identification methods have been developed to improve the diagnostic accuracy for tuberculosis (TB) and to speed up presumptive identification of *Mycobacterium* species. Most of these methods are validated for a limited group of microorganisms only. Raman spectroscopy was compared with 16S rRNA sequencing for the identification of *M. tuberculosis* complex strains and the most frequently found strains of nontuberculous mycobacteria (NTM). Sixty-three strains, belonging to 8 distinct species were analysed. The sensitivity of the Raman spectroscopy for the identification of *Mycobacterium* species was 95.2%. All *M. tuberculosis* strains were correctly identified (7/7; 100%) as were 54 of 57 NTM strains (94%). The differentiation between *M. tuberculosis* and NTM was invariably correct for all strains.

Moreover, the reproducibility of Raman spectroscopy was evaluated for killed mycobacteria (by heat and formalin) versus viable mycobacteria. The spectra of the heat-inactivated bacteria showed minimal differences as compared to the spectra of viable mycobacteria. Therefore, identification of mycobacteria appears possible without biosafety level III precautions.

Raman spectroscopy provides an innovative answer to the need for rapid species identification of cultured mycobacteria in a clinical diagnostic setting.

INTRODUCTION

Mycobacteria cause a variety of infections in humans. Classically defined lung tuberculosis (TB) is predominantly caused by *M. tuberculosis* complex. The number of new cases is estimated at nine million per year worldwide and the disease causes more than two millions deaths annually (16). In addition, the incidence of pulmonary disease caused by nontuberculous mycobacteria (NTM) appears to be increasing worldwide (1,6). The clinical features of NTM-derived pulmonary disease are in some cases indistinguishable from those of tuberculosis. Because the treatment and the epidemiology of NTM-derived infections differ significantly from tuberculosis caused by *M. tuberculosis* complex bacteria, the timely and correct identification of causative organisms is mandatory for diagnosis, therapy and tuberculosis control.

Conventional approaches to the diagnosis of mycobacterial infection rely on tests that are far from optimal. For example; sputum smear microscopy is insensitive, laborious and time-consuming. Culture is technically complex, time-consuming, has a sensitivity of only 80-85% and is scarcely available in high prevalence settings. Chest radiography is non-specific and not widely implemented either. Tuberculin skin testing is imprecise and the results are often non-specific (3). In the last decade, a number of rapid diagnostic tests has been developed in an effort to improve the diagnostic accuracy for TB and to speed up presumptive identification. PCR and other molecular amplification techniques are the most prominent among these new tools. While promising, none are more than adjunctive to the diagnosis of TB, since the sensitivity of these tests varies widely. The most reliable results are found when tests are applied to smear-positive specimens (2,13). In addition, these tests are specific for the detection of particular micro-organisms and not applicable to diagnose a wide spectrum of causative agents.

Several commercial techniques are now available for species identification of *M. tuberculosis* complex and NTM. These techniques are fast but expensive and limited to selected, frequently encountered species, as is the case for the reverse line blot (RLB) assay (e.g. INNO-LiPA Mycobacteria; Innogenetics, Ghent, Belgium and GenoType Mycobacterium CM/AS, Hain Lifescience GmbH, Nehren, Germany), AMPLICOR nucleic acid amplification test (Roche Diagnostic Systems, Inc., Branchburg, New Jersey) and Gen-Probe Amplified *Mycobacterium tuberculosis* Direct Test (MTD) (Gen-Probe, San Diego, California) (2).

For more rarely encountered *Mycobacterium* isolates, DNA sequencing of the 16S rRNA gene is mostly used at mycobacteria reference laboratories. However, at peripheral laboratories implementation of 16S rRNA gene sequencing in routine practise has many drawbacks like high costs, complexity, lack of peer-reviewed databases and clear unambiguous interpretations.

In view of these limitations, there is a continuing need for fast, simple alternatives which have the potential to be directly applicable to bacteria in clinical material, enabling identification of a wide spectrum of micro-organisms.

Vibrational spectroscopies (infrared and Raman spectroscopy) have been developed for the rapid identification of clinically important microorganisms (11). Important features of these methods are the relative ease by which measurements can be performed, the limited amount of sample handling involved, the small amounts of biomass required and the high degree of reproducibility. Fourier-Transform Infrared spectroscopy proved to be a convenient approach to classify NTM at the species level (14). However, the identification of *M. tuberculosis* complex has not yet been evaluated.

Raman spectroscopy is an optical method, enabling spectroscopic fingerprints to be obtained from biological samples in a few seconds. These fingerprints represent the molecular composition of a sample and are therefore ideally suited for identification of micro-organism at both the species- and strain level (7,9,10).

In general, viable micro-organisms are used for the identification by Raman spectroscopy. To work with viable *M. tuberculosis* complex, a biosafety level III (BSL III) of containment is required. To bypass this specific requirement, various methods to kill mycobacteria have been described in the literature, such as heat- and formalin inactivation. However, the effect of the inactivation on the spectroscopic fingerprints of mycobacteria has not been reported previously.

Here we present the results of the first study on the use of Raman spectroscopy for the identification of *M. tuberculosis* complex- and the most frequently encountered NTM species.

The aim of this study is (i) to evaluate the reproducibility of the Raman spectroscopy for killed mycobacteria versus viable mycobacteria and (ii) to compare the performance of this method with identification on the basis of 16S rRNA sequencing.

MATERIALS AND METHODS

Strains

In a pilot study, a set of 12 strains representing 6 different, frequently encountered NTM species was used to evaluate the effect of two inactivation methods on species discrimination and spectroscopic reproducibility. The set included *M. avium, M. chelonae, M. gordonae, M. xenopi, M. kansasii* and *M. malmoense*. Of each species two different strains were included. In this pilot study only NTM requiring BSL II precautions were used.

Table 1: *Mycobacterium* strains included in the identification study.

Mycobacterium species	Number of isolates
M. tuberculosis	7
M. avium	9
M. chelonae	4
M. gordonae	5
M. kansasii	9
M. malmoense	10
M. xenopi	6
M. lentiflavum	13

In the subsequent identification study a collection of 63 *Mycobacterium* strains, comprising 8 different *Mycobacterium* species, was tested (Table 1). These strains represent a variety of the NTM species most frequently isolated from humans in The Netherlands, as well as *M. tuberculosis*. Furthermore, 13 *M. lentiflavum* strains that were recently isolated from patients in Zambia were included (4). All strains were identified to the species level by 16S rRNA gene sequencing and RLB-assay (INNO-LiPA *Mycobacterium* system, Immunogenetics, Ghent, Belgium) (8). *M. lentiflavum* was identified at the species level by 16S rRNA gene sequencing only. Cultures were stored at –80°C in a 10% glycerol containing medium until use.

As a reference method for identification we have used the 16S rRNA sequence. This method has been found to be very useful for identification and taxonomy of mycobacteria (8) and is applied in many tuberculosis reference laboratories (5,15). In recent years, several new *Mycobacterium* species have been identified by 16S rRNA sequencing which could not have been identified by conventional methods. In the recently published new diagnostic criteria for nontuberculous mycobacterial diseases by the American Thoracic Society, 16S rRNA sequencing of mycobacteria is one of the recommended methods for identification (6). Therefore, this method was also used as the gold standard in the present study.

Culture

A loop (1 µm) of biomass was taken from a *Mycobacterium* culture on Middlebrook 7H10-agar or Löwenstein-Jensen and suspended in a Mycobacteria Growth Indicator Tube (MGIT; Becton Dickinson Microbiology Systems, Cockeysville, Md.). The vials were incubated in a semi-automated incubation system (BACTEC MGIT 960 system). This system continuously measures the oxygen levels in the culture vials and a change in the oxygen concentration over a preset threshold is an indication of bacterial growth. Vials positive for microbial growth were indicated by the incubation system.

Inactivation methods

In the pilot study, two inactivation methods for mycobacteria (heating at 80°C for 20 min and suspension in 10% formalin for at least one hour) were compared to direct application of Raman spectroscopy to viable mycobacteria. Positive cultures were centrifuged for 15 min at 3660xg and the sediment was divided into three equal portions. One third of the sediment was suspended in 1.0 ml normal saline and stored at 4°C (in case of viable mycobacteria); one third was suspended in 1 ml 10% formalin and stored at 4°C; and the third part was suspended in 1 ml normal saline and heated for 20 min at 80°C and thereafter stored at 4°C. To check whether the bacteria had truly been inactivated, 500 µl of the heated and formalin-inactivated suspensions was inoculated in a MGIT culture tube and incubated at 37°C for 12 weeks.

In the identification study, all *Mycobacterium* cultures were heat-killed and Raman measurements were performed directly or after storage at 4°C for less than 2 days.

Raman spectroscopy

Before Raman measurements were performed, the samples were washed three times with aquadest (AD). The sediment was suspended in 10 µl of AD and 4 µl was transferred to a fused silica glass slide and air dried, resulting in small pellets of biomass.

Raman spectra were collected using a Model 2500 High Performance Raman Module (HPRM) (River Diagnostics BV, Rotterdam, The Netherlands), coupled to a custom-build inverted microscope stage, with an automated XY-stage (River Diagnostics) and operated using RiverICon software, version 1.63. The microscope contained a custom-designed microscope objective with a numerical aperture of 0.7, optimized for Raman experiments in the 750-1000 nm wavelength region, which focused laser light emitted by the Model 2500 HPRM in the samples on the fused silica slide. The objective also collected Raman scattered light from the samples. Samples were excited using laser light from a 785 nm diode laser (Sacher Lasertechnik, Marburg, Germany), delivering approximately 150 mW to the sample. The spectrometer was calibrated according to the manufacturer's guidelines.

Automated data collection and signal pre-treatment was performed using the RiverICon software, requiring approximately 1 minute per sample. Pre-treatment consisted of correction for the signal contribution of the fused silica substrate and scaling of all spectra using the extended multiplicative signal correction (EMSC) approach described by Martens and Stark (12). Briefly, all spectra were fitted to a representative *Mycobacterium* reference spectrum using a 7^{th} order polynomial to correct for varying spectral backgrounds.

Identification and hierarchical cluster analysis (HCA)

The similarity between samples was calculated using the squared Pearson's correlation coefficient (R^2) between the representative spectra and than multiplied by 100 to be expressed as percentages.

To evaluate the possibilities for species identification, a leave-one-out approach was used. In this approach the similarity of a sample was compared to all other measured samples by calculating the R^2 values. The predicted species of a sample was assumed to be identical to the species of the sample with which the highest correlation occurred. This procedure was repeated for all measured samples. HCA on spectra was performed using the pair wise similarity matrix as distance matrix in combination with Ward's cluster algorithm.

Reproducibility of Raman spectroscopic measurements

In the pilot study all strains were cultured three times. The similarity between the spectra obtained from these replicates was used to evaluate the reproducibility of the Raman procedure compared to the inter-species similarity. Ideally, the intra-strain similarity is less than the intra-species similarity. To determine the intra-strain Raman reproducibility, we used the spectra obtained from the three parallel cultures of one isolate. First the pair wise correlation coefficients between these three spectra were calculated (between spectrum 1 and 2, between spectrum 1 and 3, between spectrum 2 and 3). The mean of these three correlation coefficients is a measure for the intra-strain reproducibility.

To determine the inter-species similarity, we calculated the mean spectrum of the first two isolates belonging to the same species. Then the pair wise correlation coefficients between this spectrum and the spectra of the other isolates in the pilot study were calculated. The mean value of these correlation coefficients is a measure for the similarity between the first species and all other species present in the study.

Data analysis

All data analysis algorithms were programmed using MATLAB version 7.1 (The Mathworks, Natick, MA, USA) and the PLS toolbox 2.0 (Eigenvector Research Inc., Manson, WA, USA). To compare the intra-strain and inter-species similarities an unpaired Student t-test was used. A p-value of less than 0.05 was considered significant.

RESULTS

1. Pilot study

Comparison of Raman spectroscopy spectra for inactivated and viable mycobacteria

For biosafety reasons, the inactivation procedures were validated prior to further experiments. None of the heat-killed or formalin-inactivated *Mycobacterium* suspensions revealed growth in a liquid culture after incubation for 12 weeks.

Raman spectra of a *M. kansasii* isolate after inactivation by formalin or heating in comparison to the procedure without inactivation are shown in Fig.1. No significant changes in the Raman spectra were seen after inactivation by heating, whereas formalin inactivation had a major influence on the Raman spectra.

Furthermore, the effects of formalin inactivation on the mycobacteria proved to be a source of large spectral variance (data not shown). Therefore, heat inactivation was selected as the method of choice for an evaluation of species identification capabilities of Raman spectroscopy.

Classification of heat-killed and viable mycobacteria

A HCA of the spectra obtained from the isolates used in the pilot study is shown in figure 2. This figure represents spectra obtained from viable as well as heat-killed mycobacteria. It was possible to obtain good discrimination between the different species used in the

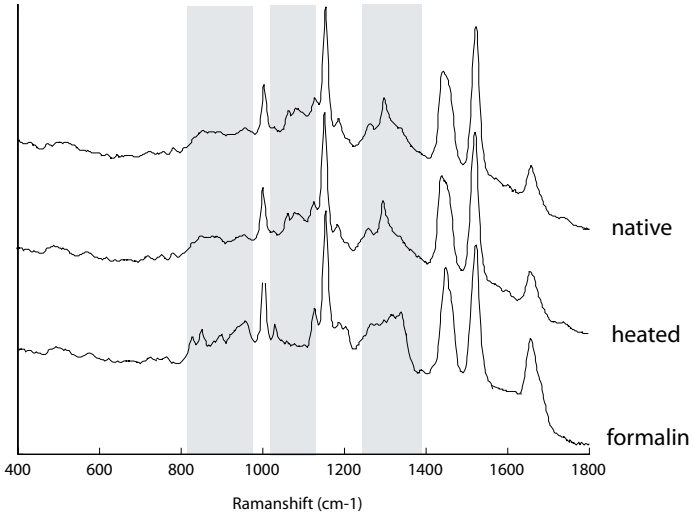

Figure 1: Raman spectra of *M. kansasii* after inactivation with formalin and heating in comparison to the procedure without inactivation. Shaded areas indicate spectral region in which significant effects of formalin inactivation can be observed.

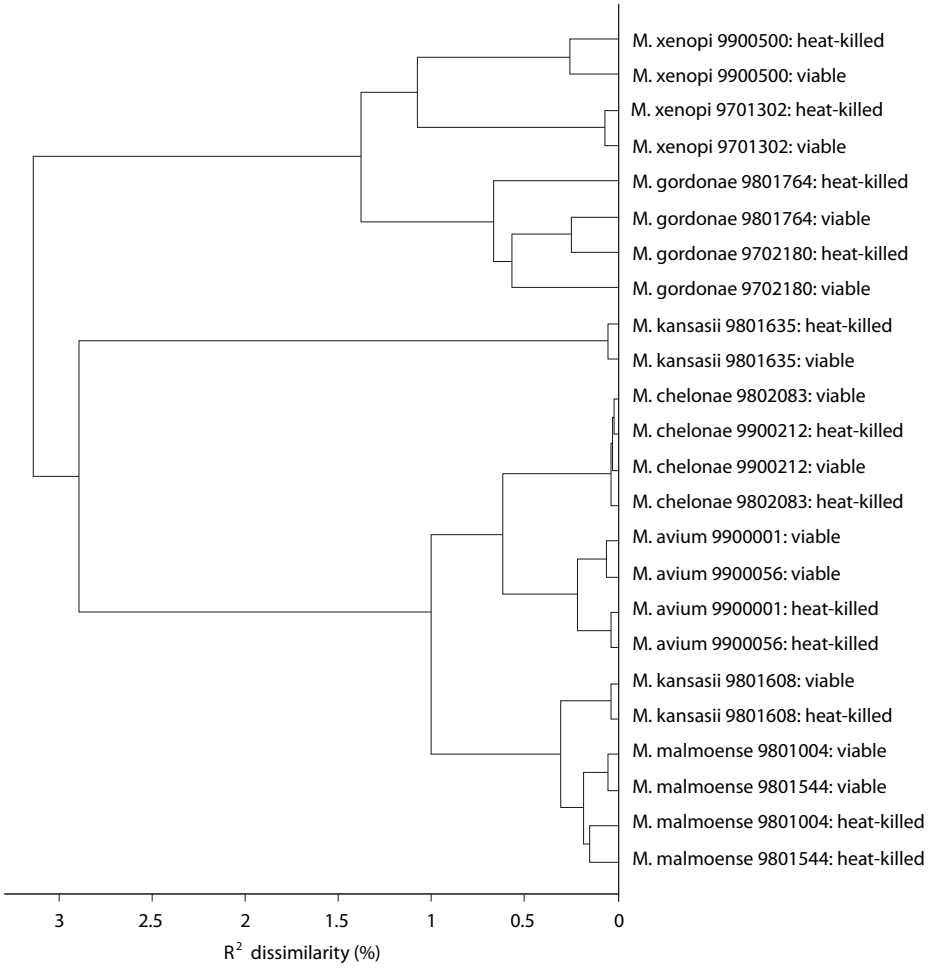

Figure 2: Dendrogram of hierarchical cluster analysis of Raman spectra of the isolates used in the pilot study. Numbers refer to isolates of the collection of the national tuberculosis reference laboratory at the National Institute for Public Health and the Environment.

pilot study. For all isolates spectra obtained from viable and heat-killed samples show low dissimilarities, indicating that the overall classification is not influenced by the pre-treatment.

Reproducibility

In Figure 3 the mean intra-strain similarity between replicate cultures of one isolate and the mean inter-species similarity between isolates of different species are given for both viable and heat-killed mycobacteria. To obtain a reliable discrimination between the different species, the intra-strain similarity should preferably be as high as possible, whereas the inter-species similarity should be much lower.

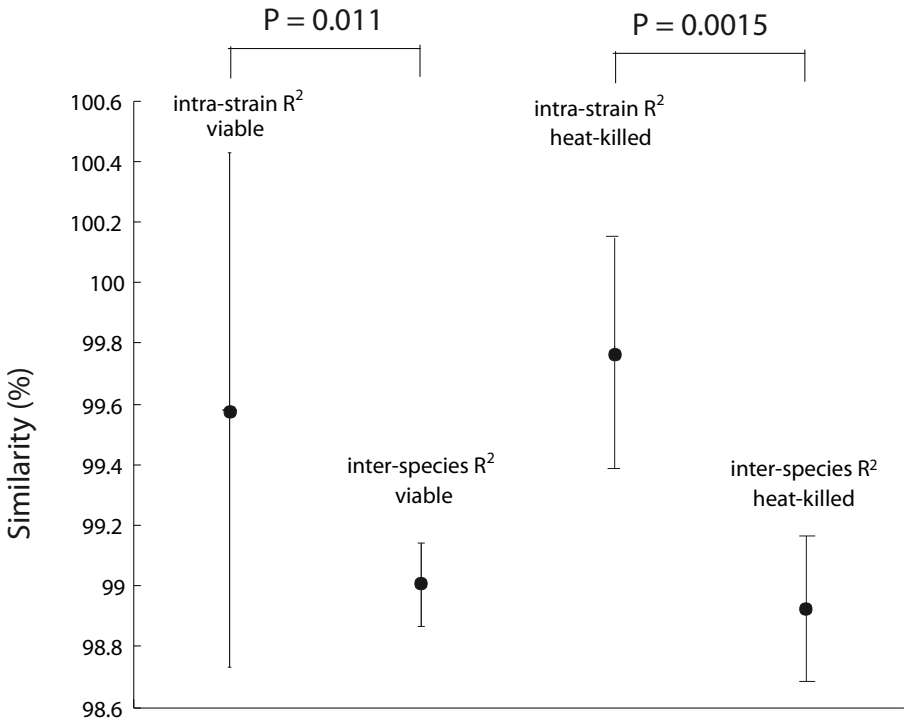

Figure 3: The average intra-strain and inter-species similarities between spectra obtained from native and heat-killed suspensions of 6 *Mycobacterium* strains. The error bar shows the 95% confidence interval.

For all isolates the intra-strain similarity was significantly higher than the inter-species similarity with a p-value of 0.011 when only the viable samples were included and a p-value of 0.0015 for the heat-killed samples.

2. Identification study

Raman spectra of mycobacteria

Representative Raman spectra for the 8 *Mycobacterium* species used in the identification study are shown in Figure 4. Main differences were found in the intensity of the peaks at 1150 cm^{-1} and 1520 cm^{-1}, due to carotenoids. Intense peaks were found for *M. gordonae*, *M. xenopi* and *M. lentiflavum* due to pigmentation of these species.

Classification of mycobacteria based on Raman Spectroscopy

Figure 5 shows the dendrogram resulting from HCA performed on the Raman spectra in the identification set. The *M. tuberculosis, M. gordonae, M. avium* and *M. chelonae* isolates formed separate species-specific clusters. For both *M. lentiflavum* and *M. xenopi*

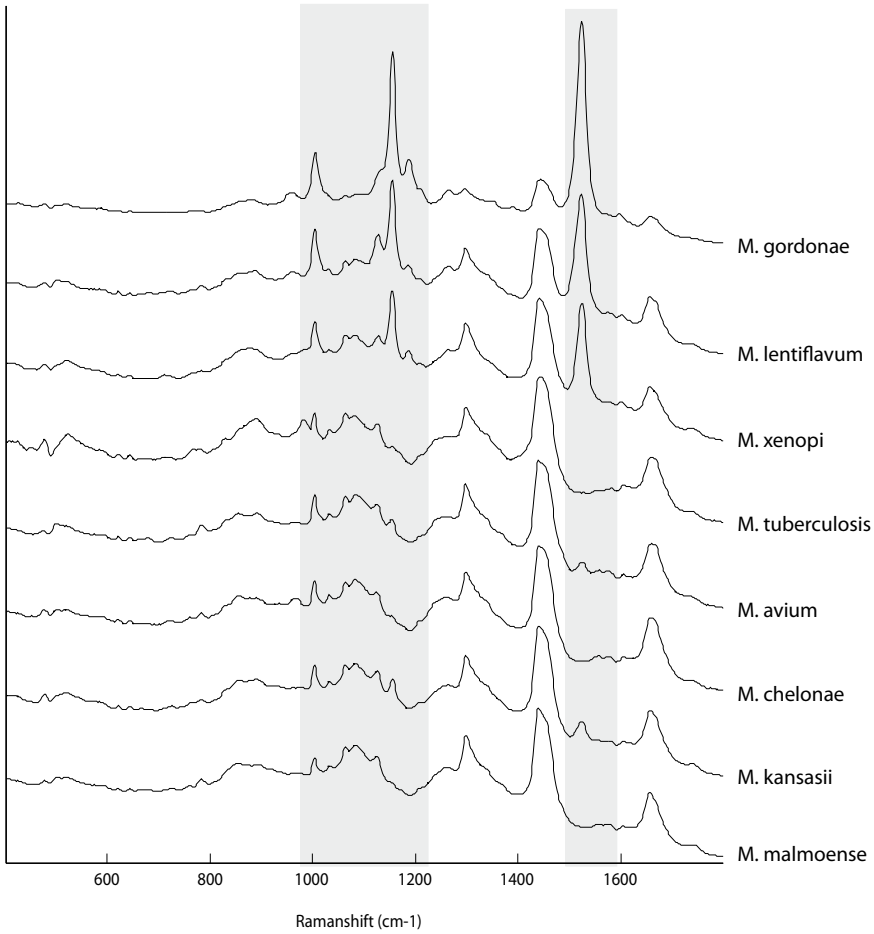

Figure 4: Representative Raman spectra from the 8 *Mycobacterium* species used in the identification study.

two clusters were found. The spectra of *M. malmoense* and *M. kansasii* overlap, but sub clusters on species level can be found.

Identification study

For species identification the R^2 value was calculated for each isolate spectrum with every other isolate spectrum in the dataset. Each isolate to be classified is matched to the isolate in the dataset with the highest R^2 value. The species identity of the isolate with which the highest R^2 occurred determined the species of the tested isolate. The species identification obtained by 16S rRNA gene sequencing was used as the gold standard. This leave-one-out approach simulates the situation in a diagnostic setting where a new measurement is compared to an existing database.

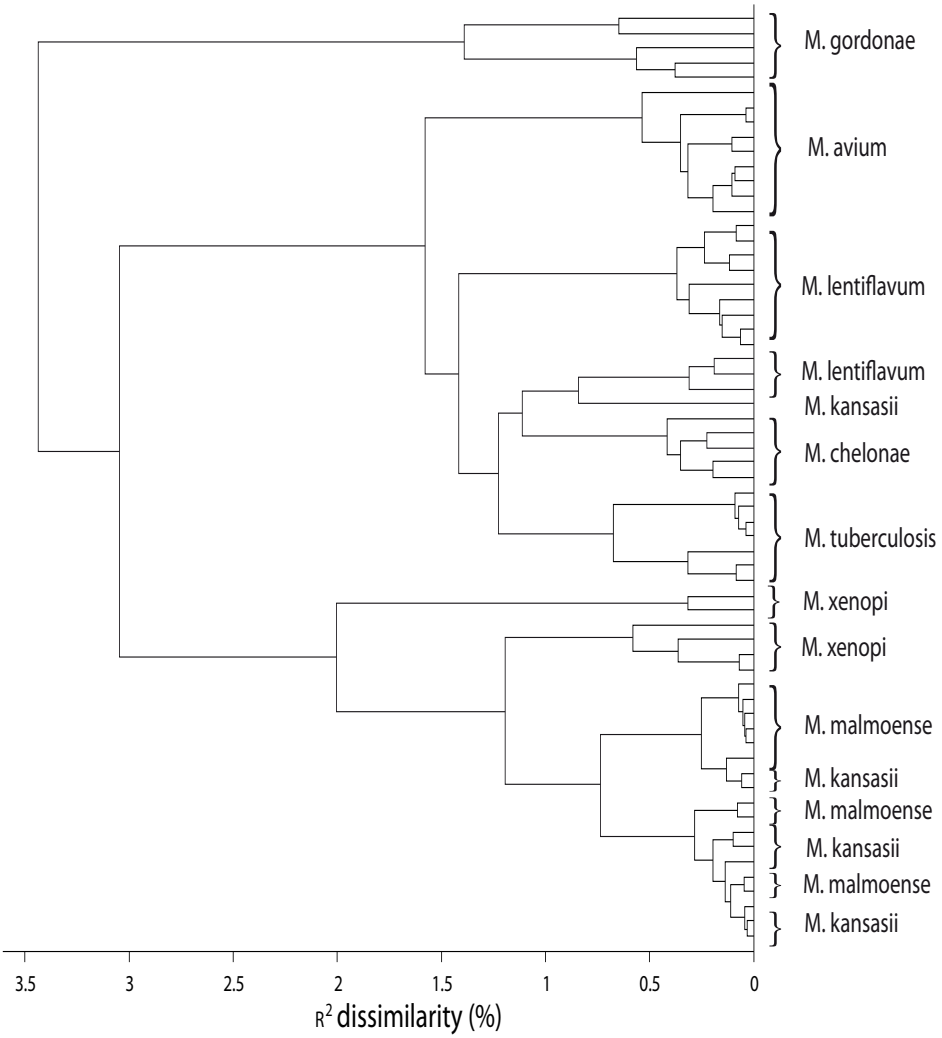

Figure 5: Dendrogram resulting from hierarchical cluster analysis of Raman spectra of the isolates used in the identification.

The overall sensitivity of this model was 95.2% (60 out of 63 measurements, table 2). The differentiation between *M. tuberculosis* and NTM was correct for all strains. Within the group of NTM isolates three strains were misidentified: *M. xenopi* misidentified as *M. malmoense*; *M. kansasii* as *M. lentiflavum*, and *M. gordonae* as *M. lentiflavum*.

Table 2: Classification of *Mycobacterium* species by 16S rRNA sequencing and Raman spectroscopy.

	Raman identification								
16S rRNA sequencing	*M. tuberculosis*	*M. avium*	*M. chelonae*	*M. gordonae*	*M. kansasii*	*M. malmoense*	*M. xenopi*	*M. lentiflavum*	Total
M. tuberculosis	7 (100%)								7
M. avium		9 (100%)							9
M. chelonae			4 (100%)						4
M. gordonae				4 (80%)				1 (20%)	5
M. kansasii					8 (88.9%)			1 (11.1%)	9
M. malmoense						10 (100%)			10
M. xenopi						1 (16.7%)	5 (83.3%)		6
M. lentiflavum								13 (100%)	13
Total	7	9	4	4	8	11	5	15	63

DISCUSSION

Our Raman measurements indicate that efficient discrimination between *Mycobacterium* species can be made. Isolates belonging to a single species were grouped correctly into different clusters, corresponding to *M. tuberculosis*, the most relevant clinical species of NTM and *M. lentiflavum*. Overall, correct species identification was achieved in 95.2% of the samples within three hours of a positive signal of the automated culture system. The differentiation between *M. tuberculosis* and NTM was 100% accurate. As the treatment of NTM disease differs significantly from the treatment of TB, both the rapidity and the accuracy of this new assay are important assets.

The spectra of the strains inactivated by heat-killing showed minimal differences when compared to the spectra of viable mycobacteria. Therefore, identification of mycobacteria was possible without biosafety III precautions during Raman measurements.

For 60 out of the 63 *Mycobacterium* strains analyzed, Raman spectroscopic identification corresponded to the molecular identification test. None of the three misidentifications can currently be explained. We suggest that although the DNA identification methods grouped these bacteria within single species, there still are considerable phenotypic differences between strains in a single species. More detailed DNA sequencing of bacteria sub-grouped by Raman may confirm this hypothesis. Further Raman studies with *Mycobacterium* isolates may reveal the accuracy of the method in discriminating more species. An extended spectral database containing more spectra of other *M. tuberculosis* complex and NTM strains with a larger number of isolates per species has to be established, as was already performed for other microorganisms (7,10,11). In addition, in our study only seven *M. tuberculosis* strains were analysed. The Raman spectroscopy correctly classified all strains, but larger studies will be necessary to confirm and extend these results.

Fourier-Transform Infrared microspectroscopy was successfully used recently to differentiate NTM at the species level (14). To our knowledge, however, our data prove for the first time that Raman spectroscopy can be used for identification of mycobacteria, including *M. tuberculosis* complex. Apart from enabling rapid identification, vibrational spectroscopic techniques require virtually no sample handling or consumables and are, therefore, very cost-effective. This is in sharp contrast to other rapid identification techniques. Although regarded as the gold standard, 16S rRNA sequencing is not appropriate for routine analysis, due to its complexity and high costs.

We conclude that Raman spectroscopy holds much promise for a rapid, accurate, and easy-to-use alternative for the identification of clinically relevant *Mycobacterium* species.

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Chapter 4

Prospects for clinical application of electronic-nose technology to early detection of *Mycobacterium tuberculosis* in culture and sputum

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ABSTRACT

Ziehl-Neelsen (ZN) staining for the diagnosis of tuberculosis (TB) is time-consuming and operator dependent and lacks sensitivity. A new method is urgently needed. We investigated the potential of an electronic nose (EN) (gas sensor array) comprising 14 conducting polymers to detect different *Mycobacterium* spp. and *Pseudomonas aeruginosa* in the headspaces of cultures, spiked sputa, and sputum samples from 330 culture-proven and human immunodeficiency virus-tested TB and non-TB patients. The data were analyzed using principal-component analysis, discriminant function analysis, and artificial neural networks. The EN differentiated between different *Mycobacterium* spp. and between mycobacteria and other lung pathogens both in culture and in spiked sputum samples. The detection limit in culture and spiked sputa was found to be 1×10^4 mycobacteria ml^{-1}. After training of the neural network with 196 sputum samples, 134 samples (55 *M. tuberculosis* culture-positive samples and 79 culture-negative samples) were used to challenge the model. The EN correctly predicted 89% of culture-positive patients; the six false negatives were the four ZN-negative and two ZN-positive patients. The specificity and sensitivity of the described method were 91% and 89%, respectively, compared to culture. At present, the reasons for the false negatives and false positives are unknown, but they could well be due to the nonoptimized system used here. This study has shown the ability of an electronic nose to detect *M. tuberculosis* in clinical specimens and opens the way to making this method a rapid and automated system for the early diagnosis of respiratory infections.

INTRODUCTION

The World Health Organization (WHO) has declared tuberculosis (TB) a global emergency. It is estimated that one-third of the world's population is infected with *Mycobacterium tuberculosis*. An estimated 8 to 9 million new cases occur each year, with 2 to 3 million deaths (4). The majority of these new infections and deaths occur in developing countries. The human immunodeficiency virus (HIV) epidemic has massively contributed to the worldwide tuberculosis problem.

The usual method of diagnosing TB in low-income countries is by detection of acid-fast bacteria in sputum by direct microscopy. When done properly, 60 to 70% of all adults with pulmonary TB can be identified using the Ziehl-Neelsen (ZN) staining procedure, followed by microscopic examination (11,19). However, in areas of endemicity, laboratories are often overloaded with samples for smear examination. Therefore, a new simple and rapid diagnostic test should directly replace microscopy with similar specificity and sensitivity (19). In the past, research was mainly focused on the development of either antibody/antigen detection assays or the development of nucleic acid amplification reactions.

Against this background, we have investigated the potential of a gas sensor array ("electronic nose" [EN]) to detect *M. tuberculosis* in culture and sputum. It is well known that smell can be used to diagnose diseases, and it has been used by both the Greeks and the Chinese since 2,000 BC (12). Electronic nose is the colloquial name for an instrument made up of chemical sensors combined with a pattern recognition system (5). The reversible adsorption of volatile organic compounds (VOCs) to the sensor surface leads to a change of physical properties (conductivity, resistance, and frequency) of the sensor, which is measured. The key function of an EN is to mimic the human olfactory system by combining nonspecific gas sensors with a pattern recognition system to analyze and characterize complex odors without separation of the mixture into individual components. In the EN, the human olfactory receptors have their analogues in chemical sensors that produce an electrical signal (similar to nerve cells). Each sensor within the array is characterized by partial and overlapping specificities to VOCs. Due to the partial and overlapping specificities, a unique response curve is recorded during the measurement by each sensor containing the vital information to allow discrimination of the different samples. To describe this information, the response curve is described by mathematical terms expressed as maximum absorption rate, desorption rate, maximum response (or divergence), and area under the response curve. These mathematical terms are subsequently analyzed by pattern recognition software. The pattern recognition software corresponds to the cerebral cortex of the brain and is able to classify and memorize odors (1,18).

Electronic noses have been applied mainly in the food industry to characterize the odors of beverages (2,17) or olive oil (7). More recently, researchers discovered the potential of

electronic noses as a diagnostic tool for the detection of *Mycobacterium bovis* in badgers and cattle (3). A review of medical applications is given elsewhere (15,23).

The aim of this study was to investigate the potential of an electronic nose to detect *Mycobacterium tuberculosis* and other pathogens in both culture and patients' sputa as a first step toward simple breath analysis for the specific, rapid, and noninvasive diagnosis of diverse lung infections.

MATERIALS AND METHODS

Liquid cultures

All bacteria (*Mycobacterium tuberculosis*, RIVM myc 4514; *Mycobacterium avium*, RIVM myc 3875; *Mycobacterium scrofulaceum*, RIVM myc 3442; and *Pseudomonas aeruginosa*, AMC 23123) were cultured in Middlebrook 7H9 medium with oleic acid-albumin-dextrose-catalase enrichment. The bacteria were incubated at 37°C until an optical density (420 nm) of 0.30 (2 x 10^8 bacteria ml^{-1}) was reached.

Sputum samples

The study was approved by the ethics committee of the Academic Medical Center, Amsterdam, The Netherlands, and The Saint Francis Hospital in Katete, Zambia. All subjects gave written permission for sputum sampling after oral and written information was provided.

Sputum samples were collected from 280 patients with suspected TB either from The Saint Francis Hospital in Katete, Zambia (n = 80), or from the WHO Sputum Bank (n = 200) (through the WHO Specimen Bank). In addition, sputum samples were collected at the Academic Medical Center, Amsterdam, The Netherlands, from 7 patients with proven pneumonia (caused by *Streptococcus pneumoniae*) and from 50 patients with proven non-TB (serving as negative controls). All patients were examined by chest X ray, and their sputum samples were examined by ZN staining and liquid culture (either by BacT/ALERT from bioMérieux, France, or by Bactec MGIT 960 from Becton Dickenson). Culture was used as the "gold standard" in this study as both inclusion and exclusion criteria for TB. Furthermore, the HIV status and the smoking habits of the suspected TB patients were investigated. The collected sputum samples were stored at –70°C until the analysis was performed.

Spiked sputum samples

A pool of 25 sputum samples (each 1 ml) was made from the above-mentioned non-TB patients (n = 50) for spiking purposes. The sputum pool was spiked with various numbers of different bacterial isolates, including *M. tuberculosis*, *M. avium*, and *P. aeruginosa*, as

well as a mixture (50:50) of *M. tuberculosis* and *P. aeruginosa*, which served as a mixed-infection sample.

Sample preparation and headspace analysis

The liquid cultures were cooled to 4°C and allowed to equilibrate for at least 20 min to minimize the loss of volatiles during the transfer into smaller-headspace vials. Two milliliters of the "cold" culture was transferred into a 5-ml-headspace vial (Macherey and Nagel, United Kingdom) and immediately sealed with a silicon/Teflon crimp cap (Jaytee Bioscience Ltd., United Kingdom). The headspace was allowed to equilibrate for 45 min at 37°C.

The frozen sputum samples were defrosted on ice to minimize the loss of volatiles; 0.5 ml of "well-mixed" sputum was transferred into a sterile 5-ml-headspace vial, mixed with 0.5 ml of a 1 M NaCl solution (4°C), and subsequently sealed.

Spiked sputum samples were prepared by mixing 0.5 ml of non-TB sputa (individual samples or a pool) with 0.5 ml of "cold and equilibrated" bacterial suspension containing 1 M NaCl. Negative control samples were prepared by mixing 0.5 ml of individual non-TB sputum samples with 0.5 ml of a 1 M NaCl solution (4°C). All control samples were prepared in 5-ml-headspace vials as described above. All sputum samples were incubated at 37°C for 330 min prior to the headspace analysis. Two cycles of freezing and thawing had no influence on the results (not shown).

Gas-sensing system and headspace sampling

For this study, an electronic nose (Bloodhound BH-114; Bloodhound Sensors, Leeds, United Kingdom) that employed 14 conducting polymers based on polyaniline was used. The sensor unit automatically set two calibration points. The first was the baseline, which was obtained when activated-carbon-filtered (Carbon Cap 150; Whatman) air was passed over the sensor at a flow rate of 4 ml min^{-1}. The second calibration point was a reference point obtained from the headspace of a control sample vial containing 9 ml of distilled water.

The interaction of the VOCs with the conducting polymer surface produced a change in resistance over time, which was measured and subsequently displayed on a computer screen for each sensor. The curve was similar to the classical Langmuir adsorption curve. Two sensor parameters were selected to study the sensor response: divergence (maximum response) and area under the response curve. The sampling profile was set at 6 seconds of absorption and 14 seconds of desorption for the analysis of liquid samples; for the analysis of spiked sputum, we used 7 seconds of absorption and 21 seconds of desorption.

For the analysis of the unknown headspace, the sample vials were connected to the electronic nose by inserting a needle into the headspaces of the sample vials. The unknown headspace was passed over the sensor surface at a flow rate of 20 ml min^{-1}, which was automatically set by the sensor unit. Between each pair of measurements, a time delay of

2 min was set. The individual samples in each experiment were tested in a randomized, blinded fashion.

Data analysis

Principal-component analysis (PCA), discriminant function analysis (DFA), and an artificial neural network (ANN) were applied to analyze the multivariate data. The sensor response was normalized prior to the multivariate data analysis (except for the determination of the detection limit). To perform these analyses Excel add-in software (*XL*stat version 3.4) was used. Two sensor parameters, namely, the maximum response and the area under the response curve (Langmuir adsorption spectrum), were used to perform the analysis.

Principal-component analysis is a method aimed at reducing the amount of data when there is a correlation present. The idea is to find principal components (PC), which are linear combinations of the original variables (sensor responses) describing each specimen. In other words, PCA projects the original data matrix from a high-dimensional space into a lower-dimensional space (a three-dimensional space or plane) without losing essential information (variance). The relationship between samples can be visualized by plotting individual principal components against each other (13).

Discriminant function analysis is a supervised classification method aimed at finding a formal decision boundary between classes. The idea is to find linear discriminant functions (S_1, S_2, …, S_n), which are linear combinations of the original variables. The classification model (DFA) was built on the first four PC, which normally account for over 90% of the variance (information) of the original data matrix. In each case, the DFA model was cross-validated as follows. Data from individual samples (culture or sputum) were withheld, and a DFA model was built on the remaining data set (training set). The data from the withheld samples (testing set) were then inserted into the discriminant functions and subsequently assigned to the class for which the centroid had the smallest Euclidean distance to the unknown sample. The result could be visualized by plotting the individual discriminant functions against each other (13).

Artificial neural networks are attempts to mimic the neurons in the human brain and were used here for classification of sputum samples into TB and non-TB. Such networks have a number of linked layers of artificial neurons, including input, hidden, and output layers. The ANN is trained using a large training set of sputum samples from suspected TB patients. In this study, a back propagation network with a sigmoid transfer function was applied. During the training period, the weights connecting individual neurons were adjusted so that the error between output signal and target signal was minimized (13). The performance of the ANN was evaluated using the test set of sputum samples. Only the sputum samples from the suspected TB patients (n = 280) and from the proven non-TB cases (n = 50) were included in the ANN analysis.

RESULTS

Specificity of the EN

The "smells" of three *Mycobacterium* sp. cultures (*M. tuberculosis*, *M. avium*, and *M. scrofulaceum*) and one *Pseudomonas aeruginosa* culture were analyzed and compared to the "odor" of blank medium. The raw EN data were analyzed by PCA (data not shown), followed by DFA. The DFA model was built on the first four principal components. Figure 1 shows the results of the DFA analysis. It was possible to distinguish between the different bacterial classes using the first two discriminant functions (S_1 and S_2). The three different *Mycobacterium* spp. were grouped closely together but still allowed discrimination (Fig. 1, top). The DFA model was validated by the analysis of 15 "unknown" samples. All unknown samples were correctly classified as either one of the three *Mycobacterium* spp., *P. aeruginosa*, or blank medium.

Similarly, when the negative pooled sputum samples were spiked with *M. tuberculosis*, *M. avium*, *P. aeruginosa*, and a mixture of *M. tuberculosis* and *P. aeruginosa* (at a final concentration of 1×10^8 bacteria ml^{-1}) and analyzed, it was possible to distinguish between "unspiked" sputum and "spiked" sputum samples (Fig. 1, bottom). Within the spiked sputum samples, a difference in smell was observable for the different bacterial classes. The DFA model was validated by the analysis of 10 unknown samples. All unknown samples were correctly identified as unspiked sputum or spiked sputum. Within the spiked sputum samples, all unknown samples were correctly assigned to one of the four "subclusters" representing the different bacterial classes (Fig. 1, bottom).

Analytical sensitivity of the EN

Six different concentrated *M. tuberculosis* suspensions (1×10^3 mycobacteria ml^{-1} to 1×10^8 mycobacteria ml^{-1}) were analyzed and compared to blank medium. The raw EN data were analyzed by PCA (data not shown), followed by DFA. The results of the DFA analysis are shown in Fig. 2, top. The DFA model was validated by the analysis of 14 "unknown" samples. Two out of 14 unknown samples were incorrectly classified as blank medium (Fig. 2, top). Both incorrectly classified samples belonged to the group containing 1×10^3 mycobacteria ml^{-1}. All other unknown samples were correctly identified. Therefore, the detection limit was determined to be as low as 1×10^4 mycobacteria ml^{-1}.

The detection limit for *M. tuberculosis* in spiked pooled sputum samples was also determined. For this purpose, a sputum pool was divided into two parts. One part was spiked with an *M. tuberculosis* suspension containing 1×10^4 mycobacteria ml^{-1}. The raw EN data were analyzed by PCA (data not shown), followed by DFA. The bottom panel of Fig. 2 shows that it was possible to distinguish between spiked and unspiked samples. The DFA model was validated by the analysis of 12 "unknown" samples. All "unknown"

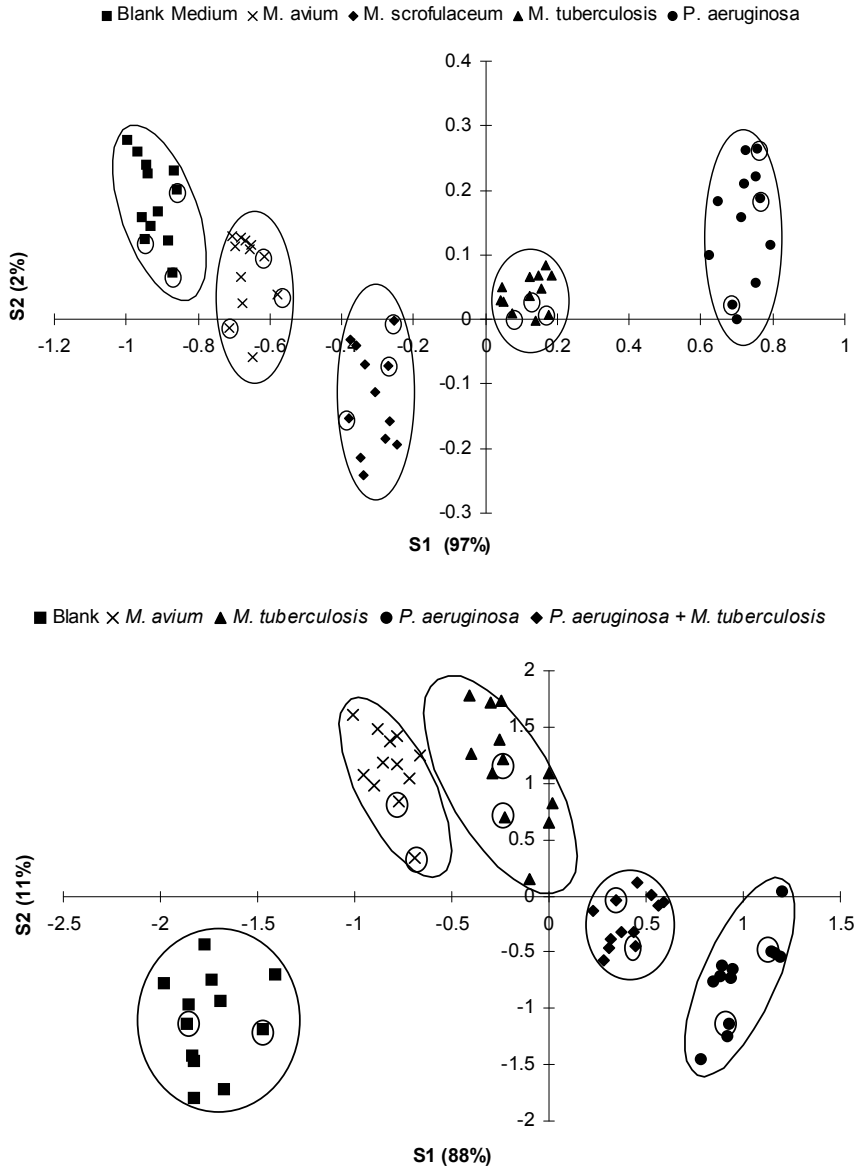

Figure 1: (Top) DFA analysis of liquid cultures of *M. tuberculosis* (12 samples), *M. avium* (12 samples), *M. scrofulaceum* (12 samples), and *P. aeruginosa* (12 samples) and blank medium (12 samples). Cross-validation: 15 samples (3 from each group) were withheld from building the DFA model but were subsequently assigned correctly once the model was built (encircled symbols). S1 and S2, discriminant functions 1 and 2. The numbers in parentheses indicate the percentages of the data matrix described by the relevant functions. The circles were added by the authors.
(Bottom) DFA analysis of sputum samples spiked with *M. tuberculosis* (12 samples), *M. avium* (12 samples), *P. aeruginosa* (12 samples), mixed infection (12 samples), and blank sputum (12 samples). Cross-validation: 10 samples (2 from each group) were withheld from building the DFA model but were subsequently assigned correctly once the model was built (encircled symbols).

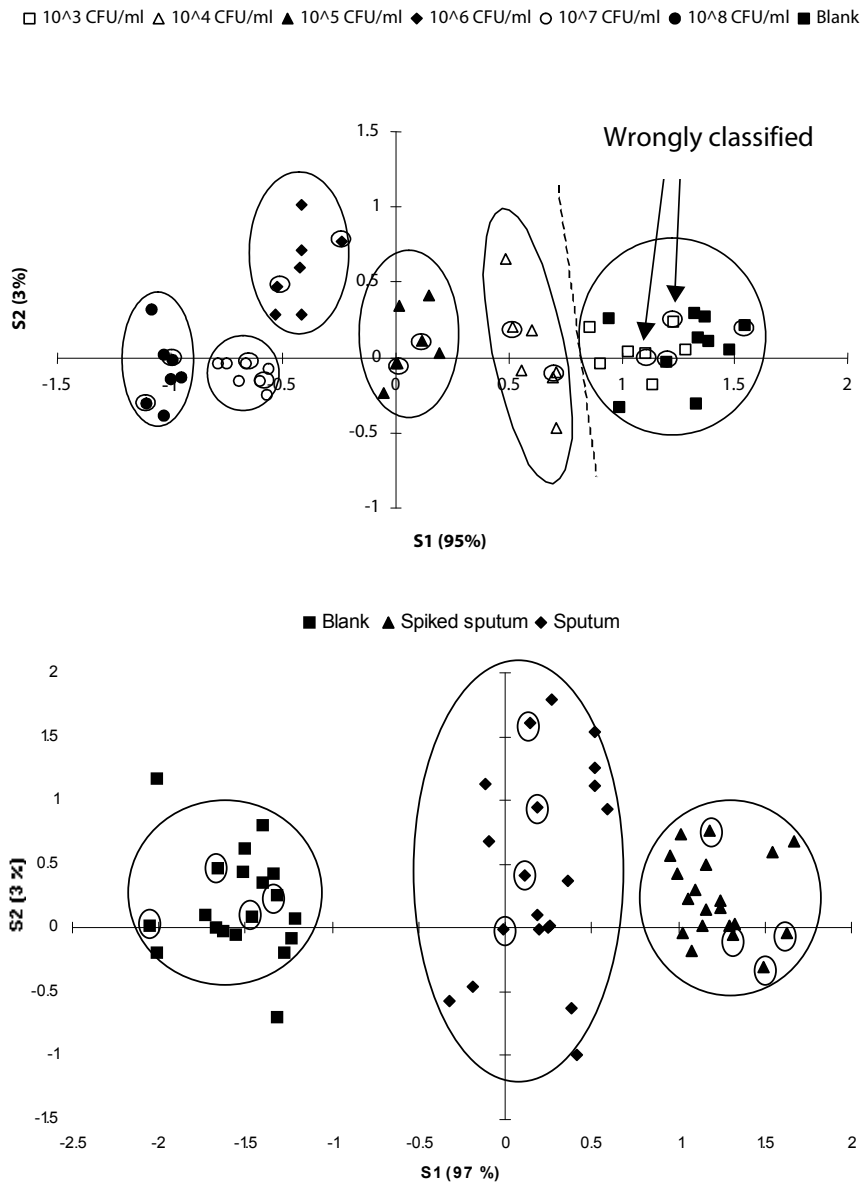

Figure 2: (Top) Determination of the detection limit of the electronic nose for *M. tuberculosis* in liquid culture. Six different concentrations ranging from 1 x 10^3 to 1 x 10^8 mycobacteria ml^{-1} were analyzed (seven samples for each concentration and seven blanks). Cross-validation: 14 samples (2 samples from each group) were withheld from building the DFA model. Samples containing more than 1 x 10^4 mycobacteria ml^{-1} were correctly assigned (encircled symbols). In contrast, blank medium and samples containing 1 x 10^3 mycobacteria ml^{-1} could not be distinguished from each other.
(Bottom) Determination of the detection limit of the electronic nose for *M. tuberculosis* in spiked sputum. The sputum was spiked with 1 x 10^4 mycobacteria ml^{-1}. Twenty replicates were analyzed for each group. Cross-validation: 12 samples (4 samples from each group) were withheld from building the DFA model but were subsequently assigned correctly once the model was built (encircled symbols).

samples were correctly classified (Fig. 2, bottom). The variability within the unspiked samples was higher than within the spiked samples.

Performance of the EN using clinical samples

The PCA analysis of 50 positive control samples (i.e., the individual non-TB sputum samples spiked with 10^8 *M. tuberculosis* cells ml^{-1}), 50 negative non-TB sputum samples from The Netherlands, and 280 clinical samples from Africa (Table 1) is shown in Fig. 3. It was possible to obtain good discrimination between TB and non-TB samples. As shown in Fig. 1, TB-negative samples were found on the left-hand side, whereas TB positive samples were on the right-hand side. Nevertheless, no complete separation could be obtained, as indicated by the overlapping circles in Fig. 3. In both groups (TB positive and TB negative), a subcluster could be identified. These subclusters contained the samples of smoking patients. However, not all smokers were present in this subcluster (89% were present). Patients suffering from pneumonia formed a separate cluster.

After training the neural network with 196 of the original samples (133 TB and 63 non-TB samples), the remaining 134 samples were used to validate the model. Among the 134 samples were 55 culture-confirmed TB samples, of which 51 were ZN positive and 4 were ZN negative. The results from the ANN are summarized in Table 2. The neural network was able to predict 49 TB-positive patients out of 55 correctly. Six suspected TB culture-positive samples gave a false-negative result. Among these six false negatives were four ZN-negative and two ZN-positive patients. Three of the four ZN-negative false negatives were HIV positive, and one was a smoker. Among the ZN-positive false negatives was one HIV-positive patient, who was also a smoker.

The neural network was also able to predict 72 suspected TB-negative patients correctly. Seven TB-negative patients gave a false-positive result. One false-positive patient was HIV positive and a smoker.

Table 1: Details of patients that provided clinical samples, showing TB status (culture), sex, HIV status, and smoking habits.

Status	Total no.	% Males (no.)	% HIV+ (no.)	% Smokers
TB+	188	67.0 (126)	53.7 (101)	31.4 (59)
TB-	142	59.2 (84)	29.6 (42)	9.2 (13)
Total	330	63.6 (210)	43.3 (143)	21.8 (72)

Table 2: Performance of the electronic-nose-neural-network system in comparison to culture.

Status	No. (%) with culture confirmed TB	No. (%) culture TB negative	Total
EN positive	49 (89.9)	7 (8.9)	56
EN negative	6 (10.9)	72 (91.1)	78
Total	55 (100)	79 (100)	134

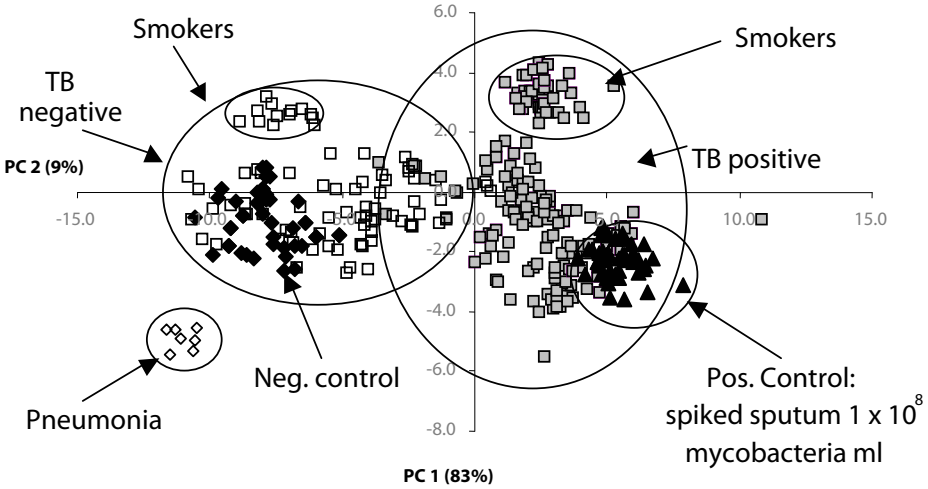

Figure 3: PCA plot showing the analysis of negative (Neg.) control samples (50 samples), positive (Pos.) control samples (50 samples), confirmed pneumonia (7 samples), and clinical samples (92 samples, TB negative; 188 samples, TB positive). PC 1 and PC 2 are the first two principal components; the numbers in parentheses represent the percentages of information described by each principal component (circles were added by the authors).

The sensitivity for the detection of culture-proven TB was 89% (95% confidence interval [CI], 80 to 97%), the specificity was 91% (95% CI, 85 to 97%), and the positive and negative predictive values were 88% (95% CI, 78 to 96%) and 92% (95% CI, 86 to 98%), respectively.

DISCUSSION

Current global TB control depends on the diagnosis of cases, followed by adequate treatment. The available laboratory methods for the detection of *M. tuberculosis* do not fully meet the need in environments with high TB and HIV prevalences (19).

This study showed that volatile detection through electronic-nose technology is able to identify *M. tuberculosis* in both cultures and sputum samples. It has long been established that smell can be used to diagnose diseases, such as diabetes and uremia (10,20). Pavlou and Turner (15) and Pavlou et al. (14,16) were among the first to apply electronic noses in medical diagnostics. They showed that different bacteria, such as *Helicobacter pylori*, *E. coli*, and *M. tuberculosis*, generate a unique "smell" and can therefore be differentiated from each other, allowing a diagnosis. Recently, the same EN used in this study was shown to be able to diagnose *M. bovis* infection in badgers and cattle (3).

Electronic-nose technology offers certain advantages, such as a low detection limit (5 to 0.1 ppm) (21), cost- and time effectiveness, robustness, simplicity, and operator

independence, in contrast to molecular or immunologically based assays. Therefore, we investigated the ability of an electronic nose to detect *Mycobacterium* spp. and other lung pathogens in culture and sputum. In this study, we showed that *Mycobacterium* spp. and *Pseudomonas aeruginosa* emit characteristic volatiles, allowing discrimination between the different bacterial classes in both culture and sputum (Fig. 1). The intragroup (class) variability is greater in sputum than in culture. The reasons for this observation are not clear. The volatility of molecules is influenced by parameters such as sample viscosity, equilibrium temperature, and concentration (22). Since the incubation parameters for liquid and sputum samples were similar, we assume that the higher viscosity, the heterogeneity, and/or a stronger background "smell" of sputum might be responsible for the variability. As shown in Fig. 1, bottom, and 2, bottom, by adding *M. tuberculosis* to sputum, an additional "odor" was introduced into the sample headspace, leading to a reduced intragroup variability. This indicates that mycobacteria release enough volatiles into the headspace, even in a complex matrix (sputum), to allow a diagnosis at low concentrations (1×10^4 mycobacteria ml^{-1}). This result is of extreme importance for clinical diagnosis, where sputum is the usual source for TB detection.

Electronic noses show a linear relationship between sensor response and concentration (8). This concentration dependency was exploited here to determine the detection limit (Fig. 2). This relationship might also reveal relevant clinical information. For the treatment itself, it is not important how many bacteria are present in sputum, but the number of bacteria present greatly influences the infectiousness of patients. This opens the possibility to predict not only the presence of TB, but also the risk for patients to transmit the disease.

The sensitivity of the ZN stain compared to culture under field conditions is at most 50 to 60% (19). With the method presented, we achieved a specificity of 91% and a sensitivity of 89% compared to culture (Table 2). The electronic nose was unable to detect four ZN-negative but culture-positive specimens (false negatives). The bacterial loads in these four specimens were most likely below the detection limit of the electronic nose under the current setup, but larger numbers of ZN-negative but culture-positive specimens need to be tested. The detection limit of the electronic nose for *M. tuberculosis* in spiked sputum was 10^4 mycobacteria ml^{-1} (Fig. 2, top). However, the two remaining false-negative specimens had a positive ZN stain (1+) and should therefore contain enough mycobacteria to cause a sufficient sensor response. At present, we do not know the reasons for either the false negatives or false positives. They could well be due to the nonoptimized system used here or to sample degradation during storage.

Clinical specimens are more diverse than spiked samples. It is assumed that the viscosity, the background smell, and especially, the heterogeneity of sputa influence the outcome of the analysis. Interestingly, smoking itself did not affect the analysis in terms of diagnosing TB. However, not all smokers were grouped in the subclusters shown in Fig.

3. The individual smoking habits (number of cigarettes per day and last cigarette before sample taking) could not be established. We assume that certain smoke ingredients give rise to a slightly different sensor response, allowing separation.

Seven cases of pneumonia were among the clinical specimens. As shown in Fig. 3, they formed a separate cluster. This indicates that the causative agent (in this case, *S. pneumoniae*) for pneumonia generates a different volatile profile (smell) than mycobacteria. This is of clinical importance, showing the potential to differentiate between TB cases and cases of other respiratory diseases.

To date, it is unknown which volatile compounds are responsible for the sensor response. We assume that the response is caused by the combined effects of (i) microbial metabolites and (ii) volatile cellular compounds. In the past, many research groups tried to identify volatile substances emitted from microorganism using gas chromatography or gas chromatography-mass spectroscopy. Each *Mycobacterium* species synthesizes a unique set of mycolic acids, among other substances, which might allow discrimination between different *Mycobacterium* species (Fig. 1) (9). In contrast, *Pseudomonas aeruginosa* emits sulfur compounds and esters (6).

The described method is not yet fully optimized for "field" application. Nevertheless, it potentially fulfils all requirements for a new diagnostic tool for TB (19), including robustness, simplicity, sensitivity, and cost-effectiveness. Among many advantages are the simple sample preparation and its amenability to automation. Together with an appropriate classification model, this method has the potential to become a rapid and automated system for the early diagnosis of respiratory diseases through sputum or even breath analysis. It might also be possible to improve or modify currently available sensors toward specific *M. tuberculosis* markers, which would simplify the optimization of such a system.

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Chapter 5

Mycobacterium szulgai causes tuberculosis-like disease in Zambia

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ABSTRACT

In Africa, the diagnosis of tuberculosis (TB) is almost invariably based on the microscopic examination of Ziehl-Neelsen-stained clinical material. However, not only *Mycobacterium tuberculosis*, but also non-tuberculous mycobacteria (NTM) yield positive results in the microscopic detection of acid-fast bacilli (AFB). Furthermore, a significant part of the patients, especially HIV-positives, may represent AFB-negative, albeit culture-positive, mycobacteriosis.

In Sesheke, Zambia, 64 (both HIV positive and negative) patients, who were chronically ill for more than two weeks, were included in a study on the etiology of tuberculosis-like disease using modern culture and identification methods. Thirty out of 64 (47%) patients yielded positive *Mycobacterium* cultures that were identified as *M. tuberculosis* (8 times), *M. szulgai* (7), *M. avium-intracellulare* (3), *M. simiae* (1) and *M. terrae* (1). Ten isolates were not suitable for identification due to contamination or re-culture problems. Twelve of the 30 (43%) culture-positive patients were also positive in microscopic examination, including four patients with NTM isolates. Seven of the 22 NTM isolates belonged to *M. szulgai*. The patients with *M. szulgai*- positive sputum manifested symptoms extremely similar to tuberculosis caused by *M. tuberculosis.* DNA fingerprinting analysis revealed four different patterns among the seven *M. szulgai* isolates, excluding the possibility of a common source of infection or a laboratory cross-contamination. Three out of five patients with *M. szulgai* infection responded to anti-tuberculosis treatment.

The contribution of NTM, and especially of *M. szulgai*, to tuberculosis-like diseases in both HIV-positive and negative patients in Africa may be underestimated.

INTRODUCTION

Tuberculosis remains an important global public health problem that has been exacerbated by the HIV epidemic. In the year 2000 an estimated number of 8-9 million new tuberculosis (TB) cases occurred. Worldwide, an estimated 11% of new adult tuberculosis was co-infected by HIV, while in Sub-Saharan Africa about 38% of new TB cases were attributable to HIV(1).

The clinical presentation of tuberculosis in HIV-infected individuals depends on the severity of suppression of immunological functions. In the early stages of HIV infection, it resembles that of HIV-negative individuals with more pulmonary involvement and localized lesions. The clinical presentation tends to be more generalized with the progressive suppression of immunological functions. In many Third World countries the diagnosis of tuberculosis is based on clinical assessment, a sputum smear, and radiological features. Symptoms are often non-specific in HIV-positive patients and a sputum smear is positive in only 50-80% of individuals with culture-confirmed cases of pulmonary tuberculosis, resulting in the delay or misdiagnosis of tuberculosis in these countries (2).

Furthermore, in developed countries it has been reported that HIV-infected patients are susceptible to infections by non-tuberculous mycobacteria (NTM), such as *Mycobacterium avium* complex (3). The isolation of these bacteria generally raises questions concerning their clinical significance and the criteria described in a Statement by the American Thoracic Society (ATS) are widely used to guide the diagnostic process (4,5). The incidence of disseminated *Mycobacterium avium* complex infections in HIV patients in the Western world has decreased significantly with the introduction of more potent anti-HIV treatments. In Africa, HIV treatment is not yet widely available. However, the isolation of NTM is rarely reported, although the prevalence of HIV infection is high in many areas. This may be due to a lack of representative studies and/or more generally to the absence of suitable diagnostic tools.

In Yeta District Hospital in Sesheke, Zambia, during the late 1990s an increasing number of HIV-positive patients, clinically suspected of pulmonary tuberculosis, had a negative sputum smear but reacted positively to tuberculosis treatment. This suggested an increasing prevalence of sputum smear-negative tuberculosis. On the other hand, NTM can cause a broad spectrum of diseases, often mimicking tuberculosis, and the contribution of NTM to tuberculosis-like diseases in Zambia is unknown.

The aim of this pilot study was to evaluate the role of NTM in the development of tuberculosis-like disease in Africa and the confusion that this may cause in the diagnosis

of tuberculosis by microscopy. This is especially relevant in the light of the increasing prevalence of HIV.

MATERIALS AND METHODS

All chronically ill patients admitted to Yeta District Hospital in Sesheke, Zambia, who did not respond to anti-malaria treatment or routine antibiotics in the period May to September 2000 were included in this study. Patients were considered chronically ill if they were suffering from chronic complaints for more than two weeks. Signs and symptoms in any part of the body were considered because tuberculosis can affect all parts of the body. In total 64 patients were included who produced sputum for a smear and culture. Most of the patients included complained about coughing and/or chest pain (75%) and general malaise (19%). The remaining 6% of the patients had lymphadenopathy or abdominal complaints.

Informed written consent to participate in the study was obtained from all patients.

The medical officer in charge documented the medical history and a general physical examination was performed. A daily follow-up of signs and symptoms was done.

A sputum specimen was cultured for mycobacteria. The sputum was divided into two equal parts. One half was decontaminated using 6% sulphuric acid and cultured on Löwenstein-Jensen (LJ) medium and the other half was stored at −20°C in Zambia. In The Netherlands the latter half was decontaminated with *N*-acetyl-L-cysteine (NALC)-NaOH and cultured in Mycobacteria Growth Indicator tubes (Becton Dickinson Microbiology Systems, Cockeysville, Md.) (6). Serological testing for HIV was performed using a particle agglutination test (Serodia HIV1/2, Fujirebio Inc., Tokyo, Japan) and the AxSYM HIV Ag-Ab Combination Assay (Abbott GmbH Diagnostika, Wiebaden-Delkenheim, Germany). Mycobacterial isolates were identified by the Accuprobe culture confirmation test for the *M. tuberculosis* complex (Accuprobe, bioMérieux, Marcy l'Etoile, France) or by 16S rRNA gene sequencing (7).

Patients suspected of pulmonary tuberculosis were treated with anti-tuberculosis drugs according to the Zambian government policy. The treatment protocol for newly diagnosed patients who had smear positive sputum tests for AFB was two months of isoniazid, rifampicin, pyrazinamide and ethambutol and six months of isoniazid and ethambutol (2HRZE/6EH). In cases of smear negative results the same protocol was applied without pyrazinamide in the first two months (2HRE/6EH).

Drug susceptibility of the mycobacterial isolates was determined using the minimal inhibition concentration method (MIC) (8). Series of dilutions of anti-tuberculosis drugs were added to melted 7H10 agar and poured into 25-well plates. Ten µl of suspension with a turbidity of McFarland 1 were inoculated into each well. As a control also 10 µl of a 1:100 diluted suspension was inoculated on 7H10 without additives to determine a 1% cut-off. The growth on these wells was compared after 5, 12, and 19 days of incubation. The MIC is the concentration at which more than 99% of the growth is inhibited.

Typing of all *M. tuberculosis* isolates was performed by IS*6110* RFLP analysis and the seven *M. szulgai* isolates were subjected to random amplified polymorphic DNA (RAPD) analysis using rapd 1, ERIC2, 1749, 1750 and 1752 as primers (9,10). Apart from the seven Zambian *M. szulgai* isolates, two independent *M. szulgai* isolates from The Netherlands were included as controls.

RESULTS

The study population consisted of 64 Zambian patients, of which 51 (79%) were HIV-positive. Thirty-five female and 20 male adults over 15 years of age and nine children were included.

Thirty out of 64 (47%) patients yielded positive *Mycobacterium* cultures of their sputum (see Table 1). *M. tuberculosis* was isolated from eight patients, of whom four patients were HIV-positive, and NTM were isolated from 22 patients, of whom 16 were HIV-positive and two had an unknown HIV-status. The NTM cultures were identified as *M. szulgai* (7), *M. avium-intracellulare* (3), *M. simiae* (1) and *M. terrae* (1). Ten NTM isolates were not suitable for identification due to contamination or failure to re-culture.

The Ziehl-Neelsen smears of sputum were positive in 12 of the 30 culture-positive patients (40%), of which seven (58%) were HIV-positive. In four patients with a positive smear NTM were isolated and in eight patients *M. tuberculosis* was identified. All smears

Table 1: Positive *Mycobacterium* cultures.

Ziehl-Neelsen (ZN)	Culture result		Total
	M. tuberculosis	NTM	
ZN+	8 (4 HIV+)	4 (3 HIV+)	12
ZN-	0	18 (13 HIV+ and 2 HIV?[a])	18
Total	8	22	30

[a]HIV?: Unknown HIV-status.

for the patients with a positive *M. tuberculosis* culture were positive, and of these eight, four (50%) were HIV-positive. Of the four patients with a positive smear and NTM in the culture, three were HIV-positive.

The NTM isolates could be identified of 12 patients, including nine adults and three children.

Nine of these 12 patients were female. The median age of the adult patients was 39.5 years. The most frequent symptoms were coughing and/or chest pain (10 patients); abdominal discomfort with diarrhea and a subcutaneous abscess on the leg were each presented by one patient. The four patients with a positive smear and NTM in the culture had all respiratory complaints.

Anti-tuberculosis treatment was provided to seven of 12 NTM positive patients and comprised isoniazid, rifampicin, pyrazinamide and ethambutol according to the World Health Organization's recommended first-line regimens for tuberculosis treatment (11). The anti-tuberculosis treatment was started by the physician on the basis of clinical symptoms without knowing the culture results. All 12 patients did not improve on two courses of empiric antibiotics. Four patients responded clinically in the first 3 months and three patients did not respond to the anti-tuberculosis treatment. From three of the five patients who did not receive anti-tuberculosis treatment during the study period *M. avium-intracellulare* was isolated from their sputum. The two other patients who did not receive anti-tuberculosis treatment had *M. szulgai* in the sputum culture and were HIV-negative.

From seven admitted patients, *M. szulgai* was cultured from the sputum (see Table 2). Six patients complained of a productive cough and/or chest pain without night sweats and one patient had an abscess on the leg without pulmonary problems. Two patients were HIV-negative; one with respiratory complaints and the other with an abscess on her leg. The median duration of complaints was one month. Physical examination was consistent with infiltration in three out of the six patients with pulmonary complaints. The sputum smear was positive in two patients with respiratory complaints, one of whom was HIV-positive and one was negative. All patients were first treated with two courses of routine empiric antibiotics without result, where after anti-tuberculosis treatment was prescribed to five patients. Three of the five patients, who were HIV-positive, responded well. The other two patients, who were HIV-negative, showed no improvement on the anti-tuberculosis treatment.

The drug susceptibility of the identified NTM was also determined. The seven *M. szulgai* strains had reduced sensitivity to isoniazid (MIC 11 μg mL^{-1}) and were sensitive to

Table 2: NTM isolated from sputum in Zambian patients.

Case	Isolate	Male / Female	Age	HIV	Complaints[a]	Duration[b] (wks)	ZN[c]	Two courses of antibiotics	Anti-tuberculosis treatment
1	*M. szulgai*	female	38	pos	resp	4	pos	no improvement	improvement
2	*M. szulgai*	male	4	neg	resp	4	pos	no improvement	no improvement
3	*M. szulgai*	male	13	pos	resp	156	neg	no improvement	improvement
4	*M. szulgai*	female	22	pos	resp	4	neg	no improvement	no anti-tuberculosis treatment during study
5	*M. szulgai*	female	29	pos	resp	2	neg	no improvement	improvement
6	*M. szulgai*	female	43	pos	resp	8	neg	no improvement	no anti-tuberculosis treatment during study
7	*M. szulgai*	female	43	neg	leg abscess	20	neg	no improvement	no improvement

[a]Complaints = reason for visiting the hospital. Resp = complaints/symptoms of the tractus respiratorius.
[b]Duration = duration of complaints at the time of visiting a hospital and being included in study.
[c]ZN = Ziehl-Neelsen microscopy of sputum.

rifampicin, ethambutol, streptomycin, pyrazinamide, protionamide, amikacin, ciprofloxacin, clofazimine and claritromycin.

To examine the possibility of cross-contamination or a common source of infection, all *M. tuberculosis* and *M. szulgai* isolates were typed by IS*6110* RFLP- and by RAPD typing, respectively. The isolated *M. tuberculosis* strains all showed unique RFLP patterns. RAPD on the seven *M. szulgai* isolates was repeated several times, using two different primer sets. Four distinct patterns were found with both primer sets (see Figure 1). The RAPD patterns that were found among *M. szulgai* isolates from Zambia were different from the RAPD patterns of strains isolated in the Netherlands (not in Figure 1).

DISCUSSION

Since the early 1970s, *M. szulgai* has been recognized as a cause of serious infections and cases have been reported in several parts of the world. However, Africa is the only continent from which no *M. szulgai* isolation has been described so far (12,13). In this study, 30 out of 64 (47%) patients yielded positive *Mycobacterium* cultures. These 30 *Mycobacterium* cultures were identified in eight cases as *M. tuberculosis* (27%) and in 22 cases as NTM (73%). Of the 12 NTM isolates suitable for identification, seven were identified as *M. szulgai*. As far as it is known, these are the first documented cases of *M. szulgai* infection in Africa.

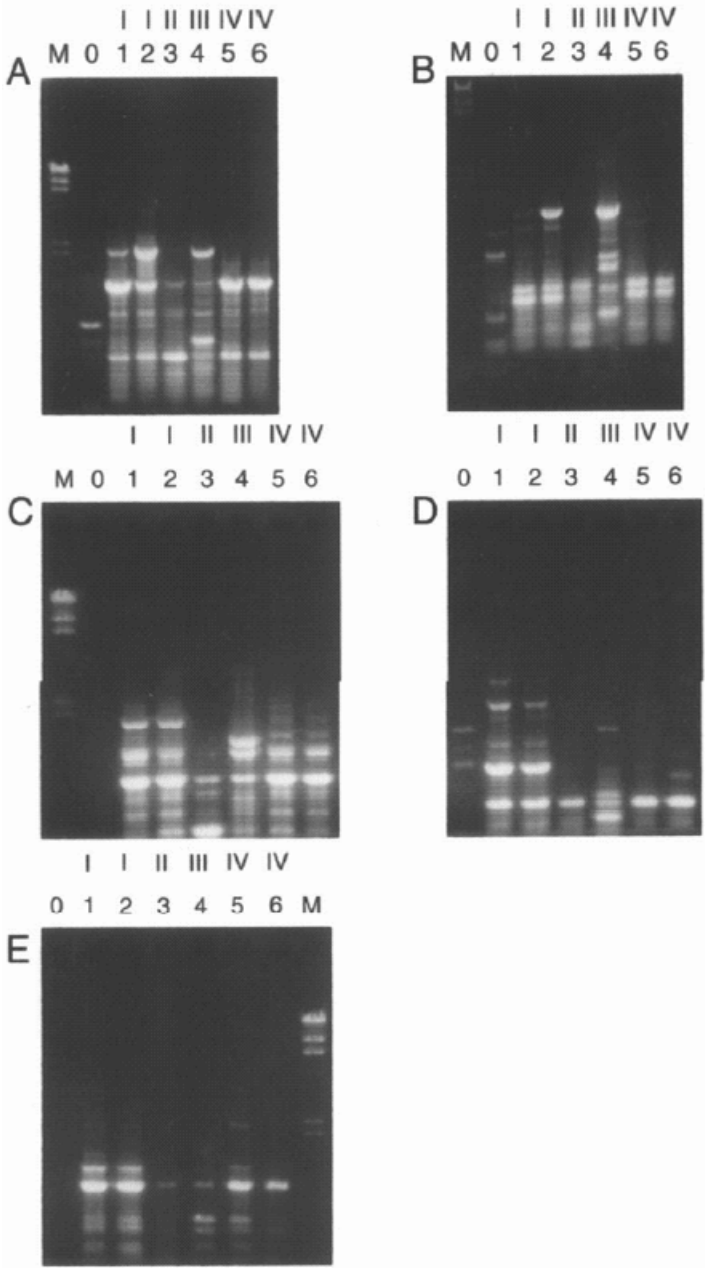

Figure 1: RAPD analysis of *Mycobacterium szulgai* strains.
Figure 1A shows the RAPD fingerprints generated using the primer RAPD1 (5'-ggttgggtgagaattgcacg), figure 1B applying primer ERIC2 (5'-aagtaagtgactggggtgagcg), figure 1C primer 1749 (5'-tgccgggtgg), figure 1D primer 1750 (5'-gcgattgggg) and figure 1E primer 1752 (5'-gccgaaccgg).
The M indicates the lane with a molecular marker (λ-DNA cleaved with HindIII). Lane 0 represents a control reaction without DNA template. Lanes 1-6 show the RAPD patterns of 6 different *M. szulgai* isolates. Four different RAPD genotypes, marked I-IV, were identified among the 6 isolates.

Similar to other mycobacteria, *M. szulgai* is associated with a wide spectrum of diseases. The reported cases of human infection caused by *M. szulgai* revealed that pulmonary disease, mimicking tuberculosis, was the commonest type of infection caused by this organism (12,13). Nevertheless, skin infection (14,15), olecranon bursitis (12), septic arthritis (16), osteomyelitis (17,18), tenosynovitis (19), cervical adenitis (12), and disseminated infections (14,20) have also been recorded. Most infections have been described in patients with chronic obstructive pulmonary disease (COPD) and healed tuberculosis (13,21,22).

Furthermore, *M. szulgai* is one of the most rarely isolated, known mycobacterial pathogens. Although *M. szulgai* is also seen as an environmental mycobacteria, recovery from the environment is exceptional. There are a limited number of reports on isolation of *M. szulgai* from a snail, tropical fish, aquarium water and swimming pool water. (21)(13). Most literature on this *Mycobacterium* suggests that it is an unlikely laboratory contaminant; it is unlikely to act as a commensal and should be considered clinically significant when isolated (22).

In this study, six of the seven patients from whom *M. szulgai* was isolated showed pulmonary disease indistinguishable from that caused by *M. tuberculosis*. Physical examination was consistent with infiltration of the lung in three of the six patients. Unfortunately, no chest X-ray was available. From these seven patients one sputum was cultured and the Ziehl-Neelsen was positive in two cases. Five of the seven patients were HIV-positive. No improvement was achieved after two routine empiric antibiotic courses in all patients. In contrast, three out of five patients with a *M. szulgai* infection recovered on anti-tuberculosis treatment. An explanation could be that in at least a part of these patients with chronic respiratory complaints (cases1, 3 and 5) their lung disease was associated with the presence of *M. szulgai*. However, not all the conditions described in this study completely fulfil the ATS criteria regarding NTM pulmonary disease. The diagnosis 'pulmonary disease' due to NTM instead of colonization according to the ATS is based on a combination of clinical and radiological observation, exceeding all bacteriological criteria. In the African setting it was not possible to perform a bronchial wash. All possible efforts were made to prevent laboratory cross-contamination and therefore a single sputum from each patient was cultured. The equipment for chest X-rays was unfortunately out of order for some time.

In this study the cumulative results of the cultures on Löwenstein-Jensen medium and the Mycobacteria Growth Indicator tubes were provided. Therefore, the separate results of the two culture methods and the contamination rates could not be deduced. Moreover, the number of colonies on the LJ tubes cultured in Zambia were not reported. In fact, a single colony on the LJ tube could have indicated colonization. Therefore, the distinction

between colonization and infection or disease could not be deduced from the culture results. In addition, the respective patients could also have represented culture-negative TB cases, as the sputum was only subjected to culture and not tested by molecular methods for the presence of *M. tuberculosis.* However, the performance of the nucleic acid amplification (NAA) tetst is in general good in clinical respiratory specimens that are AFB smear-positive but less in specimens that contain fewer organisms or are AFB-negative. The NAA-test would in a part of the cases be better in comparison to culture to detect a mixture of NTM and *M. tuberculosis*.

Although *M. szulgai* is rarely recovered from the environment, contamination with NTM should be considered in all cases of suspected nosocomial infection due to NTM and careful surveillance must be applied to identify possible outbreaks. Many species of non-tuberculous mycobacteria, such as *Mycobacterium gordonae, M. xenopi, M. kansasii*, and *M. abscessus*, have been reported to cause pseudo-infection (23-26). The isolates causing contamination have been traced to a variety of sources, such as PANTA PLUS (Becton Dickinson Microbiology Systems, Cockeysville, Md.), the hospital water and ice supplies, bronchoscopes, and a variety of laboratory handling errors (24-26). Recently, a *M. szulgai* pseudo-infection originating from hospital water has also been described (27). In this study the possibility of a pseudo-infection and a laboratory cross-contamination was considered highly unlikely because DNA fingerprinting analysis revealed four different patterns among the seven *M. szulgai* isolates.

In contrast to other NTM, *M. szulgai* has shown in vitro and in vivo susceptibility to most primary anti-tuberculosis drugs. However, several reports describe variable susceptibility to anti-tuberculosis drugs, probably because standardized protocols for susceptibility testing of *M. szulgai* are lacking. The organism is inherently resistant to pyrazinamide and streptomycin. In vitro resistance to isoniazid, rifampicin and ethambutol have been shown in some cases (28). Resistance to clarithromycin was observed and susceptibility to ciprofloxacin was noted (29). In previously reported cases, anti-tuberculosis drugs were reported successful when combinations of more than two drugs were used (30). Nevertheless, the optimal treatment for *M. szulgai* infections has not yet been established; most authors recommend the use of 3 or 4 drug regimens based on *in vitro* susceptibility for 9-12 months after the culture has become negative (5,30).

In this study the seven *M. szulgai* strains had reduced sensitivity to isoniazid and were sensitive to rifampicin, ethambutol and ciprofloxacin. Five of the seven patients were treated with a combination of isoniazid, rifampicin, ethambutol and, in cases of a positive smear, also with pyrazinamide, because an infection with *M. tuberculosis* was expected. Three of the five patients, who were HIV-positive, responded well. The other two patients, who were HIV-negative, showed no improvement on the anti-tuberculosis

treatment. One of these patients had a positive smear and was suspected of pulmonary disease. Probably the combination of two *in vitro* sensitive anti-tuberculosis drugs was not optimal. Moreover, the clinical response to anti-tuberculosis treatment of patients in this study was evaluated for 3 months and this may have been too short. The other patient had an abscess on her leg and no respiratory complaints. The abscess fluid was not cultured for mycobacteria. It is most likely that the isolated *M. szulgai* in the sputum was not the etiology of her disease.

The study focused on the seven patients with a positive culture for *M. szulgai*. In addition, there were five more patients with an identified NTM in the sputum culture. Instead to cultures yielding *M. szulgai* which have almost always a clinical significance, the significance of positive *M. simiae* or *M. terrae* cultures is more controversial, as these bacteria are uncommon causes of pulmonary disease (5). *M. avium-intracellulare* is a frequently encountered, clinically significant NTM. However, *M. avium-intracellulare* is also common in many environmental sources. Distinguishing between colonization, contamination and disease in case of isolation of these NTM is complicated and can be a real challenge. Therefore, in this study we concentrated on the seven patients with *M. szulgai* cultures.

The contribution of NTM, and especially of *M. szulgai*, to tuberculosis-like diseases in both HIV-positive and negative patients in Africa may have been underestimated.

This could have important consequences for the use of various diagnostic methods to establish the diagnosis of a mycobacterial infection as well as for the possibilities for adequate treatment. In addition, more extended studies are needed to determine the magnitude of this problem. To discriminate between colonization and infection or disease, studies with a control group from the same area should be examined for natural prevalence of different NTM and sputum of patients with probable NTM disease should also be subjected to a direct molecular test to detect possible presence of *M. tuberculosis* complex.

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Chapter 6

Geographic differences in the isolation of non-tuberculous mycobacteria in Zambia; a pilot study

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ABSTRACT

To access the possible role of non-tuberculous mycobacteria (NTM) as a cause of tuberculosis-like diseases, 167 chronically ill patients, hospitalised in three hospitals in Katete, Sesheke and Chilonga, Zambia, were investigated.

The percentage of patients with a positive culture for *M. tuberculosis* complex was equal in the three geographic locations (19 to 25%). In contrast, the percentage of sputum cultures positive for NTM differed significantly between the villages and ranged from 78% in Katete, 65% in Sesheke and 21% in Chilonga. Furthermore, the distribution of NTM species was different at the three geographic sites.

Two sputum specimens were cultured from each of in total 101 patients. In 15 patients (15%) the same NTM species was found in both sputum specimens. In almost half (7) of these there was a basis for a suspicion of NTM-associated disease. In five patients *M. lentiflavum* and in two patients *M. intracellulare* was isolated twice.

The demographic and clinical characteristics of patients with positive and negative NTM cultures were analysed. The odds ratio for an NTM culture positive sputum was significantly higher for patients living in Katete and Sesheke. Furthermore, females and patients with a chest X-ray suspected of tuberculosis were independently associated with an NTM culture positive sputum.

In association with clinical complaints of patients admitted at three different geographic sites in Zambia, a high percentage of positive NTM cultures was found. A proportion almost fulfilled the criteria of the American Thoracic Society. This finding may be due to the extremely high rate of HIV positivity (between 69-79%) among the patients examined. Although not examined in this study a low CD4+ T-lymphocyte count in HIV positives is sometimes associated with NTM infection. More extended studies are needed to determine the magnitude and nature of NTM infection on the African continent.

INTRODUCTION

Tuberculosis is almost invariably caused by *Mycobacterium tuberculosis*. However, infections by some of the non-tuberculous mycobacteria (NTM) can also mimic this disease. NTM are distinguished from members of the *M. tuberculosis* complex by the fact that they are not obligate pathogens, but natural inhabitants of a wide variety of environmental reservoirs, including natural and municipal water, soil, aerosols, protozoans, domestic and wild animals, milk and food products (20).

A large geographical variation in the distribution of NTM has been reported. In the United States, NTM lung disease is most commonly attributable to *M. avium* complex, with *M. kansasii* being the second most important (18). In the United Kingdom, especially in England and Wales, *M. kansasii* is the pathogen most commonly associated with NTM lung disease, while *M. malmoense* is the most commonly encountered NTM in Scotland. *M. xenopi* predominates in Southeast England (2). In Japan, the most common cause of NTM pulmonary disease is *M. avium* complex, followed by *M. kansasii* (24). However, in most parts of the world the distribution of NTM is not yet fully known.

Reported rates of NTM colonization and disease are likely underestimates, with the former probably less accurate than the latter, given that people without significant symptoms will not commonly undergo intensive investigations to detect possible NTM infection. Also the lack of systematic reporting of NTM isolation in most nations limits the ability to derive accurate estimates of infection. NTM disease is a problem in certain populations, including patients with underlying lung disease and individuals with impaired immunity. Northern American rates of colonization and disease have been reported to range from approximately 1-15 per 100,000 and 0.1-2 per 100,000, respectively. Generally, similar rates have been reported in European studies, with the exception of extremely high rates in an area of the Czech Republic where mining is the dominant industry (14). These studies have also shown marked geographic variability in NTM prevalence. Rates in Japan and Australia were similar to those reported in Europe and North America.

Most data reporting high rates of infection with NTM come from northern European countries or the United States. In African countries, despite the fact that environmental exposure to NTM is very high, infection by NTM seem to be rare, even among patients with AIDS (16). However, it is not clear whether this is due to a true low prevalence of NTM infection or that the magnitude of this problem has not yet been unravelled.

A number of studies on the epidemiology of NTM has been performed in South Africa. The results of mycobacterial studies of sputum cultures from large random samples of South African native people were reported in two studies (3,11). In 1,196 Zulus examined

in Natal, prevalence rates of 750 and 1,400 per 100,000 for *M. tuberculosis* and NTM were found, respectively (3). In the other study, sputum from 2,230 Xhosa people in the Transkei region was examined and 69 specimens grew *M. tuberculosis* and 150 NTM, yielding prevalence rates of 4,300 and 6,700 per 100,000, respectively (11). Furthermore, two other South African NTM studies focussed on a population of gold miners (8,9). Within the framework of one of these projects the records of a cohort of HIV-negative gold miners, investigated for suspected pulmonary mycobacterial disease, were reviewed (9). Annual rates of NTM infection were found to be 101 per 100,000 with the two most common organisms being isolated *M. kansasii* (66/100,000) and *M. scrofulaceum* (12/100.000), respectively.

A considerable part of the NTM has proven to be to a larger or smaller degree clinically relevant, stimulating interest in these NTM in recent years (22). The criteria set in a Statement by the American Thoracic Society (ATS) and those formulated by the British Thoracic Society (BTS) may be used to differentiate between true NTM infection, colonization, pseudo-infection and contamination (1,2). This differentiation between NTM infection or disease and no NTM infection or disease is made on the basis of clinical, radiological and microbiological features.

The goal of this pilot study was to assess the possible role of NTM in tuberculosis-like diseases in various geographic locations in Zambia.

MATERIAL AND METHODS

Chronically ill patients, hospitalised in three hospitals in different provinces of Zambia from March to August 2001, were the subject of this study. The hospitals included were St. Francis Hospital in Katete (Eastern Province), Yeta District Hospital in Sesheke (Western Province), and Our Lady's Hospital in Chilonga (Northern Province). St. Francis Hospital and Our Lady's Hospital are referral hospitals and Yeta District Hospital is a first level hospital. All the hospitals serve a rural population.

The distance between the hospitals in Katete and Chilonga amounts to approximately 250 kilometres and both villages are about 950 km apart from Sesheke. All patients above the age of 15 years who were chronically ill with complaints of any part of the body for more than two weeks and visited the hospital for clinical examination were included in this study.

The medical history of the patients was retrieved, and a detailed physical examination was performed. Sputa of the patients were examined for the presence of acid-fast bacteria

and subjected to culture in Mycobacteria Growth Indicator tubes (Becton Dickinson Microbiology Systems, Cockeysville, Md.). Decontamination of the sputum was done using *N*-acetyl-L-cysteine (NALC)-NaOH and 6% sulfuric acid after dividing the sputum specimen into two equal parts to compare decontamination procedures for the detection of mycobacteria (4). In St. Francis Hospital in Katete and Yeta District Hospital in Sesheke, sputum from patients was collected and cultured on two consecutive days. In Our Lady's Hospital in Chilonga only a single sputum was collected and cultured because of logistic reasons. Serological testing for HIV was performed using a qualitative immunoassay (Abbott Determine HIV-1/2) and the Vidas HIV DUO assay (bioMérieux, Marcy l'Etoile, France). Chest X-rays were taken and evaluated blind in The Netherlands. The Accuprobe culture confirmation test for *M. tuberculosis* complex (Accuprobe, bioMérieux, Marcy l'Etoile, France) and/or 16S rRNA gene sequencing were used to identify mycobacterial isolates (13).

Data analysis

Data were entered in SPSS 6 and analysed using STATA vs 8.0 (Stata corporation College Station, TX, USA). A student t-test was used to assess different means between groups and proportions were compared using chi-square testing.

Univariate odds ratios (ORs) with 95% confidence intervals (CIs) were calculated to assess associations of potential risk factors of participants being NTM-positive. A stepwise backward regression approach was used for a multivariate analysis.

RESULTS

General characteristics

In total, 167 chronically ill patients were included in the study; 63 in Katete, 51 in Sesheke and 53 in Chilonga. The patient characteristics are shown in Table 1. The sex and age distribution of the patients was comparable between the villages. The ratio of HIV-positive patients varied between 69 and 79 percent in the three hospitals. The occupation of the majority of patients in Katete and Sesheke was farming; 71% and 61% respectively. In Chilonga more variation in the occupation was observed. Nineteen (36%) patients were farmers, nine were fulltime housewives, seven were in business, five were teachers, four were working in construction and the remaining patients were working as a taxi driver, office worker or guard. The minority of patients were smokers and alcohol drinkers in Katete and Sesheke. Insufficient data on this issue were available from Chilonga. The patients with tuberculosis in the past were comparable between the three hospitals, between 19% and 29%. In Katete, most patients (52%) retrieved their water from a borehole. In Sesheke and Chilonga the main source of water was a nearby river which was used in combination

Table 1: Characteristics of included patients with chronic complaints for at least two weeks in Katete, Sesheke and Chilonga.

	Katete	Sesheke	Chilonga	p-value	All
Number of included patients, n (%)	63	51	53		167
Female, n (%)	37 (59)	26 (51)	19 (36)	0.05	82 (49)
Age, median (range)	36 (18-76)	37 (18-67)	39 (7-74)	0.9	38 (7-76)
HIV, n (%)	48 (79)	38 (76)	34 (69)	0.3	120 (75)
Farmer, n (%)	45 (71)	31 (61)	19 (36)	<0.001	95 (57)
Smoking, n (%)	1 (2)	11 (22)	-*	0.001	12 (11)
Using alcohol, n (%)	4 (7)	15 (31)	-*	0.01	19 (18)
Tuberculosis Rx in past, n (%)	12 (19)	15 (29)	15 (28)	0.4	42 (25)
Water source, n (%)					
Well	16/60 (27)	7 (14)	5/39 (13)		29 (19)
Borehole	31/60 (52)	0	2/39 (5)		33 (22)
Tap	5/60 (8)	21 (41)	15/39 (40)		41 (27)
River	8/60 (13)	23 (45)	16/39 (42)	<0.001	47 (31)
Un-boiled milk, n (%)	1 (2)	35 (69)	-*	<0.001	36 (33)
Died during study period, n (%)	9 (14)	1 (2)	5 (9)	0.07	15 (9)

*Data on smoking and using alcohol or (un)boiled milk were not available in Chilonga.

with water from the tap (40 to 45%). Patients who were milk consumers in Katete usually boiled their milk prior to use. The patients in Sesheke mainly used un-boiled milk. No data were available on this item from Chilonga. A relatively high, but not significant number of patients (14%), died during the study period in Katete in comparison with Sesheke (9%) and Chilonga (2%).

Most of the 167 patients included were complaining of regular coughing and/or chest pain (79%). Complaints of the digestion tract was found in 13% of the patients and the other 8% of the patients had either skin infections/abscesses, lymphadenopathy, tractus urogenitalis or central nervous system complaints.

Culture results

The percentage of patients with a positive culture for *M. tuberculosis* complex was equal in the three locations and varied between 19 and 25% (see Table 2). Moreover, a high percentage of positive sputum cultures were found for NTM. The number of patients with positive NTM cultures was significantly higher in Katete (78%) and Sesheke (65%) in comparison to Chilonga (21%). However, in Chilonga only one sputum specimen was cultured, while in Katete and Sesheke two sputum specimens were subjected to culture. Taking only the first sputum specimen in Katete and Sesheke into account, the number of patients with positive NTM cultures was still significantly higher in Katete and Sesheke in comparison with Chilonga. The sputum smear was positive in 19 out of 38 patients (50%)

Table 2: Patients with positive *Mycobacterium* cultures in Katete, Sesheke and Chilonga.

	Katete, n (%)	Sesheke, n (%)	Chilonga, n (%)	p-value	All, n (%)
Number of included patients	63	51	53		167
M. tuberculosis complex	12 (19)	13 (25)	13 (25)	0.7	38 (23)
NTM in any sputum	49 (78)	33 (65)	11 (21)	<0.001	93 (56)
NTM only in 1st sputum	42 (67)	25 (49)	11 (21)	<0.001	78 (45)
NTM in 1st and 2nd sputum	28 (44)	12 (24)	-*	0.02	40 (35)
Same NTM in both sputa	13 (21)	5 (10)	-*	0.1	18 (16)
NTM and *M. tuberculosis*	8 (13)	7 (14)	3 (6)		18 (10)

*Only a single sputum specimen per patient was cultured in Chilonga.

with a sputum culture positive for *M. tuberculosis* complex and in only 3 out of 76 (4%) patients with sputum culture positive for only NTM.

In all three hospitals, the collected sputum specimens were split in two equal parts before decontamination and culturing to test the performance of two decontamination methods. The result below summarizes the sum of both approaches.

In Katete, 217 sputum specimens were cultured from 63 patients (see Table 3). From 140 of the 217 (65%) sputum specimens mycobacteria were isolated. *M. tuberculosis* was found in 16 of the 217 (7%) cultured sputum specimens and NTM in 124 (57%). The most isolated NTM were *M. lentiflavum* (42) and *M. intracellulare* (19).

The number of mycobacteria isolated in Sesheke was comparable with the number isolated in Katete. In contrast, no *M. lentiflavum* was found in Sesheke. Mycobacteria were

Table 3: Isolated mycobacteria from sputum specimens in Katete, Sesheke and Chilonga.

Culture result	Isolates Katete, n (%)	Isolates Sesheke, n (%)	Isolates Chilonga, n (%)
Negative	77 (35)	55 (30)	67 (65)
M. tuberculosis	16 (7)	25 (14)	18 (17)
M. avium complex			
M. intracellulare	19 (9)	19 (10)	0
M. avium	0	0	1 (1)
M. chelonae	8 (4)	0	0
M. lentiflavum	42 (19)	0	0
Various unknown *Mycobacterium* species	18 (8)	29 (16)	5 (5)
Various other *Mycobacterium* species*	7 (3)	14 (8)	6 (6)
AFB without identification	30 (14)	42 (23)	6 (6)
Total number of sputum specimens	217	184	103

*Various other *Mycobacterium* species in Katete include *M. mucogenicum*, *M. elephantis, M. gordonae*, *M. gilvum* and *M. rhodesia*.
Various other *Mycobacterium* species in Sesheke include *M. fortuitum, M. mucogenicum, M. asiaticum, M. terrae* and *M. triplex*.
Various other *Mycobacterium* species in Chilonga include *M. mucogenicum*, *M. triplex, M. obuense* and *M. gordonae*.

isolated in 129 of the 184 (70%) sputum specimens cultured. *M. tuberculosis* was found in 25 of the 184 (14%) cultured sputum specimens and NTM in 104 (57%). The mainly isolated NTM in Sesheke was *M. intracellulare* (19).

In Chilonga mainly one sputum specimen was collected, which was also divided and pre-treated in two ways before culturing. From the 53 patients included in the study, 103 sputum specimens were cultured. In contrast to Katete and Sesheke, only 36 (35%) cultures were positive for mycobacteria. *M. tuberculosis* was found in 18 of the 103 (17%) cultured sputum specimens and NTM in the other 18 (17%) specimens. *M. intracellulare* and *M. lentiflavum* were not isolated in Chilonga. *M. avium* was isolated only in a single culture.

Comparison of patients with and without NTM in their sputum

The demographic and clinical characteristics of patients with positive and negative NTM cultures were analysed. The medical history, physical examination and pathology of chest X-ray did not show significant differences between these two patient groups (Table 4). Table 5 shows the analysis of possible risk factors for patients with NTM in their sputum. The odds ratio (OR) for NTM culture positive sputum was significantly higher for patients living in Katete and Sesheke. Furthermore, females and patients with a chest X-ray suspected of TB were independently associated with a NTM culture positive sputum. A higher OR, but not significant in the multi-variate analysis, was seen for farming and

Table 4: Medical history, physical examination and chest X-ray of NTM culture-positive and culture-negative patients.

167 chronically ill patients	NTM positive	NTM negative	p-value	All
Number of patients	93	74	-	167
Female sex, n (%)	55 (59)	27 (36)	0.004	82 (49)
Age, median (range)	37 (17-76)	38 (7-74)	0.9	38 (7-76)
HIV-positive, n (%)	71 (79)	49 (70)	0.2	120 (75)
BMI, mean	17.3	17.7	0.6	17.5
Weakness, n (%)	75 (82)	63 (88)	0.3	138 (84)
Vomiting, n (%)	19 (21)	18 (25)	0.5	37 (23)
Diarrhoea, n (%)	20 (22)	15 (21)	0.9	35 (22)
Dysuria, n (%)	10 (11)	13 (18)	0.2	23 (14)
Hepatomegaly, n (%)	19 (22)	13 (22)	0.9	32 (220
Splenomegaly, n (%)	21 (24)	10 (17)	0.3	31 (21)
Lymph nodes, n (%)	52 (56)	44 (60)	0.6	96 (58)
Chest X-ray compatible with TB in the absence of *M. tuberculosis* in culture, n (%)	25 (47)	15 (32)	0.1	40 (40)
Previous TB treatment, n (%)	23 (25))	19 (26)	0.9	42 (25)
Died, n (%)	8 (9)	7 (9)	0.8	15 (9)

Table 5: Risk factors for positive NTM culture from sputum.

	Univariate analysis, OR (95% CI)	Multivariate analysis, OR (95% CI)
Location		
Chilonga	1	1
Sesheke	7.0 (2.9-16.8)	4.7 (1.6-13.7)
Katete	13.4 (5.5-32.6)	11.8 (4.5-30.8)
Sex (female)	2.5 (1.5-4.3)	2.0 (1.0-4.5)
Chest X-ray suggesting TB	1.9 (1.0-3.9)	2.5 (1.1-5.9)
Age ≥ 25	0.8 (0.3-2.1)	ns
Farmer	2.0 (1.1-3.8)	ns
Water (in comparison to tap water = 1):		ns
Borehole	3.1 (1.2-8.2)	
Well	2.6 (0.9-7.0)	
River	1.2 (0.5-2.8)	
Un-boiled milk	1.0 (0.4-2.4)	ns
Smoking	0.4 (0.1-1.3)	ns
Using alcohol	0.7 (0.2-1.9)	ns
M. tuberculosis isolated from sputum	0.6 (0.3-1.2)	ns
Tuberculosis Rx in past	1.0 (0.5-1.9)	ns
HIV-positive	1.6 (0.8-3.3)	ns
Underweight (BMI < 18)	1.0 (0.5-2.1)	ns

Definition of abbreviations: ns = not (statistically) significant, OR = odds ratio, CI = confidence interval.

using water from the borehole. The HIV-status and the influence of un-boiled milk were not statistically significant.

Repeated isolation of NTM from sputum

The diagnostic criteria for NTM pulmonary disease according to the ATS and BTS include both imaging findings consisted with pulmonary disease and repeated isolation of the same mycobacteria from sputum samples in symptomatic patients.

In this study, two sputum specimens were collected and cultured from a total of 101 patients. In 21 (21%) of these patients *M. tuberculosis* and in 66 patients (65%) NTM were isolated from at least one sputum specimen. In 10 patients *M. tuberculosis* in combination with NTM were isolated from the sputum specimens.

In 56 patients (55%), NTM (presumably) representing different species were found in both sputum specimens. In 29 of these 56 patients the NTM in at least one sputum could not be identified. In 15 patients (15%) the same species of NTM was found in both sputum specimens and in three more patients the same species of NTM in both sputum samples together with a *M. tuberculosis* were found. (see Table 6 for an overview of all 18 patients with same species NTM in both sputum samples). Nine of these 15 patients with the same species NTM, without *M. tuberculosis*, in both sputum samples (60%) were female and the median age was 41 years (range 25-67). Thirteen patients were visiting the

Table 6: Cases with twice the same NTM in the sputum culture.

Case	Isolate sputum	ZN	Male/ female	Age	BMI[a]	HIV	Temp[b]	Complaints[c]	Duration (wks)[d]	Chest X-ray	Died	Remarks[f]
1 (11)	*M. lentiflavum*	pos	female	50	20	indiff	?	resp	21	TB	yes	*M. lentiflavum* also in urine, started with TB-Rx
2 (13)	*M. lentiflavum*	pos	male	35	24	pos	39	resp	34	TB	no	
3 (15)	*M. lentiflavum*	neg	female	30	18	pos	38.5	resp	5	TB	no	TB in past, improvement on TB-Rx
4 (34)	*M. lentiflavum*	neg	female	25	?	pos	38.5	resp	4	TB	yes	TB in past
5 (18)	*M. lentiflavum*	neg	male	29	14	pos	38.5	resp	3	no pathology	no	started with TB-Rx
6 (14)	*M. lentiflavum*	neg	female	40	17	pos	38.5	resp	13	no pathology	no	TB in past
7 (38)	*M. intracellulare*	neg	male	45	16	pos	36	dig	43	no pathology	no	
8 (41)	*M. chelonae*	neg	female	41	14	pos	38	resp	32	no pathology	no	
9 (50)	*M. intracellulare*	neg	male	57	14	neg	36.5	resp	17	not suspected of TB	no	
10 (146)	*M. intracellulare*	neg	female	40	16	pos	?	resp	57	no pathology	no	started with TB-Rx
11 (33)	*M. lentiflavum*	neg	female	67	?	neg	35.5	dig	8	no chest X-ray	no	*M. lentiflavum* cultured from lymph node
12 (135)	*M. intracellulare*	neg	female	43	?	?	?	resp	4	no chest X-ray	no	started with TB-Rx
13 (138)	*M. intracellulare*	neg	female	49	17	pos	36.6	resp	78	no chest X-ray	no	
14 (145)	*M. intracellulare*	neg	male	59	21	neg	?	resp	46	no chest X-ray	no	started with TB-Rx
15 (163)	UMS[e] 12	neg	male	36	17	pos	39	resp	8	no chest X-ray	no	TB in past
16 (1)	*M. lentiflavum*	neg	female	63	20	neg	36.5	resp	3	TB	no	also *M. tuberculosis* in sputum, started with TB-Rx
17 (19)	*M. lentiflavum*	pos	male	34	13	pos	38	resp	6	TB	no	also *M. tuberculosis* in sputum, started with TB-Rx
18 (37)	*M. chelonae*	pos	male	24	?	pos	37	resp	20	TB	no	also *M. tuberculosis* in sputum, started with TB-Rx

[a]BMI = Body mass index. ? = BMI not known. [b]Temp = temperature at time of inclusion. ? = temperature at time of inclusion not known. [c]Complaints = reason for visiting the hospital. Dig = complaints/symptoms of the tractus digestivus. Resp = complaints/symptoms of the tractus respiratorius. [d]Duration = duration of complaints at the moment of visiting hospital and inclusion in study. [e]UMS = unknown *Mycobacterium* species. [d]Remarks: TB-Rx = anti-tuberculosis treatment.

hospital because of respiratory complaints and two because of complaints of the gastrointestinal tract. Six patients (40%) had suffered from tuberculosis in the past, of whom five presenting at the time of inclusion in the study had respiratory complaints. Ten of the 14 tested patients (71%) were HIV-positive. Two of these 15 patients died during the study period.

A chest X-ray was made in 10 of these 15 cases (Table 6). In 4 patients, the pathology on the chest X-ray suggested tuberculosis. *M. lentiflavum* was isolated in both sputum samples from all 4 patients. The sputum smears were microscopically positive in two patients.

In six patients, the chest X-ray showed no signs indicative of tuberculosis. *M. lentiflavum* was isolated from both sputum specimens in three patients, *M. intracellulare* in another two patients and *M. chelonae* in one patient.

Unfortunately, from five patients no chest X-ray was made. In one patient, *M. lentiflavum* was cultured from the lymph node biopsy and from two sputum samples. In three of these five patients *M. intracellulare* was identified in both sputum specimens. In the last patient without an X-ray, mycobacteria were cultured from both sputum specimens but could not be identified to the species level. The highest phylogenetic agreement, based on rRNA gene sequences, demonstrated a high degree of similarity with *M. intracellulare*. One of these five patients had a history of tuberculosis and two did start with antituberculosis treatment.

Case reports of patients suspected of NTM disease

In four of the 166 cases (i.e. four of the ten patients with a chest X-ray of the 15 patients with same species NTM in both sputum samples) included in this study, mycobacteria were repeatedly and consistently isolated from sputum in addition to lesions on the chest X-ray. Patient one was a 50-year-old female who presented herself with a productive cough with haemoptysis for 24 weeks. Furthermore, she was having pain passing urine. She was not responding to antibiotics. The HIV tests were indifferent. Her body mass index (BMI) was 20. The radiographic investigation of the chest revealed a pleural and pericardial effusion and an alveolar consolidation. The sputum smear was positive and treatment with rifampin, isoniazid, ethambutol and pyrazinamide was started. *M. lentiflavum* was isolated from two consecutive sputum specimens. Also from two consecutive urine samples *M. lentiflavum* was isolated. The patient died three months after the treatment was started.

The second case concerned a 35-year-old HIV-positive man. He complained about a cough and night sweats that started 34 weeks earlier. A relative of this patient had been found sputum-positive a few months earlier. On physical examination, enlarged axillary

and submandibular lymph nodes and an enlarged spleen were found. The temperature was 39°C and the BMI 24. He had already been treated with antibiotics without improvement. The chest X-ray did show pleural fluid. The sputum smear was positive. *M. lentiflavum* was isolated from two sputum specimens.

An HIV-positive, 30-year-old woman presented with coughing and vomiting for five weeks. She had been treated for tuberculosis in the past, but she interrupted this treatment. On physical examination, enlarged axillary and supra-clavicular lymph nodes and an enlarged spleen were found. The temperature was 35.5°C and the BMI 18. She was not improving on antibiotics before admission. Pleural effusion, alveolar infiltration, and interstitial pathology were seen on the chest X-ray. The patient clearly improved upon treatment with rifampin, isoniazid, pyrazinamide, and ethambutol. *M. lentiflavum* was cultured from two sputum specimens.

The last patient was a 25-year-old, HIV-positive woman who complained of a productive cough with haemoptysis of one month duration. She was known with asthma. Enlarged axillary and submandibular lymph nodes and an enlarged liver were found on physical examination. The temperature on admission was 38.5°C. Her chest X-ray showed pleural and pericardial effusion. Treatment with antibiotics was started. The culture of the both sputum specimens indicated the presence of *M. lentiflavum*. The patient died nine days later.

DISCUSSION

A high percentage (56%) of patients presenting with a variety of complaints at three African hospitals yielded positive NTM sputum cultures. This finding may be due to the extremely high rate of HIV positivity (between 69-79%) among the patients examined. The group of HIV-negative patients that was found was so relatively small in this study that correlations between NTM isolation and HIV status could not be studied adequately. Although not examined in this study, a low CD4+ T-lymphocyte count in HIV positives may be associated with NTM infection, whereas all HIV positives may have a much higher chance of being colonized by these bacteria.

The ratio of patients with positive NTM cultures was significantly higher in Katete (78%) and Sesheke (65%) in comparison to Chilonga (21%). Therefore, the patient characteristics of the patient populations at the three sites were compared. In Katete and Sesheke more patients were working as a farmer in comparison with Chilonga. In the literature, mining and other heavy industries are the environments most frequently cited as risk factors for NTM acquisition (10,14). Farming has not yet been mentioned as a risk factor

for NTM infections in Africa, but it is conceivable since farmers have an intensive and long lasting exposure to NTM in soil, water, and other potential environmental sources. Therefore, the observation of a lower percentage of NTM culture-positive patients in Chilonga could indeed be the consequence of the lower proportion of farmers among the patients in this area.

NTM are natural inhabitants of a wide variety of environmental reservoirs including municipal water systems and soil (20,25). Food is also a source of human exposure to NTM. Between the three villages also main differences were observed in the water supply and the consumption of (un)boiled milk. In Katete most patients (52%) retrieved their water from a borehole and in Sesheke and Chilonga a nearby river and water from the tap (40 to 45%) were the main source of water supply. Patients who were milk consumers in Katete usually boiled their milk prior to consumption, whereas patients in Sesheke mainly used un-boiled milk. This suggests that direct contact with the environment may be a risk factor for contracting a NTM infection in Zambia.

Alcohol abuse and smoking have also been associated with NTM infection in the literature (17). Unfortunately, in this study the use of alcohol and smoking was noted in the patient history in Katete and Sesheke, but not quantified.

In most NTM studies older males with underlying chronic obstructive pulmonary disease (COPD) are the predominant patient group. COPD was documented in this study but not observed as a risk factor, probably because many patients did not know if they had this underlying disease. In addition, studies have also described a patient group of elderly woman with no pre-existing pulmonary disease at increased risk of especially *Mycobacterium avium* complex and *M. kansasii* (7,18). Although other NTM have been found in Zambia than those mentioned here, in this study the female sex was also a risk factor for NTM culture positive sputum. Traditionally, in Zambia women are more involved in cropping and gardening compared to men. NTM exposure of the female patient through farming could be a possibility.

NTM have been found all over the world, although there seems to be a geographic distribution (2,18,24). Mycobacteria such as *M. kansasii, M. chelonae, M. fortuitum, M. avium* and *M. intracellulare* are responsible for the great majority of the NTM infections. In Africa little is known about the most prevalent NTM species. In South African gold miners who were investigated for suspected pulmonary mycobacterial disease, the most common micro-organisms were *M. kansasii* and *M. scrofulaceum* (9). In a study in Nigeria, 11 out of 102 (11%) mycobacterial isolates from cases with persistent symptoms of lower respiratory tract infections were identified as NTM (12). Six were classified as *M. avium*, four as *M. kansasii* and one as *M. fortuitum*.

In Kenyan adults with acute pneumonia, 11 of the 53 positive mycobacterial cultures belonged to NTM; *M. fortuitum/M. chelonae* (3 cultures), *M. szulgai* (2), *M. kansasii* (2), *M. terrae* (1), and other NTM (3) (21).

In this study clear geographic differences of isolated NTM species were observed.

In Katete most commonly *M. lentiflavum* and *M. intracellulare* were isolated, but in Sesheke mainly *M. intracellulare* was isolated and no *M. lentiflavum* was found. Neither *M. intracellulare* nor *M. lentiflavum* were isolated in Chilonga. Interestingly, in all three villages not previously identified mycobacteria were found (between 5-16% of the cultured sputum specimens). These data indicate there may be another distribution of mycobacteria in Africa compared with the United States and Europe.

The incidence of NTM colonization and disease is almost completely unknown in the major part of Africa. North American and European rates of colonization and disease have been reported to range from approximately 1-15 per 100,000 and 0.1-2 per 100,000, respectively. In two population-based studies in South Africa prevalence rates of NTM colonization were 1,400 and 6,700 per 100,000, respectively (3,11).

In a population of South African gold miners annual rates of NTM colonization were measured at 101 per 100,000 and rates of disease with the two most common organisms were 66 (*M. kansasii*) and 12 (*M. scrofulaceum*) per 100,000, respectively (8,9).

In this study NTM were isolated from at least one sputum specimen in 98 of the 167 (59%) hospitalised and chronically ill patients. Two sputum specimens were collected and cultured from 101 patients. In 15 patients the same species NTM was found in both sputum specimens. A chest X-ray was made in 10 of these 15 patients (Table 6). The chest X-ray was suspected for TB in four patients with repeated *M. lentiflavum* isolates from their sputum, which may indicate pulmonary infection. Chronic respiratory symptoms not reacting to antibiotics, radiological features suggesting tuberculosis and also two positive sputum cultures are nearly sufficient for fulfillment of the ATS diagnostic criteria. In six patients the chest X-ray did not suggest tuberculosis. In five of these 15 patients no chest X-ray was made because of technical problems with the X-ray equipment. At least three patients without a chest X-ray were suspected of pulmonary disease by NTM. In one patient *M. lentiflavum* was isolated from two sputum specimens and from a (normally sterile) lymph node. In two other patients with chronic respiratory complaints and no response to antibiotics, anti-tuberculosis treatment was started. *M. intracellulare* was isolated from consecutive sputum specimens in both patients. The combination of symptoms, together with the positive cultures, is an important indicator of NTM infection and is suggestive of NTM pulmonary disease.

However, the ATS criteria are not completely fulfilled because two instead of three sputum specimens were NTM culture-positive. Furthermore, the sputum of the patients suspected for NTM disease were not tested by molecular methods for the possible

presence of *M. tuberculosis*, especially the ZN positive sputa should ideally have been subjected to such a test. Culture of the sputum was only done in MGIT tubes and not on a solid medium. This liquid culture system has the disadvantage that it does not allow the quantitative detection of colony-forming units (CFUs). A low number of CFUs could have been an indication of colonization of specimens by NTM. Therefore, the liquid culture was not helpful in discriminating between colonization and infection or disease. Moreover, NTM are found ubiquitously in the environment and colonization or infection by NTM may result from drinking contaminated water or showering with it. Recently, *M. lentiflavum* was isolated from public water distribution systems (23). Isolation of the same NTM from two different sputum specimens collected at consecutive days could therefore be a result of colonization with the NTM, because of exposure to drinking water contaminated with NTM.

Additionally, disease caused by *M. lentiflavum* is difficult to treat with standard anti-tuberculosis drugs. In most cases clarithromycin is added to the treatment but the management of this NTM infection remains difficult (15). The improvement of patient number three in our study on anti-tuberculosis treatment without clarithromycin may therefore be indicative of disease caused by *M. tuberculosis* instead of *M. lentiflavum*.

Overall, for almost half (7 /15) of the patients with the same NTM in both sputum specimens there was a basis for the suspicion of NTM-associated disease. In five patients *M. lentiflavum* (cases 1,2,3,4 and 11) and in two patients *M. intracellulare* (cases 12 and 14) was isolated twice. Overall, at least in a part of these patients with the same NTM in both sputum specimens a NTM associated disease was considered.

In this study the estimated rate of colonization is 58% (59/101) with a striking rate of disease of about 7% (7/101). The fact that all patients were presented at the hospitals with chronic complaints could partially explain such a high rate. The limited sample size of this study lacks the power to generalise these findings to the population as a whole. Moreover, the patients were examined over six months of the year and seasonal influences may have played a role as well (6,19). The study was performed from March to August during the cool and dry (May to August) and warm and rainy (mid-November through to the end of April) seasons. Patients were included during six consecutive months and the variation during one year or between years could not be measured. The first study was started in Katete and the study in Chilonga was the last one finalized. In summary; differences in climate could have influenced the number and species of isolated NTM.

Furthermore, differences in the incidence of NTM colonization/disease and geographic differences in the isolation of NTM between the three villages could possibly be clarified by the variation in geographical characteristics. The three villages of this study are located in three different districts in Zambia. Katete and Chilonga are about 250km apart from each other and both villages are about 950km apart from Sesheke. Katete and Sesheke

are situated at an altitude of between 1000 and 1500m and Chilonga between 1500 and 2000m. Furthermore, mean annual temperatures are between 19-22°C, reaching their maximum annual range in the extreme south-west (14-26°C in Sesheke). The mean annual rainfall decreases from over 1000mm in the North (for instance in Chilonga) to less than 700mm in the South. The agricultural potential correlates with these different ecological zones.

The ubiquitous presentation of NTM in the environment contributes to the difficulties in interpreting positive culture results. NTM can be associated with either colonization, serious infection, or with pseudo-outbreaks with a wide variety of presentations (26). In this study laboratory cross-contamination was prevented by all possible efforts, including that all materials needed for collection and cultivation were imported from The Netherlands. All handling was carried out according to standard operating procedures that were introduced by experienced staff from The Netherlands. Also the fact that in a significant proportion of the cases from consecutive sputa the same NTM were isolated indicates that the quality of NTM isolation was adequate. Furthermore, molecular analysis revealed that the mycobacterial isolates represented several evolutionary lineages and strain variation (5). In addition, at the three villages the same materials were used, while different species NTM were isolated. Therefore, laboratory cross-contamination is considered highly unlikely in this study.

High rates of NTM colonization and disease are reported in this study. Furthermore, differences in the distribution of specific species of NTM in chronically ill patients in three villages in Zambia were observed. So far, only a few studies on the epidemiology of NTM have been performed in Africa, mainly South Africa, and benchmark data are not available. The current study in the Zambian patient population at least indicates that more extended studies are needed to determine the magnitude of NTM infection in the African continent.

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Chapter 7

Isolation of nontuberculous mycobacteria in Zambia: eight case reports

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ABSTRACT

The isolation of nontuberculous mycobacteria (NTM) raises the question of their clinical significance, especially in an African setting. We found a high percentage of NTM isolated from various specimens, including ones that are normally sterile, among 213 patients in Zambia. Because tuberculosis can affect all parts of the body, we decided to include patients who had signs and symptoms in any part of the body for more than 2 weeks. Most patients had tractus respiratorius (80%) and tractus digestivus (10%) symptoms. During three consecutive days, sputum was collected and two separate sputum specimens were cultured for mycobacteria. Depending on the clinical picture, pleural effusion, ascites, abscess material, or enlarged lymph nodes were also cultured for mycobacteria. A specimen from one sterile body site was collected from 25 patients (60% human immunodeficiency virus [HIV] positive). NTM were isolated from 8 of these 25 specimens. *Mycobacterium lentiflavum* was isolated from four patients, and *Mycobacterium goodii* was isolated from one patient. In order to exclude the possibility of laboratory cross-contamination, a novel amplified fragment length polymorphism DNA typing method for *M. lentiflavum* was developed. Genetic variation was detected, rendering the likelihood of laboratory contamination unlikely. Clinically relevant infection due to NTM occurs in both HIV-positive and HIV-negative patients in Zambia, and their clinical impact seems to be underestimated. This is the first report of *M. lentiflavum* and *M. goodii* infections in Africa.

INTRODUCTION

Mycobacterium tuberculosis and human immunodeficiency virus (HIV) co-infections are increasingly noted in third-world countries (8). In developed countries, it has been reported that HIV-infected patients are susceptible to infections by nontuberculous mycobacteria (NTM) (14). The isolation of NTM generally raises questions of their clinical significance, especially in an African setting, and assessment of this significance should be guided by the diagnostic criteria of the American Thoracic Society (1). According to these criteria, the diagnosis of NTM pulmonary disease must be based on solid clinical, radiographic, and bacteriologic factors. A culture of a tissue biopsy is one of the bacteriologic criteria and is considered positive if there is any growth from a usually sterile extra-pulmonary site. Many infections occur without the identification of causal microorganisms, especially in African settings. An improved understanding of the aetiology of these infections and which microorganisms play a role is of the utmost importance. Because only microscopic examination is usually available to confirm the diagnosis of tuberculosis in Africa, the involvement of acid-fast NTM in tuberculosis-like syndromes might result in the misdiagnosis of tuberculosis.

Mycobacterium avium complex bacteria, as well as other NTM, are widely distributed in the environment, and colonization of humans appears to be common (16). However, genuine infection by NTM has been reported almost exclusively from developed countries and not from developing countries, although the prevalence of HIV infection is generally much higher in the latter settings (7). Disseminated disease due to *M. avium* complex bacteria does occur in HIV-positive Africans but has been reported more frequently in the United States and Europe (2). This may be due to a lack of attention to this phenomenon in Africa, where possibilities for identification of mycobacteria are generally limited.

During a tuberculosis surveillance study prior to the present study, we regularly isolated NTM from various clinical specimens from 84 Zambian patients with pulmonary syndromes. A positive *Mycobacterium* culture was found in 51% of the included patients who were clinically suspect for tuberculosis, and 73% of these mycobacteria were NTM. The clinical significance of some of these NTM was questionable, and the aim of the present study was to resolve this enigma. To examine the clinical relevance of NTM in Zambian patients, eight patients from whom NTM were cultured from sterile body sites are described in detail.

CASE REPORTS

Case 1.

A 67-year-old woman who was HIV negative was admitted with a painful abdominal swelling on the left side. Pain started a month earlier, after a herpes zoster infection. On physical examination, an enlarged axillary lymph node and enlarged liver and spleen, but no chest abnormalities, were found. *Mycobacterium lentiflavum* was cultured from the lymph node biopsy and from two sputum samples.

Case 2.

A 37-year-old female HIV-negative patient who was suffering for 2 months with a painfully swollen abdomen was admitted. She was also complaining about night sweats and a poor appetite. The chest X ray revealed an enlarged heart and pleural effusion at the left and right sites. The ultrasound showed an enlarged liver, ascites, a splenic abscess, and pericardial fluid. The splenic abscess and heart failure were treated with metronidazole and diuretics. After 2 weeks of treatment, the splenic abscess was reduced in size, but the liver seemed to have enlarged further. Treatment with rifampin, isoniazid, and pyrazinamide was started. Mycobacteria were cultured from the ascites and subsequently identified by 16S rRNA gene sequencing as an unknown *Mycobacterium* phylogenetically related to *Mycobacterium gilvum* (Fig. 1). One month later, the patient was readmitted because of heart failure.

Case 3.

The third case concerns a 38-year-old HIV-negative woman. She complained about chills and abdominal distension that started 7 months earlier. She had already been treated with diuretics without improvement. The white blood cell count was 6.200/mm^3 with 18% eosinophils. No abnormalities were seen on the chest X ray. The patient was treated with antibiotics, anthelminthics, diuretics, and draining of the ascites. *M. lentiflavum* was cultured from the ascites.

Case 4.

A 21-year-old female HIV-positive patient presented herself with a distended abdomen and edema of the ankles that started 3 weeks earlier. Furthermore, she was having a productive cough and pain passing urine for 4 months. On physical examination, an icteric patient was seen with enlarged cervical, supraclavicular, and submandibular lymph nodes and abdominal ascites. The chest X ray did not show any abnormality. *M. tuberculosis* was isolated from sputum and urine, and *Mycobacterium fortuitum* was isolated from the ascites. The patient died 4 days later.

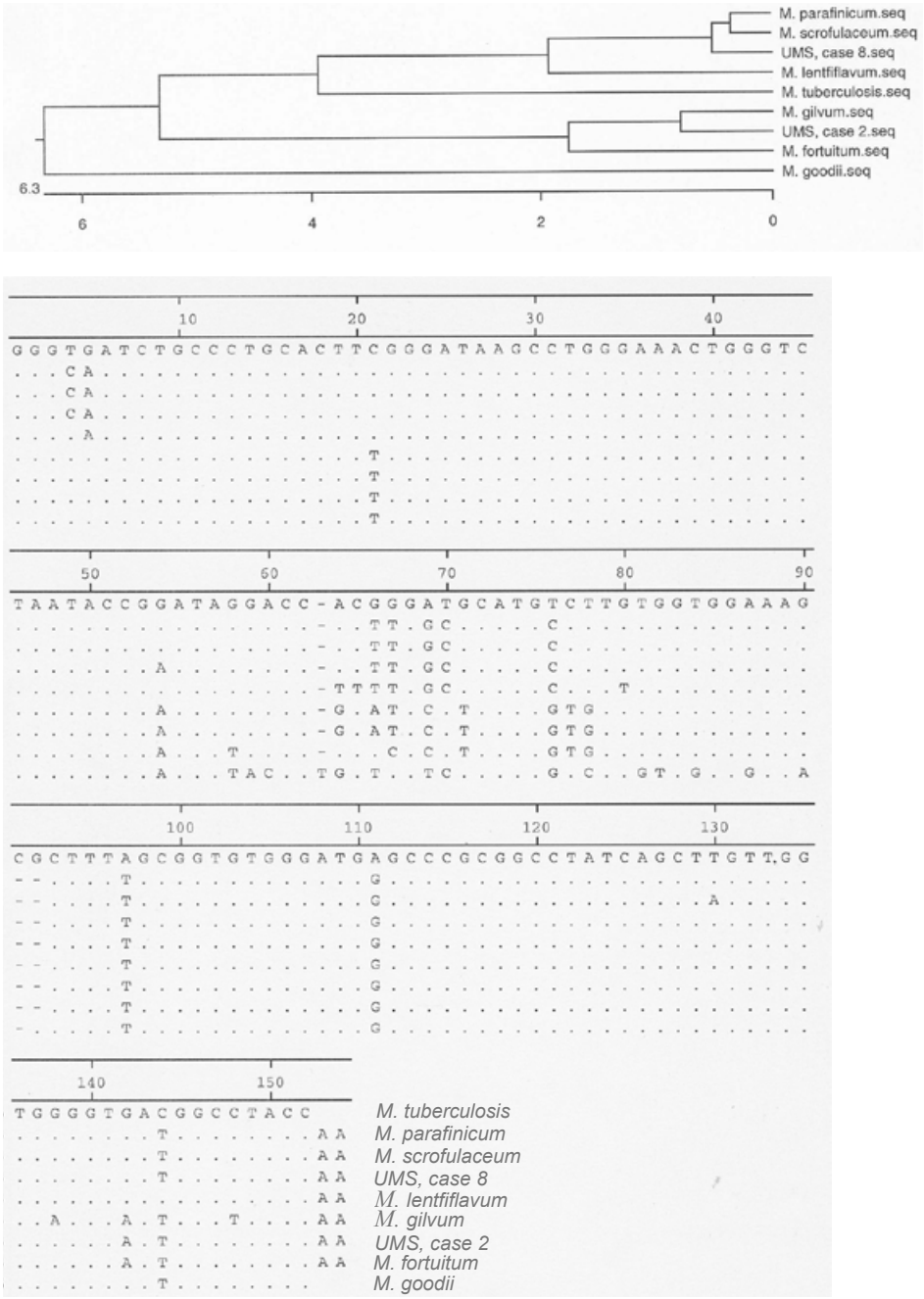

Figure 1: Phylogenetic tree on the basis of similarity in the 16S rRNA gene sequences and respective sequences of the mycobacterial isolates.
UMS = unknown *Mycobacterium* species.

Case 5.

The patient was a 36-year-old HIV-positive woman who suffered from recurrent abscesses for 2 years. The patient presented with fever and multiple cutaneous abscesses on both legs and in the neck area. *M. lentiflavum* was isolated from the pus and from one of two sputum samples.

Case 6.

A 35-year-old HIV-negative woman was admitted previously because of cough and left pleural effusion. Symptomatic improvement was achieved with amoxillin. She subsequently presented with a nonproductive cough and left chest pain. The radiographic investigation of the chest revealed a pleural effusion and alveolar consolidation on the left side. The culture of the pleural effusion proved the presence of *M. lentiflavum*. The patient improved upon treatment with rifampin, isoniazid, pyrazinamide, and ethambutol.

Case 7.

An HIV-negative 66-year-old man presented with a productive cough, chest pain, and diarrhea for 3 days. The patient's medical history showed that he had pneumonia 1 year ago which improved with amoxicillin treatment. Pleural effusion, a left alveolar infiltration, and peribronchial pathology were seen on the chest X ray. Hookworms were found in the stool. The culture of the pleural effusion indicated the presence of *M. goodii*.

Case 8.

The patient was a 26-year-old HIV-positive woman who complained of a productive cough with haemoptysis and chest pain of 1-month duration. She had been treated for tuberculosis twice in the past. Her chest X ray showed pleural effusion and possible interstitial pathology. Mycobacteria were cultured from the pleural effusion and could not be identified to the species level (Fig. 1). The highest phylogenetic similarity, based on rRNA gene sequences, was demonstrated with *M. parafinicum* and *M. scrofulaceum.*

MATERIALS AND METHODS

The study population consisted of 213 Zambian patients, 99 female and 114 male adults over 15 years of age. All patients who were admitted with chronic complaints to St. Francis Hospital in Katete (Eastern province), Yeta District Hospital in Sesheke (Southern province), or Our Lady's Hospital in Chilonga (Northern province) during the period of March to August 2001 were included in the study. Because tuberculosis can affect all parts of the body, we decided to include patients who had signs and symptoms in any part of the body for more than 2 weeks. Most of the included patients had tractus respiratorius

(80%) and tractus digestivus (10%) symptoms. The other 10% of the patients had skin infections/abscesses, lymphadenopathy, or tractus urogenitalis or central nervous system complaints.

The medical history of the patients was retrieved, and detailed physical examination was performed. During three consecutive days, sputum was collected from patients with a productive cough. The first two sputum specimens were cultured for mycobacteria, and the third one was stored at −20°C. Depending on the clinical symptoms, samples of pleural effusion, ascites, abscess material, or enlarged lymph nodes were obtained in a sterile manner. All specimens were cultured for mycobacteria. Materials needed for collection and cultivation were imported from The Netherlands, and an experienced Dutch technician performed the work in the Zambian laboratory. All possible efforts were made to prevent laboratory cross-contamination, including the collection of a specimen in a container that was not used before and that was imported from The Netherlands. The stock of decontamination fluid was sterilized twice a week, and each day a fresh, sterile tube or bottle was opened, the samples in the laboratory were processed one by one in the absence of other samples, and negative control cultures were included in each batch of samples. Microscopic slides were also prepared one at a time. All work associated with this project was conducted by the same experienced laboratory technician. All culture procedures were performed in a new class I biosafety cabinet.

A specimen from a sterile body site was collected from 25 patients. Ten of these specimens were cultured without decontamination, and 15 specimens were divided into two equal parts: one half was decontaminated with *N*-acetyl-L-cysteine (NALC)-NaOH and the other half was decontaminated using 6% sulfuric acid to compare decontamination procedures for the detection of mycobacteria (4). The specimens from a sterile body site were first cultured without decontamination, but to avoid any confusion in the handling of the samples in the laboratory, all collected specimens were decontaminated after the first weeks of inclusion. Serological testing for HIV was performed using a qualitative immunoassay (Abbott Determine HIV-1/2) and the Vidas HIV DUO assay (bioMérieux, Marcy l'Etoile, France). Chest X rays were taken and evaluated in The Netherlands in a blinded manner regarding the origin of the pictures and any additional clinical information. The specimens were cultured in Mycobacteria Growth Indicator tubes (Becton Dickinson Microbiology Systems, Cockeysville, Md.) according to the instructions of the manufacturer and the guidelines described previously by Master (17). Mycobacterial isolates were identified by the Accuprobe culture confirmation test for the *M. tuberculosis* complex (Accuprobe, bioMérieux, Marcy l'Etoile, France) or by 16S rRNA gene sequencing (15).

Molecular analysis of 12 *M. lentiflavum* isolates was performed by high-throughput amplified fragment length polymorphism (AFLP) (htAFLP) analysis according to a method described previously by van den Braak et al. (27) and Melles et al. (18). In

short, DNA was isolated from pure cultures (28), and htAFLP was performed according to standard procedures. One enzyme combination was used to digest the mycobacterial DNA: Csp6I/NIaIII. The general primers for preamplification were 5′-GTAGACTGCGTACCTAC-3′ for Csp6I and 5′-GAGTCCTGAGCATG-3′ for NIaIII and eight different sets of primers (+G/AA, +G/AG, +G/AT, +G/CA, +C/AA, +C/AG, +C/AT, and +C/CA) suited for selective amplification of subsets of restriction fragments. Apart from the 10 Zambian *M. lentiflavum* isolates, we included two independent isolates from The Netherlands and *M. tuberculosis* control strain H37Rv (6). Nine isolates were tested in duplicate to define the test reproducibility. Banding patterns of coded samples were scored visually by an independent observer. DNA fragments from 150 to 550 bp in length were evaluated.

The study was approved by the research ethical committee of the University of Zambia and by the Central Board of Health and the Ministry of Health in Zambia. Informed written consent to participate in the study was obtained from all patients. At the time of the study, the estimated Zambian HIV seroprevalence was 20%, and the case notification rate for tuberculosis was about 500/100,000 (20).

RESULTS

Clinical microbiology

Overall, out of 213 patients, 69% were HIV positive. *M. tuberculosis* was isolated from 44 patients, 77% of whom were HIV positive, and NTM were isolated from 90 patients. Without knowing the results of the study, treatment for tuberculosis was given to 73 patients, 10 of whom were treated for extra-pulmonary tuberculosis.

A specimen from a sterile body site was collected from 25 patients (60% HIV positive). Eight of these 25 specimens were positive for NTM (Table 1), while in another five specimens, the presence of *M. tuberculosis* was demonstrated. Twelve specimens were culture negative. The Ziehl-Neelsen smears were all negative except for one (case 1). In contrast, 21 of the 44 patients with a positive culture for *M. tuberculosis* had a positive Ziehl-Neelsen smear of their sputum. The restriction fragment length polymorphism patterns of the isolated *M. tuberculosis* strains were all different (data not shown), essentially excluding the possibility of laboratory cross-contamination for these strains.

Typing of *M. lentiflavum*

Fifty-five *M. lentiflavum* isolates were cultured from 149 specimens obtained from 38 of the 70 patients admitted to St. Francis Hospital in Katete. This mycobacterial species has so far been rarely identified from clinical materials (see Discussion). In order to exclude the possibility that laboratory cross-contamination was the source of these

Table 1: NTM isolated from sterile body sites in patients from three hospitals in Zambia[j].

Case	Date of inclusion[a]	Specimen	Isolate, no decontamination	Isolate, sulphuric acid	Isolate, Nalc-NaOH	Location (hospital-ward)[c]	Male / female	HIV	Temp[d] (°C)	Complaints[e]	Duration[f] (wk)	Gram[g]	ZN[h]
1	22-Mar	Lymphnode	*M. lentiflavum*			Katete-F	F	-	?	Dig	8	L, no m.o.	+
2	04-Apr	Ascites	UMS[b]			Katete-F	F	-	36,0	Dig	11	No gram	-
3	05-Apr	Ascites		*M. lentiflavum*	Negative	Katete-F	F	-	36,5	Dig	30	L, no m.o.	-[i]
4	10-Apr	Ascites		*M. fortuitum*	Negative	Shesheke-F	F	+	36,3	Dig	3	L, no m.o.	-
5	30-Mar	Cutaneous abscess		*M. lentiflavum*	Negative	Katete-F	F	+	39,5	Skin inf	104	PMN, gram neg cocci	-
6	27-Mar	Pleural eff		*M. lentiflavum*	Negative	Katete-F	F	-	?	Resp	4	L, no m.o.	-[i]
7	26-Jun	Pleural eff		Negative	*M. goodii*	Chilonga-M	M	-	35,0	Resp	2	PMN, no m.o.	-
8	03-Jul	Pleural eff		UMS[b]	Negative	Chilonga-F	F	+	36,0	Resp	4	PMN, gram pos cocci	-

[a]Date of inclusion indicates the date of inclusion in study in 2001, and the time that the specimens were collected. Mar = March; Apr = April; Jun = June.
[b]UMS = unknown *Mycobacterium* species.
[c]Location of hospital and ward where patients were admitted. F = internal medicine female ward; M = internal medicine male ward.
[d]Temp = temperature of patient at the time of inclusion. ? = temperature at the time of inclusion was unknown.
[e]Complaints for visiting the hospital. Dig = complaints/symptoms of the tractus digestivus; Resp = complaints/symptoms of the tractus respiratorius; Skin inf = infection of skin/abscess.
[f]Duration of complaints at the moment of visiting the hospital and inclusion in the study.
[g]Gram stain of normally sterile specimen. L = lymphocytes; PMN = polymorphonuclear leukocytes; No m.o. = no microorganisms.
[h]ZN = direct Ziehl-Neelsen smear of a normally sterile specimen.
[i]Two out of three acid-fast bacteria were seen in the slide.
[j]Note that the nontuberculous mycobacteria isolated from cases 1, 6, 7, and 8 are medically relevant on the basis of the diagnostic criteria of the American Thoracic Society.

Table 2: htAFLP results for *M. lentiflavum* strains isolated from Zambian patients admitted to St. Francis Hospital, including Dutch control strains[b].

Strain	Strain name	Ward	Origin of strain	Marker Pattern[a]							Overall htAFLP type
				I	II	III	IV	V	VI	VII	
1.	037 UA	1	Zambia	+	–	–	–	–	+	–	A
2.	00-1863	X	The Netherlands	–	+	–	–	–	–	–	B
4.	049 SpA	1	Zambia	+	–	+	–	–	–	–	C
5.	019 Sp2A	1	Zambia	–	+	–	–	–	–	+	D
7.	001 SpA	2	Zambia	–	+	–	–	–	–	+	D
8.	021 Sp2N	3	Zambia	–	+	–	–	–	–	+	D
9.	033 biopsy	2	Zambia	–	+	–	–	–	–	+	D
13.	033 Sp2A	2	Zambia	–	+	–	–	–	–	+	D
10.	011 U2A	4	Zambia	–	+	–	+	–	–	+	E
11.	045 Plv2A	2	Zambia	–	+	–	–	–	–	+	D
12.	062 SpN	2	Zambia	–	+	–	–	–	–	+	D
15.	01-0014	X	The Netherlands	–	+	–	–	+	–	–	F

[a]For an example of a marker pattern, see Fig. 2.

[b]Note that strains 9 and 13 are derived from two specimens obtained from a single patient. Strains 9, 13, and 11 are derived from cases 1 and 6 as described in this paper. The column denominated "Ward"identifies the ward of admission of the patient with "1"indicating the internal medicine ward for males, "2"indicating the internal medicine ward for females, "3"indicating the surgery ward for males, and "4"indicating the tuberculosis ward. Materials are identified in the strain code, with "U"being urine, "Sp"being sputum, "biopsy" being a lymph node biopsy, and "Plv"being pleural effusion.

positive cultures, a random set of 12 of these isolates was subjected to htAFLP analysis. The *M. lentiflavum* strains from cases 1 and 6 were included in this random set (strains 9, 11, and 13 in Table 2). Evaluation of the DNA fragments resulted in 256 scorable DNA markers. Reproducibility of the variable markers as deduced from the duplo analyses appeared to be 100% (results not shown), and two strains from two specimens from a single patient were indistinguishable.

Although most of the markers were not variable among the *M. lentiflavum* isolates, a part of the AFLP restriction fragments revealed a considerable level of variation. Any variable combination of markers received a marker pattern number (I to VII) (Table 2). Consequently, a marker pattern is corroborated by various marker fragments, thereby constituting a very reliable genotype. *M. tuberculosis* appeared to yield completely distinct AFLP patterns (see Fig. 2 for an example of the experimental output). Among the 12 *M. lentiflavum* strains, six different types were documented. The types obtained for the two Dutch strains (types B and F) were clearly different from those generated for the Zambian isolates (types A, C, D, and E) (Table 2).

Type D strains were isolated from six patients (patients 019, 001, 021, 033, 045, and 062), and the sputum and biopsy isolates from patient 033 yielded the same type. Although other strains from patients visiting the same hospital were genetically distinct,

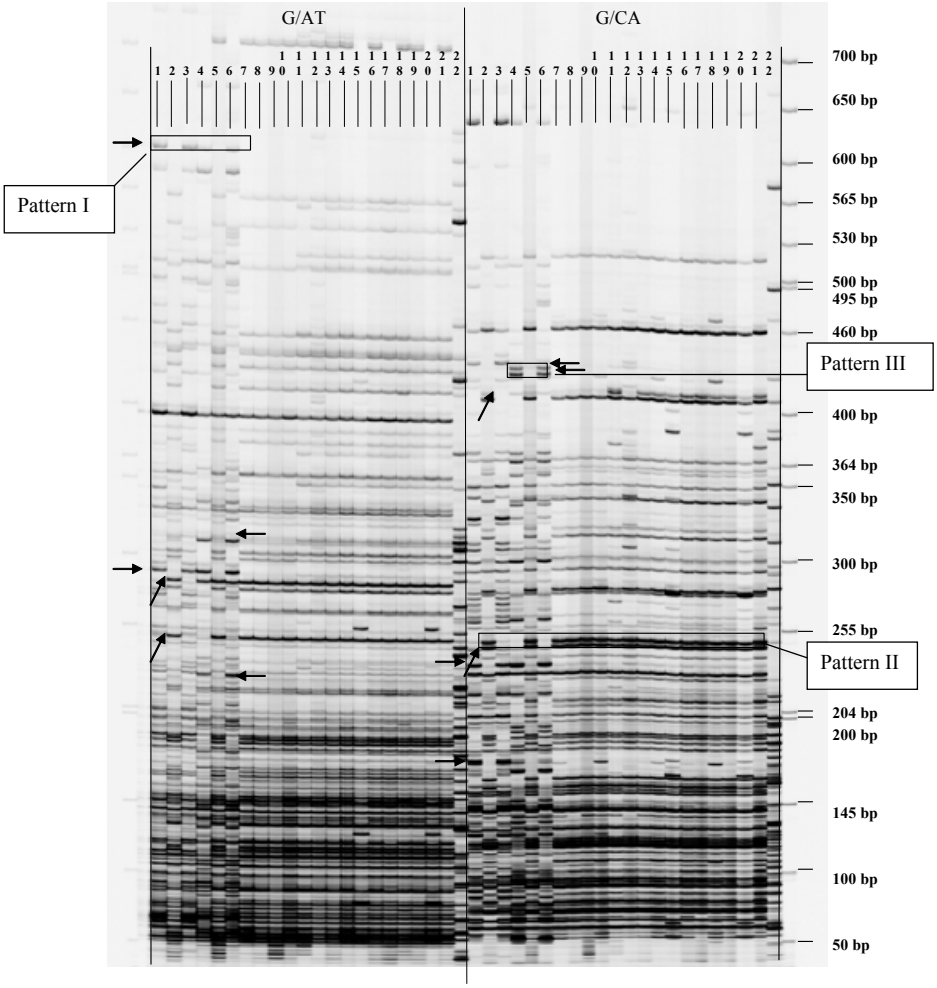

Figure 2: AFLP analysis of *M. lentiflavum* strains.
Two separate fragment patterns obtained by two amplifications steps with the primers G/AT and G/CA are demonstrated. Shown are the fingerprints for all strains listed in Table 2. Some strains are included in duplicate. Lane order is (from left to right): 1 = 037 UA, 2 = 00-1863, 3 = 037 UA, 4 = 049 SpA, 5 = 019 Sp2A, 6 = 049 SpA, 7 = 001 SpA, 8 = 021 Sp2N, 9 = 033 biopsy, 10 = 011 U2A, 12 = 062 SpN, 13 = 033 Sp2A, 14 = 001 SpA, 15 = 01-0014, 16 = 033 Sp2A, 17 = 00-1863, 18 = 011 U2A, 19 = 033 Sp2A, 20 = 01-0014, 21 = 045 Plv2A, 22 = *M. tuberculosis*. Three different types of arrows (pointing up, to the right or left) identify three main marker patterns. The marker patterns are mentioned in table 2. The arrow pointing up identifies pattern II, the arrow pointing to the right identifies pattern III and the arrow pointing to the left identifies pattern I. The boxed regions highlight the pattern belonging to the specific arrow. Molecular weight in bp on the right side of the figure.

ruling out full-blown laboratory contamination, we cannot exclude such an event for the six patients infected by a type D strain. On the other hand, this region of Zambia might be an area where type D is endemic.

DISCUSSION

NTM are ubiquitously present in the environment and can therefore be associated with either colonization, serious infection, or pseudo-outbreaks with a wide variety of presentations (10,22,23,25). NTM should be considered in all cases of nosocomial infection, and careful surveillance must be applied to identify possible outbreaks. Presumed nosocomial outbreaks of NTM can be investigated by molecular typing of mycobacterial isolates. In cases where identical DNA fingerprints are found, laboratory cross-contamination cannot always be excluded. However, in our study, all possible measures were taken to avoid any laboratory cross-contamination. The NTM were cultured from patients with compatible clinical syndromes and were isolated from specimens that do not normally contain microorganisms. Molecular analysis revealed that the mycobacterial isolates represented several evolutionary lineages and more subtle strain variations. Therefore, in our study, laboratory cross-contamination is considered highly unlikely.

On basis of the diagnostic criteria of the American Thoracic Society, the medical relevance of the NTM isolations reported here appears certain for at least half of the cases (cases 1, 6, 7, and 8). Their signs and symptoms were compatible with pathology found on physical examination or chest X ray, and the NTM were isolated from pleural effusions and a lymph node. In the other four cases (cases 2, 3, 4, and 5), NTM were isolated from ascites and a cutaneous abscess, and in these cases, it is not completely clear whether these mycobacteria were the most important causes of the pathologies. These last patients had other conditions that could have explained their nonspecific symptoms and the physical findings.

In none of the eight patients diagnosed with NTM infection of normally sterile body sites was the diagnosis made before hospital discharge or death. No specific therapy was given for the mycobacteria isolated, except for patient 2, who received empirical treatment with rifampin, isoniazid, and pyrazinamide before the results of the culture became available. In five of the six specimens of sterile body sites, which were decontaminated with sulfuric acid and NALC-NaOH, NTM were cultured only after decontamination with sulfuric acid. However, the commonly used decontamination method is the use of NALC-NaOH. It has been shown that the decontamination method used affects the recovery of NTM (4).

Only a few cases of human disease caused by *M. lentiflavum* have been reported previously. The first case, concerning an 85-year-old woman with spondylodiscitis that improved upon anti-tuberculous treatment, was reported in 1996 (24). Four children with lymphadenitis due to *M. lentiflavum* were described between 1997 and 2002 (5,12,26), as were cases of disseminated infection in an HIV-infected patient (21) and in a patient undergoing steroid therapy (13). Finally, *M. lentiflavum* was isolated from a patient with a chronic pulmonary disease. *M. goodii* was first described in 1999 and was isolated from

patients with traumatic osteomyelitis following iatrogenic infections and from patients with respiratory infections (3). Recently, a patient with bursitis due to an *M. goodii* infection was described (9). All of these cases occurred in Europe and the United States. To our knowledge, here we describe the first cases of *M. lentiflavum* and *M. goodii* infection in African patients. In fact, no clinically relevant NTM isolations in sub-Saharan Africa were reported before the 1990s (11,19).

Our htAFLP-mediated strain-typing method further corroborates the clinical relevance of the isolates; genetic diversity was observed among the strains. This degree of strain diversity shows that (i) the typing procedure is as adequate as can be expected when small numbers of strains are used and (ii) it is not the case that laboratory cross-contamination is the source of all of the *M. lentiflavum* isolates. However, multiple isolates of genotype D (Table 2) were identified. Whether this is due to laboratory contamination or a genuine NTM outbreak still needs to be resolved. It has to be emphasized that the respective type D strains were derived from patients nursed in different wards. The elevated incidence of the type D *M. lentiflavum* isolates (7/10 [70%] of the Zambian isolates) suggests that local dissemination of a certain type occurred or that *M. lentiflavum* as a species is quite clonal. The latter hypothesis is not in contradiction with the well-conserved features of the htAFLP fingerprints, even when isolates from The Netherlands and Zambia are compared (Fig. 2). In addition, both restriction fragment length polymorphism analysis and randomly amplified polymorphic DNA analysis performed for a subset of strains also revealed additional DNA polymorphisms among *M. lentiflavum* isolates (results not shown).

We conclude from our study that clinically relevant infection due to NTM seems to occur in HIV-positive as well as in HIV-negative patients in Zambia. The role of NTM in human disease in Africa may well be underestimated and should be examined in more detail and on a larger scale. Information is urgently needed with regard to the proper diagnostic procedures and the possibilities for adequate treatment of NTM-induced disease.

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Chapter 8

Clinical relevance of non-tuberculous mycobacteria in Katete, Zambia

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ABSTRACT

Some of the tuberculosis-like syndromes in Africa may be caused by NTM, as is the case in Western countries. Due to the spread of HIV, the role of NTM in mycobacterial infections may be particularly underestimated. In this study, the clinical relevance of the isolation of non-tuberculous mycobacteria (NTM) from HIV-positive as well as HIV-negative patients in Zambia with a high prevalence of HIV in the population is evaluated.

The study was performed in 2003 in St. Francis Hospital, which is located in the district of Katete in Zambia. One hundred and eighty chronically-ill patients and 385 controls were included. From these patients and controls their baseline characteristics were recorded and sputum or gargle specimens were cultured for mycobacteria.

The proportion of NTM-positive sputum samples from the patients was significantly higher in comparison to the controls, 11 and 6% respectively ($p<0.05$). The most frequently isolated NTM in the population of patients and controls was the *Mycobacterium avium* complex. However, in both groups also many (32%) not previously identified mycobacteria were isolated.

Two consecutive sputum samples were cultured from 29 of the 31 NTM-positive patients. In 25 of the 29 patients only a single culture was positive. The remaining 4 NTM-positive patients had two positive cultures for NTM of whom one patient had pulmonary disease. In two patients *M. intracellulare* was isolated twice, in one patient *M. avium* was isolated twice and in one patient two different species NTM together with *M. tuberculosis* were isolated. In three more NTM-positive patients one of the two sputum cultures contained AFB which could not be identified; two of these patients probably had pulmonary disease with *M. intracellulare*.

In contrast, only one control out of the 61 NTM-only positive controls had two sputum or gargle samples positive with (non-identical) NTM.

The 93 patients and controls with NTM cultured from the sputum had significantly different clinical and radiological features compared to the 472 patients and controls without NTM in the sputum. They were more likely to complain of vomiting and diarrhoea and were more often underweight (Body Mass Index < 18). In addition, the chest X-ray had more frequently changes consistent with TB.

NTM probably play an important role in the aetiology of mycobacterioses in Zambia. In this study two consecutive sputum samples were cultured from 154 patients and 383 controls. The estimated rate of colonization in the patient population was 14/154 (9%) with

a rate of disease of 3/154 (about 2%). The estimated rate of colonization in the general population was 61/383 (16%). More extended studies, in duration and size, are needed to determine the magnitude of NTM infection and colonization in the African population.

INTRODUCTION

The genus *Mycobacterium* can be divided into the *Mycobacterium tuberculosis* complex, *M. leprae,* and the atypical mycobacteria or non-tuberculous mycobacteria (NTM). These NTM are also named 'environmental mycobacteria', since they are frequently isolated from environmental sources, including surface water, tap water, and soil (21).

There are indications that NTM infections in humans are increasing (1,3). In addition, there appears to be marked geographic variability in the distribution of NTM both in terms of the prevalence of NTM lung disease and causative mycobacterial species (1,3,27). However, most data reporting high rates of NTM infection come from Northern European countries and the United States. In Africa, studies reporting NTM infections are rare, despite the fact that environmental exposure to NTM was found to be very high (17).

Tuberculosis is a problem of enormous dimension in Africa and *M. tuberculosis* is the most important causative agent. However, it is known that NTM also play a significant role in industrialised countries in the aetiology of tuberculosis-like syndromes. In Africa, the contribution of NTM to the clinical problem of tuberculosis has so far been examined only on a very small scale.

In Zambia, a country with historically high prevalence rates of tuberculosis, patients with acid-fast bacilli (AFB)-positive sputum on direct microscopic examination, or those displaying chest radiographic findings that suggest active tuberculosis but do not respond to antibiotics, are generally presumed to have pulmonary tuberculosis. In principle these patients are treated empirically with a combination of anti-tuberculosis drugs for a period of six months. Therefore, an inconclusive diagnosis of pulmonary tuberculosis could lead to the inappropriate or unnecessary treatment of patients. A number of the tuberculosis-like syndromes in Africa could be caused by NTM, as is the case in many industrialised countries. However, even if NTM are cultured and identified in respiratory specimens, NTM lung disease still requires differentiation from contamination or colonization. Thus, the detection of AFB in respiratory specimens, or the isolation of NTM, may pose a diagnostic problem for the clinician.

A useful guide to the diagnosis of NTM disease in patients with or without HIV co-infection are the set of criteria set out by the American Thoracic Society (ATS), published in a statement in 1997 and the similar guidelines formulated by the British Thoracic

Society (BTS) (1,3). The diagnostic criteria for NTM pulmonary infections include both the imaging findings consistent with pulmonary disease and the repeated isolation of the same mycobacteria from sputum or bronchial wash in a symptomatic patient.

In a pilot study performed in three different hospitals in Zambia in 2001, high rates of NTM culture positive sputa were observed (7). Therefore, in the present study, the clinical relevance of the isolation of NTM from HIV-positive as well as HIV-negative patients in Zambia is examined in detail.

MATERIAL AND METHODS

The study was performed in St. Francis Hospital, which is located in the district of Katete in Zambia, from August 2002 to March 2003. It serves a rural population of over 200,000 within a 60 km radius and receives specialist referrals from all over the Eastern Province (about 1.5 million residents).

The study population comprised of adults (≥15 years old) with chronic (defined as ≥ two weeks) complaints and a productive cough who were admitted to the department of internal medicine of the hospital. Most of the included patients had respiratory tract symptoms (96%). The other 4% of the patients had skin infections/abscesses or lymph adenopathy.

For each eligible admitted patient who consented to participate in the study, two healthy community controls were recruited randomly from the neighbouring community. These controls were not adjusted regarding age or other characteristics of the patients.

On enrolment, each patient and control was interviewed in their own language, and their medical records were reviewed using a standard form. A detailed physical examination was performed. Chest X-rays were evaluated blind in The Netherlands without any additional clinical information. Films were scored for mediastinal adenopathy, cavitation, pleural and pericardial fluid, miliary pathology, alveolar infiltration, interstitial pathology, pathology other than mentioned, or no pathology. The conclusions of the scoring system were chest X-rays labelled with no pathology, pathology not suggestive of TB, and pathology consistent for TB. Over three consecutive days, sputum was collected from patients with a productive cough. The controls were asked to gargle with normal saline if they could not produce sputum. The first two sputum or gargle specimens were cultured for mycobacteria, and the third one was stored at −20°C.

The study was approved by the research ethical committee of the University of Zambia, the Central Board of Health, and the Ministry of Health in Zambia. Informed consent was obtained from all patients and controls before recruitment.

Laboratory methods

Sputum or gargle specimens were divided into two equal parts: one half was decontaminated with *N*-acetyl-L-cysteine (NALC)-NaOH and the other half was decontaminated using 6% sulfuric acid in order to compare decontamination procedures for the culture of mycobacteria (6). The specimens were cultured in Mycobacteria Growth Indicator tubes (Becton Dickinson Microbiology Systems, Cockeysville, Md., US) according to the manufacturer's instructions and the guidelines described previously by Master (16). Mycobacterial isolates were identified by the Accuprobe culture confirmation test for the *M. tuberculosis* complex (Accuprobe, bioMérieux, Marcy l'Etoile, France) or by 16S rRNA gene sequencing (14). Serological testing for HIV was performed using a qualitative immunoassay (Abbott Determine HIV-1/2) and the Vidas HIV DUO assay (bioMérieux, Marcy l'Etoile, France).

Materials needed for collection and culture were imported from The Netherlands, and an experienced Dutch technician performed the work in the Zambian laboratory. All possible efforts were made to prevent laboratory cross-contamination, including the collection of a sputum specimen in a brand-new container imported from The Netherlands. The stock of decontamination fluid was sterilized twice a week, and each day a fresh, sterile tube or bottle was opened. The samples in the laboratory were processed one by one, away from other samples, and negative control cultures were included in each batch of samples. Microscopic slides were also prepared one at a time. All work associated with this project was conducted by the same experienced laboratory technician. All culture procedures were performed in a new class I biosafety cabinet.

Data analysis

Data were entered in SPSS 6 and analysed using STATA vs 8.0 (Stata Corporation College Station, TX, USA). Student t-tests were used to assess different means between groups; proportions were compared using chi-square testing. Univariate odds ratios (OR) with 95% confidence intervals (CI) were calculated to assess the associations of potential risk factors of participants being NTM-positive. A stepwise backward regression approach was used for a multivariate analysis.

Body Mass Index (BMI) was calculated as weight in kg divided by squared height in meters. Underweight was defined as a BMI of less than 18.

Clinical significance of isolated NTM: disease, infection, colonization

The Diagnostic Criteria for NTM Lung Disease were issued in 1997 by the American Thoracic Society (ATS) (1). In the year 2000, the British Thoracic Society (BTS) published its guidelines for the management of NTM lung disease (3). According to the British Thoracic Society guidelines, NTM lung disease is indicated when positive cultures develop from specimens of sputum obtained at least seven days apart in a patient whose chest radiograph suggests mycobacterial infection and who may or may not manifest symptoms or signs. The American Thoracic Society guidelines include diagnostic criteria that are stricter than those of the British Thoracic Society statement. The BTS criteria include symptomatic patients with infiltrate, nodular, or cavitary disease, who have had three positive sputum/bronchial wash cultures with negative AFB smears, or two positive cultures and one positive AFB smear, in the last 12 months.

However, in developing countries the criteria of the ATS or BTS may need some modification in order to be implementable, because the collection, examination and culturing of three consecutive samples within a certain time period is hampered by major logistic problems. In this study patients were therefore diagnosed as having NTM lung disease if they exhibited respiratory symptoms, abnormal chest radiography suggestive of mycobacterial infection and two positive sputum cultures with the same NTM. Patients or controls with positive NTM cultures who did not meet these criteria were considered colonized.

RESULTS

Between August 2002 and February 2003, 180 patients and 385 controls were included in the study. Two sputum samples were cultured from a total of 154 patients and 383 controls. The median age of the patients and controls was 35 (range 16-80) and 30 (range 15-78), respectively. Females represented 55% (99/180) of the patient group and 69% (265/385) of the control group. Of the patients, 128 (71%) were HIV-positive and of the controls 87 (23%).

Culture results of patients

Of the 180 patients, 60 (33%) with exclusively *M. tuberculosis* were isolated from the sputum samples, 12 (7%) yielded both *M. tuberculosis* and NTM, and 19 (11%) only NTM (see Table 1). The Ziehl-Neelsen smear microscopy of the sputum sample was positive in 46 of the 69 (67%) patients with *M. tuberculosis* and in one patient with a NTM-positive culture.

Table 1: Culture results of patients and controls.

Culture results	Patients	Controls
Number	180	385
Culture exclusively *M. tuberculosis*	60 (33%)	2 (0.5%)
Culture *M. tuberculosis* and NTM	12 (7%)	1 (0.3%)
Culture exclusively NTM[a]	19 (11%)	61 (16%)
Culture NTM	31 (17%)	62 (16%)
Two sputum or gargle samples cultured	154	383
Two sputum or gargle samples cultured in NTM positive person	29 (of 31 NTM-positive patients)	62 (of 62 NTM-positive controls)
Single NTM positive culture in NTM positive person with two samples cultured	25 (of 29 NTM-positive patients)	61 (of 62 NTM-positive controls)
Two NTM positive cultures in NTM positive person with two samples cultured	4 (of 29 NTM-positive patients)	1 (of 62 NTM-positive controls)
Two NTM positive cultures in persons with two samples cultured[b]	4 (of 154 patients with two samples cultured)	1 (of 383 controls with two samples cultured)

[a]The proportion of patients with exclusively NTM was comparable with the controls (p=0.2).
[b]Significantly more patients in comparison to controls had two sputum or gargle cultures positive with NTM ($p<0.05$).

Two consecutive sputum samples were cultured from 29 of the 31 NTM-positive patients. In 25 of the 29 patients only a single culture was positive. The remaining 4 NTM-positive patients had two positive cultures for NTM of whom one had pulmonary disease. In two patients *M. intracellulare* was isolated twice, in one patient *M. avium* was isolated twice and in one patient two different species NTM together with *M. tuberculosis* were isolated.

In three more NTM-positive patients one of the two sputum cultures contained AFB which could not be identified. Two patients had *M. intracellulare* in one sputum sample and from one patient *M. porcinum* was isolated.

Case reports for patients suspected of NTM disease

The characteristics of the patients with NTM in both sputum samples and NTM together with AFB which could not be identified are shown in Table 2. In three (cases 1, 2 and 4) there was a solid basis for the suspicion of NTM-associated disease because of the combination of symptoms, positive cultures and pathology on the chest X-ray consistent with TB. However, only Case 1 fulfilled the criteria of this study for NTM lung disease. In all three of these patients *M. intracellulare* was isolated.

One patient (Case 4) was a 32-year-old HIV-positive man who complained of a productive cough with haemoptysis of 17 weeks' duration. Furthermore, he was vomiting and had diarrhoea. The BMI was 15. He had been treated for tuberculosis in the past. The chest X-ray was consistent with TB and showed alveolar infiltration and interstitial

Table 2: Cases with NTM in two consecutive sputum samples.

Case	Isolate 1st sputum	Isolate 2nd sputum	ZN	Male/female	Age	HIV	Temp[b]	Complaints[c]	Duration[d] (wk)	Chest X-ray	Died	BMI[e]
1[a] (624)	*M. intracellulare*	AFB not identified	1+	Male	55	pos	34.6	resp	17	suspected of TB	yes	17
2[a] (691)	AFB not identified	*M. intracellulare*	neg	Female	45	pos	36.1	resp	487	suspected of TB	no	20
3 (643)	*M. intracellulare*	*M. intracellulare*	neg	Male	43	pos	36.3	resp	3	no pathology	no	19
4[a] (684)	*M. intracellulare*	*M. intracellulare*	neg	Male	32	pos	36.8	resp	17	suspected of TB	yes	15
5 (645)	AFB not identified	*M. porcinum*	neg	Female	50	neg	36.6	resp	8	not suspected of TB	no	21
6 (703)	*M. avium*	*M. avium*	neg	Male	33	pos	37.4	resp	17	no chest X-ray	no	16
7 (644)	*M. avium*	*M. peregrinum* + *M. tuberculosis*	neg	Female	24	pos	35.0	resp	13	no pathology	yes	?

[a]Patients assumed to have pulmonary NTM disease.

[b]Temp = Temperature at time of inclusion.

[c]Complaints = Reasons for visiting the hospital. Resp = Complaints/symptoms of the tractus respiratorius.

[d]Duration = Duration of complaints at the point of visiting hospital and inclusion in study.

[e]BMI = Body mass index. ? = BMI not known.

pathology. He did not improve on antibiotics and started for the second time with an anti-tuberculosis treatment. Mycobacteria were cultured from both sputum samples and identified as *M. intracellulare*. He died five weeks later.

Another patient (Case 1), suffering from *M. intracellulare* pulmonary disease, was a 55-year-old man who was HIV-positive. He was admitted with a productive cough with haemoptysis that he had had for 17 weeks. Diarrhoea was also present and his BMI was 17. He had been receiving anti-tuberculosis treatment for four months. The sputum obtained before treatment was started was AFB negative. The radiographic investigation of the chest revealed cavities and alveolar consolidation. No improvement was achieved while on antibiotics. The sputum was examined again and the smear this time was positive for AFB. The first sputum culture revealed *M. intracellulare* and, unfortunately, in the second sputum culture the mycobacteria could not be identified due to logistic reasons. The patient died three weeks later.

The last patient (Case 2) with *M. intracellulare* pulmonary disease was an HIV-positive 45-year-old woman who presented with respiratory complaints that she had had for more than one year. She was known to have asthma. On physical examination enlarged submandibular, supraclavicular and axillary lymph nodes were found. Her BMI was 20. Alveolar infiltration was seen on the chest X-ray. The culture of the first sputum indicated the presence of mycobacteria which could not be identified; in the second sputum *M. intracellulare* was identified.

Culture results of controls

In three of the 385 (0.8%) controls, *M. tuberculosis* was cultured from the sputum or gargle specimen, and NTM was also isolated from one of these three patients (see Table 1). In 61 (16%) controls exclusively NTM were isolated and this number was comparable with the proportion of patients among whom exclusively NTM were isolated (11%, p=0.2). In 61 out of 62 NTM positive controls, only one sputum or gargle specimen was culture positive for NTM. In one control, who was HIV-negative, two different mycobacteria, *M. porcinum* and an unknown *Mycobacterium*, were isolated. No chest X-rays suggestive of tuberculosis were observed in any of the controls.

From 383 controls, two sputum or gargle samples were subjected to *Mycobacterium* culture. Significantly more patients in comparison to these controls had two sputum or gargle cultures positive for NTM, four out of 154 patients and one out of the 383 controls ($p<0.05$).

Isolated Mycobacterium species

The collected sputum or gargle samples from all patients and controls were split in two equal parts before decontamination in order to compare the influence of the decontamination

Table 3: Culture results of sputum of patients and controls.

Culture result	Patients	Controls
Negative	362 (57%)	1428 (93%)
M. tuberculosis	201 (32%)	5 (0.3%)
M. avium complex	15 (2%)	5 (0.3%)
M. intracellulare	12 (2%)	0
M. avium	3 (0.5%)	5 (0.3%)
M. gordonae	4 (0.6%)	0
M. peregrinum	2 (0.3%)	4 (0.3%)
M. goodii	1 (0.2%)	4 (0.3%)
M. porcinum	1 (0.2%)	3 (0.2%)
M. lentiflavum	1 (0.2%)	0
Various unknown *Mycobacterium* species	13 (2%)	42 (3%)
Various other *Mycobacterium* species*	3 (0.5%)	7 (0.5%)
Unidentified AFB	32 (5%)	34 (2%)
Total number of sputum samples	635	1532

*Various other *Mycobacterium* species in the patients include *M. fortuitum, M. neoaurum,* and *M. simiae*. Various other *Mycobacterium* species in the controls include *M. fortuitum, M. asiaticum, M. aurum,* and *M. conspicuum.*

method on the yield of mycobacteria (6). From the 180 patients included, a total of 635 sputum samples were cultured and in total 1,532 sputum or gargle samples were analysed from the 385 controls. The results of the cultures are depicted in Table 3. The relative number of NTM (72) isolated from the 635 sputum samples of patients was significantly more in comparison with the number of NTM (99) isolated from 1,532 sputum or gargle samples from the controls: 11 and 6 percent, respectively ($p<0.001$).

From 273 (43%) of the 635 sputum samples of patients, mycobacteria were isolated. *M. tuberculosis* was found in 201 of the 273 (74%) positive sputum specimens and NTM in 72 (26%). The most frequently isolated NTM was *M. intracellulare* (12).

Mycobacteria were isolated from 104 of the 1,532 (6.8%) sputum or gargle samples cultured from the controls. *M. tuberculosis* was found in 5 of the 104 (4.8%) positive cultures and NTM in 99 (95%). The predominant NTM isolated from the controls were *M. avium* (5), *M. goodii* (4) and *M. peregrinum* (4).

In both groups also many (55 of the 171 NTM = 32%) mycobacteria not previously identified were found.

Comparison of persons with and without NTM in their sputum or gargle sample

The 93 patients and controls with NTM-positive cultures had significantly different clinical and radiological features compared to the 472 patients and controls without NTM in the sputum (Table 4). They were more likely to report vomiting and diarrhoea. Furthermore, they were more often underweight (BMI < 18). Overall, a clinical picture of general

Table 4: Clinical features of NTM-positive and negative persons.

	Any sample NTM-positive	NTM-negative	p-value	All
Subjects, n (%)	93 (16.5)	472 (83.5)	-	565
HIV-positive, n (%)	41 (45.6)	174 (38.5)	0.2	215 (39.7)
M. tuberculosis, n (%)	13 (14.0)	62 (13.1)	0.8	75 (13.3)
BMI, mean (sd)	20.2 (4.2)	20.8 (3.7)	0.2	20.7 (3.8)
Underweight, n (%)	26 (29.6)	92 (20.6)	0.06	118 (22.1)
Vomit, n (%)	8 (8.6)	18 (3.8)	0.04	26 (4.6)
Diarrhoea, n (%)	12 (12.9)	11 (2.3)	<0.001	23 (4.7)
Lymph nodes, n (%)	21 (22.8)	146 (30.9)	0.1	167 (29.6)
Chest X-ray compatible with TB but culture negative for *M. tuberculosis*, n (%)	5 (26.3)	28 (7.1)	0.003	33 (8.0)
Died, n (%)	9 (9.7)	26 (5.5)	0.1	35 (6.2)

Table 5: Background characteristics of NTM-positive and negative persons.

	NTM-positive, n (%)	NTM-negative, n (%)	p-value	All, n (%)
Subjects	93 (16.5)	472 (83.7)	-	565
Female	61 (65.6)	303 (64.2)	0.8	364 (64.4)
Age (mean, sd)	36.7 (13.1)	34.8 (14.6)	0.2	35.1 (14.4)
Farmer	48 (51.6)	245 (51.9)	1.0	293 (51.9)
Water				
Well	27 (29.4)	158 (33.6)		185 (32.9)
Borehole	40 (43.5)	235 (49.9)		275 (48.9)
Tap	23 (25.0)	62 (13.2)		85 (15.1)
River	1 (1.1)	9 (1.9)		10 (1.8)
Swamp	1 (1.1)	7 (1.5)	0.1	8 (1.4)
Un-boiled milk	15 (16.1)	62 (13.1)	0.4	77 (13.6)
Smoker	5 (5.7)	41 (8.9)	0.3	46 (8.4)
Alcohol	9 (10.3)	51 (11.2)	0.8	60 (11.0)
Hospitalised	31 (33.3)	149 (31.6)	0.7	180 (31.9)
Previous TB treatment	9 (9.7)	28 (5.9)	0.2	37 (6.6)

malaise was more often observed in the NTM-positive person. In addition, the chest X-ray more frequently revealed changes consistent with TB, such as consolidation and interstitial pathology. The HIV-status and the presence of *M. tuberculosis* in the sputum culture was not significantly different between the NTM culture positive and negative persons. Moreover, there were no significant differences in age (mean 36.7 versus 34.8 yrs; p=0.2), sex, smoking habits, alcohol, and previous tuberculosis treatment between the groups (Table 5). The percentage of farmers, source of water supply and the use of (un)boiled milk were comparable. A subgroup analysis, restricted to patients with NTM versus patients without NTM in the sputum, yielded broadly similar results (data not shown).

Table 6: Crude and adjusted risk factors (OR, 95% CI) for NTM isolated from sputum.

	Univariate analysis, OR (95% CI)	Multivariate analysis, OR (95% CI)*
Hospitalised	1.1 (0.7-1.7)	ns
Age >=25	1.3 (0.8-2.5)	ns
Sex (female)	1.1 (0.7-1.7)	ns
Underweight (BMI < 18)	1.6 (1.0-2.7)	1.7 (1.0-2.9)
M. tuberculosis	1.1 (0.6-2.0)	ns
Previous TB treatment	1.7 (0.8-3.7)	ns
HIV-positive	1.3 (0.8-2.1)	ns
Tap water to other sources of water supply	2.2 (1.3-3.8)	2.0 (1.1-3.5)
Alcohol	0.9 (0.4-1.9)	ns
Smoking	0.6 (0.2-1.6)	ns
Un-boiled milk	1.3 (0.7-2.3)	ns
Farmer	1.0 (0.6-1.5)	ns
Chest X-ray compatible with TB but culture negative for *M. tuberculosis*, n (%)	4.7 (1.6-13.9)	ns

*stepwise backward elimination.
Definition of abbreviations: ns = not (statistically) significant, OR = odds ratio, CI = confidence interval.

The independent risk factors for NTM culture positive sputum were determined using multivariate analysis. Two factors, underweight (BMI<18) and water supply from the tap, were independently associated with having a NTM-positive sputum culture (see Table 6).

DISCUSSION

The purpose of this study was to compare the prevalence of NTM in sputum between hospitalised, chronically-ill patients and community controls and to determine the clinical significance of the isolation of NTM.

The proportion of patients and controls with a positive sputum or gargle culture for NTM were comparable, 11 and 15%, respectively. However, the proportion of NTM-positive sputum samples from the patients was significantly higher in comparison to the controls, 11 and 6% respectively. This suggests persistent NTM are associated with the chronic complaints of these patients.

Gargle is not the same as a normal sputum sample and it is not well known whether culture results are influenced by these different ways of obtaining specimens. In a specimen obtained by gargle especially the flora of the oropharyngeal mucosa is obtained, while sputum also represents the flora of the lower airways. In the patient group more subjects

were capable of producing sputum and this may have influenced the yield of positive NTM cultures.

In the literature, many risk factors for NTM have been determined. These risk factors can be divided in underlying medical conditions, the living and working environment, and demographic features. Patients with cystic fibrosis and HIV-positives are two important high-risk groups (15,18). Other important factors includes the following: underlying chronic lung disease, working in the mining industry, advanced age, and being male (5,12,15,22). Studies in immune competent patients recognised two patient profiles as being at increased risk of NTM: 'elderly females' and 'middle-aged males' (9,19). In this study, in an African setting, HIV, sex and age were not determined as risk factors. In fact, the only two true risk factors for a positive NTM culture were being underweight and consuming of tap water. It is known from the literature that NTM are natural inhabitants of municipal water systems and soil. However, the demographic figures in Africa may not be comparable to that in the industrialised world.

It became clear in this study that patients and controls with NTM in the sputum or gargle sample had more frequent symptoms and signs of general malaise and that the chest X-rays in these NTM-positive persons more frequently showed pathology in comparison with NTM culture-negative persons. Clinical symptoms caused by NTM can be distinguished in four main categories: pulmonary disease, superficial lymphadenitis, skin and soft tissue infection, and disseminated disease. Pulmonary disease accounts for up to 90% of all cases. Because one of the inclusion criteria for this study concerned chronic complaints with a productive cough, it is conceivable that most of the included patients were having respiratory symptoms. However, besides the respiratory symptoms and signs, patients with a positive NTM culture also more frequently revealed a clinical picture of general malaise, including diarrhoea, vomiting, and being underweight. These symptoms and signs may not be specific for NTM infection, but may reflect the poor health of the patient more generally.

Differences in the geographic distribution of NTM species have been reported (3,19,25). The most commonly encountered NTM from clinical specimens in the industrialised world are *M. avium* complex (MAC) and *M. kansasii*. In Africa limited studies have been performed and the distribution of NTM is not fully known. In the literature, MAC is less frequently isolated from clinical specimens (11,17).

In contrast, in this study, the most isolated NTM in the population of patients and controls was MAC. However, in both groups a part (32%) of the NTM found in Zambia have not yet been identified on a species level and named in other countries. This study indicates that the distribution of NTM in Africa may be different from Europe and the United States of America. The not-yet identified NTM at least cause colonization in

Africans and in some patients they also cause disease. The magnitude of this problem, in addition to the problem of tuberculosis, is unknown at the moment, but deserves more attention.

In a former study in Katete during the end of the warm and rainy season in 2001, a high rate of *M. lentiflavum* among patients was reported (7). However, in the present study, performed during the cool and dry season as well as in the warm and rainy season, there was only one patient from whom *M. lentiflavum* was isolated. It is known from the literature that several factors influence the occurrence of mycobacteria in the environment. Soil and geologic factors such as chemical composition of the substrates and pH play an important role in the distribution of mycobacteria, in general, and of each mycobacterial species, in particular. Furthermore, seasonal variations in the composition of the mycobacterial flora are linked to climatic conditions and rates of recovery of mycobacteria in the environment are higher in the dry than in the wet season (8,20). Another possible explanation for the differences in the number of *M. lentiflavum* isolated in the two studies may be found in man-made artificial environments of NTM. NTM such as *M. avium*, *M. kansasii* and *M. xenopi* have frequently been isolated from drinking and hospital water distribution systems and also *M. lentiflavum* was isolated from these water systems (24,26). In the year 2001 an environmental reservoir contaminated with *M. lentiflavum* may have resulted in colonization of the respiratory or digestive tract of the patients and, hence, have caused disease in a part of them.

Also the rates of NTM colonization and disease that have been reported seem to be variable in different geographic areas. In North America and Europe, rates of colonization and disease in the general population range from about 1-15 per 100,000 and 0.1-2 per 100,000, respectively. In the major part of Africa these rates are largely unknown. In South Africa, prevalence rates of NTM colonization in the population of between 1,400 and 6,700 per 100,000 have been reported (4,13). In South Africa gold miners' rates of NTM colonization were calculated at 101 per 100,000 and rates of disease were reported as 66 and 12 per 100,000 for *M. kansasii* and *M. scrofulaceum*, respectively (10,11).

In this study two sputum samples were collected and cultured from 154 of the 180 chronically-ill patients included.

NTM were isolated from at least one sputum sample in 29 patients. In four of these 29 patients NTM was isolated in both sputum samples. In three more NTM-positive patients one of the two sputum cultures contained AFB which could not be identified (Table 1).

NTM lung disease was definitely diagnosed in one and probably in three patients (cases 1, 2 and 4). From the sputum samples of these HIV-positive patients *M. intracellulare* was isolated. These patients presented with respiratory complaints and the chest X-ray

showed pathology compatible with tuberculosis. Unfortunately, in two patients one of two sputum samples with mycobacteria could not be identified due to contamination and re-culture problems. The combination of symptoms, positive cultures and pathology on the chest X-ray are significant characteristics of NTM infection and are suggestive of NTM pulmonary disease.

However, the ATS criteria are not completely fulfilled because two instead of three sputum samples were cultured on consecutive days. Furthermore, two of these patients suspected for NTM lung disease were treated for tuberculosis in the past. The sputum specimens of these patients were cultured twice in liquid medium after splitting each specimen for decontamination with two different methods. These sputa were not tested with molecular amplification techniques for the possible presence of *M. tuberculosis* and for the presence of multi-drug resistant *M. tuberculosis* which should have been excluded (2,23). However, the performance of these nucleic acid amplification (NAA) tests is in general good in clinical respiratory specimens that are AFB smear-positive but less in specimens that contain fewer organisms or are AFB-negative. The NAA-test would in a part of the cases be better in comparison to culture to detect a mixture of NTM and *M. tuberculosis*. Moreover, sputum specimens were not cultured on a solid medium and therefore it was not possible to count the number of colony-forming units to distinguish between colonization and infection or disease.

The estimated rate of colonization in this study in the patient population was 14/154 (9%) with a rate of disease of 3/154 (about 2%).

From 383 controls, two sputum or gargle specimens were collected and cultured. NTM were isolated from both specimens in one of the 61 controls with at least one sample being NTM culture positive. The control was not suspected of NTM pulmonary disease. The estimated rate of colonization in the general population on the basis of this figure is 61/383 (16%).

NTM probably play an important role in the aetiology of mycobacterioses in Zambia. More extended studies, both in terms of duration and size, will be needed to determine the extension of NTM infection in the African continent.

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Chapter 9

Only half of the tuberculosis cases diagnosed in Zambia by sputum Ziehl-Neelsen microscopy and/or chest X-ray are confirmed by culture

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ABSTRACT

Diagnosis of tuberculosis (TB) in resource-poor countries is almost exclusively based on microscopy of Ziehl-Neelsen stained (ZN) sputum smears; in some settings supplemented by chest X-ray. The aim of this study was to determine the accuracy of this diagnosis of TB in Zambia in the era of increasing HIV prevalence. By applying MGIT liquid culture technique, this study distinguished between (i) TB cases confirmed by positive *Mycobacterium tuberculosis* cultures, (ii) mycobacteriosis caused by non-tuberculous mycobacteria (NTM) and, (iii) tuberculosis-like disease caused by organisms other than mycobacteria.

Patients (15 years and above) who had been diagnosed with TB on the basis of: respiratory complaints lasting two or more weeks, failure to improve on two courses of routine empiric antibiotics, a positive ZN sputum smear and/or findings on the chest X-ray consistent with TB were included in this study. Sputum of the diagnosed TB cases was additionally subjected to MGIT liquid culture. *Mycobacterium* isolates were identified using a nucleic acid amplification method and 16S DNA sequencing.

Only in 47% of the 187 diagnosed TB cases *M. tuberculosis* was cultured. In another 19% of the cases, exclusively NTM were cultured, whereas from 12% of the TB cases a combination of *M. tuberculosis* and NTM was isolated. In the remaining 29% of cases in which TB was diagnosed, no mycobacteria were cultivable. HIV positivity was significantly correlated with the isolation of NTM from sputum and inversely associated with the isolation of exclusively *M. tuberculosis* from sputum ($p<0.05$).

Basing the diagnosis of tuberculosis on symptoms, sputum smear and/or chest X-ray may lead to significant numbers of false-positive cases of tuberculosis in Zambia, due to the increased prevalence of HIV.

INTRODUCTION

The increase in tuberculosis (TB) rates is closely associated with the spread of the HIV epidemic throughout sub-Saharan Africa. The clinical presentation of TB in HIV-infected individuals various with the degree of immunosuppression, and TB symptoms in HIV infected persons are often non-specific, resulting in a delayed- or misdiagnosis.

Sputum smear examination for acid-fast bacteria (AFB) is still the cornerstone of diagnosis of pulmonary TB in low-income countries. Smear examination by experienced technicians can result in the diagnosis of at most 50-60% of TB cases. However, in these high-prevalence settings considerably lower rates of AFB detection in TB patients are observed because of poor access to good quality microscopes, the shortage of trained staff and the work load. Moreover, the sputum smears of HIV-positive patients often present as Ziehl-Neelsen (ZN) negative. Unfortunately, although sputum culture remains the 'gold standard' for the diagnosis of pulmonary TB, this diagnostic tool is rarely available in low-income countries. Moreover, the traditional solid medium culture of AFB takes 3-8 weeks, limiting the usefulness of the culture as a first-line diagnostic test. In the last decade, liquid culture methods have become available in the industrialized world, with a shorter turn-around time of 1-3 weeks (2,16).

According to the International Standards for Tuberculosis Care, all persons with an unexplained productive cough lasting two to three weeks or more should be evaluated for TB (10). These patients should submit at least two, and preferably three, sputum specimens for microscopic examination. If such patients have a sputum smear positive for acid fast bacilli, they are officially diagnosed with TB. The diagnosis of sputum smear-negative pulmonary TB is based on at least three negative sputum smears, chest radiography findings consistent with TB, and lack of response to broad-spectrum antimicrobial agents.

However, in areas with a high prevalence of HIV and tuberculosis infection, the diagnosis of smear-negative TB is problematic, given the atypical presentation of pulmonary TB in HIV-infected patients. Furthermore, smear-negative patients can also present with normal or only slightly abnormal chest X-rays. Moreover, in low income countries, access to high quality radiography and interpretation is often limited (14). The accurate diagnosis of smear-negative pulmonary TB may therefore be complicated when reliance is just placed on clinical and radiological features.

Patients for whom there is a strong clinical suspicion of TB are often treated empirically with a combination of anti-tuberculosis drugs for six to nine months. Therefore, the inaccurate diagnosis of tuberculosis leads to unnecessary treatment of patients, diverting assets and personnel from true TB cases.

The aim of this study was to determine the accuracy of the clinical diagnosis of TB, based on sputum smears and chest X-ray in Zambia in the era of increasing HIV prevalence. Using a modern liquid culture technique a distinction was made between (i) TB cases confirmed by positive *Mycobacterium tuberculosis* cultures, (ii) mycobacterioses caused by non-tuberculous mycobacteria (NTM) and, (iii) tuberculosis-like diseases caused by pathogens other than mycobacteria.

MATERIAL AND METHODS

This study was conducted in St. Francis Hospital in Katete, Yeta District Hospital in Sesheke and Our Lady's Hospital in Chilonga. Ethical approval for the study was provided by the research ethical committee of the University of Zambia, the Central Board of Health, and the Ministry of Health in Zambia.

All adults aged 15 years and more, admitted to the internal medicine ward with chronic complaints lasting two or more weeks, were screened for TB and HIV after informed consent had been obtained. The study was performed between 2001 and 2003 during consecutive time periods of several months.

The medical history of the patients was retrieved, and detailed physical examination was performed. During three consecutive days, sputum was collected from patients with a productive cough. The first two sputum specimens were cultured for mycobacteria, and the third one was stored at −20°C. Sputa specimens were examined in the normal routine flow of the hospital by microscopy for the presence of acid-fast bacteria after Ziehl-Neelsen staining and all were subjected to culture in Mycobacteria Growth Indicator tubes (Becton Dickinson Microbiology Systems, Cockeysville, Md., US). Decontamination of the sputum was done using *N*-acetyl-L-cysteine (NALC)-NaOH and 6% sulfuric acid after dividing the sputum specimen into two equal parts to compare decontamination procedures for the detection of mycobacteria (7).

Chest X-rays were evaluated in the Netherlands on a blinded manner without any additional clinical information. Films were scored for mediastinal adenopathy, cavitation, pleural and pericardial fluid, miliary pathology, alveolar infiltration, interstitial pathology, pathology other than mentioned, or no pathology. The conclusions of the scoring system were defined as: chest X-rays labelled with no pathology, pathology not consistent with TB, or pathology consistent with TB.

Serological testing for HIV was performed using a qualitative immunoassay (Abbott Determine HIV-1/2) and the Vidas HIV DUO assay (Biomérieux, Marcy l'Etoile, France). The Accuprobe culture confirmation test for *M. tuberculosis* complex (Accuprobe, bio-Mérieux, Marcy l'Etoile, France) and/or 16S rRNA gene sequencing were used to identify mycobacterial isolates (12).

RESULTS

In this study, 320 chronically ill patients were enrolled. The median age of the population was 36 (range 16-80) and 52% were female. Of these patients 313 were HIV-tested and 75% of them were HIV-positive. The median duration of their complaints before admission to the hospital was eight weeks.

From the 320 patients, 187 were diagnosed as having TB on the basis of: respiratory complaints lasting two or more weeks, no improvement on two courses of routine empiric antibiotics (e.g. chloramphenicol, cotrimoxazole), a positive ZN sputum smear and/or abnormalities on the chest X-ray consistent with TB. At least two sputum smears for AFB were microscopically examined in 170 (91%) patients and at least two sputum specimens were cultured from 151 (81%) patients. In this cohort in the group of patients diagnosed having TB, the HIV-positivity, median duration of complaints and the median age were all comparable with that in the whole study group.

Of the 187 patients diagnosed with TB on the basis of sputum smears and chest X-ray, 72 (39%) had a positive ZN smear and 174 (95%) had a chest X-ray consistent with TB (Table 1 and 2). In 47% (88) of these 187 patients *M. tuberculosis* was isolated from one of the at least two sputum specimens. In another 19% (36) of these 187 cases, exclusively NTM were cultured, whereas from 12% (23) of the TB cases a combination of *M. tuberculosis* and NTM was isolated. In the remaining 29% (54) cases in which TB was diagnosed, the culture for mycobacteria remained negative. In five patients diagnosed with TB and having a positive ZN sputum microscopy, only NTM were isolated. However, most of the patients who were diagnosed as TB and from whom only NTM were cultured from the sputum had a negative ZN.

Of the 187 diagnosed TB patients, 183 were tested for HIV and 134 (73%) were HIV-positive (Table 2). Significantly more HIV-positive patient had a positive culture for NTM in comparison with HIV-negative patients; 38% (50/134) and 18% (9/49) respectively ($p<0.05$). Furthermore, 30% (40/134) of the HIV-positive TB patients had a positive culture for exclusively *M. tuberculosis*, in comparison to 51% (25/49) of the HIV-negative TB patients ($p<0.05$).

Table 1: Ziehl-Neelsen (ZN) of sputum, chest X-ray, culture results and HIV status of diagnosed TB patients.

ZN sputum	Chest X-ray	Culture result					Total
		negative	*M. tuberculosis*	*M. tuberculosis* and NTM	NTM only	AFB not identified	
Negative	Consistent with TB	53 (39 HIV+ and 3 HIV? [a])	18 (16 HIV+)	6 (5 HIV+)	31 (24 HIV+)	7 (5 HIV+ and 1 HIV? [a])	115
Positive	No abnormalities	1 (HIV-)	5 (4 HIV+)	2 (2 HIV+)	0	1 (HIV-)	9
	Consistent with TB	0	41 (20 HIV+)	14 (13 HIV+)	3[b] (3 HIV+)	1 (HIV-)	59
	No chest X-ray	0	1 (HIV-)	1 (HIV+)	2[c] (2 HIV+)	0	4
Total		54 (29%)	65 (35%)	23 (12%)	36 (19%)	9 (5%)	187

[a]HIV? = HIV status not known.
[b]Two of these three patients with a sputum positive ZN and only NTM isolated from the sputum were receiving anti-tuberculosis treatment.
[c]One of these two patients with a sputum positive ZN and only NTM isolated from the sputum was receiving anti-tuberculosis treatment.

Table 2: Culture results, HIV status and whether was started with or without anti-tuberculosis treatment of diagnosed TB patients.

Culture result	Patients, n (%)	HIV status, n (%)		Anti-tuberculosis treatment, n (%)	
		Positive	Negative	Yes	No
Negative for mycobacteria	54 (29)	39 (29)	12 (25)	29 (22)	25 (47)
M. tuberculosis only	65 (35)	40 (30)	25 (51)	60 (45)	5 (9)
M. tuberculosis and NTM	23 (12)	21 (16)	2 (4)	23 (17)	0
NTM only	36 (19)	29 (22)	7 (14)	18 (13)	18 (34)
AFB not identified	9 (5)	5 (3)	3 (6)	4 (3)	5 (9.5)
Subtotal		134 (73)	49 (27)	134 (72)	53 (28)
Total	187	183		187	

Anti-tuberculosis treatment was initiated in 134 of the 187 (72%) patients in the research group diagnosed with TB on the basis of a positive sputum smear or a chest X-ray consistent with TB (Table 2). Of the 88 patients with *M. tuberculosis* positive sputa, 83 (94%) were treated. However, also 18 of the 36 (51%) of the patients with only NTM in their sputum and 29 of the 54 (54%) patients with a negative culture for mycobacteria were also treated.

In three of the 72 patients with a positive ZN sputum smear no anti-tuberculosis treatment was started. In the first patient, therapy was withheld because only one of the three smears was positive and very few AFB were seen. Also the chest X-ray of this patient

was read as normal. Sputum cultures were all negative. The second untreated patient was infected by HIV, received several courses of antibiotics without improvement, and the chest X-ray was consistent with tuberculosis. However, only one of the smears was microscopically weakly positive, and culture of the sputum specimens yielded in both sputum specimens *M. lentiflavum*. The last patient who was not treated for TB had one sputum smear positive for AFB and the culture yielded only *M. gordonae*. This patient, who was HIV-positive, died a few days after admission.

In five patients with a positive sputum smear who were treated with anti-tuberculosis treatment, no *M. tuberculosis* was isolated. In three of these patients only NTM were recovered, and in two patients the acid fast organisms could not be identified to the species level. The first patient treated with anti-tuberculosis treatment from whom only NTM were cultured from the ZN-positive sputa was infected with HIV and had suffered from TB in the past. The chest X-ray was consistent with tuberculosis. The NTM were identified as *M. intracellulare* and also a not yet identified *Mycobacterium* was isolated. The second patient, who was also HIV-positive, was receiving anti-tuberculosis treatment for four months without improvement. She was already treated for TB in the past. The sputum culture revealed *M. lentiflavum*, *M. intracellulare* and AFB which could not be identified. This patient died one week after admission. The third patient treated for TB with a positive smear for AFB from which only NTM were cultured from the sputum was also infected with HIV. The chest X-ray was consistent with tuberculosis and the sputum culture yielded *M. intracellulare* and other AFB which could not be identified to species level. The patient died three weeks after admission. In the last two patients with a positive sputum smear and treated for TB the cultured yielded acid fast bacilli which could not be identified. Both patient were HIV-negative and were treated for TB in the past. One of these patients had a chest X-ray consistent with tuberculosis and the chest X-ray of the other patient was read as normal.

There were in total 99 patients diagnosed with TB on basis of the sputum smear and/or chest X-ray without a positive culture of *M. tuberculosis* (Table 3). The culture was negative for mycobacteria in 54 (55%) of these patients and in the remaining 55 patients NTM were isolated. Eight (8%) of these patients had a positive ZN of the sputum and 95 (96%) had abnormalities on the chest X-ray consistent with tuberculosis. The HIV-positivity as well as the median duration of complaints, the median age and percentage of women in this patient group were comparable with the 187 patients diagnosed with TB in the whole study group. A history of TB was documented in 29 (29%) of the patients which was also similar to the entire group of TB patients diagnosed. However, significantly more patients died in the group of patients diagnosed with TB and a negative culture for *M. tuberculosis* in comparison with the whole group of 187 patients diagnosed with TB, 28% and 16% respectively ($p<0.05$).

Table 3: Characteristics of 99 patients diagnosed with TB and a negative *M. tuberculosis* culture.

Culture result	ZN pos	Chest X-ray TB[a]	HIV positive	TB treatment[b]	TB in past[c]	Died	Total
Negative	1	53	39 (and 3 ? [d])	29	11	10	54 (55%)
Single NTM positive culture	2	20 (and 1 ? [d])	16	8	7	6	21 (21%)
Different NTM in both sputa	2	9 (and 1 ? [d])	8	7	3	9	10 (10%)
Same NTM in both sputa	1	5	5	3	2	2	5 (5%)
AFB not identified	2	8	5 (and 1?)	4	6	1	9 (9%)
Total	8 (8%)	95 (96%)	73 (74%)	51 (52%)	29 (29%)	28 (28%)	99

[a]Chest X-ray consistent with tuberculosis.
[b]Anti-tuberculosis treatment started by clinician without knowing culture results.
[c]History of tuberculosis.
[d]? = Chest X-ray or HIV status unknown.

Table 4: History of TB and patients who died during study period in patients with culture positive for *M. tuberculosis*, NTM, and negative culture.

	Culture			p-value
	M. tuberculosis (88)	NTM (36)	Negative (54)	
Died, n (%)	9 (10%)	10 (28%)	10 (19%)	<0.05
History of TB, n (%)	11 (13%)	12 (33%)	11 (20%)	<0.05

There were significant differences between patients with *M. tuberculosis* and NTM in their culture regarding presence of history of TB and the proportion of patients that died during the study period (see Table 4). Ten percent (9/88) of the patients with *M. tuberculosis* isolated from the sputum died during the study period compared to 28% (10/36) of the patients with exclusively NTM in their sputum and 19% (10/54) of the patients with a negative culture for mycobacteria ($p<0.05$). A history of tuberculosis was observed in 13% (11/88) of the *M. tuberculosis* culture-positive patients. In the patients with only NTM isolated from the sputum and a negative culture for mycobacteria these percentages were 33 (12/36) and 20 (11/54), respectively ($p<0.05$).

The collected sputum samples from all patients were split in two equal parts before decontamination in order to compare the influence of the decontamination method on the yield of mycobacteria (7). From the 187 diagnosed TB patients, a total of 670 sputum samples were cultured. The results of the cultures are depicted in Table 5. From 382 (57%) of the 670 sputum samples of patients, mycobacteria were isolated. *M. tuberculosis* was found in 236 of the 382 (62%) positive sputum specimens and NTM in 146 (38%). The most frequently encountered NTM were *M. lentiflavum* (21) and *M. intracellulare* (18).

Table 5: Isolated mycobacteria from sputum of patients diagnosed with TB.

Culture result	Isolates
Negative	288 (43%)
M. tuberculosis	236 (35%)
M. lentiflavum	21 (3%)
M. avium complex	20 (3%)
M. intracellulare	18 (3%)
M. avium	2 (0.3%)
M. gordonae	5 (0.7%)
M. chelonae	4 (0.6%)
M. fortuitum	2 (0.3%)
M. mucogenicum	2 (0.3%)
Various unknown *Mycobacterium* species	32 (5%)
Various other *Mycobacterium* species*	5 (0.7%)
Unidentified AFB	55 (8%)
Total number of sputum samples	670

*Various other *Mycobacterium* species include *M. goodii*, *M. terrae*, *M. neoaurum*, *M. peregrinum*, and *M. obuense*.

Table 6: Patients with positive NTM culture(s) from whom two consecutive sputum samples were cultured.

	Culture result		Total (%)
	M. tuberculosis and NTM	NTM only	
Single NTM positive culture	17	16	33 (63)
Different NTM in both sputa	1	10	11 (21)
Same NTM in both sputa	3[a]	5[b]	8 (15)
Total	21	31	52

[a]NTM identified as *M. lentiflavum* (2x) and *M. chelonae*.
[b]NTM identified as *M. lentiflavum* (4x) and *M. intracellulare*.

In 59 of the 187 (32%) diagnosed TB patients NTM were isolated from the sputum; 36 (61%) with exclusively NTM and 23 (39%) with cultures yielding a combination of *M. tuberculosis* and NTM (Table 6). Two consecutive sputum samples were cultured from 52 of the 59 NTM-positive patients. In 33 of the 52 patients only a single culture was positive. The remaining 19 NTM-positive patients had two positive cultures for NTM; 11 patients had different NTM species in the sputum samples and eight patients had the same NTM in both sputum samples. In four of the eight patients with same NTM in both sputum samples *M. lentiflavum* was isolated and in one patient *M. intracellulare*. In the remaining three patients *M. lentiflavum* (2) and *M. chelonae* were isolated twice together with *M. tuberculosis*.

In five of the 187 cases diagnosed with TB, only NTM were repeatedly and consistently isolated from sputum in addition to the finding of lesions on the chest X-ray (Table 7).

Table 7: Cases with the same NTM in two consecutive sputum samples.

Case	Isolate sputum	ZN	Male/female	Age	BMI[a]	HIV	Temp[b]	Complaints[c]	Duration (wks) [d]	Chest X-ray	Died	Remarks
1 (11)	*M. lentiflavum*	pos	female	50	20	? [e]	? [e]	resp	21	TB	yes	*M. lentiflavum* also in urine, started with TB treatment
2 (13)	*M. lentiflavum*	pos	male	35	24	pos	39	resp	34	TB	no	
3 (15)	*M. lentiflavum*	neg	female	30	18	pos	38.5	resp	5	TB	no	TB in past, improvement on TB treatment
4 (34)	*M. lentiflavum*	neg	female	25	? [e]	pos	38.5	resp	4	TB	yes	TB in past
5 (684)	*M. intracellulare*	neg	male	32	15	pos	36.8	resp	17	TB	yes	

[a]BMI = Body mass index.

[b]Temp = Temperature at time of inclusion.

[c]Complaints = Reasons for visiting the hospital.

Resp = Complaints/symptoms of the tractus respiratorius.

[d]Duration = Duration of complaints at the point of visiting hospital and inclusion in study.

[e]? = Temperature, HIV status or BMI not known.

Patient one was a 50-year-old female who presented herself with a productive cough with haemoptysis for 24 weeks. Furthermore, she was having pain passing urine. She was not responding to antibiotics. The HIV tests were indifferent. Her body mass index (BMI) was 20. The radiographic investigation of the chest revealed a pleural and pericardial effusion and an alveolar consolidation. The sputum smear was positive and treatment with rifampin, isoniazid, ethambutol and pyrazinamide was started. *M. lentiflavum* was isolated from two consecutive sputum specimens. Also from two consecutive urine samples the same bacterium was isolated. The patient died three months after the treatment was started.

The second case with the same NTM in both sputum samples concerned a 35-year-old HIV-positive man. He complained about a cough and night sweats that started 34 weeks earlier. A relative of this patient had been found sputum-positive a few months earlier. On physical examination, enlarged axillary and sub-mandibular lymph nodes and an enlarged spleen were found. The temperature was 39°C and the BMI 24. He had already been treated with antibiotics without improvement. The chest X-ray revealed pleural fluid. The sputum smear was positive and *M. lentiflavum* was isolated from two sputum specimens.

The third patient was an HIV-positive, 30-year-old woman presenting with coughing and vomiting for five weeks. She had been treated for tuberculosis in the past, but she interrupted this treatment. On physical examination, enlarged axillary and supra-clavicular lymph nodes as well as an enlarged spleen were found. The temperature was 35.5°C and the BMI 18. She was not improving on antibiotics before admission. Pleural effusion, alveolar infiltration, and interstitial pathology were seen on the chest X-ray. The patient clearly improved upon treatment with rifampin, isoniazid, pyrazinamide, and ethambutol. *M. lentiflavum* was cultured from two sputum specimens.

The fourth patient with in consecutive sputum samples exclusively the same NTM was a 25-year-old, HIV-positive woman who complained of a productive cough with haemoptysis with one month duration. She was known with asthma. Enlarged axillary and sub-mandibular lymph nodes and an enlarged liver were found on physical examination. The temperature on admission was 38.5°C. The chest X-ray showed pleural and pericardial effusion. Treatment with antibiotics was started. The culture of both sputum specimens revealed *M. lentiflavum*. The patient died nine days later.

The last patient was a 32-year-old HIV-positive man who complained of a productive cough with haemoptysis of 17 weeks' duration. Furthermore, he was vomiting and suffered from diarrhoea. The BMI was 15. He had been treated for tuberculosis in the past. The chest X-ray was consistent with TB and showed alveolar infiltration and interstitial pathology. He did not improve on antibiotics and started with anti-tuberculosis treatment. Mycobacteria were cultured from both sputum samples and identified as *M. intracellulare.* He died five weeks later.

Table 8: Performance of algorithm for diagnosing TB on basis of Ziehl-Neelsen sputum smear and/or chest X-ray.

	M. tuberculosis culture-positive	*M.* tuberculosis culture-negative	Total
ZN+ and/or X-ray +[a]	88	99	187
ZN- and X-ray -[b]	5	67	72
Total	93	166	259

Sensitivity 88/93 = 95%
Specificity 67/166 = 40%
Positive predictive value 88/187 = 47%
Negative predictive value 67/72 = 93%

[a]72 patients who had respiratory complaints lasting two or more weeks, had not improved on two courses of routine empiric antibiotics but had negative sputum smears and a chest X-ray without pathology.
[b]187 patients who had respiratory complaints lasting two or more weeks, had not improved on two courses of routine empiric antibiotics but had a positive sputum smear and/or chest X-ray suspected for TB.

From the above described data it can be deduced that the positive predictive value of the algorithm for diagnosing TB on the basis of: respiratory complaints for two or more weeks, not improving on two courses of routine empiric antibiotics, a positive sputum smear and/or pathology on the chest X-ray consistent with TB was 47% (88/187) in this study (Table 8). However, it included 72 patients who had respiratory complaints lasting two or more weeks, had not improved on two courses of routine empiric antibiotics, had negative sputum smears and a chest X-ray without pathology. Of these 72 patients, five had a culture positive for *M. tuberculosis* (with or without NTM) which makes the negative predictive value of this algorithm 93%. The sensitivity and specificity were 95% (88/93) and 40% (67/166), respectively.

DISCUSSION

The results of this study show that *M. tuberculosis* was not cultured from the sputum of 53% of the patients diagnosed with TB on the basis of ZN microscopy and chest X-ray results in Zambia. In 48% of the cases exclusively NTM or a negative culture for mycobacteria was observed and in the remaining 5% acid fast organisms, which could not be identified to the species level, were found. Among the patients diagnosed with TB, irrespective of the *Mycobacterium* culture results, an extremely high rate of HIV positivity was observed: 73%.

The spectrum of HIV-related morbidity in adults in sub-Saharan Africa shows some differences to that observed in Europe and north America. This includes a greater incidence of tuberculosis, a greater incidence of bacterial diseases in general, but a probably lower incidence of *Pneumocystis jirovecii* pneumonia (5,6). However, less is known about the

effect of HIV on NTM disease in developing countries. There are only a few, controversial reports from developing countries on NTM from HIV/AIDS patients. However, the results in this study indicate that these opportunistic bacteria may be important pathogens regarding the diagnosis and perhaps the aetiology of tuberculosis-like infections in Zambia. HIV positivity was found to be significantly correlated with the isolation of NTM from sputum. In the patients diagnosed with TB the culture was exclusively positive for NTM in 19% of the cases. In 32% of the cases NTM (together with or without *M. tuberculosis*) was isolated. Therefore, the role of NTM in tuberculosis-like disease should be considered in smear-negative and smear-positive patients and seems more relevant than considered so far in Zambia.

NTM can cause both colonization and clinical disease in humans. To guide the diagnosis in a patient from whom NTM has been isolated from a specimen, the recently published new diagnostic criteria for non-tuberculous mycobacterial diseases by the American Thoracic Society will provide support (11). This is especially important because the treatment of NTM disease is generally not directly analogous to the treatment of TB. Drug therapy of NTM infection involves multiple drugs, most of the time including a newer macrolide (e.g. azithromycin, clarithromycin) (11). In this study probably five patients would meet the criteria for NTM pulmonary disease (Table 7). From two consecutive sputum samples of these HIV-positive patients *M. lentiflavum* and *M. intracellulare* were isolated. These patients presented with respiratory complaints and the chest X-ray showed abnormalities compatible with tuberculosis. According to criteria of the ATS for diagnosing NTM lung disease these patients suffice. However, these sputa were not additionally tested with molecular amplification techniques for the possible presence of *M. tuberculosis*. Furthermore, it was not possible to count the number of colony-forming units cultured from the sputum to discriminate colonization and infection/disease, because the sputum samples were not cultured on a solid medium.

The presentation of NTM infections typically mimic TB, confounding the diagnosis of TB. In this study, in five of the 72 (7%) patients with a positive sputum smear only NTM was isolated. In the light of this, methods for distinguishing *M. tuberculosis* and NTM in clinical material should be implemented on a broader scale in Africa. The direct detection and identification of *M. tuberculosis* in clinical specimens are made possible by methods using nucleic acid amplification (NAA) techniques (3,9). However, the performance of the test is good in clinical respiratory specimens that are AFB smear-positive (sensitivity 95% and specificity 98%) but far less in specimens that contain fewer organisms, or are AFB-negative (sensitivity 50% and specificity 95%) (1). Fortunately, recent development of an 'enhanced' nucleic acid amplification test in patients who are clinically suspected of having TB (AFB smear-positive and negative) showed a higher sensitivity (8). The NAA-

tests may provide a reliable way of increasing the specficity of the TB diagnosis (ruling in disease) in this algorythm. The challenge to overcome is the implementation of such delicate techniques in an African setting.

NTM are widely distributed all over the world, although there seems to be a geographic distribution (4,11,15,18). *M. avium* complex (MAC) is the most common NTM species causing disease, but many other NTM species have been found associated to severe infections. In Africa little is known on the prevalence of different NTM species. In this study, the most isolated NTM in the population of patients diagnosed as TB were *M. lentiflavum* and MAC. Interestingly, a part (22%) of the NTM found in Zambia has not yet been identified on a species level. This suggests the distribution of NTM in Africa may be different from Europe and the United States of America and the evolutionary divergence of phylogenetic sub-lineages of bacteria with in species may merit more detailed taxonomic studies.

In this study 29% of the patients diagnosed with TB had a culture negative for mycobacteria. Causative micro-organisms of respiratory illness in HIV-positive and negative patients besides *M. tuberculosis* may also be either bacterial (most commonly *Streptococcus pneumoniae* and *Haemophilus influenzae*), viral or *Pneumocystis jirovecii*. No diagnostic methods in this study were used to demonstrate these micro-organisms because the goal of this study was not to determine all the causes of tuberculosis-like diseases in Zambia. However, in a follow-up study a broader microbiological diagnostic approach would be interesting.

Another reason for negative cultures may be found in the lack of sensitivity of culture. For mycobacteria this amounts 80-85% (with a specificity of approximately 98%) (13,17). As a result, for a limited number of the patients no indications were collected regarding the possible causative agent.

Anti-tuberculosis treatment was initiated by the clinician in 134 of the 187 (72%) patients diagnosed with TB. In almost all patients (94%) who were *M. tuberculosis* culture positive, treatment was started in comparison to approximately half of the patients with positive NTM cultures (51%) or the ones with a negative culture for mycobacteria (54%). The clinicians' diagnosis of TB and the decision to treat often deviated from the protocol used in Zambia, especially for the patients with NTM or a negative culture for mycobacteria. Probably more factors play a role in the decision of the clinician to start with an anti-tuberculosis treatment in a patient suspected of TB than the official algorithm. In this study differences in the history of TB and mortality were observed between the group of patients with a culture positive for *M. tuberculosis* and the patients yielding a positive

NTM culture. In the study period significantly more patients with a positive NTM culture died in comparison with patients with a positive *M. tuberculosis* culture. Probably treatment could not be started because the patient died before the diagnosis had been made due to immunosuppression because of HIV/AIDS. Moreover, significantly more patients with a positive culture for NTM instead of *M. tuberculosis* had a history of TB. It is likely that the knowledge that the patient had TB in the past discouraged the clinician from re-starting the antituberculosis treatment.

We conclude that the traditionally-defined diagnosis of tuberculosis has become highly inaccurate in Zambia, due to the increased prevalence of HIV. Misdiagnosis of TB can be reduced by the introduction of new diagnostic techniques in addition to AFB microscopy, and this would also favour the diagnosis of NTM-causes pulmonary disorders. A logical next step is the introduction of knowledge in Africa to treat NTM infections in an accurate way.

Al in all, with the changing situation in Africa diagnostic scoring systems and algorithms should be developed and validated to assist clinicians working in resource-poor settings with a high prevalence of HIV.

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Chapter 10

Polymorphism in monocyte chemoattractant protein-1 is associated with increased susceptibility to tuberculosis in Zambian patients

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ABSTRACT

Single nucleotide polymorphisms (SNPs) in genes encoding *monocyte chemoattractant protein 1* (*MCP-1*), *mannose-binding lectin* (*MBL*), *tumor necrosis factor* (*TNF*)-*α*, and *interferon* (*IFN*)-*γ* were examined in 46 tuberculosis patients and 119 healthy tuberculin-positive controls in Zambia. The odds of having tuberculosis was 2.7-fold higher in carriers of *MCP-1* genotype *AG* than in those carrying the homozygous genotype *AA* (95% confidence interval (CI): 1.3-5.5). This association was maintained among human immunodeficiency virus-negative patients and controls (Odds ratio 3.0 (95% CI 1.0-8.7)). In contrast, none of the *MBL*, *TNF-α*, and *INF-γ* variant alleles or genotypes were associated with tuberculosis susceptibility. The *MCP-1* gene is one of the relevant genes influencing susceptibility to tuberculosis in African people.

INTRODUCTION

Tuberculosis (TB), which is mainly caused by *Mycobacterium tuberculosis*, is a major cause of morbidity and mortality worldwide, and a recent World Health Organization report estimated that there were 8.8 million new cases of clinical TB diagnosed in the year 2005, with 1.6 million deaths directly attributable to this disease (30).

Progression to clinical disease is far from being an inevitable consequence of infection, and it is generally accepted that 90% of *M. tuberculosis* infected subjects will never develop TB. One-in-two people of the remaining 10% will progress to disease during the first two years after infection (6).

The factors determining an individual's risk of infection and breakdown to active disease are multi-factorial and involve host-pathogen interactions and environmental conditions. Recent evidence supports the role of multiple host genetic factors in susceptibility to TB. For instance, the mannose-binding lectin (MBL) is an important factor in the innate immune response and acts in concert with the complement system to opsonize and facilitate phagocytosis of microorganisms (26). In mycobacteria, MBL binds to mannose residues in the lipoarabinomannan membrane (LAM), covering the bacteria (7). Three structural variants of the *MBL* gene, located separately at codons 52, 54, and 57 of exon 1, have been shown to produce variant (amounts of) MBL proteins. Several studies have indicated a partial protective effect of heterozygosity for MBL variant alleles against TB (8,10,11), whereas others have pointed toward an increased susceptibility for TB for homozygous carriers of MBL variant alleles (24). Low serum MBL levels, due to heterozygosity for MBL variant alleles, may be protective against TB by limiting complement activation and uptake of bacilli by complement receptors (25).

In addition, host protective immunity to mycobacterial infection is dependent on the precise coordination of T-lymphocyte sensitisation and monocyte recruitment. Several cytokine and chemokine signals play crucial roles in the normal immune response to TB, thereby contributing to the initial protection against infection. These factors include pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, interferon (IFN)-γ, and chemokines with selective chemotactic properties for leukocytes such as Monocyte Chemoattractant protein 1 (MCP-1) (1).

TNF-α is involved in the recruitment of cells forming a protective granuloma. A TNF-independent production of chemokines result in a dysregulated inflammatory response unable to contain *M. tuberculosis*. Reactivation of clinical tuberculosis was observed in patients undergoing treatment for rheumatoid arthritis or Crohn disease with a humanized monoclonal antibody to TNF (13). Polymorphism in the *TNF-α* promoter region, single nucleotide polymorphisms (SNP) located at nucleotides -238, -308, and –376, were

found to be associated with susceptibility to several diseases including cerebral malaria, mucocutaneous leishmaniasis, asthma and leprosy (2,17,18,22).

Macrophage activation by IFN-γ is essential for protective immunity, as mice and humans that lack components of the IFN-γ signalling pathway are highly susceptible to tuberculosis. A genetic defect in the production of IFN-γ in individuals homozygous for the +874 A allele contributed to an increased risk of developing tuberculosis (15,21).

Chemokines are key signal molecules that direct leukocyte subsets to sites of inflammation. The regulated interaction of chemokines with their respective receptors is thought to mediate the controlled recruitment of specific leukocyte subpopulations specifically required during local host defense at the site of infection (9). MCP-1 is a prototypic chemokine and is a chemoattractant for monocytes and T-lymfocytes. In an unmatched case-control study it was found that the *G* allele of the *MCP-1* promoter region was strongly associated with increased odds of developing active pulmonary tuberculosis in Mexicans and Koreans (5). Furthermore, individuals with the –2518G variant expressed higher levels of MCP-1 which inhibited IL12p40 production in response to *M. tuberculosis* and increased the likelihood of TB infection progressing to active disease. Therefore, in the present study the *MCP-1* promoter gene polymorphisms in association with susceptibility to develop tuberculosis was investigated in Zambia. In addition, the influence of SNPs in the *TNF-α* gene, *IFN-γ* and *MBL* gene on manifestation of TB were examined.

MATERIAL AND METHODS

Subjects

During an unmatched case-control study in Zambia, tuberculosis patients (between 15 and 78 yr of age) were recruited at St. Francis Hospital in Katete (Eastern province of Zambia). Unrelated, healthy persons of the same age and living in a neighbouring community were recruited as controls. Demographic data, previous history of TB and/or previous contact wit individuals with TB, and the presence or absence of a scar characteristic of bacilli Calmette-Guérin (BCG) vaccination was recorded. Documented socio-economic status features included smoking cigarettes, drinking alcohol and being employed. Furthermore, the body mass index (BMI) and HIV-status for each included person were determined.

Tuberculosis was confirmed by at least two consecutive sputum smears positive for acid-fast bacilli and/or a positive sputum culture for *M. tuberculosis*. Patients were subjected

to an extensive clinical examination, and an anterior-posterior chest radiograph was produced for each individual included in the study.

Controls were healthy adults who had at least two gargle specimens with normal saline or sputum specimens negative for acid-fast bacteria (AFB) in the smear, together with a negative culture for *M. tuberculosis*. Furthermore, the chest radiographic findings of the included controls were without abnormalities indicative of tuberculosis. A tuberculin skin test was administered to all controls, using the Mantoux method to deliver 5 tuberculin Units of purified protein derivative RT/23 (Statens Serum Institute, Copenhagen, Denmark) intradermally. The diameter of induration was measured 48 h after inoculation. All included tuberculin reactor controls had tuberculin reactions of ≥ 5 mm of induration.

The study was approved by the research ethical committee of the University of Zambia, the Central Board of Health, and the Ministry of Health in Zambia, and all participants gave written consent.

Genotyping

Genomic DNA was purified from peripheral blood leukocytes. The sequence containing the –2518 *G* to *A* transition locus in the *MCP-1* promoter region was amplified from 100 ng of genomic DNA using the forward primer (5'-GCTCCGGGCCCAGTATCT-3') and reverse primer (5'-ACAGGGAAGGTGAAGGGTATGA-3') and a Hot Start PCR (Applied Biosystems) according to Flores-Villanueva et al. (5). Restriction fragment length polymorphism after PCR was used for the identification of *MCP-1* alleles. The allele *G* creates a *Pvu*II restriction site yielding two fragments of 182 and 54 bp, respectively. The allele *A* was identified by the presence of a 236 bp undigested fragment. Genotyping of the –308 and –238 TNF-α, +874 INF-γ and MBL codons 54 and 57 positions were performed according to cited references (16,29,31,32). Restriction enzymes were obtained from New England Biolabs (Leusden, The Netherlands) or Fermentas (St. Leon-rot, Germany). All restriction endonucleases were used as recommended by the manufacturer.

Statistical analyses

Analysis was done with SPPS version 11.0 (SPSS, Inc., Chicago, IL) and STATA version 8.0 (Stata corporation College Station, TX, USA). Controls were tested for Hardy-Weinberg equilibrium (HWE) by use of a 3 x 2 χ^2 test with 1 degree of freedom. Associations of genotype with disease were tested using χ^2 test. Univariate odds ratio's were calculated by logistic regression.

RESULTS AND DISCUSSION

Demographic and clinical features

We recruited 46 tuberculosis patients and 119 healthy controls (Table 1). A positive sputum smear was found in 33 of these 46 (72%) patients, the remaining ones were diagnosed by a positive culture with *M. tuberculosis*. Chest radiographs were consistent with tuberculosis in 41 (89%) of the tuberculosis patients. Genotyping was successful in all patients and controls. The group of patients and controls were similar in demographics, being employed, having a BCG-scar, consumption of alcohol and smoking of cigarettes. However, the percentage of HIV-positivity (65%) was significantly higher in the group of tuberculosis patients (p<0.001). In addition, a lower BMI was observed in the patients with tuberculosis (p<0.001) and a history of tuberculosis was more frequently documented among patients.

Table 1: Demographic and clinical features of tuberculosis cases and controls.

Parameter	TB cases[a] (n=46)	Healthy PPD+[b] (n=119)	p-value
Female, n (%)	28 (61)	89 (75)	0.08
Age (yr), mean ±SD	37 ± 13	33 ± 15	0.1
BMI (kg/m2)	17.5 (SD 2.1)	21.9 (SD 3.8)	<0.001
TB in past, n (%)	6 (13)	4 (3)	0.02
HIV-positive, n (%)	30 (65)	17 (14)	<0.001
Smokers, n (%)	1/42 (2)	6 (5)	0.5
Alcohol use, n (%)	2 (5)	8 (7)	0.6
Employed, n (%)	7 (15)	13 (11)	0.4
BCG-scar, n (%)	37/44 (84)	98/117 (84)	1.0
Contact with TB patient, n (%)	17 (37)	53 (45)	1.0

[a]TB cases: cases of pulmonary tuberculosis, culture positive for *M. tuberculosis*.
[b]Healthy PPD+: healthy controls, tuberculin positive.

The –2518 *MCP-1* promoter polymorphism

In Zambian control subjects, genotypes of the *MCP-1*, *TNF-α*, *INF-γ* and *MBL* gene were in Hardy-Weinberg equilibrium, but none of the *TNF-α*, *INF-γ* and *MBL* variants or genotypes were associated with tuberculosis (see Table 2 and 3 for overview). In contrast, allele *G* of the *MCP-1* gene was associated with tuberculosis compared with healthy tuberculin reactors (p=0.04). Carriers of the *MCP-1* genotypes *AA* were significantly over-represented among healthy tuberculin reactors. The genotypes *AA* and *AG* were equally distributed in the tuberculosis cases. The genotype *GG* was rarely observed in both groups. The odds ratio (OR) for heterozygous *AG* in tuberculosis cases versus tuberculin-positive controls was 2.7.

Table 2: The genotype frequencies of various MBL, MCP-1, TNF-α, and INF-γ polymorphisms.

Gene and genotype[a]			TB cases[b] (n=46) n (%)	Healthy PPD+[c] (n=119) n (%)	HWE[d]	p-value	OR (95% CI)[e]
MBL							
	54	ww	46 (100)	119 (100)	-	-	-
		wm	0	0			-
		mm	0	0			-
	57	ww	33 (71.7)	77 (64.7)	0.45	0.7	1
		wm	12 (26.1)	39 (32.8)			0.7 (0.3-1.5)
		mm	1 (2.2)	3 (2.5)			0.8 (0.1-7.8)
MCP-1							
	-2518	AA	22 (47.8)	83 (69.7)	0.66	0.02	1
		AG	23 (50.0)	32 (26.9)			2.7 (1.3 - 5.5)
		GG	1 (2.2)	4 (3.4)			0.9 (0.1-8.9)
TNF-α							
	-238	GG	42 (91.3)	103 (86.6)	0.08	0.6	1
		GA	4 (8.7)	14 (11.8)			0.7 (0.2-2.3),
		AA	0	2 (1.7)			-
	-308	GG	38 (82.6)	104 (87.4)	0.49	0.4	1
		GA	8 (17.4)	15 (12.6)			1.5 (0.6-3.7)
		AA	0	0			-
INF-γ							
	874	AA	32 (69.6)	93 (78.2)	0.29	0.5	1
		AT	13 (28.3)	23 (19.3)			1.6 (0.7-3.6)
		TT	1 (2.2)	3 (2.5)			1.0 (0.1-9.6)

[a]w/w = wild/wild, w/m = wild/mutant and m/m = mutant/mutant.
[b]TB cases: cases of pulmonary tuberculosis, culture positive for *M. tuberculosis.*
[c]Healthy PPD+: healthy controls, tuberculin positive.
[d]HWE: Hardy-Weinberg equilibrium.
[e]OR (95% CI): Odds ratio with 95% confidence interval.

MCP-1 polymorphism in HIV-negative patients and controls

In the patient group, a high percentage of HIV-positivity was observed. Therefore, analyses of the HIV-negative subpopulation were performed. In total 16 patients and 102 healthy tuberculin reactors were HIV-negative. Similar (but not significant) results were found; carriers of *MCP-1* genotypes *AA* were again more often found in the controls and the genotypes *AA* and *AG* were equally distributed in the tuberculosis cases. The OR for the heterozygous AG type in the HIV-negative tuberculosis cases versus HIV-negative tuberculin-positive controls was 3.0 (95% CI 1.0-8.7). Furthermore, no association between HIV-status and *MCP-1 genotypes* was observed. Again, none of the *TNF-α*, *INF-γ* and *MBL* alleles or genotypes were associated with tuberculosis in these HIV-negative persons.

Table 3: The allele frequency of the various MBL, MCP-1, TNF-α, and INF-γ polymorphisms.

Gene		Allele[a]	TB cases[b] (92 alleles) n (%)	Healthy PPD+[c] (238 alleles) n (%)	p-value
MBL					
	54	w	92 (100)	238 (100)	-
		m	0	0	
	57	w	78 (84.8)	193 (81.1)	0.42
		m	13 (14.1)	45 (18.9)	
MCP-1					
	-2518	A	67 (72.8)	198 (83.2)	0.04
		G	25 (27.2)	40 (16.8)	
TNF-α					
	-238	G	88 (95.7)	220 (92.4)	0.46
		A	4 (4.3)	18 (7.6)	
	-308	G	84 (91.3)	223 (93.7)	0.47
		A	8 (8.7)	15 (6.3)	
INF-γ					
	874	A	77 (83.7)	209 (87.8)	0.37
		T	15 (16.3)	29 (12.2)	

[a]w = wild and m = mutant.
[a]TB cases: cases of pulmonary tuberculosis, culture positive for *M. tuberculosis.*
[b]Healthy PPD+: healthy controls, tuberculin positive.

Our results are similar to those of Flores-Villanueva et al., who examined tuberculosis patients and healthy controls in Mexico and Korea (5). In this study 435 and 129 patients and 510 and 162 controls were included, respectively. The odds of developing tuberculosis were 2.3- and 5.8-fold higher in carriers of *MCP-1 genotypes AG* as compared to homozygous *AA*. Although a smaller number of patients and controls were included in the present cohort, already a significant association was disclosed of susceptibility to TB and the *AG* polymorphism in this gene. Furthermore, the *G* allele was dominant in patients with TB, which emphasizes the power and reliability of the observed association. In contrast, Jamieson et al. found no association of the *MCP-1* –2518 allele *G* with susceptibility to tuberculosis in a study of cases and controls derived from families in Brazil (12).

Various studies have looked at the correlation between serum MBL concentrations, *MBL* gene polymorphisms and susceptibility to infections. Infections by intracellular agents seem to occur more frequently in patients with increased MBL levels (8,11,25). The serum MBL level was not measured in the present study and the promotor haplotype was not assessed, limiting the reliability of the interpretation of our current results.

Inconsistent results in the literature have been found for polymorphisms in the *TNF-α* associated with susceptibility to tuberculosis in different populations (4,20,23,27). In

addition, data suggest that the more common polymorphism at position +874 of the *IFN-γ* gene is associated with the risk of tuberculosis in several populations (3,14,15,21). However, results of the present and other studies suggest that the *IFN-γ +874T/A* mutation has no association with TB susceptibility (19,28). The comparison of the production of IFN-γ with the genotypes needs to be studied more extensively (15,28). Functional variation in the closely linked alleles of the *INF-γ* gene may also influence the cytokine production.

Our data were also analyzed for a subgroup of patients suffering from non-tuberculous mycobacterial (NTM) infections versus the controls. Numbers of NTM infected patients turned out to be too low to perform reliable analyses (data not shown).

To evaluate genetic factors associated with susceptibility to tuberculosis, we selected new sputum smear-positive and/or culture-confirmed tuberculosis patients.

As controls, healthy tuberculin reactors were selected. Of these controls 82% were vaccinated with BCG and 53% had been in recent contact with a tuberculosis patient. The positive Mantoux-test could have been a consequence of an infection with *M. tuberculosis* or the BCG vaccination. On the other hand, these Zambian controls were more likely exposed to *M. tuberculosis*, given that they are living in a country with an estimated TB incidence of 650 per 100.000. To exclude the possibility of tuberculosis disease in the controls, the smear and culture of two sputum or gargle specimens had to be negative for AFB and *M. tuberculosis*, and the chest radiograph without findings consistent with tuberculosis. However, we were not fully confident that the controls were in no case infected with *M. tuberculosis*. In any case, we made sure they were not suffering from pulmonary tuberculosis. Moreover, if there would have been cases of active TB among the controls, the correlations between polymorphism in susceptibility genes in the TB patients and manifestation of the disease would even be stronger than recorded in this study.

To our knowledge, this is the first study investigating the polymorphism of the *MCP-1* gene in African people; and this may be a first indication that especially this gene may play a crucial role in increased susceptibility to this infectious disease in particular human populations. Further studies with sufficient statistical power are required to compare the true contribution of the *MCP-1* gene to the susceptibility to develop tuberculosis among Africans and populations in other geographic regions.

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Chapter 11

Summary and General discussion

Tuberculosis, especially due to the high prevalence of HIV, remains a problem of large dimension in Africa and *Mycobacterium tuberculosis* is the most important causative agent. However, it is known that in developed countries also nontuberculous mycobacteria (NTM) play a significant role in the aetiology of tuberculosis-like syndromes, especially in HIV-positive patients. In Africa, the contribution of NTM to the problem of tuberculosis has hardly been examined.

Southern Africa is the most affected sub-region in Africa concerning HIV/AIDS, where HIV prevalence rates have stabilized at high levels, exceeding 25% in some countries, while in other African countries, the epidemic is still growing. In Zambia the estimated adult (15-49 yrs) prevalence of HIV/AIDS amounted 13.5-20% in 2003 (61).

NTM disease was one of the major AIDS-defining diseases in industrialized countries before the era of highly active antiretroviral therapy (HAART). Such infections have been thought to be less frequent in sub-Saharan Africa, but with the remaining doubt that the unavailability of the laboratory facilities and sophisticated radiological tools required to diagnose such disease may lead to underestimating of their true frequency (9,12,24,27,37,41,47). Whereas an international prospective study showed that NTM prevalence for disease was five to 10-fold lower in Kenya than in the United States of America or Northern Europe (24), a few hospital-based studies have reported a different prevalence for disease than reported in Kenya (9,12,27,37,41,47). For an overview of the literature on prevalence/incidence rates of colonization/infection/disease of NTM in Africa see Table 1 in the introduction of this thesis.

Because the clinical relevance of the isolation of NTM in HIV-positive as well as in HIV-negative patients in Africa may be underestimated in this study, the meaning of NTM was examined in more detail in Zambia.

While progress in tuberculosis diagnostics has been made in developed countries, in the rest of the world the techniques used for diagnosing tuberculosis have remained relatively unchanged for the past one hundred years. Since the discovery of tuberculosis, the basis for its definitive diagnosis has been the detection of the bacillus in clinical specimens. Following microscopic detection of the organism in 1882, technical advances have allowed the detection of fewer and fewer organisms in a specimen. Unfortunately, however, the level of sophistication and cost associated with more sensitive techniques has, to date, made their general applicability unfeasible in developing countries. Microscopic detection is simply but cumbersome, while the newer tools are still expensive and too complex for wide scale implementation in poor resource settings.

Therefore, the basis for tuberculosis diagnosis in developing countries has continued to be microscopy of stained smear of expectorated sputum to visualize acid-fast bacteria

(AFB). Fortunately, this technique detects most infectious patients. According to the International Standards for Tuberculosis Care all patients suspected of having pulmonary tuberculosis should have at least two, and preferably three, sputum specimens examined by microscopy. When possible, at least one early morning specimen should be included (23).

Smear examination by experienced technicians can result in the detection of 50-60% of the tuberculosis cases. However, in low-income countries often much lower rates of AFB detection in tuberculosis patients are observed because of poor access to good quality microscopes, heavy workload, and shortage of trained staff. Moreover, microscopy is usually negative in cases with less advanced disease and certain groups of patients, such as those with HIV co-infection and patients with extra-pulmonary disease. The proportion of cases detected by microscopy is often as low as 20-30% of all cases (20,36,48).

In the absence of positive sputum smears for AFB and no improvement to treatment with broad-spectrum empiric antibiotics, most cases of pulmonary tuberculosis in low-income countries are diagnosed on the basis of clinical and radiological features.

Various criteria, clinical scoring systems, tools, and algorithms have been developed to facilitate the diagnosis of pulmonary tuberculosis in people with suspected tuberculosis who have repeated negative sputum smears (8,30,49-51,59).

All persons with unexplained productive cough lasting for two-three weeks or more should be evaluated for tuberculosis, according to current evidence and WHO guidelines. The diagnosis of sputum smear-negative pulmonary tuberculosis should be based on at least three negative sputum smears, chest radiography findings consistent with tuberculosis, and lack of response to a trial of broad-spectrum anti-microbial agents. However, in areas with high prevalence of HIV and tuberculosis infection, the diagnosis of smear-negative tuberculosis is complicated, given the atypical presentation of pulmonary tuberculosis in HIV-infected patients.

For instance, clinicians are less likely to find typical radiological signs of pulmonary tuberculosis in areas with high prevalence of HIV infection than in those with lower rates, as patients with HIV and smear-negative tuberculosis are less likely to reveal cavities on the chest X-ray than patients with smear-positive tuberculosis. Furthermore, smear-negative patients can also present with normal or only slightly abnormal chest X-rays. Numerous studies have shown that no radiographic pattern is fully characteristic of tuberculosis (7,20,30,32,43). To establish the tubercular aetiology of an abnormality on the chest X-ray further examination is necessary. The experience of many decades of data collection and analysis indicate that chest radiography for diagnosis or follow-up of pulmonary tuberculosis cases, with or without HIV co-infection, is unreliable (31).

The accurate diagnosis of smear-negative pulmonary tuberculosis may even be more complicated when reliance is solely placed on clinical and radiological features.

Furthermore, the involvement of acid-fast NTM in tuberculosis-like syndromes might result in the misdiagnosis of tuberculosis. To guide the diagnosis in a patient from whom NTM is isolated from a specimen, the diagnostic criteria for nontuberculous mycobacterial diseases by the American Thoracic Society (ATS) can provide support (29). The key difference between the former and new (2007) diagnostic ATS criteria lies in the more lenient bacteriological criteria, single NTM culture from bronchial washing fluid or two positive sputum cultures, in a symptomatic patient with nodular or cavitary opacities in chest radiograph, now suffice. More patients tend to meet the new criteria, which may result in increasing numbers of patients receiving anti-mycobacterial treatment. Its authors, however, repeatedly state that meeting these criteria does not, *per se*, necessitate the institution of therapy. Noteworthy is the exclusion of positive smear(s) of sputum or bronchial wash and the exclusion of extra-pulmonary NTM isolates from the diagnostic criteria. The consequences of these new diagnostic criteria are that difficulties may arise regarding the eventual decision to start treatment in patients and the clinical significance of the isolation of NTM, especially from extra-pulmonary samples.

In this light methods for the distinction of *M. tuberculosis* and NTM in clinical material should be implemented on a broader scale in Africa. Nucleic acid amplification tests can enhance diagnostic certainty and the LiPA Rif-B test allows to detect *M. tuberculosis* complex bacilli as well as resistance to rifampicin (1,22,28,58). Furthermore, the knowledge on treatment of NTM infections and the basis underlying the decision to initiate the treatment should be widely implemented.

PART I: DIAGNOSIS OF TUBERCULOSIS

Culture is considered the gold standard for the diagnosis of tuberculosis, but most clinical sputum samples contain a variety of micro-organisms that may overgrow *M. tuberculosis*. Decontamination of these samples is therefore crucial in preventing contamination of the mycobacterial culture. However, decontamination inhibits the recovery of mycobacteria. In the study described in **Chapter 2**, we compared the effect of two decontamination procedures, NaOH-N-actetyl cysteine (NaOH-NALC) and sulphuric acid, on the sensitivity to detect *M. tuberculosis* complex and NTM in sputum samples. The recovery of NTM in the sulphuric acid group was significant higher than in the NaOH-NALC group ($p = 0.001$). In contrast, no significant difference was found for the recovery of *M. tuberculosis*.

The choice of the decontamination method applied definitely influences the recovery of mycobacteria and this has clear consequences for the quality of both microbiological diagnosis and treatment of patients. In the light of the results obtained in this study it

seems best to decontaminate specimens of patients suspected of NTM lung disease with a sulphuric acid instead of NaOH-NALC to optimise the detection of NTM.

Unfortunately, although sputum culture is still the gold standard for the diagnosis of pulmonary tuberculosis, the application of this diagnostic tool in most hospitals in low-income countries is not standard practise. Moreover, classical solid culture for mycobacteria requires 3-8 weeks, which limits the usefulness of culture as a first-line diagnostic test. In the last decade, liquid culture methods have become available in the Western world, with a shorter turn-around time of 1-3 weeks. However, liquid culture systems have hardly been applied in Africa because of the costs and maintenance of the equipment involved (5,38,39). In addition, culture of mycobacteria requires specialized personnel and a dependable supply of water and electricity.

A number of rapid diagnostic tests have been developed in an effort to improve the diagnostic accuracy for tuberculosis and to speed presumptive identification prior to the report on the cultures. Molecular amplification is the most prominent among these new tools. Amplification tests, such as the Amplified *Mycobacterium tuberculosis* Direct Test (MTD) (Gen-Probe, San Diego, California) and the Amplicor *Mycobacterium tuberculosis* Test (Amplicor) (Roche Diagnostic Systems, Inc., Branchburg, New Jersey), are promising because they have a high sensitivity and potential for good specificity and can be applied directly to clinical specimens such as sputum (2,17). The specificity of amplification test can be 98-100% and the sensitivity is higher than 95% in sputum that is AFB smear-positive and 60-70% in smear-negative, culture positive specimens (4,16,60). Recently developed molecular amplification tests may have better sensitivity in smear-negative specimens while retaining the same high degree of specificity (13,35). Furthermore, these tests are promising for materials other than sputum (blood, lymph, bone marrow, gastric aspirate, cerebrospinal fluid, urine, bronchial aspirate and lavage) altough results have considerable variability (45,46). Perhaps the application of molecular amplification methods to multiple samples, as is common practise for culture since decades, will sufficiently increase the sensitivity of these tests in comparison to culture.

In any cases, at the moment, the high cost of equipment and material, complexity in use, and low specificity under field conditions make them inappropriate for resource-poor settings.

One of the genomic regions often used for *Mycobacterium* species identification is the 16S ribosomal RNA (rRNA) gene and at present sequencing of this gene is the gold standard. The micro-organism is identified by comparison of the nucleotide sequence with reference sequences in several internet databases. Despite its accuracy, 16S rRNA gene sequence analysis lacks widespread use beyond the large and reference laboratories because of technical and cost considerations. An additional problem may be that the bacteria

of *Mycobacterium* species in Third world setting presumably have another evolutionary development than the ones in the Western world. This implies new data bases are needed, specific for high prevalence areas, containing genetic information of the local population of mycobacteria to facilitate an adequate and accurate identification.

Another method for the identification of *Mycobacterium* species is based on line probe hybridization, e.g. the Inno-Lipa *Mycobacterium* system (Innogenetics, Ghent, Belgium), which targets the 16S-23S rRNA gene spacer region or the GenoType Mycobacterium CM/AS (Hain Lifescience GmbH, Nehren, Germany) which targets the 23S gen regio. This method is currenty used for the identification of positive cultures but has potential to be used to identify *Mycobacterium* species directly in clinical specimens. However, also this method is currently based on the most frequently encountered species in the Europe and perhaps an African version of this method should be produced.

In view of these limitations, there is a need for fast, less complicated tests that are in principle directly applicable to bacteria in clinical material and have the flexibility to detect any micro-organism, especially in poor resource settings.

It is expected that in the coming years molecular diagnosis of tuberculosis will slowly be introduced in Africa. There are already nucleic acid amplification methods that amplify DNA without thermal cycling, all reactions can be conducted under isothermal conditions ranging from 60 to 65°C by using only one type of enzyme.

Loop-mediated isothermal amplification (LAMP) is a novel nucleic acid amplification method in which reagents react under isothermal conditions with high specificity, efficiency, and rapidity. LAMP was used for detection of *M. tuberculosis* complex, *M. avium*, and *M. intracellulare* directly in sputum specimens as well as for identification of culture isolates grown in a liquid medium or on a solid medium.

Due to the easy operation of LAMP without sophisticated equipment, it will be simple enough to use in small-scale hospitals, primary care facilities, and clinical laboratories in developing countries if the remaining issues such as sample preparation, nucleic acid extraction, and cross-contamination controls are addressed (11,34).

A new and promising diagnostic tool for the rapid identification of mycobacteria is Raman spectroscopy, described in **Chapter 3**. The results obtained clearly show the possibility to discriminate between 63 different *Mycobacterium* strains and to group them correctly into 8 clusters corresponding to *M. tuberculosis*, the most relevant clinical species of NTM in the Netherlands and *M. lentiflavum*. Moreover, the spectra of the viable (and infectious) strains and strains inactivated by heat-killing were compared and showed minimal differences. Therefore, identification of mycobacteria was being possible

without biosafety level III precautions. Raman spectroscopy is an optical method, providing spectroscopic fingerprints from samples in a few seconds. The produced fingerprints represent the molecular composition of a sample and are therefore suited for identification of micro-organism at both the species- and strain level. Important features of these methods are the relative ease with which measurements can be performed, the limited amount of sample handling involved, the little amount of biomass required and the high reproducibility. In the future, Raman analysis of clinical specimens may be applicable. In the past several years there has been a remarkable progress in lowering the costs of Raman systems, the equipment has become smaller and less vulnerable and therefore application in Africa could become reality in the future.

The electronic nose (gas sensor array) also shows potential for the direct detection of mycobacteria in clinical samples. In **Chapter 4** we evaluated the electronic nose (EN), comprising 14 conducting polymers, to detect different *Mycobacterium* spp. and *Pseudomonas aeruginosa* in the headspaces of cultures, spiked sputa, and sputum samples from 330 culture-proven and human immunodeficiency virus-tested tuberculosis and non-tuberculosis patients. The EN differentiated between different *Mycobacterium* species and between mycobacteria and other lung pathogens both in culture and in spiked sputum samples. The specificity and sensitivity of the described method were 91% and 89%, respectively, compared to culture. The detection limit in culture and spiked sputa was found to be 1×10^4 mycobacteria ml^{-1}.

This study has shown the potential of an electronic nose to detect *M. tuberculosis* in clinical specimens and opens the way to make this method a rapid and automated system for the early diagnosis of respiratory infections. Therefore, this method in principle can become a cheap and wide-spread screening method for large populations in poor-resource settings.

PART II: CLINICAL RELEVANCE OF NTM

Most data reporting high rates of NTM infections come from Northern European countries and the United States. In African countries, despite the fact that environmental exposure to NTM is very high, infection by NTM seems to be rare, even among patients with AIDS (42). However, it is not clear whether this is due to a true low prevalence of NTM infection or that the magnitude of this problem has not yet been unravelled. In Africa the contribution of NTM to the clinical problem of tuberculosis has so far only been examined at a very small scale.

A number of studies on the epidemiology of NTM has been performed in South Africa. The results of sputum cultures from a large random sample of South African native

people were reported in two studies (6,25). Prevalence rates of NTM colonization/infection amounted; 1,400 and 6,700 per 100,000, respectively.

Furthermore, two other South African NTM studies focussed on a specific population of gold miners, investigated for suspected pulmonary mycobacterial disease (18,19). Annual rates of NTM infection/disease were found to be 101 per 100,000 with the two most common organisms isolated being *M. kansasii* (66/100,000) and *M. scrofulaceum* (12/100,000), respectively.

In Yeta District Hospital in Sesheke, which is located in the Western Province of Zambia, during the late 1990s an increasing number of HIV-positive patients, clinically suspected of pulmonary tuberculosis, had a negative sputum smear but reacted positively to tuberculosis treatment. This suggested an increasing prevalence of sputum smear-negative tuberculosis. On the other hand, the contribution of NTM to tuberculosis-like diseases in Zambia had not been investigated previously.

The aim of the first pilot study described in **Chapter 5** was to evaluate the role of NTM in the development of tuberculosis-like disease in Zambia and the confusion this may cause in the diagnosis of tuberculosis by microscopy. In Sesheke, 64 (both HIV positive and negative) patients, who were chronically ill for more than two weeks, were investigated using modern liquid culture and identification methods. Thirty out of 64 (47%) patients yielded positive *Mycobacterium* cultures that were identified as *M. tuberculosis* (8 times), *M. szulgai* (7), *M. avium-intracellulare* (3), *M. simiae* (1) and *M. terrae* (1). Ten isolates were not suitable for identification due to contamination or re-culture problems. Twelve of the 30 (43%) culture-positive patients were also positive in microscopic examination, including four patients with NTM isolates. Seven of the 22 NTM isolates belonged to *M. szulgai*. The patients with *M. szulgai*- positive sputum manifested symptoms extremely similar to tuberculosis caused by *M. tuberculosis.* DNA fingerprinting analysis revealed four different patterns among the seven *M. szulgai* isolates, excluding the possibility of a common source of infection or a laboratory cross-contamination. Three out of five patients with *M. szulgai* infection responded to anti-tuberculosis treatment. This suggests that in at least a part of the patients with chronic respiratory complaints their lung disease was caused by *M. szulgai*.

In several parts of the world *M. szulgai* has been recognized as a cause of serious infections in humans. However, Africa is the only continent from which no *M. szulgai* isolation has been described so far. Recovery of *M. szulgai* from the environment is exceptional and most literature on this *Mycobacterium* suggests that it is an unlikely laboratory contaminant (3,56). Therefore, *M. szulgai* should be considered clinically significant when isolated (10). Similar to other mycobacteria, *M. szulgai* is associated with a wide spectrum

of diseases. The reported cases of human infection caused by *M. szulgai* revealed that pulmonary disease, mimicking tuberculosis, was the commonest type of infection caused by this micro-organism (54,56).

Because the contribution of NTM, and especially of *M. szulgai*, to tuberculosis-like diseases in both HIV-positive and negative patients in Sesheke seemed to be underestimated (**chapter 5**) the subsequently pilot study was to assess the possible role of NTM in tuberculosis-like diseases in various geographic locations in Zambia, **Chapter 6**. In 2001, in three hospitals in Katete, Sesheke and Chilonga 167 hospitalised chronically ill patients were investigated. An extremely high rate of HIV positivity (between 69-79%) among the patients was observed. The percentage of patients with a positive sputum culture for *M. tuberculosis* complex was equal in the three geographic locations (19 to 25%). In contrast, the percentage of sputum cultures positive for NTM differed significantly between the villages and ranged from 78% in Katete, 65% in Sesheke and 21% in Chilonga. Furthermore, the distribution of NTM species was different at the three geographic sites.

Nearly half (7/15) of the patients almost fulfilled the criteria of the American Thoracic Society for NTM disease and had the same NTM species isolated from both sputum specimens.

In the study described in chapter 6 we did not only find a high percentage of patients with positive NTM isolates from sputum specimens but also from specimens that are normally sterile, like lymph nodes and pleural effusion. Eight patients from whom NTM were cultured from normally sterile body sites are described in detail in **Chapter 7**. From chronically ill patients who had signs and symptoms in any part of the body for more than two weeks, pleural effusion, ascites, abscess material, or enlarged lymph nodes were cultured for mycobacteria. A specimen from one sterile body site was collected from 25 patients (60% HIV-positive); NTM were isolated from eight of these 25 specimens. *M. lentiflavum* was isolated from four patients, and *M. goodii* was isolated from one patient. In order to exclude the possibility of a laboratory cross-contamination, a novel amplified fragment length polymorphism DNA typing method for *M. lentiflavum* was developed. Genetic variation was detected, rendering the likelihood of laboratory cross-contamination unlikely. To our knowledge, we describe the first cases of *M. lentiflavum* and *M. goodii* infection in African patients.

M. lentiflavum is a slowly growing acid-fast bacillus that has biochemical characteristics identical to those of organisms belonging to the *Mycobacterium avium* complex and mycolic acid and fatty acid chromatography patterns very similar to those of *Mycobacterium simiae*, so genetic analysis is necessary for conclusive identification (53). This organism has been isolated from normally sterile clinical samples in Italy, Switzerland, Germany, France and Spain and from sputum samples in Brazil and Italy (21,33,40,44,53,57). Cases

of human disease have been reported, including chronic pulmonary disease, cervical lymphadenitis, liver abscess and fatal disseminated infection (15,33,40,55). In addition, *M. lentiflavum* was isolated from two patients co-infected with *M. avium* (54).

In the initial microbiological report of *M. goodii*, most of the 28 isolates were recovered from non-pulmonary sources, including skin and soft tissue infections and osteomyelitis following penetrating trauma or surgery (14). Additional isolates were associated with nosocomial infection (catheter-related sepsis, pacemaker site infection, and other surgical wounds), and other isolates were from patients with lipoid pneumonia or other types of pulmonary infection. The next report on *M. goodii* describes a case of olecranon bursitis in a patient with type 2 diabetes mellitus and benign monoclonal gammopathy (26). The patient did not have any history of penetrating trauma, but the possibility of bacterial introduction with intrabursal injections or subsequent surgery was raised. Recently, a patient with an abdominal wall abscess after an inguinal hernia repair was described. He presented with abdominal wall- and preperitoneal abscesses three weeks after surgery (52).

High rates of NTM culture positive specimens were observed in the pilot study in Zambia, described in **chapter 5 and 6**. Therefore, the clinical relevance of isolation of NTM from HIV-positive as well as HIV-negative patients was examined in detail in the study described in **Chapter 8**. In 2003, in St. Francis Hospital, 180 chronically ill patients and 385 controls were included in the study. The proportion of NTM positive sputum samples from the patients was significantly higher in comparison to the controls, 11% and 6% respectively ($p<0.05$). In both groups, many (55 of the 171 NTM = 32%) not previously identified mycobacteria were isolated.

From 154 patients and 383 controls two consecutive sputum samples were cultured. Four patients had two positive cultures for NTM of whom one had pulmonary disease caused by *M. intracellulare*. In three more NTM-positive patients one of the two sputum cultures contained AFB which could not be identified of whom two had probable pulmonary disease caused by *M. intracellulare*. In contrast, only one control had two sputum samples positive for (none identical) NTM. Significantly more patients in comparison to controls had two sputum cultures positive for NTM ($p<0.05$).

NTM probably play an important role in aetiology of tuberculosis-like disease in Zambia. In our study the estimated rate of colonization in the patient population was 14/154 (9%) with a rate of disease of 3/154 (about 2%). The estimated rate of colonization in the general population was 61/383 (16%). In addition, it became clear in this study that patients and controls with NTM in the sputum were more often having symptoms and signs of general malaise and that the chest X-rays in these NTM-positive persons more frequently showed pathology in comparison with NTM culture-negative persons.

Moreover, patients with a positive NTM culture also more frequently revealed a clinical picture of general malaise (including diarrhoea, vomiting) and underweight (BMI < 18).

Finally, in **Chapter 9**, the accuracy of clinical diagnosis of tuberculosis in Zambia in the era of increasing HIV prevalence is summarized. In Zambia patients are diagnosed with tuberculosis on basis of respiratory complaints for two or more weeks, no improvement on two courses of routine empiric antibiotics, a positive Ziehl-Neelsen (ZN) sputum smear and/or pathology on the chest X-ray consistent with tuberculosis. Within the frame work of this study, sputum of the diagnosed tuberculosis cases were additionally subjected to MGIT liquid culture to distinguish between (1) tuberculosis cases confirmed by positive *M. tuberculosis* cultures, (2) mycobacteriosis caused by non-tuberculous mycobacteria and, (3) tuberculosis-like disease caused by of organisms other than mycobacteria. Only in 47% of the 187 diagnosed tuberculosis cases, *M. tuberculosis* was cultured. In another 19% of the cases, exclusively NTM were cultured, whereas from 12% of the tuberculosis cases a combination of *M. tuberculosis* and NTM was isolated. In the remaining 29% of cases in which tuberculosis was diagnosed clinically, no mycobacteria were cultivable. HIV positivity was found significantly correlated with the isolation of NTM from sputum and inversely correlated with the isolation of exclusively *M. tuberculosis* from the sputum ($p<0.05$). Basing the diagnosis of tuberculosis on symptoms, sputum smear and/or chest X-ray may lead to significant numbers of clinically-defined false-positive cases of tuberculosis in Zambia, due to the increased prevalence of HIV.

A critical approach has to be made to the clinical relevance of the NTM isolated in the patients described in chapter 5, 6, 8 and 9. Not all the conditions described in these studies completely fulfilled the ATS criteria regarding NTM pulmonary disease because of reasons related to the African conditions of work. Moreover, the number of colonies on the solid medium tubes cultured in Zambia were not reported or culturing was only performed in liquid medium. A low number of colony-forming units could have been an indication of colonization of specimens by NTM. Therefore, the distinction between colonization and infection or disease could not be deduced from the culture results; some patients could also have represented culture negative TB cases.

Sputum of suspected cases was only subjected to culture and not tested by a nucleic acid amplifications (NAA) test or for instance the LiPA-Rif TB test on direct sputum to detect the presence of *M. tuberculosis* or multi-drug resistant *M. tuberculosis* (1,58). However, the performance of the NAA-test is good in clinical respiratory specimens that are AFB smear-positive (sensitivity 95% and specificity 98%) but less in specimens that contain fewer organisms or are AFB-negative (sensitivity 50% and specificity 95%). The NAA-test would be in a part of the cases be better in comparison to culture to detect a mixture of NTM and *M. tuberculosis*.

In addition, no studies in this thesis were performed on environmental mycobacteria isolated from natural and artificial (e.g. tap water) sources in areas where patients and controls were living.

The factors determining an individual's risk of infection and development of active disease are multi-factorial and involve host-pathogen interactions and environmental components. Substantial epidemiological evidence supports the role of host genetic factors in susceptibility to tuberculosis and mycobacteriosis. Host protective immunity to mycobacterial infection is dependent on the precise coordination of T-lymphocyte sensitisation and monocyte recruitment. Several cytokine and chemokine signals play crucial roles in the immune response to tuberculosis, by contributing to the initial control of the infection. One of these factors is monocyte chemoattractant protein-1, MCP-1, a chemokine strongly involved in the inflammatory process. In the study described in **Chapter 10**, the MCP-1 promotor gene polymorphism in association with susceptibility to development of tuberculosis after infection was investigated in Zambian people. The allele G of the MCP-1 promotor-enhancing region was found associated with increased odds of developing active pulmonary tuberculosis after infection. Persons with the MCP-1 genotype AG were 2.7-fold more likely to develop tuberculosis than those with the AA genotype.

Although a small number of patients and controls were included in this study, already a significant association was disclosed of susceptibility to tuberculosis and a particular polymorphism in this gene. To our knowledge, this is the first study investigating the polymorphism of the MCP-1 gene in African people, and this may be a first indication that especially this gene may play a crucial role in increased susceptibility to this infectious disease in particular human populations. Further studies with sufficient power are needed to compare the true contribution of the MCP-1 gene to the susceptibility to develop tuberculosis among Africans and populations in other geographic regions.

DIRECTIONS FOR FUTURE RESEARCH

High rates of NTM colonization and NTM disease in Zambia are reported in this thesis. Furthermore, differences in the distribution of specific species of NTM in chronically ill patients in three villages in Zambia were observed. So far, only a few studies on the epidemiology of NTM have been performed in Africa, mainly South Africa, and benchmark data are barely available. More extensive studies; in duration and size, will be needed to determine the true magnitude of NTM colonization and infection on the African continent. Furthermore, studies on the environmental flora of areas were patients and controls are living will be needed. The limited sample size of our studies lacks the

power to generalise findings across the population as a whole. Moreover, patients were examined during certain months of the year and seasonal influences may have played a role as well.

Consistent data of patients have to be collected and the minimum data required should include the criteria of the ATS for evaluation of a patient suspected for NTM disease: (1) chest radiograph with nodular or cavitary opacities, or an high-resolution computed tomography (HRCT) scan that shows multi-focal bronchiectasis with multiple small nodules, (2) three or more sputum specimens for AFB analysis and (3) exclusion of other disorders, such as tuberculosis. The preferred staining procedure is the fluorochrome microscopy and specimens should be cultured on both liquid and solid media. It is difficult to determine the clinical significance of the isolate without species identification and therefore NTM should be identified to the species level. Introduction of diagnostic tools, adequate laboratory facilities and training of laboratory technicians will therefore be mandatory in Africa. It is advisable to use a nucleic acid amplification method directly on clinical specimens from TB suspected patients, in addition to sputum smear microscopy and culture, to investigate the possibility of being a true TB case despite the absence of positive culture for *M. tuberculosis*.

To further investigate the occurrence of NTM by culture, the use of MGIT tubes in combination with a solid medium is recommended. This would provide the number of colonies grown on solid medium and would help to deduce the clinical relevance of a positive culture. At least one advanced reference laboratory is required in each country and preferable should have an affiliation with a laboratory in the industrialized world.

In general NTM infections in Africa are not treated at this moment and indications have been found that at least a part of the NTM isolated in African patients is clinically relevant. Patients diagnosed with NTM disease should be evaluated for the initiation of therapy and outcome should be critically followed for extended time periods. Furthermore, patients suspected of having NTM lung disease but not meeting the diagnostic criteria should be followed until the diagnosis is established or excluded. The treatment of NTM disease is generally not directly analogous to the treatment of tuberculosis. Multi-drug regimes include most of the time a newer macrolide (azithromycin, clarithromycin) (29). *In vitro* susceptibilities for many NTM do not correlate well with clinical response to antimycobacterial drugs but recommendations for routine *in vitro* susceptibility testing of NTM isolates are limited. The duration of therapy for most pulmonary NTM pathogens is at least one year until sputum cultures are consecutively negative while on therapy.

Certain conditions like HIV infection tend to facilitate NTM infection and patients with such risk factors in future research deserve special attention. In HIV positive patients,

disseminated NTM infections typically occurs only after the CD4+ T-lymphocyte number has fallen below 50/µl. Lung disease due to NTM occurs commonly in structural lung disease, such as chronic obstructive pulmonary disease (COPD), bronchiectasis, CF, pneumoconiosis, and previous tuberculosis. NTM lung disease also occurs in women without clearly recognized predisposing factors. Additional, extended case control studies to identify possible risk factors for NTM colonization and infection would be a logical next step.

In conclusion, tuberculosis classically defined on basis of ZN-smear of sputum and chest X-ray may increasingly be over-diagnosed in Zambia, due to the increased prevalence of HIV and inaccuracies in the diagnosis of tuberculosis, as described in this thesis. Furthermore, a part of the tuberculosis-like syndromes in Africa is probably caused by NTM and the treatment of such infections should be considered. Misdiagnosis of tuberculosis can be reduced by the introduction of new diagnostic techniques in addition to AFB microscopy, which incorporate the diagnosis of NTM pulmonary disorders. Furthermore, diagnostic scoring systems and algorithms must be developed and validated to assist clinicians working in resource-poor settings with high prevalence of HIV.

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Chapter 12

Samenvatting en Algemene discussie

Tuberculose (TB) behoort met HIV/AIDS en malaria tot één van de belangrijkste doodsoorzaken ter wereld. Deze drie infectieziekten samen zijn namelijk verantwoordelijk voor ongeveer 10 procent van alle sterfte in de wereld, die zich voor het overgrote deel manifesteert in ontwikkelingslanden. Het afgelopen decennium is het aantal tuberculosepatiënten sterk toegenomen in Afrika als gevolg van de HIV-epidemie en de daarmee gepaard gaande immuunsuppressie.

Wereldwijd is één derde van de wereldbevolking, twee miljard mensen, latent geïnfecteerd met tuberculose. Slechts 1 op de 10 geïnfecteerde mensen krijgt actieve tuberculose en wordt ziek. Jaarlijks ontwikkelen wereldwijd naar schatting zo'n negen miljoen mensen daadwerkelijk de ziekte tuberculose.Tuberculose eist bijna twee miljoen doden per jaar, hetgeen betekent dat elke dag ongeveer vijfduizend mensen aan tuberculose sterven, ondanks het feit dat tuberculose te genezen is. Bijna alle sterfgevallen aan tuberculose, 98 procent, komen voor in de ontwikkelingslanden.

Tuberculose is de belangrijkste doodsoorzaak onder mensen met een verzwakt immuunsysteem door een HIV-infectie. Wereldwijd zijn ongeveer 34 miljoen mensen geïnfecteerd met HIV/AIDS. Hiervan kent Afrika het hoogste aantal geïnfecteerde mensen. In sommige landen heeft één op de vijf volwassenen HIV/AIDS. Een kwart miljoen patiënten die per jaar aan tuberculose sterven is HIV-geïnfecteerd of ook AIDS-patiënt. De meeste van hen wonen in Afrika. HIV is de voornaamste oorzaak van de dramatische toename van tuberculose in Afrika gedurende de afgelopen vijftien jaren. In sommige delen van Afrika heeft wel tachtig procent van de tuberculosepatiënten een HIV-infectie.

Tuberculose, in de volksmond ook 'tering' of 'witte pest' genoemd, is een infectieziekte die meestal wordt veroorzaakt door de tuberculosebacterie, *Mycobacterium tuberculosis*. Echter in de Westerse wereld is bekend dat ook atypische mycobacteriën, non-tuberculeuze mycobacteriën (NTM), een oorzakelijke rol spelen in tuberculose-achtige ziektebeelden. Normaliter maken deze NTM de mens niet ziek. Echter, in geval van een slechte afweer zoals bij AIDS kunnen sommige NTM wel ziekte veroorzaken. In Afrika is de bijdrage van NTM aan het tuberculose probleem nog nauwelijks onderzocht. De gedachte was dat deze infectie weinig in sub-Sahara Afrika voorkomt. Uitgebreide laboratorium faciliteiten en radiologische apparatuur die nodig zijn om de ziekte te diagnosticeren ontbreken echter vrijwel overal in Afrika, waardoor een onderschatting van het werkelijke probleem is ontstaan.

In Zambia heeft naar schatting 13,5-20% van de volwassen (15-49 jaar) HIV/AIDS. Daarom werd juist in dit land de klinische relevantie van NTM in zowel in HIV-positieve als HIV-negatieve patiënten onderzocht.

De afgelopen jaren zijn in de Westerse landen aanzienlijke vorderingen gemaakt in de tuberculose diagnostiek. Helaas zijn juist in gebieden waar heel veel TB voorkomt, zoals Afrika, de technieken om tuberculose te diagnosticeren vrijwel onveranderd gebleven in de afgelopen eeuw. In 1882 werd de tuberkelbacil voor het eerst microscopisch aangetoond als zuurvaste staaf in een sputumuitstrijk gekleurd volgens Ziehl-Neelsen. Deze Ziehl-Neelsen microscopie is in Afrika en in veel andere ontwikkelingsgebieden nog steeds de basis van de diagnose van tuberculose. Helaas zijn gevoeligere technieken zoals kweek en DNA/RNA amplificatie zo complex en duur dat ze in het algemeen niet toegepast worden in ontwikkelingslanden. De basis voor TB diagnostiek in ontwikkelingslanden is daarom nog steeds het microscopisch onderzoek naar de zuurvaste staven (mycobacteriën) in sputum. Volgens internationale standaarden, zoals International Standards for Tuberculosis Care, zouden van alle verdachte tuberculose patiënten twee, en bij voorkeur drie, sputum specimens microscopisch op tuberkelbacillen onderzocht moeten worden, indien mogelijk inclusief een ochtendsputum.

Een ervaren analist vindt 50-60% van de tuberculose patiënten door middel van microscopisch onderzoek van sputum. In ontwikkelingslanden ligt dit percentage veel lager omdat de kwaliteit van de microscopen slechter is, de werkdruk hoger ligt en er een tekort is aan getraind personeel. Verder is de microscopie vaak negatief in patiënten met recente tuberculose en bepaalde groepen patiënten zoals die met een HIV co-infectie en patiënten met extra-pulmonale TB. Het percentage TB patiënten dat door middel van microscopie wordt gediagnosticeerd kan daardoor teruglopen tot 20-30%.

Indien sprake is van een negatief microscopisch onderzoek van het sputum en geen reactie op breedspectrum antibiotica wordt waargenomen worden de meeste patiënten met pulmonale TB in ontwikkelingslanden op basis van klinische en radiologische kenmerken gediagnosticeerd.

Volgens huidige richtlijnen moeten alle personen met een onverklaarde productieve hoest die 2-3 weken of langer duurt op TB onderzocht worden. De diagnose sputumuitstrijk-negatieve pulmonale TB wordt gebaseerd op tenminste drie keer een negatief microscopisch onderzoek op zuurvaste staven, radiologische afwijkingen passend bij TB en geen reactie op een kuur breedspectrum antibiotica.

Echter, de diagnose 'sputumuitstrijk negatieve pulmonale TB' is in gebieden met hoge prevalentie HIV en TB infectie gecompliceerd ten gevolge van de atypische presentatie van pulmonale TB in HIV-geïnfecteerde patiënten. Typische radiologische afwijkingen, passend bij pulmonale TB, zijn minder vaak te vinden in gebieden met een hoge HIV prevalentie. Verder presenteren HIV positieve en sputumuitstrijk-negatieve TB patiënten zichzelf minder vaak met caviteiten in de longen. Tevens kunnen sputumuitstrijk-negatieve patiënten zichzelf ook presenteren met een normale of minimaal afwijkende thorax foto. Om vervolgens de etiologie van de afwijkende thorax foto te achterhalen is verder

onderzoek noodzakelijk, omdat uit jarenlang onderzoek is gebleken dat radiologisch onderzoek voor de diagnose of follow-up van pulmonale TB niet betrouwbaar is.

Een bijkomend probleem is dat het voorkomen van zuurvaste NTM bij patiënten met tuberculose-achtige ziektebeelden kunnen resulteren in het misdiagnosticeren van TB. De recentelijk gepubliceerde diagnostische criteria voor NTM ziekte van de American Thoracic Society (ATS) kunnen de diagnose ondersteunen indien bij een patiënt NTM uit een specimen wordt geïsoleerd. Het grootste verschil tussen de oude en nieuwe diagnostische ATS criteria ligt in de minder harde bacteriologische criteria. Een enkele positieve bronchusspoelselkweek voor NTM, of twee positieve sputumkweken in een symptomatische patiënt met nodulaire of cavitaire afwijkingen op de thorax foto, zijn nu voldoende om een NTM infectie vast te stellen. Waarschijnlijk zullen nu meer patiënten dan voorheen aan de ATS criteria voor een echte NTM infectie voldoen, waardoor een toename verwacht wordt van het aantal patiënten dat voor een anti-mycobacteriële behandeling in aanmerking komt. Door de auteurs van de nieuwe ATS criteria wordt wel duidelijk naar voren gebracht dat patiënten die aan de criteria voldoen niet *per se* met behandeling hoeven te beginnen. Het klinisch beeld blijft van belang. Noemenswaardig is ook de exclusie van een positieve sputumuitstrijk of bronchusspoelsel en de exclusie van extra-pulomonale NTM isolaten in de diagnostische criteria. De consequenties van deze nieuwe criteria zijn waarschijnlijk dat er moeilijkheden gaan ontstaan betreffende initiatie van therapie en vaststellen van de klinische significantie van de geïsoleerde NTM, met name afkomstig uit een extra-pulmonaal specimen.

In dit kader zouden er in Afrika op korte termijn methoden geïntroduceerd moeten worden die onderscheid kunnen maken tussen *M. tuberculosis* en NTM in klinische materialen. Wel blijft de gevoeligheid van dergelijke testen bij directe toepassing op klinische materialen een punt van zorg.

Met de huidige kennis lijkt onderzoek naar de diagnostiek en behandeling van NTM infecties in Afrika van belang te zijn, zeker in het licht van de toenemende HIV prevalentie.

DEEL I: DIAGNOSTICEREN VAN TUBERCULOSE

De kweek wordt gezien als de gouden standaard voor het aantonen van het oorzakelijke agens *M. tuberculosis.* De meeste klinische monsters bevatten echter een variëteit aan micro-organismen die *M. tuberculosis* kunnen overgroeien. Decontaminatie van deze monsters is dan ook essentieel om contaminatie van de mycobacteriële kweek te voorkomen.

De decontaminatie heeft echter ook invloed op het kweken van mycobacteriën; deze worden ook in de groei geremd en zelfs deels geïnactiveerd. In het onderzoek dat beschreven is in **Hoofdstuk 2** wordt het effect van twee decontaminatie procedures; de NaOH-N-actetyl cysteine (NaOH-NALC) en de zwavelzuur methode op het kweken van *M. tuberculosis* complex en NTM in sputum samples vergeleken. De detectie van NTM met de zwavelzuurmethode was significant beter dan met de NaOH-NALC methode (p = 0.001). Er werd echter geen significant verschil gevonden voor de detectie van *M. tuberculosis*.

De decontaminatiemethode die wordt gebruikt heeft dus een duidelijke invloed op de detectie van mycobacteriën, hetgeen uiteindelijk belangrijke consequenties heeft voor zowel de microbiologische diagnose als de behandeling van de patiënt. Op basis van dit onderzoek lijkt het van belang om klinische monsters afkomstig van patiënten die mogelijk aan NTM longziekte lijden, te decontamineren met zwavelzuur in plaats van NaOH-NALC om zo de detectie van NTM te optimaliseren.

Hoewel sputumkweek de gouden standaard is voor de diagnose van pulmonale TB wordt deze diagnostische test in de meeste ziekenhuizen in ontwikkelingslanden niet standaard gebruikt, men heeft over het algemeen alleen de microscopie beschikbaar. Verder wordt het gebruik van de kweek als eerstelijns diagnostische test beperkt doordat kweken van mycobacteriën op een vast medium gemiddeld 3-8 weken duurt. De laatste decennia zijn er in de Westerse wereld vloeibare kweekmethoden beschikbaar gekomen die in 1-3 weken een uitslag kunnen geven. De vloeibare kweeksystemen worden echter nog nauwelijks in Afrika toegepast ten gevolge van de kosten en onderhoud van apparatuur. Tevens vereist het kweken van mycobacteriën gespecialiseerd personeel en betrouwbare voorziening van water en elektriciteit.

Om de diagnostiek van TB te versnellen zijn er de laatste decennia een aantal testen ontwikkeld, waarvan de moleculaire amplificatie technieken het meest prominent zijn. Deze amplificatie testen, zoals de Amplified Mycobacterium Tuberculosis Direct Test (MTD) (Gen-Probe, San Diego, California) en de Amplicor Mycobacterium Tuberculosis Test (Amplicor) (Roche Diagnostic Systems, Inc., Branchburg, New Jersey) hebben een hoge sensitiviteit en specificiteit en zijn direct toepasbaar op klinische specimens zoals sputum. De specificiteit van deze testen zou 98-100% zijn en de sensitiviteit meer dan 95% in microscopisch positief sputum, terwijl dit 60-70% zou zijn voor microscopisch negatief, kweek positief materiaal. Recentelijk ontwikkelde moleculaire amplificatie testen hebben een betere sensitiviteit in microscopisch negatieve monsters, terwijl de specificiteit blijft behouden. Tevens zijn deze testen waarschijnlijk ook toepasbaar op specimens anders dan sputum, zoals bloed, lymfklieren, beenmerg, liquor, urine, broncho-alveolaire lavage, hoewel de resultaten aanzienlijk verschillen. Vermoedelijk zal de toepassing van de moleculaire amplificatie methode op monsters van diverse aard de sensitiviteit verhogen in

vergelijking met kweek. Niettemin, op dit moment zijn deze testen in minder ontwikkelde gebieden niet geschikt om toe te passen ten gevolge van de hoge kosten van zowel apparatuur als materiaal, de complexiteit in gebruik en lage specificiteit in het veld.

Eén van de regio's op het genoom van mycobacteriën die vaak voor de species identificatie wordt gebruikt is het 16S ribosomaal RNA (rRNA) gen. Sequencen van dit gen wordt tegenwoordig gezien als gouden standaard voor de identificatie. Het organisme wordt geïdentificeerd door vergelijking van de nucleotide sequence met referentie sequenties in verscheidene internet databases. Vanwege de grote nauwkeurigheid wordt 16S rRNA gen sequentie analyse door vrijwel alle referentie laboratoria gebruikt. In perifere laboratoria en vooral in ontwikkelingslanden wordt deze techniek niet gebruikt ten gevolge van technische overwegingen en kosten. Een bijkomend probleem is dat *Mycobacterium* species in ontwikkelingslanden waarschijnlijk een andere evolutionaire ontwikkeling hebben doorgemaakt dan de species in de Westerse wereld. Daardoor zijn nieuwe databases nodig die genetische informatie van de lokale populatie mycobacteriën bevatten, zodat een adequate en nauwkeurige identificatie kan plaatsvinden.

Een andere methode voor de identificatie van *Mycobacterium* species is gebaseerd op line probe hybridisatie welke zich richt op de 16S-23S rRNA gen spacer regio of 23S gen regio, zoals respectievelijk de Inno-Lipa Mycobacteria system (Innogenetics, Ghent, Belgium) en GenoType Mycobacterium CM/AS (Hain Lifescience GmbH, Nehren, Germany). Deze methode wordt momenteel gebruikt voor de identificatie van positieve kweken maar heeft de potentie om *Mycobacterium* species direct in klinisch materiaal te identificeren. Ook deze methode is echter op de meest voorkomende species in Europa gebaseerd en het is nog niet duidelijk of deze methode zondermeer in Afrika te gebruiken is.

In het kader van deze beperkingen is er met name in minder ontwikkelde gebieden behoefte aan snelle, minder gecompliceerde diagnostische testen die in principe direct toepasbaar zijn op bacteriën in klinisch materiaal.

Naar verwachting zal in de komende jaren de moleculaire diagnostiek van TB langzaam in Afrika worden geïntroduceerd. Er zijn al amplificatie methoden die DNA kunnen amplificeren zonder thermische cycli, zodat alle reacties onder isothermische condities, varierend van 60 tot 65°C, kunnen worden uitgevoerd. Loop-gemedieerde isothermische amplificatie (LAMP) is een nieuwe nucleinezuur amplificatie techniek waarin de reagentia onder isothermische condities met hoge specificiteit, efficiëntie en snelheid met elkaar reageren. LAMP is al gebruikt voor de detectie van *M. tuberculosis* complex, *M. avium* en *M. intracellulare* zowel in sputum samples als in kweek. Door de gemakkelijke uitvoering van LAMP zonder gecompliceerde apparaten zal deze techniek eenvoudig genoeg zijn

om in kleinere ziekenhuizen en klinische laboratoria in ontwikkelingslanden te introduceren.

Een nieuwe diagnostische techniek voor de snelle identificatie van mycobacteriën is de Raman spectroscopie, beschreven in **Hoofdstuk 3**. De resultaten van dit onderzoek laten duidelijk zien dat het mogelijk is de 63 verschillende *Mycobacterium* stammen van elkaar te onderscheiden en te groeperen in 8 clusters behorende tot *M. tuberculosis*, de meest relevante klinische NTM species in Nederland en *M. lentiflavum*. Tevens toonden de spectra van levende (en dus infectieuse) stammen en warmte-geïnactiveerde (ongevaarlijke) stammen minimale verschillen. Het was daarom mogelijk om de identificatie van mycobacteriën uit te voeren zonder biosafety level III inperkingsmaatregelen. Raman spectroscopie is een optische methode die het mogelijk maakt binnen enkele seconden spectroscopische fingerprints van samples te verkrijgen. De fingerprints zijn een afspiegeling van de moleculaire samenstelling van het sample en daarom ideaal voor de identificatie van micro-organismen op zowel species als stam niveau. Belangrijke kenmerken van de methode zijn de eenvoud waarmee het sample gemeten kan worden, het geringe aantal handelingen, de geringe hoeveelheid aan benodigd materiaal en de hoge mate van reproduceerbaarheid. In de nabije toekomst zal de Raman analyse waarschijnlijk direct op klinische materialen toepasbaar worden. De laatste jaren zijn de kosten van Raman systemen aanzienlijk verminderd en de apparatuur is kleiner en minder kwetsbaar geworden en daarom wordt de bruikbaarheid in Afrika realiteit in de toekomst.

Ook de elektronische neus (gas sensor array) heeft de potentie om op zeer snelle wijze mycobacteriën direct in klinisch materiaal te detecteren. In **Hoofdstuk 4** wordt het gebruik van de elektronische neus (EN) om verschillende *Mycobacterium* species en *Pseudomonas aeruginosa* in kweken, kunstmatige besmet sputum en sputum samples van 330 kweek-positieve tuberculose and non-tuberculose patiënten te detecteren besproken. De EN differentieerde de verschillende *Mycobacterium* species en ook mycobacteriën ten opzichte van andere pathogenen, zowel in kweek als in het kunstmatig besmet sputum. De specificiteit en sensitiviteit waren respectievelijk 91% en 89% in vergelijking tot kweek. De detectie limiet in kweek en kunstmatig besmet sputum was 1×10^4 mycobacteriën ml^{-1}.

Dit onderzoek toont aan dat het in principe mogelijk is om *M. tuberculosis* direct in klinische samples aan te tonen. Deze methode kan in de toekomst een goedkope, snelle en geautomatiseerde test zijn voor de vroege diagnose van respiratoire infecties in minder ontwikkelde gebieden.

DEEL II: KLINISCHE RELEVANTIE VAN NTM

De meeste data betreffende prevalentie van NTM infecties zijn afkomstig van Noord Europese landen en de Verenigde Staten van Amerika. In Afrika zijn weinig NTM infecties gerapporteerd, ondanks de hoge omgevingsblootstelling en het hoge percentage patiënten met AIDS. Het is echter niet duidelijk of hier sprake is van een daadwerkelijke lage prevalentie van NTM infectie of dat de omvang van het probleem nog niet voldoende in kaart is gebracht. In Afrika is de bijdrage van NTM aan het ziektebeeld tuberculose tot nu toe alleen zeer kleinschalig onderzocht.

In Zuid-Afrika is de epidemiologie van NTM in een aantal onderzoeken beschreven. In twee mycobacteriële studies werden de sputum kweek resultaten van Zuid-Afrikaanse personen gerapporteerd. De NTM prevalentie voor kolonisatie/infectie bedroeg in deze studies 1.400 and 6.700 per 100.000. Twee andere Zuid-Afrikaanse NTM studies concentreerden zich op een specifieke populatie goudmijnwerkers verdacht voor pulmonale mycobacteriële ziekte. Er werd een NTM incidentie voor infectie/ziekte van 101 per 100.000 gevonden. De incidentie van de twee meest geïsoleerde organismen was voor *M. kansasii* 66/100.000 en *M. scrofulaceum* 12/100.000.

In het Yeta District Ziekenhuis in Sesheke, gelegen in de Westelijke Provincie van Zambia, werden gedurende de jaren 90 een toenemend aantal HIV-positieve patiënten, verdacht voor pulmonale TB, met een negatieve sputumuitstrijk gezien die reageerde op anti-tuberculose behandeling. Deze bevinding suggereerde een toenemende prevalentie van sputumuitstrijk negatieve TB. Echter, de bijdrage van NTM aan tuberculose-achtige ziektebeelden was in Zambia nog niet eerder onderzocht.

Het doel van het eerste pilot onderzoek dat beschreven is in **Hoofdstuk 5** was het evalueren van de bijdrage van NTM in de ontwikkeling van tuberculose-achtige ziektebeelden in Zambia en de verwarring die NTM mogelijk veroorzaken in de diagnostiek van TB, gebaseerd op microscopie. In Sesheke werden 64 (zowel HIV positieve als negatieve) patiënten, die chronisch ziek waren gedurende meer dan twee weken, op mycobacteriën onderzocht met een vloeibare kweek en moderne identificatie methoden. Dertig van de 64 (47%) patiënten hadden een positieve kweek voor mycobacteriën die werden geïdentificeerd als *M. tuberculosis* (8 keer), *M. szulgai* (7), *M. avium-intracellulare* (3), *M. simiae* (1) en *M. terrae* (1). Tien isolaten konden niet worden geïdentificeerd ten gevolge van contaminatie of kweek problemen. Twaalf van de 30 (43%) kweek positieve patiënten hadden ook een positieve sputumuitstrijk, inclusief vier patiënten met NTM isolaten. Zeven van de 22 NTM isolaten behoorden tot *M. szulgai*. De patiënten met een *M. szulgai* positief sputum presenteerden zich met symptomen die zeer vergelijkbaar waren met TB veroorzaakt door *M. tuberculosis*.

Door middel van DNA fingerprinting analyse was het mogelijk vier verschillende patronen onder de zeven *M. szulgai* stammen te onderscheiden, waardoor de mogelijkheid van een gezamenlijke bron of laboratorium kruis-contaminatie werd uitgesloten. Drie van de vijf patiënten met een *M. szulgai* infectie reageerden op anti-tuberculose behandeling. In dit hoofdstuk wordt gesuggereerd dat bij een deel van de patiënten met chronische pulmonale klachten *M. szulgai* het oorzakelijke agens zou kunnen zijn.

M. szulgai wordt in de meeste delen van de wereld herkent als een oorzaak van infecties bij de mens. Afrika was tot nu toe het enige continent waar de isolatie van *M. szulgai* niet was beschreven. *M. szulgai* wordt niet vaak uit de omgeving geïsoleerd en de meeste literatuur suggereert dat het zelden een laboratoriumcontaminant is. Indien *M. szulgai* wordt geïsoleerd, moet deze *Mycobacterium* daarom als klinisch significant worden beschouwd. *M. szulgai* is net als andere NTM geassocieerd met een breed spectrum aan klinische beelden. Pulmonale ziekte, lijkend op TB, is het meest voorkomende ziektebeeld.

Aangezien de bijdrage van NTM, met name *M. szulgai*, aan tuberculose-achtige ziektebeelden in zowel HIV-positieve als HIV-negatieve patiënten in Sesheke leek te worden onderschat (**hoofdstuk 5**) was het volgende pilot onderzoek gericht op de bijdrage van NTM aan tuberculose-achtige ziektebeelden in verschillende geografische locaties in Zambia; zie **Hoofdstuk 6**. In 2001 werden 167 chronisch zieke patiënten in drie ziekenhuizen in Katete, Sesheke en Chilonga onderzocht. Een extreem hoog percentage van deze patiënten was HIV positief; tussen de 69 en 79%. Het percentage patiënten met een positieve kweek voor *M. tuberculosis* was vergelijkbaar op de drie geografische locaties (19 tot 25%). Echter, het percentage NTM positieve sputumkweken verschilde significant tussen de drie ziekenhuizen en varieerde van 78% in Katete, 65% in Sesheke, tot 21% in Chilonga. Verder was de verdeling van de NTM species tussen de drie plaatsen verschillend.

Een deel van de patiënten waarbij NTM uit het sputum werd geïsoleerd voldeed bijna aan de criteria van de American Thoracic Society voor NTM ziekte: 7 van de 15 patiënten hadden twee maal dezelfde NTM in beide sputum monsters.

In deze studie, beschreven in hoofdstuk 6, vonden we niet alleen een hoog percentage patiënten met positieve NTM isolaten uit het sputum, maar ook uit klinische monsters die normaal gesproken steriel behoren te zijn, zoals lymfklieren en pleuravocht. In **Hoofdstuk 7** worden 8 patiënten in detail beschreven bij wie uit normaliter steriele klinische materialen NTM werd gekweekt.

Er werden van chronisch zieke patiënten met klachten en symptomen die langer dan twee weken aanhielden, pleuravocht, ascites, abces materiaal of een vergrote lymfklier gekweekt op mycobacteriën. In totaal werden monsters van een normaliter steriel materiaal van 25 patiënten (60% HIV-positief) verzameld. NTM werden uit acht van de 25

monsters geïsoleerd. *M. lentiflavum* werd uit 4 patiënten en *M. goodii* werd bij één patiënt geïsoleerd. Om vervolgens de mogelijkheid van een laboratorium kruis-contaminatie uit te sluiten werd een nieuwe amplified fragment length polymorphism DNA typeringsmethode voor *M. lentiflavum* ontwikkeld. Het was mogelijk door middel van deze methode genetische variatie aan te tonen waardoor een laboratorium kruis-contaminatie onwaarschijnlijk werd. Deze studie beschrijft naar ons beste weten voor de eerste keer patiënten met een *M. lentiflavum* en *M. goodii* infectie in Afrika.

M. lentiflavum is een langzaam groeiende zuurvaste bacterie met biochemische karakteristieken identiek aan mycobacteriën behorend tot het *Mycobacterium avium* complex. Deze bacterie heeft een mycolinezuur en vetzuur chromatografie patroon vergelijkbaar met *Mycobacterium simiae*, waardoor genetische analyse noodzakelijk is voor de identificatie. *M. lentiflavum* werd reeds geïsoleerd uit normaal gesproken steriele samples in Italië, Zwitserland, Duitsland, Frankrijk en Spanje en uit sputum samples in Brazilië en Italië. Patiënten met chronisch longlijden, cervicale lymphadenitis, leverabces en gedisseminеerde infectie ten gevolgde van *M. lentiflavum* zijn in de literatuur beschreven.

In het eerste publicatie over *M. goodii* werden de meeste van de 28 isolaten van extrapulmonale monsters geïsoleerd, zoals infecties van de huid en subcutane weefsel en een osteomyelitis na een penetrerend chirurgisch trauma. Later gerapporteerde isolaten waren geassocieerd met nosocomiale infecties (catheter gerelateerde sepsis, infectie van de pacemaker site en andere chirurgische wonden) en pulmonale infectie. Een case-report beschrijft een olecranon bursitits in een patiënt met diabetes mellitus type 2 en een goedaardige monoklonale gammo-globulinopathie. Intrabursale injecties of chirurgie werden als mogelijke verklaringen van de bacteriële introductie gezien. Recentelijk is er nog een patiënt beschreven met een buikwandabces dat drie weken na een inguinale hernia operatie ontstond.

In de pilot onderzoeken beschreven in **hoofdstuk 5 en 6** worden hoge percentages van NTM kweek positieve specimens in Zambia gevonden. De klinische relevantie van de geïsoleerde NTM van zowel HIV positieve als HIV negatieve patiënten werd daarom meer gedetailleerd onderzocht in het onderzoek dat beschreven is in **Hoofdstuk 8**. In 2003 werden in het St. Francis Ziekenhuis in Katete 180 chronisch zieke patiënten onderzocht. Tevens werden 385 controle personen geïncludeerd in de studie. Het percentage NTM positieve sputum samples afkomstig van patiënten was significant hoger dan van de controle personen, respectievelijk 11% and 6% ($p<0.05$). In beide groepen werden veel (55 of the 171 NTM = 32%) niet eerder geïdentificeerde mycobacteriën geïsoleerd.

Van 154 patiënten en 383 controle personen werden twee opeenvolgende sputum samples gekweekt. Vier van deze 154 patiënten hadden twee positieve NTM kweken waarvan

één patiënt een pulmonale ziekte had, veroorzaakt door *M. intracellulare*. Verder waren er nog drie NTM positieve patiënten, waarbij één van de twee sputumkweken zuurvaste staven bevatte die niet konden worden geïdentificeerd, waarvan er twee pulmonale ziekte hadden die mogelijk veroorzaakt werd door *M. intracellulare*.

In tegenstelling hiermee had maar één controle persoon twee sputum samples positief voor (zelfs niet identieke) NTM. Dus patiënten hadden significant vaker twee positieve NTM kweken dan controle personen ($p<0.05$).

Waarschijnlijk spelen NTM een belangrijke rol in de etiologie van tuberculose-achtige ziektebeelden in Zambia. In ons onderzoek was de geschatte prevalentie kolonisatie in de patiënten populatie 9% (14/154), met een percentage ziekte van ongeveer 2 (3/154). In de controle populatie was het percentage kolonisatie 16 (61/383). Verder werd in deze studie duidelijk dat zowel patiënten als controle personen met NTM in het sputum vaker klachten en symptomen van algehele malaise (inclusief diarree en braken) en ondergewicht (BMI <18) hadden en dat de thoraxfoto in deze NTM positieve personen vaker pathologie vertoonde dan in de NTM negatieve personen.

In **Hoofdstuk 9** wordt de nauwkeurigheid van de klinische diagnose TB, gedurende een periode van toenemende HIV prevalentie in Zambia, beschreven. In Zambia worden patiënten met TB gediagnosticeerd op basis van respiratoire klachten die langer dan twee weken bestaan, geen verbetering tonen na twee kuren breed spectrum antibiotica, of een positieve ZN sputumuitstrijk hebben en/of pathologie op de thoraxfoto passend bij TB. In deze studie werd vervolgens het sputum van de gediagnosticeerde TB patiënten gekweekt in MGIT vloeibaar medium zodat onderscheid mogelijk werd tussen (1) tuberculose patiënten met geconfirmeerde positieve *M. tuberculosis* kweken, (2) mycobacteriosis veroorzaakt door non-tuberculeuze mycobacteriën en (3) tuberculose-achtige ziektebeelden van niet mycobacteriële oorsprong.

In maar 47% van de 187 gediagnosticeerde TB patiënten werd *M. tuberculosis* gekweekt. In 19% van deze patiënten werd uitsluitend NTM gekweekt, terwijl in 12% een combinatie van zowel *M. tuberculosis* als NTM werd geïsoleerd. In de overige 29% van de klinisch gediagnosticeerde TB patiënten werden geen mycobacteriën gekweekt. Een positieve HIV status bleek significant gecorreleerd te zijn met de isolatie van NTM uit het sputum en omgekeerd evenredig gecorreleerd te zijn met uitsluitend de isolatie van *M. tuberculosis* uit het sputum ($p<0.05$). Indien de diagnose TB wordt gebaseerd op symptomen, sputumuitstrijk en/of thoraxfoto leidt dit in Zambia waarschijnlijk tot aanzienlijke aantallen foutpositief gediagnosticeerde TB patiënten ten gevolge van de toegenomen prevalentie HIV.

Bij het bepalen van de klinische relevantie van de geïsoleerde NTM in de patiënten beschreven in hoofdstuk 5, 6,8 en 9 was het niet altijd mogelijk om de ATS criteria afdoende

te toetsen. Dit had veelal betrekking op de werkcondities in Zambia. Vaak ontbraken klinische gegevens, of was het röntgenapparaat buiten werking. Het bleek buitengewoon moeilijk om meervoudige sputummonsters te analyseren. Een follow-up van de patiënten was in de praktijk bijna nooit mogelijk Verder werd in Zambia het aantal kolonies op het vaste kweekmedium niet gerapporteerd of werd er uitsluitend in een vloeibaar medium gekweekt. Een zeer beperkt aantal gekweekte kolonies zou een indicatie kunnen zijn van kolonisatie.

Verder zouden deze patiënten ook kweek-negatieve TB patiënten kunnen zijn. Helaas was het bij dit onderzoek niet mogelijk om met moleculaire amplificatie testen te screenen op de aanwezigheid van *M. tuberculosis*. Het blijft ook de vraag of dit veel bij zou dragen. De gevoeligheid van dergelijke testen om *M. tuberculosis* in sputum aan te tonen is veelal niet groter dan de gevoeligheid van de kweek. Wel zou een moleculaire test bij de aanwezigheid van een mengsel van NTM en *M. tuberculosis* de laatstgenoemde bacteriën kunnen aantonen, terwijl dit bij een kweek vrijwel onmogelijk is.

Tevens werd nog geen onderzoek gedaan naar aanwezigheid van NTM in de omgeving, in de gebieden waar de patiënten en gezonde Zambianen leefden. Dit zou informatie kunnen hebben opgeleverd over de blootstelling aan bepaalde NTM. Toch zou ook bij veelvuldige blootstelling aan NTM in het milieu dezelfde vraag over klinische relevantie van isolatie van NTM van verdachte personen overeind blijven.

De factoren die iemands risico op infectie en ontwikkeling naar actieve ziekte bepalen zijn multi-factorieel en bevatten zowel gastheer-pathogeen interacties als omgevingselementen. Epidemiologische data ondersteunen de rol van gastheer genetische factoren in de vatbaarheid voor TB en mycobacteriosis. Gastheer immuniteit voor mycobacteriële infecties is afhankelijk van een precies samenspel van T-lymfocyten sensibilisatie en monocyten recrutering. Verscheidene cytokine en chemokine signalen spelen hierbij een cruciale rol. Eén van deze factoren is de monocyte chemoattractant protein-1, MCP-1, een chemokine dat sterk betrokken is bij het ontstekingsproces.

In het onderzoek beschreven in **Hoofdstuk 10** wordt in Zambiaanse personen onderzocht of polymorfisme in het MCP-1 promotor gen geassocieerd is met de vatbaarheid voor het ontwikkelen van TB na infectie. De resultaten demonstreerden dat het G allel van de MCP-1 promotor regio was geassocieerd met een toegenomen risico op de ontwikkeling van actieve pulmonale TB na infectie. Personen met MCP-1 genotype AG hadden 2.7 keer meer kans op het ontwikkelen van TB dan degene met een AA genotype. Alhoewel er een klein aantal patiënten en controle personen in deze studie werden geïncludeerd kon echter al een significante associatie tussen de vatbaarheid voor het ontwikkelen van TB en een specifiek polymorfisme in dit gen worden aangetoond. Dit is voor zover bekend de eerste studie die het polymorfisme van het MCP-1 gen in Afrikaanse personen onderzocht heeft en dit zou een eerste indicatie kunnen zijn dat met name dit gen een

cruciale rol speelt in de vatbaarheid voor TB in bepaalde populaties. Er zijn meer studies met voldoende power nodig om de daadwerkelijke bijdrage van het MCP-1 gen aan de vatbaarheid voor het ontwikkelen van TB in Afrikaanse populaties in kaart te brengen.

SUGGESTIES VOOR VERDER ONDERZOEK

In dit proefschrift worden hoge percentages van NTM kolonisatie en NTM ziekte in Zambia gerapporteerd. Tevens werden er verschillen geobserveerd in de geografische verdeling van geïsoleerde NTM species in chronisch zieke patiënten in de drie ziekenhuizen in Zambia. Tot dusver zijn er maar een paar epidemiologische studies betreffende NTM prevalenties verricht in Afrika, en hoofdzakelijk Zuid-Afrika, waardoor referentie data nauwelijks beschikbaar zijn. Om de daadwerkelijke omvang van NTM kolonisatie en infectie in Afrika te bepalen zullen meer uitgebreide onderzoeken in zowel duur als grootte nodig zijn. Tevens is onderzoek naar het voorkomen van NTM in de omgeving waar de patiënten en gezonde Zambianen leven van belang. Door het beperkte aantal geïncludeerd patiënten en controle personen in onze studies is het niet mogelijk de bevindingen te generaliseren naar de algehele populatie. Tevens werden de patiënten gedurende bepaalde maanden van het jaar onderzocht waardoor seizoensinvloeden ook een rol kunnen hebben gespeeld.

Data van patiënten zullen consistent moeten worden verzameld. Verder zou een minimale hoeveelheid data geïncludeerd moeten worden om de criteria van de ATS te kunnen toetsen: (1) thoraxfoto met nodulaire afwijkingen of holtevorming of een high-resolution computed tomography (HRCT) scan die multifocale bronchiectasieën met multipele kleine noduli laat zien, (2) drie of meer sputum samples voor analyse van zuurvaste staven en (3) uitsluiting van andere pathologie zoals TB. De voorkeur gaat uit naar Fluorescentie microscopie en specimens zouden zowel in een vloeibaar als op een vast medium moeten worden gekweekt. NTM zouden tot op species niveau geïdentificeerd moeten worden, omdat het anders moeilijk wordt om de klinische significantie te bepalen. Introductie van diagnostische methoden, adequate laboratorium faciliteiten en het trainen van analisten zal daarom noodzakelijk zijn in Afrika. Het is aan te raden om klinisch materiaal van patiënten die verdacht zijn van TB met moleculaire amplificatie methoden te testen op de aanwezigheid van *M. tuberculosis*, naast het uitvoeren van de microscopie en kweek op mycobacteriën, om zo een aantal werkelijke TB patiënt te identificeren ondanks het ontbreken van een positieve kweek op *M. tuberculosis*.

Het gebruik van een vloeibaar medium in combinatie met een vast medium is hierbij aan te bevelen, omdat het aantal kolonies op het vaste medium een indicatie van ziekte of kolonisatie kan zijn.

Om het bovenstaande te bereiken, zal er in de Afrikaanse setting tenminste één goed uitgerust referentielaboratorium in elk land nodig zijn met bij voorkeur een langdurige samenwerking met een laboratorium in de Westerse wereld.

In het algemeen worden NTM infecties momenteel in Afrika niet behandeld terwijl er aanwijzingen zijn dat tenminste een deel van geïsoleerde NTM in Afrikaanse patiënten klinisch relevant is. Patiënten die gediagnosticeerd zijn met NTM ziekte zouden moeten worden geëvalueerd voor behandeling en de respons zou gedurende langere tijd moeten worden vervolgd. Tevens zouden patiënten die verdacht zijn voor pulmonale NTM ziekte, maar niet voldoen aan de diagnostische criteria, moeten worden vervolgd totdat de diagnose wordt bevestigd of verworpen.

De behandeling van NTM ziekte is in het algemeen niet gelijk aan de behandeling van TB. Meestal wordt in de geneesmiddelen regimes een macrolide (azithromycin, clarithromycin) geïncludeerd. *In vitro* gevoeligheid voor veel NTM correleert niet goed met de klinische respons op anti-mycobacteriele geneesmiddelen, aanbevelingen voor *in vitro* routine testen van gevoeligheden zijn echter beperkt. De duur van de behandeling van de meeste pulmonale NTM infecties is tenminste een jaar, indien de opeenvolgende sputumkweken gedurende therapie negatief zijn geworden.

Bepaalde condities, zoals een HIV infectie, lijken het krijgen van een NTM infectie te bevorderen en aan patiënten met zulke risicofactoren zal in de toekomst extra aandacht moeten worden besteed. In HIV positieve patiënten treedt een gedissemineerde NTM infectie typisch op nadat het CD4+ T-lymfocyten getal onder de 50/µl is gedaald. Longziekte ten gevolge van NTM treedt hoofdzakelijk op in reeds beschadigde longen zoals bij chronisch obstructieve longziekte (COPD), bronchiectasieën, CF, pneumoconiosis en eerdere longtuberculose. NTM longziekte ziet men ook in vrouwen zonder duidelijke herkenbare predisponerende factoren. Aanvullende case-control studies zijn nodig om mogelijke risico factoren voor NTM kolonisatie en infectie te identificeren.

Concluderend kan men stellen dat de klassieke diagnose tuberculose, gedefinieerd op basis van de ZN sputumuitstrijk en thoraxfoto, in toenemende mate leidt tot foutpositief gediagnosticeerde patiënten ten gevolge van de toegenomen prevalentie HIV en onnauwkeurigheden in de diagnostiek van TB in Afrika, zoals beschreven in dit proefschrift. Verder wordt een belangrijk deel van de tuberculose-achtige ziektebeelden in Afrika waarschijnlijk veroorzaakt door NTM en behandeling van deze infecties dient overwogen te worden. Misdiagnose van TB kan worden gereduceerd door de introductie van nieuwe diagnostische technieken, naast ZN sputumuitstrijk, die ook pulmonale NTM pathologie kunnen aantonen. Daarnaast moeten diagnostische scorings systemen en algoritmes

worden ontwikkeld en gevalideerd die de clinicus begeleiden in de benadering van NTM infecties in ontwikkelingsgebieden met een hoge HIV prevalentie.

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Curriculum Vitae

Patricia Buijtels werd op woensdag 18 maart 1970 als eerste kind van Harry en Jo Buijtels in het zuidelijk gelegen, Brabantse dorpje Luyksgestel geboren.

In 1989 behaalde zij in Valkenswaard haar VWO diploma aan het Hertog-Jan College. Hetzelfde jaar startte zij haar studie Geneeskunde aan de Rijksuniversiteit te Leiden. In 1993 werkte zij voor haar afstudeeronderzoek een half jaar bij het St. Jospeh Hospital in Kilgoris, Kenia. Vervolgens liep ze gedurende vier maanden een klinische stage op de afdelingen Interne Geneeskunde en Kindergeneeskunde in de Medical University of South Africa (Medunsa) te Pretoria.

Na het behalen van haar Artsexamen in het voorjaar van 1997 werkte zij in het kader van de tropenopleiding een jaar als arts-assistent op de afdeling Chirurgie van het Kennemer Gasthuis te Haarlem en vervolgens een jaar op de afdeling Obstetrie/Gynaecologie van het Eemland Ziekenhuis te Amersfoort. Dit werd gevolgd door aanstellingen als IVF-arts bij het Catharina Ziekenhuis te Eindhoven (1999) en het Universitair Medisch Centrum Utrecht (2000).

In november 2000 startte ze met haar promotie-onderzoek op de afdeling Medische Microbiologie van het Medisch Centrum Rijnmond-Zuid onder leiding van Pieter Petit, met Dr. D. van Soolingen van het RIVM als co-promotor en Prof. H.A. Verbrugh van het Erasmus Medisch Centrum als promotor. In oktober 2002 werd gestart met de opleiding tot arts-microbioloog in de cluster Rotterdam (opleider Prof. H.A. Verbrugh) die zij in september 2007 heeft afgerond.

Abbreviations

Abbreviations

AD	Aquadest
AFB	Acid-fast bacilli
AFLP	Amplified fragment length polymorphism
AIDS	Acquired immune deficiency syndrome
AMC	Academic Medical Centre, Amsterdam
ANN	Artificial neural network
ATS	American thoracic society
BAL	Broncho-alveolar lavage
BCG	Bacillus Calmette-Guerin
BMI	Body mass index
BSL	Biosafety level
BTS	British Thoracic Society
CDC	Centers for Disease Control
CF	Cystic fibrosis
CFU	Colony-forming units
CI	Confidence interval
COPD	Chronic obstructive pulmonary disease
DFA	Discriminant function analysis
DNA	Deoxyribonucleic acid
EMSC	extended multiplicative signal correction
EN	Electronic nose
HAART	Highly active antiretroviral therapy
HCA	Hierarchical cluster analysis
HIV	Human immunodeficiency virus
HRCT scan	High-resolution computed tomography scan
HPLC	High-performance liquid chromatography
HPRM	High performance Raman module
HWE	Hardy-Weinberg equilibrium
IFN-γ	Interferon-γ
KIT	Royal Tropical Institute / Koninklijk Instituut voor de Tropen
LAM	Lipoarabinomannan membrane
LAMP	Loop-mediated isothermal amplification
LJ	Löwenstein-Jensen
MAC	*M. avium* complex

MBL	Mannose-binding lectin
MCP-1	Monocyte chemoattractant protein-1
MCRZ	Medisch Centrum Rijnmond-Zuid
MGIT	Mycobacteria Growth Indicator Tube
MIC	Minimal inhibition concentration
MTD	*Mycobacterium tuberculosis* Direct Test
NAA	Nucleic acid amplification
NALC	*N*-acetyl l-cysteine
NTM	Non-tuberculous mycobacteria
OR	Odds ratio
PC	Principal component
PCA	Principal component analysis
PCR	Polymerase chain reaction
PPD	purified protein derivative
PRA	Polymerase chain reaction restriction analysis
R^2	Squared Pearson's correlation coefficient
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RIVM	National institute for Public Health and Environment / Rijksinstituut voor Volksgezondheid en Milieu
RLB	Reverse line blot
RNA	Ribonucleic acid
rRNA	ribosomal RNA
SDS	Sodium dodecyl sulfate
SNP	Single nucleotide polymorphisms
TB	Tuberculosis
TNF-α	Tumor necrosis factor-α
VOC	Volatile organic compound
WHO	World Health Organisation
ZN	Ziehl-Neelsen