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Diagnostics of non-tuberculous mycobacteria

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Diagnostics of Non-Tuberculous Mycobacteria

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1 Introduction.

General introduction.

Mycobacteria belong to the family *Mycobacteriaceae* and are members of the CMN group (*Corynebacteria, Mycobacteria and Nocardia*). The family *Mycobacteriaceae* are Gram-positive, nonmotile, catalase-positive, have a rodlike to filamentous morphology and can be pleomorphic. As a group, they produce characteristic long chain fatty acids termed mycolic acids. Mycobacteria are acid-fast rods of variable appearance, approximately 0.2-0.6 by 1-10 micrometer.

The genus *Mycobacterium* consists of 127 species (excluding subspecies) according to the latest approved list of bacterial species *(1)*. Mycobacteria other than *Mycobacterium tuberculosis* are commonly referred to as atypical or non-tuberculous mycobacteria (NTM). Two of these cause disease in normal hosts and are thus primary pathogenic: *M. leprae, M. ulcerans*. They are often not regarded as NTM. The remaining species are considered nonpathogenic or opportunistic pathogens and cause disease when host-defences are compromised. Mycobacteria can be arranged into four groups according to the Runyon classification:

- Group 1 – Photochromogens: slow growers and form pigment when exposed to light (eg, *M. kansasii, M. marinum, M. simiae*)
- Group 2 – Scotochromogens: slow growers and form pigment in the dark (eg, *M. scrofulaceum, M. szulgai, M. gordonae*)
- Group 3 – Nonphotochromogens: slow growers and not pigmented (eg, *M. malmoense, M. xenopi, M. avium*-complex, *M. ulcerans, M. haemophilum*)
- Group 4 - Rapid growers (eg, *M. fortuitum, M. chelonae, M. abscessus*)

Most slow-growing species have been associated with disease in humans while only few species of group 4 (the ones mentioned above) are disease associated.

Since the advent of AIDS and the application of recent developments in molecular biology for the detection and identification of NTM, NTM infections are increasingly detected. This in its turn created a higher awareness for mycobacterial involvement in a variety of clinical conditions and NTM diseases have been increasingly recognized in immunocompetent patients as well.

Taxonomy of the genus Mycobacterium.

Unidentified species are constantly being discovered and mycobacterial taxonomy is continuously changing. The species belonging to a species group (sometimes referred to as a species-complex) can be very different in virulence or pathogenesis.
Several previously considered species appear to consist of several closely related species, as biochemical and mainly genetic analyses have demonstrated in, for instance, the *M. tuberculosis*-complex. The complex species can be identified using differences in size of genomic sequences: Region of Difference1 (RD1), RD2, RD4, RD9 and RD12, analysed in PCR *(2)*. This complex now consists of *M. tuberculosis, M. africanum, M. microti* and *M. bovis*, the latter of which has been further differentiated into *M. bovis*, *M. caprae, M. pinnipedii* and *M. canetii,* all named after their original host *(2)*. A similar phenomenon can be found in the *M. avium*-complex. Originally, one species, but meanwhile divided into the species *M. avium*, *M. intracellulare* and *M. scrofulaceum.* Previous serovars belonging to this complex have been re-arranged *(3, 4, 5)* and several subspecies of *M. avium* have been identified: *M. avium subsp. paratuberculosis*, *subsp. silvaticum* and *subsp. avium*. This identification was based on molecular and biochemical criteria, that is High Performance Liquid Chromatography (HPLC) of mycolic acids and sequencing of, for instance, Internal Transcribed ribosomal Regions (ITS). *M. avium subsp. avium*, has recently been divided in *subsp. avium* and *subsp. hominissuis* using Restriction Fragment Length Polymorphism (RFLP) of a insertion sequence *IS*1245 *(6)*. The majority of human infections is caused by *M. hominissuis (6)*.

The identification of a new species was conventionally based on the description of the Runyon classification, the biochemical properties of the strain(s) and the degree of DNA-DNA hybridization. With the growing amount of species and subspecies with various biochemical properties, new methods were developed and this created a new standard for species differentiation *(7*). For the acceptance of a new species, the old and new ways of identification are all included: biochemical characteristics, growth and pigmentation characteristics, HPLC analysis and a unique genetic composition determined by the sequence of genes that allow species differentiation, such as the 16S rRNA gene, the *hsp65* gene and the ITS region, as applied and subsequently published in the International Journal of systematic Bacteriology *(1, 8, 9).* Sequencing of at least two targets as mentioned above must be included, but the choice of targets is not specified. From 1990 to 1999, 28 new species have been recognized *(9)* and from 2000 to September 2007, 41 more species have been identified (Table 1). Of the latter 41, at least 26 were the cause of disease in humans, and at least six new species of clinical origin were rapid growers not belonging to one of the known clinical species groups. This illustrates the present discovery rate within mycobacterial taxonomy.

Table 1: New NTM determined from January 2000 to September 2007.

year	species name	source	runyon group	additional info
2000	*M. botniense*	water	2	*M. xenopi*-like
2000	*M. kubicae*	human	2	between slow / rapid growers
2000	*M. septicum*	human	4	
2000	*M. elephantis*	elephant	4	
2001	*M. heckeshornense*	human	2	*M. xenopi*-like
2001	*M. doricum*	human	2	
2001	*M. immunogenum*	human	4	*M. abscessus* group
2001	*M. frederiksbergense*	contaminated soil	4	
2002	*M. palustre*	human / other	2	
2002	*M. lacus*	human	3	
2002	*M. vanbaalenii*	contaminated soil	4	
2002	*M. holsaticum*	human	4	
2003	*M. pinnipedii*	seal		*M. tuberculosis*-complex
2003	*M. shottsii*	striped bass	3	no growth >30 ^{0}C
2003	*M. montefiorense*	eels	3	no growth >30 ^{0}C
2004	*M. saskatchewanense*	human	2	
2004	*M. parascrofulaceum*	human	2	
2004	*M. parmense*	human	2	
2004	*M. nebraskense*	human	2	
2004	*M. chimaera*	human	3	MAC group
2004	*M. psychrotolerans*	pond water	4	
2004	*M. canariasense*	human	4	
2004	*M. cosmeticum*	human	4	
2004	*M. pyrenivorans*	contaminated soil	unknown	
2004	*M. massiliense*	human	4	*M. abscessus* group
2005	*M. florentinum*	human	unknown	slow growing pigment forming
2005	*M. pseudoshottsii*	striped bass	1	
2006	*M. arupense*	human	3	rapid growing at 30 ^{0}C
2006	*M. phocaicum*	human	4	
2006	*M. neworleansense*	unknown	4	*M. fortuitum* group
2006	*M. houstonense*	unknown	4	*M. fortuitum* group
2006	*M. aubagnense*	human	4	*M. mucogenicum* group
2006	*M. bolletii*	human	4	*M. abscessus* group
2006	*M. boenickei*	unknown	4	*M. fortuitum* group
2006	*M. conceptionense*	human	4	*M. fortuitum* group
2006	*M. fluoranthenivorans*	soil	unknown	
2006	*M. kumamotonense*	human	3	*M. terrae* group
2006	*M. colombiense*	human	3	MAC group
2006	*M. monacense*	human	4	
2006	*M. brisbanense*	unknown	4	*M. fortuitum* group
2007	*M. seoulense*	human	2	

Disease caused by NTM.

The most common sites where mycobacterial disease occurs are the lungs, the lymph nodes and skin. However, as *M. tuberculosis* is mostly known to cause the well-established pulmonary manifestation but is capable of infecting virtually all tissue types, the NTM species follow the same behaviour: the range of clinical manifestations is extensive *(10)*.

Pulmonary infections.

Pulmonary involvement is most common in immunocompromised patients. *M. avium* or *M. kansasii* infection are the predominant species known in AIDS patients *(11)*, but nowadays *M. avium* is frequently encountered in immunocompetent patients, probably due to the improved diagnostic tools and clinical awareness. While NTM infection in immuno competent patients is increasingly common, a high bacterial burden or a damaged epithelial are usually cofactors for infection in these patients *(12)*.

Studies at the reference laboratories in Australia revealed 80% of clinical NTM isolates to be derived from pulmonary sources. However, as stated by the reference laboratories, the significance of an isolate is often doubtful when isolated from pulmonary sources and in Australia only 10% of all pulmonary isolates are associated with disease (clinically significant). In contrast, almost all NTM isolates (91-98%) from lymphatic, bone or soft tissue (skin and joint) origin were clinically significant. Because many NTM species are ubiquitous, the detection of NTM in clinical materials is not per sé proof of the identification of the cause of disease. This is especially true for the detection in non-sterile materials. The criteria for the clinical significance of positive NTM diagnostics have been described by the American Thoracic Society (ATS) *(13)*.

Still, pulmonary infection with NTM can take several forms which are evidently associated with disease *(13, 14)*. The first, or classical, form is radiographically indistinguishable from tuberculosis. It is characterized by nodular opacities in the apices, cavitation and/or apical pleural thickening *(12)*. 80%-90% of the patients are elderly Caucasian males and it is often found secondary to other lung disease. The most common species are *M. avium, M. kansasii* and *M. malmoense (12)*. Risk factors include smoking, alcoholism, cardiovascular disease, chronic liver disease, and previous gastrectomy. Symptoms include coughing (60-100%), weight loss, fever (10-13%), weakness, and hemoptysis (15-25%), but are often mild or completely absent *(15)*. The second form is non-classical, does not resemble tuberculosis and *M. avium* is primarily the species involved. This infection is characterized by an interstitial and/or nodular pattern instead of a cavitary pattern involving mostly the lingula or middle lobe of the right lung. Risk factors are poorly understood. It is not necessarily related to smoking or any underlying chronic lung disease and is found most often in middle-aged or elderly women (Lady Windermere syndrome) *(16, 17, 15)*. The third form of mycobacterial pulmonary disease is the “hot-tub lung” affecting middle-aged patients, male and female. It is mostly caused by species belonging to the *M. avium*-complex and is often recognized in metal-fluid workers and indoor swimming pool staff. Primary differences to other forms of pulmonary mycobacterial disease are the diffuse nodular presentation and the acute

manifestation instead of chronic manifestation *(18, 19, 20)*. Already the most common species involved in pulmonary tract infections in immunocompromised patients, the incidence proportion of *M. avium* in immunocompetent patients is increasing rapidly *(21)*.

Skin infections.

Cutaneous NTM infections result from external inoculation at sites of trauma, the spread of a deeper infection from the joints or other tissues, or haematogenous spread of a disseminated infection. There are a few species-specific infections (fish tank or swimming pool granuloma, due to *M. marinum*, Buruli ulcer, caused by *M. ulcerans* and Leprosy, caused by *M. leprae*). Most species, however, produce a nonspecific clinical picture, like *M. haemophilum* or *M. abscessus* and are mostly encountered in industrialised countries. Lesions occur in various forms as suppurative nodules, ulcers, abscesses, sporotrichoid lesions, folliculitis, furunculosis and indurated plaques. In immunocompetent patients the infection is normally localized, superficial and limited to the extremities. In immunosuppressed patients the number of lesions is often multiple and cutaneous involvement is often accompanied by disseminated disease *(22)*. Abscesses and ulceration are also more frequently observed in immunosuppressed patients.

The risk factors for NTM infection include: 1) HIV infection, lymphoma, leukemia or immunosuppressive therapy. Immunosuppression is responsible for the increase of cutaneous infections by a large variety of species, particularly in industrialized countries. 2) The natural environment is directly responsible for the emergence of cutaneous infections caused by a small number of species including *M. avium* and *M. marinum* in Europe and North America, and *M. ulcerans* in the tropics. 3) The medical environment when sterilization is inadequate is also not uncommonly responsible *(23).*

Different histopathological patterns can be noted in biopsy specimens from cutaneous nontuberculous mycobacterial infections. The evolution of the disease and the immunologic status of the host may explain this spectrum of morphological changes. Tuberculoid, palisading and sarcoid-like granulomas, a diffuse infiltrate of histiocytic foamy cells, acute and chronic panniculitis, non-specific chronic inflammation, cutaneous abscesses, suppurative granulomas and necrotizing folliculitis can be detected. Suppurative granulomas are the most characteristic feature in skin biopsy specimens from cutaneous NTM infections. A marked granulomatous inflammatory reaction is more common in immunocompetent than in immunosuppressed patients *(24)*. Both sexes are equally affected but males predominate in *M. marinum* infection and females predominate in rapid growers. All ages can be affected, but most cases involve middle-aged people. Cervical lymphadenitis and cutaneous abscesses are the common manifestations of rapid-grower infections. Hyperkeratotic verrucous plaques (tuberculosis verrucosa cutis-like) and sporotrichoid lesions are the common manifestations of slow-grower infection *(25)*.

Lymphadenitis.

NTM lymphadenitis is seen in immunocompromised patients, but is mostly known as the most common manifestation of NTM disease in immunocompetent patients and usually

affects children under the age of 12 as chronic cervicofacial lymphadenitis. It is more common in industrialized countries and is suggested to be a “prosperity disease” *(26)*. This age group may also be more susceptible because of a lack of a fully-developed immune system *(27)*.

Occurrence of lymphadenitis in immunocompromised patients is often accompanied by disseminated mycobacterial disease *(28)* and affects lymph nodes at different body sites. In lymphadenitis in “healthy” children, involvement of only one single lymph node is common except in *M. haemophilum* infection, where the involvement of multiple lymph nodes is more common (56%) *(29)*. Involvement of submandibular lymph nodes are seen in 75% of the patients, while preauricular or periparotid sites of infection account for 12% to 25% of NTM adenitis cases. The clinical features include non-tender enlargement of the lymph node and violaceous skin discoloration of the overlying skin. After several weeks to months, caseous necrosis develops and when untreated, spontaneous drainage can occur leaving scars. The ports of entry are the pharyngeal mucosa, tonsils, conjunctiva, gingiva, and salivary glands. Ingestion of contaminated soil or water is speculated to be the source of infection. In salivary gland infection, the possibility of retrograde passage of the mycobacteria along the duct exists, thumb sucking being a possible risk factor in children *(30, 31)*.

Studies reported an annual incidence varying from 1,21 to 1.78 cases per 100.000 children which is increasing *(32, 33, 34, 35)*. In the Netherlands, the estimated annual incidence of NTM cervicofacial infections is 0.77 per 100.000 children *(36)*. Earlier publications describe a higher incidence of cervical lymphadenitis in winter and spring *(37, 38),* but this is contradicted by Lindeboom et al who did not observe a seasonal difference in the Netherlands: autumn (29% of patients), winter (25%), spring (25%), and summer (21%) *(29).*

The number of mycobacterial species is increasing and several newly identified species have first been encountered in lymphadenitis patients *(39, 40)*. However, approximately 70% of the cervicofacial lymphadenitis cases are caused by *M. avium (41)*. Other frequently involved species depend on the geographical distribution of mycobacterial species. In India *M. scrofulaceum* is commonly involved, and it used to be the most prevalent species in the United States. In Israel, Australia and The Netherlands, *M. haemophilum* is the second most common species *(14, 32, 42, this thesis chapters 3 and 4)*. In the rest of Europe this is *M. malmoense (12)*. The switch in species prevalence is thought to be caused by variability in their presence in natural sources *(21, 43)*.

<u>Disseminated disease.</u>

Disseminated NTM infection in HIV or otherwise immunodeficient patients appears to originate from a primary infection of either the skin or respiratory or gastrointestinal tracts.

These infections may involve any organ, but most commonly occur in the lungs, liver, spleen, lymph nodes or bone marrow. Common symptoms include prolonged fevers (often accompanied by night sweats), weight loss and occasional abdominal pain or diarrhea. This disease is most commonly seen in patients with less than 50 CD4 cells *(13)*. The primary *Mycobacterium* species associated with disseminated infections in HIV infected patients is

M. avium. However, *M. chelonae, M. abscessus, M. kansasii, M. haemophilum, M. genavense* and sporadically *M. scrofulaceum* have also been implicated *(44)*.

Skeletal infections.

NTM (usually slow-growing species) can cause skeletal infections as well, which often affect the synovium or osteoarticular components of the extremities, but may occur at other skeletal sites (i.e. osteomyelitis), particularly when there is underlying immunosuppression. Monoarthritis is the most common, but polyarthritis has been reported as well *(45, 46, 47, 48, 49)*.

Gastrointestinal infections.

Both slow-growing and rapid-growing species, have been isolated from intestinal specimens from patients with Inflammatory bowel disease (Crohn's disease), ulcerative colitis, and non-inflammatory bowel diseases *(50)*. It is still controversial which role mycobacterial infections have in the etiology of Crohn's disease. Several studies suggested *M. avium* spp *paratuberculosis* (MAP) as the primary cause in the etiology of Crohn's disease and reported positive cultures for MAP and PCRs or high specific immune responses *(51, 52, 53, 54, 55)*. A significant correlation between Crohn's disease and MAP has even been established *(56*), but the exact role of the mycobacterium remains to be defined *(56)*. One of the popular theories on Crohn's disease is the autoimmune theory which suggests that the disease results from inappropriate ongoing activation of the mucosal immune system driven by the presence of normal luminal flora *(57)*. This would place the infectious agent in a secondary position but, nevertheless, in an active role.

Foreign body related and nosocomial infections.

Keratitis is another manifestation of NTM disease. This eye infection, usually caused by rapid growers, can cause significant damage when not treated properly. The typical clinical features consist of irregular corneal infiltrates with radiating projections, indistinct fluffy lesion margins, satellite lesions and associated epithelial defect *(58)*. More than 150 cases have been reported to date, the majority of which in Asian countries. The major risk factor is injury to and the presence of foreign bodies in the cornea, frequent use of lens fluid and surgical trauma *(58, 59, 60, 61)*. Infection following laser-assisted in situ keratomileusis (LASIK) has been more commonly described in recent years *(62, 63, 64, 65)*.

Other infections with NTM subsequent to surgical or other invasive medical procedures have been reported as well, predominantly caused by rapid-growing species. *(66, 67, 68, 69)*. Postsurgical inflammatory complications with NTM present a difficult challenge because of the resistant nature of mycobacteria against disinfectants used in the cleaning of hospital equipment. NTM have been encountered in hospital water supplies *(70, 71)* and have in some cases been linked to pseudo-outbreaks *(72, 73)*.

Table 2: clinical manifestations and the most commonly encountered NTM species. Adapted from Wagner et al. 2004) *(12, 14, 74, RIVM personal correspondence)*

clinical manifestation	**common species**	**less common species**	
pulmonary disease	*M. avium*-complex	*M. simiae*	*M. asiaticum*
	M. kansasii	*M. szulgai*	*M. shimodii*
	M. abscessus	*M. fortuitum*	*M. smegmatis*
	M. chelonae	*M. celatum*	*M. haemophilum*
	M. xenopi	*M. gordonae*	
	M. malmoense		
lymphadenitis	*M. avium*-complex	*M. fortuitum*	*M. interjectum*
	M. malmoense	*M. kansasii*	*M. heidelbergense*
	M. haemophilum	*M. abscessus*	*M. scrofulaceum*
		M. chelonae	*M. bohemicum*
		M. lentiflavum	
skin and soft-tissue disease	*M. ulcerans*	*M. kansasii*	
	M. marinum	*M. malmoense*	
	M. haemophilum	*M. chelonae*	
	M. abscessus	*M. smegmatis*	
	M. avium-complex	*M. fortuitum*	
disseminated disease	*M. avium*-complex	*M. celatum*	*M. scrofulaceum*
	M. kansasii	*M. conspicuum*	*M. abscessus*
	M. haemophilum	*M. malmoense*	*M. simiae*
	M. fortuitum	*M. genavense*	
	M. chelonae	*M. xenopi*	
skeletal infection	*M. avium*-complex	*M. chelonae*	*M. marinum*
	M. abscessus	*M. kansasii*	*M. smegmatis*
	M. fortuitum	*M. scrofulaceum*	*M. nonchromogenicum*
		M. haemophilum	*M. malmoense*
		M. xenopi	*M. szulgai*
Gastrointestinal infection	*M. avium*-complex	*M. mucogenicum*	
		M. kansasii	
foreign body-related infections and nosocomial infections	*M. fortuitum*	*M. mucogenicum*	*M. smegmatis*
	M. abscessus	*M. neoaurum*	*M. avium*-complex
	M. chelonae	*M. aurum*	
		M. gordonae	
		M. simiae	

Natural reservoirs.

NTM are saprophytes and ubiquitous and can exist in soil, dust, food (eggs, raw milk, vegetables) and water *(43, 75)*. Animal reservoirs are also proposed to be involved in the etiology of human disease *(46)*: *M. avium* has been recovered from the lymph nodes of swine and domestic fowl; *M. genavense, M. fortuitum,* and *M. avium* from birds; *M. chelonae* from fish and frogs *(12) M. haemophilum* from cockroaches *(76)* and *M. ulcerans* from mosquitoes *(77)*. Many species have been isolated from natural water and drinking water systems and appear highly capable of forming biofilms, are able to sustain disinfecting treatment and are present in aerosols *(78).*

Pathogenesis and host defences.

Mycobacteria are thermoresistant, endure most disinfectants and have the ability to form biofilms. This is due to their thick acid-fast cell wall. No human-to–human transmission has been recognised for mycobacteria other than *M. tuberculosis* and *M. leprae*. Humans are thought to get infected through the inhalation of aerosols (showering, swimming) or direct contact with the bacteria by skin or mucosa with affected integrity *(79)*. Pulmonary infections can occur in patients with impaired ventilation systems but without specific immunity problems. Children with cervical lymphadenitis also are considered healthy. Therefore, the mycobacteria need to possess ingenious mechanisms to evade the host defences.

Mycobacteria have the capacity to thrive inside macrophages. As part of the immune system, macrophages are capable of destroying a wide variety of bacterial pathogens. Mycobacteria, however, are one of the few types of bacteria that are not only able to survive the antibacterial effects of macrophages, but actually grow and multiply inside them.

Considerable research has been done to try and understand how mycobacteria flourish in - what is thought to be- the hostile intracellular environment of macrophages. Two properties of mycobacteria explain their resistance to being killed by macrophages: The first is the cord factor that can neutralize the antibacterial chemicals produced inside macrophages and inactivate mitochondrial membranes of phagocyts *(80)*. Cord factor (trehalose 6, 6'-dimycolate) is a glycolipid in the cell wall of mycobacteria. They are mostly known as the molecules responsible for the serpentine cord-forming growth characteristics of *M. tuberculosis*, but comparable growth phenomena are encountered in non-tuberculous species caused by variable forms of cord factor in the cell wall as well. The glycolipid is widely distributed as a potent immunomodifier among non-tuberculous mycobacteria and related micro-organisms such as *Corynebacterium (81)*.

The second is the chemically unique mycobacterial cell wall that is resistant to destruction or penetration. The cell wall of mycobacteria is composed of a mixture of lipids and polysaccharides. The lipids in the cell wall inhibit the migration of macrophages, have the capacity to disrupt phagosomal membranes of alveolar macrophages and disrupt normal

cytokine signalling that is responsible for the ineffective cell-mediated immune respons *(82, 83, 84, 85).*

Pathological properties of NTM might vary greatly between species. This is illustrated by three closely related species, *M. marinum, M. ulcerans* and *M. haemophilum*: All three cause necrotizing skin disease, are taxonomically related *(86)* and share common reservoirs (stagnant or slow-flowing water) *(87)*, but pathogenic differences are noted. *M. ulcerans* is highly pathogenic for humans and causes specific large necrotic ulcers, while *M. marinum* and *M. haemophilum* are responsible for mostly self-limiting and slowly progressing granulomatous lesions *(87)*. *M. marinum* causes disease- but seldom death- in fish, while *M. haemophilum* infection in fish results readily in death *(88)*. Infections with *M. marinum* and *M. ulcerans* are almost always restricted to the skin, but *M. haemophilum* often causes disease in deeper tissues. This is in contrast with the in vitro growth charachteristics of the three species: *M. haemophilum* has great difficulties to grow at higher temperatures, *M. marinum* and *M. ulcerans* grow at normal culture temperatures of 35-37˚C but faster at lower temperatures (30-32 ˚C). Deep tissue infections would logically be restricted to species able to grow at higher temperatures.

Iron uptake of mycobacterial species also differs. Because mycobacteria require iron in pathogenesis and the iron levels in inflammation processes are elevated, the strong cellular immune response of the host is induced by mycobacteria *(89, 90)*. *M. haemophilum* however, is the only species that requires iron-additives added to the culture and this demonstrates the differences in iron-management between NTM.

Pathogenic properties of NTM can be transferred between species. An example of a potentially hazardous change is the transfer of the toxin produced by *M. ulcerans*. This mycolactone causes the destructing properties of this pathogenic species. Previously, only *M. ulcerans* was known to harbour a virulence plasmid with the gene for mycolactone *(91)*. Recently, in strains of *M. marinum* and *M. pseudoshottsii,* responsible for death in fish, a mycolactone variant has been identified. This gene is suspected to have been spread by horizontal transfer *(92, 93)*.

Secundary to the pathogenic properties of the mycobacteria themselves, impaired host defences are thought to be responsible for the susceptibility of healthy patients to NTM because, due to the environmental presence, humans are continuously exposed to the bacteria in low levels (50-500 bacilli per day) *(43, 85)*. Acquired human resistance is cell-mediated, antibodies do not have a protective role. T lymphocytes lyse infected macrophages directly or activate them via soluble mediators to destroy intracellular bacteria. Mutations in the genes responsible for this mechanism create resistance disorders. Mutations in the interferon-gamma receptor ligand-binding chain (IFN gamma R1), interferon-gamma receptor signalling chain (IFN gamma R2), Signal Transducer and Activator of Transcription-1 (STAT-1), interleukin-12 p40 subunit (IL-12 p40), and interleukin-12 receptor beta 1 chain (IL-12R beta 1) genes have all been identified as predisposing factors for NTM infections *(94, 85)*. Dominant or recessive alleles causing complete or partial

cellular defects have been found to define nine different inheritable disorders for the susceptibility of patients for opportunistic pathogens *(95, 96, 97)*.

Treatment of NTM.

Guidelines for diagnosis and treatment have been produced for NTM by the British Thoracic Society (BTS) and the American Thoracic Society (ATS) *(13, 98)*. Diagnosis is addressed in chapter 2 of this thesis. Criteria for the treatment of NTM infection is based on the species involved, the immune characteristics of the patient and the clinical manifestation of the infection.

Two basic rules apply to mycobacterial disease: 1) For all mycobacterial infections long therapy is necessary (2-24 months), which is a direct consequence of the slow-growing properties of the genus. 2) Mycobacteria rapidly obtain resistance to the most common antibiotics and therefore dual or multiple combinations of antibiotic groups are common regimens *(99, 100).*

Side effects to antituberculous drugs and NTM regimens are common due to the toxicity of the agents and include hepatitis, cutaneous reactions, gastrointestinal intolerance, haematological reactions and renal failure *(101, 102)*. This results in modification or discontinuing of the therapy *(103)*. The only alternative for antibiotic regimens is surgical excision in some cases. Treatment with steroids is not a safe alternative therapy *(104)*. Mycobacteria are intrinsically resistant to most common antibiotics and while *M. tuberculosis* is resistant to macrolides, NTM are often resistant to first-line antituberculous drugs which emphasises the importance of species identification in mycobacterial disease *(99)*.

NTM infection with slow growing species in immunocompetent patients is often treated by a three-component or dual therapy of oral clarithromycin, rifabutin, ciprofloxacin, rifampicin and ethambutol *(105)*. The normal duration of the therapy is 4-24 months depending on the clinical manifestation (e.g. 4-6 months in lymphadenitis, 24 months in bone-infections) *(13, 98)*. In children with lymphadenitis in the Netherlands, surgical excision of the affected lymph nodes was the treatment with the highest cure rate. A combination of clarithromycin and rifabutin appeared less effective in these patients *(106)*. In other countries, the same treatment for lymphadenitis is recommended *(107, 108)*. For cutaneous or localised lung disease, surgical treatment is preferred in many cases as well, taking scarring and other complications into account. However, for *M. marinum* infection medical therapy is the treatment of choice *(25)*.

Treatment in immunocompromised patients is often given for a longer duration than in immunocompetent patients: most treatments are administered for many years. Also, sometimes profylaxis is given in AIDS patients: lifelong azithromycin, clarithromycin or azithromycin + rifabutin *(13)*. Drawbacks of antimycobacterial therapy in AIDS patients are the interactions with antiretroviral therapy, which need to be closely monitored *(13, 98)*.

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2 Diagnosis of NTM disease.

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

Clinical diagnosis.

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

Immunological tests.

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

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

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

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

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

Histopathological diagnosis.

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

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

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

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

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

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

Conventional microbiological diagnosis.

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

Acid-fast staining.

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

Decontamination.

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

Culture.

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

Species identification.

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

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

Molecular identification of NTM species.

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

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

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

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

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

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

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

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

Molecular detection of NTM in clinical specimens.

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

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

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

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

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

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

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

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

Susceptibility testing.

Conventional methods.

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

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

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

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

Molecular approaches for susceptibility testing.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

Typing of NTM.

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

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

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

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

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

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

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

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

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

Table 6 part 2.

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

Conclusions and design of the thesis.

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

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

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

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

References: 2 Diagnosis of NTM disease.

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Chapter 3.

Real-time PCR assay using fine-needle aspirates and tissue biopsy specimens for rapid diagnosis of mycobacterial lymphadenitis in children.

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Abstract.

A real-time PCR assay was developed to diagnose and identify the causative agents of suspected mycobacterial lymphadenitis. Primers and probes for the real-time PCR were designed on the basis of the internal transcribed spacer sequence, enabling the recognition of the genus *Mycobacterium* and the species *Mycobacterium avium* and *M. tuberculosis*. The detection limit for the assay was established at 1,100 CFU/ml of pus, and the specificity tests showed no false-positive reaction with other mycobacterial species and other pathogens causing lymphadenitis. From 67 children with suspected mycobacterial lymphadenitis based on a positive mycobacterial skin test, 102 samples (58 fine-needle aspirates [FNA] and 44 tissue specimens) were obtained. The real-time PCR assay detected a mycobacterial infection in 48 patients (71.6%), whereas auramine staining and culturing were positive for 31 (46.3%) and 28 (41.8%) of the patients. The addition of the real-time PCR assay to conventional diagnostic tests resulted in the recognition of 13 more patients with mycobacterial disease. These results indicate that the real-time PCR is more sensitive than conventional staining and culturing techniques (P = 0.006). The *M. avium*-specific real-time PCR was positive for 38 patients, and the *M. tuberculosis*-specific real-time PCR was positive for 1 patient. Analysis of 27 patients from whom FNA and tissue biopsy specimens were collected revealed significantly more positive real-time PCR results for FNA than for tissue biopsy specimens (P = 0.003). Samples from an age-matched control group of 50 patients with PCR-proven cat scratch disease were all found to be negative by the real-time PCR. We conclude that this real-time PCR assay with a sensitivity of 72% for patients with lymphadenitis and a specificity of 100% for the detection of atypical mycobacteria can provide excellent support for clinical decision making in children with lymphadenitis.

Introduction.

Nontuberculous mycobacterial (NTM) lymphadenitis appears to be an emerging disease in children (6, 13). The portals of entry are the pharyngeal mucosa, tonsils, conjunctiva, gingiva, salivary glands, and skin (7, 14). Cervicofacial lymphadenitis, the most frequent head and neck manifestation of NTM infection, often presents as chronic, unilateral lymphadenopathy with characteristic violaceous overlying skin changes. It may be difficult to differentiate NTM lymphadenitis from lymphadenitis caused by other microorganisms, such as staphylococci, streptococci, and *Bartonella* species. The *Mycobacterium avium* complex accounts for 80% of NTM lymphadenitis cases, followed by *M. scrofulaceum* in the United States and *M. malmoense* in Europe (1, 8). The exact incidence of NTM lymphadenitis in children in The Netherlands is unknown but is estimated to be 0.4 patients per 100,000 inhabitants annually.
Currently, the most commonly used treatment for NTM lymphadenitis is excision of the infected lymph node. Surgery is preferred because of a higher risk of sinus tract formation or recurrence of infection when other conservative therapies are used (1, 14, 20, 21). A number of recent case reports, however, suggested that effective treatment of NTM lymphadenitis is

possible with specific antimycobacterial agents (2, 17, 29). Since culture results for mycobacteria can take as long as 12 weeks, a rapid diagnostic technique is required to institute appropriate antimycobacterial therapy.

Several molecular assays have been developed for the detection or identification of mycobacteria, but most of them are for *M. tuberculosis* only (5, 11, 19, 27, 31). The existing molecular methods for the identification of atypical mycobacteria are mainly applied on cultured mycobacteria and lack specificity or sensitivity when used directly on clinical materials (15, 18, 24). A real-time PCR technique for the detection of mycobacteria is able to detect a specific sequence during amplification and needs no hybridization or further processing time for analysis. It is easy to use and able to attain high sensitivity mainly because of the use of short amplicons.

In this study, a real-time PCR assay for the genus *Mycobacterium* and the mycobacterial species *M. avium* and *M. tuberculosis* was developed for direct use on fine-needle aspirates (FNA) and biopsy specimens from patients with suspected mycobacterial lymphadenitis. The target chosen for PCR was the internal transcribed spacer (ITS) sequence between the 16S and the 23S rRNA genes. The ITS contains sufficient sequence variations to differentiate mycobacterial species (23, 24) but is still conserved enough to allow the development of a genus *Mycobacterium*-specific PCR for the detection of all species possibly involved in lymphadenitis

Material and Methods.

Bacterial strains.

The mycobacterial strains used for sensitivity and specificity testing and for optimization of PCR are listed in Table 1. The strains were either ATCC strains or clinical isolates, identified by 16S RNA sequencing and obtained from the Medical Microbiological Laboratory (Leiden University Medical Center, Leiden, The Netherlands), the Regional Public Health Laboratory (Leeuwarden, The Netherlands; supplied by G. Noordhoek), and the National Institute of Public Health and the Environment (Bilthoven, The Netherlands). All mycobacterial strains were cultured in liquid Dubos medium. Additionally, 36 strains belonging to 28 different species were also used in specificity tests. Some of these species are established pathogens causing lymphadenitis, and others are normal inhabitants of the oropharynx. The species belonged to the genera *Streptococcus*, *Staphylococcus*, *Bordetella*, *Haemophilus*, *Neisseria*, *Chlamydia*, *Bartonella*, *Campylobacter*, *Legionella*, and *Corynebacterium* and to the family *Enterobacteriaceae*.

Patients and samples.

Samples from affected lymph nodes were obtained from patients included in the CHIMED study. The CHIMED study is a multicenter trial in The Netherlands for studying the optimal treatment of children with NTM cervicofacial lymphadenitis. Surgical excision is compared to an antimicrobial treatment consisting of clarithromycin and rifabutin. To be eligible, patients

must have a positive skin test for atypical mycobacteria (29, 30). Differential skin testing with sensitins for *M*. *avium*, *M*. *scrofulaceum*, and *M*. *kansasii* and purified protein derivative (Statens Seruminstitut, Copenhagen, Denmark) consists of two simultaneous injections each on the middle part of the ventral surface and on the dorsal surface of the right forearm.

From September 2001 until November 2003, 67 patients with affected lymph nodes in the neck were included in the study. FNA were taken from affected lymph nodes and transported in saline to the microbiological laboratory at Leiden University Medical Center. The lymph nodes removed from surgically treated patients were also investigated. Clinical samples arrived at the laboratory within 6 h. All samples were kept at 4°C until processing. An age-matched control group consisted of 50 patients with affected lymph nodes. Cat scratch disease was diagnosed by *Bartonella henselae*-specific PCR of samples from these affected lymph nodes at the Department of Medical Microbiology, St. Elisabeth Hospital, Tilburg, The Netherlands (3).

Conventional mycobacterial diagnostic tests.

Patient samples were tested for contamination with rapidly growing bacteria by culturing on standard brain heart infusion agar. When contamination was found, the aspirates or biopsy specimens were decontaminated with an NaCl-NaOH decontamination protocol (12). Auramine staining was performed on directly obtained materials or on decontaminated materials for the detection of acid-fast rods. When auramine-positive rods were detected, Ziehl-Neelsen staining was performed to confirm the presence of acid-fast rods. Culturing was done at 35°C by using Bactec bottles with liquid mycobacteria growth indicator tube (MGIT) (Becton Dickinson Microbiology Systems) medium and on solid Löwenstein-Jensen medium. Positive culture results for acid-fast bacteria were further investigated by using the Inno-Lipa assay (InnoGenetics, Gent, Belgium), a reverse hybridization assay in which the mycobacterial species is identified. When no growth was detected after 12 weeks of incubation, the culture results were listed as negative. Samples were also investigated for the presence of other bacterial pathogens by conventional bacterial culturing and by PCR for *B*. *henselae* (3).

Nucleic acid isolation for real-time PCR.

Aliquots of the clinical samples used for PCR were first decontaminated with the NaCl-NaOH method. DNA from cultured strains was extracted without decontamination. Each patient sample was divided into three aliquots. One aliquot was spiked with 2.5 x 10^4 CFU of *M*. *bovis* and used as a control for DNA extraction and PCR inhibition. The other two aliquots were tested as duplicates of the patient sample. Extraction of DNA for the real-time PCR was performed as described by Boom et al. (4). Briefly, 90 µl of decontaminated material or bacterial culture was extracted, and the DNA was eluted in 100 µl of Tris-EDTA (0.1 M; pH 8.0). The extracted DNA was stored at –20°C until used in the PCR.

Table 1. Reference strains and clinical isolates used in this study and results of real-time PCR.

strain[a]	real-time PCR results[b] for:		
	genus *Mycobacterium*	*M. avium*	*M. tuberculosis*
M. intracellulare LUMC 5	+	–	–
M. intracellulare LUMC 7	+	–	–
M. intracellulare ATCC 13950	+	–	–
M. avium LUMC 14	+	+	–
M. avium subsp. *silvaticum* LUMC 18	+	+	–
M. avium subsp. *avium* RIVM D71076	+	+	–
M. avium subsp. *avium* RIVM 160/74	+	+	–
M. avium subsp. *avium* RIVM R13	+	+	–
M. avium subsp. *paratuberculosis* ATCC 19698	+	+	–
M. avium subsp. *paratuberculosis* ATCC 43544	+	+	–
M. scrofulaceum RIVM 2002-530	+	–	–
M. scrofulaceum RIVM 2002-1933	+	–	–
M. tuberculosis ATCC 35822	+	–	+
M. bovis LUMC 1	+	–	+
M. kansasii ATCC 12478	+	–	–
M. kansasii LUMC 30	+	–	–
M. kansasii LUMC 31	+	–	–
M. kansasii LUMC 32	+	–	–
M. xenopi LUMC 59	+	–	–
M. xenopi LUMC 60	+	–	–
M. malmoense M 2148	+	–	–
M. malmoense M 2240	+	–	–
M. malmoense M 1172	+	–	–
M. haemophilum LUMC 201	+	–	–
M. fortuitum LUMC 101	+	–	–
M. gordonae ATCC 14470	+	–	–
M. marinum LUMC 48	+	–	–

[a] LUMC, Leiden University Medical Center; ATCC, American Type Culture Collection; RIVM, National Institute of Public Health and the Environment; M, Regional Public Health Laboratory. Identification of clinical strains was performed at RIVM.

[b] +, positive; –, negative.

[c] RIVM strains.

Primers and probes.

Primers were selected based on alignments of the ITS region with sequences from the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov) and the Ridom GmbH database (http://www.ridom.com) and sequences derived from clinical isolates. Sequence alignments were made to investigate the interspecies variations and the intraspecies variations. The alignments included all three subspecies of *M. avium* and five sequevars of *M. avium* (A, B, C, D, and E). From these alignments, conserved regions were

used to select primers specific for the genus *Mycobacterium*. The forward primer was selected from the ITS region; the reverse primer was selected from the 23S rRNA gene (Fig. 1). Another small conserved sequence in the ITS region was used for the Taqman probe specific for the genus *Mycobacterium*. The larger part of the ITS region contains a high degree of variation; probes specific for *M. avium* (molecular beacon) and *M. tuberculosis* (Taqman probe) were designed from this part (Fig. 1B). The choice of a molecular beacon or a Taqman probe was based on the overall stability of the secondary structures in the probe sequence.

The PCR primer and probe sequences were selected by using the Primer3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (25) and were checked with Oligo-Analyzer 3.0 (http://biotools.idtdna.com/analyzer), an online service of IDT Biotools (Coralville, Iowa), to ensure minimal self-complementarity and to prevent the presence of secondary structures.

The molecular beacon for *M. avium* was designed by using the Mfold program (http://biotools.idtdna.com/mfold) (IDT Biotools). Additional criteria for a good probe included a melting temperature at least 7°C above the melting temperatures of the primers and a relatively short amplicon, with a maximum of 200 nucleotides. The stem sequence of the molecular beacon was selected to have a melting temperature compatible with that of the beacon. The beacon formed a stable structure at the proposed annealing temperature of 55°C, with no secondary structures (26). An NCBI BLAST search was performed to check the specificity of the DNA sequences of the primers and probes. Primers were synthesized at Eurogentec (Seraing, Belgium), and probes were synthesized at Biolegio (Malden, The Netherlands). Selected primers and probes are shown in Table 2.

Table 2. Sequences of oligonucleotides used in this study.

primer or probe	**sequence (5'-3')**
Forward primer	GGGGTGTGGTGTTTGAG
Reverse primer	CTCCCACGTCCTTCATC
Mycobacterium-specific Taqman probe	Fam-TGGATAGTGGTTGCGAGCATC-Tamra
M. avium-specific molecular beacon	Fam-CCCACCGGCCGGCGTTCATCGAAATGGTGGG-DabCyl
M. tuberculosis-specific Taqman probe	Hex-GCTAGCCGGCAGCGTATCCAT-BHQ1

Sensitivity testing.

Quantified dilutions of *M. avium* and *M. bovis* were prepared by measuring optical density (spectrophotometry) and CFU. Aliquots of 20 µl, containing serial dilutions of 110 to 10^7 CFU/ml, were added to water and pooled pus samples. The influence of decontamination on the sensitivity of the assay was investigated by comparing decontamination before spiking with decontamination after spiking.

Real-time PCR.

The real-time PCR was performed with a 50-µl reaction mixture consisting of 25 µl of 2x IQ Supermix (Bio-Rad, Veenendaal, The Netherlands), 3.0 mM $MgCl_2$, 20 pmol of each primer, 10 pmol of probe, and 10 µl of template. The PCR thermal profile consisted of an initial incubation for 3 min at 95°C, followed by 50 cycles of 30 s at 95°C, 40 s at 55°C, and 30 s at 72°C (the annealing temperature for the *M. tuberculosis*-specific PCR was 52°C instead of 55°C). Amplification, detection, and data analysis were performed with an iCycler IQ real-time detection system (Bio-Rad). The same reaction mixture and the same PCR profile were used for all three probes.

Each DNA extract was tested undiluted and diluted 10-fold by the real-time PCR for detection of the genus *Mycobacterium* and the species *M. avium*. For the *M. avium*-specific PCR, *M. avium* DNA was used as a positive control instead of *M. bovis* DNA.

Statistical analysis.

All statistical calculations were performed with SPSS 10.0.7. The Wilcoxon signed rank test was used for a comparison of FNA and tissue biopsy specimens from patients from whom both materials were collected. For other calculations, a chi-square test was used.

Results.

Test characteristics in vitro. Quantified dilutions of *M. avium* and *M. bovis* were tested in water and pooled pus samples. All three real-time PCRs reached a sensitivity of 2.7 CFU when spiked in water and a sensitivity of 27 CFU when spiked in pus, establishing a detection limit for the assay of 1,100 CFU/ml of pus. The sensitivity of the assay was not influenced by the decontamination protocol.

The specificity was assessed with DNA from 11 mycobacterial species (Table 1) and 36 other human pathogens. Several pathogens that may cause lymphadenitis, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Nocardia* spp., and *Bartonella* spp., were also included in this specificity testing. No nonspecific results were obtained in the genus *Mycobacterium*-specific PCR. The *M. tuberculosis*-specific PCR detected only *M. tuberculosis* and *M. bovis*. The *M. avium*-specific PCR recognized seven isolates belonging to all three subspecies of *M. avium* and was negative for *M. scrofulaceum*, *M. intracellulare*, and other species tested (Table 1).

Patient samples. From 67 patients included in the CHIMED study, 102 samples were obtained (58 FNA and 44 tissue specimens) (Table 3). Auramine staining was positive for 31 patients (46.3%). Mycobacteria could be cultured from 28 patients (41.8%) and included *M. avium* (*n* = 22), *M. malmoense* (*n* = 2), *M. kansasii* (*n* = 1), *M. tuberculosis* (*n* = 1), and *Mycobacterium* spp. (*n* = 2). The real-time PCR assay detected a mycobacterial infection in 48 patients (71.6%). The *M. avium*-specific real-time PCR was positive for 38 patients, and the *M. tuberculosis*-specific real-time PCR was positive for 1 patient (Table 3). The remaining nine PCR-positive patients were found to be positive only by the genus-specific real-time

PCR. The real-time PCR assay was more sensitive for the detection of atypical mycobacteria in patients with lymphadenitis than were staining and culturing (*P* value determined by the chi-square test, 0.006). When all tests were combined, the diagnosis of mycobacterial infection could be verified for 55 (82.1%) of 67 patients. The mycobacterial species was not identified for 12 of these 55 patients, because the samples were found to be positive only by the genus *Mycobacterium*-specific PCR (*n* = 8) or only by auramine staining (*n* = 2) or because the cultured species could not be identified by the reverse line blot assay (*n* = 2). Samples from the age-matched control group of 50 patients were all found to be negative by the real-time PCR.

The results indicated higher recovery from FNA than from tissue specimens from lymph nodes obtained during surgical excision or biopsy (Table 4). For 27 patients from whom FNA and tissue biopsy specimens were collected, specific real-time PCRs for the genus *Mycobacterium* and the species *M. avium* both yielded significantly more positive results for FNA than for tissue biopsy specimens (*P* values determined by the Wilcoxon test, 0.001 and 0.0011, respectively). Tissue biopsy specimens never were found to be positive by the real-time PCR when FNA were found to be negative. Although acid-fast staining and culture results were also more frequently positive for FNA than for surgically removed tissue (no statistical significance), tissue biopsy specimens yielded three more positive results when only conventional diagnostic tests were applied for patients from whom both materials were obtained (Table 4).

Compared to a positive mycobacterial skin test as an indication of the presence of a mycobacterial infection, the sensitivity, specificity, positive predictive value, and negative predictive value of the real-time PCR assay for patients with lymphadenitis were 71.6, 100, 100, and 72.5%, respectively. The application of the real-time PCR assay to all collected samples revealed 66.7% sensitivity, 100% specificity, 100% positive predictive value, and 59.5% negative predictive value. In total, the PCR assay detected a mycobacterial infection in 13 more patients than when only conventional diagnostic tests were performed.

Table 3. Results of real-time PCR, auramine staining, and mycobacterial culturing of FNA and tissue samples from 67 patients with suspected NTM lymphadenitis.

positive diagnostic test	total no. (%) of:	
	patients (*n* = 67)	smples (*n* = 102)
Auramine staining	31 (46.3)	38 (37.3)
Mycobacterial culturing	28 (41.8)	35 (34.3)
Real-time PCR	48 (71.6)	68 (66.7)
Genus specific	45 (67.2)	59 (57.8)
M. tuberculosis specific	1 (1.5)	1 (1.0)
M. avium specific	38 (56.7)	53 (52.0)
Any test	55 (82.1)	76 (74.5)

Table 4. Results of diagnostic tests of materials from 27 patients from whom both FNA and tissue biopsy specimens were collected.

diagnostic test	no. (%) of patients with the following test results for FNA and excisional biopsy specimens:			
	FNA +, biopsy +	FNA +, biopsy –	FNA –, biopsy +	FNA –, biopsy –
Auramine staining	8 (29.6)	6 (22.2)	3 (11.1)	10 (37.0)
Mycobacterial culturing	6 (22.2)	5 (18.5)	2 (7.4)	14 (51.9)
Real-time PCR assay[a]	16 (59.3)	9 (33.3)	0	2 (7.4)
M. avium specific	12 (44.4)	9 (33.3)	1 (3.7)	5 (18.5)
Genus specific	10 (37.0)	13 (48.1)	1 (3.7)	3 (11.1)
Any positive diagnostic test	16 (59.3)	9 (33.3)	0	2 (7.4)

[a] Significantly better performance with FNA than with tissue samples, as determined by the Wilcoxon test. *P* values for real-time PCR assay, *M. avium*-specific real-time PCR assay, genus-specific real-time PCR assay, and any positive diagnostic test were 0.003, 0.011, 0.001, and 0.005, respectively.

Discussion.

In the past few years, real-time PCR assays have been implemented in clinical microbiological laboratories. A few assays for the detection of mycobacteria have been described (15, 19). However, assays for mycobacterial species other than *M. tuberculosis* have not been successfully applied to clinical patient material and are mainly restricted to the identification of cultured isolates. In this study, we developed a real-time PCR to identify the causative agents of mycobacterial lymphadenitis. Primers for the real-time PCR were designed to amplify an amplicon in the ITS between the 16S and the 23S rRNA genes of mycobacteria, enabling the annealing of a genus-, *M. avium*-, or *M. tuberculosis*-specific probe. The detection limit of the assay was established at 1,100 CFU/ml of pus, and the assay was shown to be specific. For patients with suspected NTM lymphadenitis, the real-time PCR was significantly more sensitive than conventional staining and culturing. All

samples were also tested for the presence of inhibition of the assay by spiking portions of the samples and by diluting the samples 10 times. We did not encounter any inhibition, but the risk of inhibition will remain, considering the nature of the clinical materials (26).

Among 67 patients with suspected NTM lymphadenitis, successful identification of the pathogen was achieved for 55 of the patients by acid-fast staining, culturing, or real-time PCR. For 48 patients, it was possible to identify the pathogen within 2 days by the real-time PCR, whereas culturing of the remaining mycobacteria took 4 to 8 weeks. *M. avium* was identified in 39 patients, i.e., 71% of all confirmed mycobacterial infections in this study. This result corresponds to those of earlier studies where the *M. avium* complex was found to be the most common species in atypical mycobacterial lymphadenitis (8, 14, 22).

Our results also indicate that FNA have a higher recovery rate than tissue samples for diagnostic testing by the real-time PCR. The high recovery rate for FNA in the PCR diminishes the need for invasive methods for diagnosis of the involved pathogen, but a higher recovery rate for tissue biopsy specimens was expected. The genus-specific PCR performed less well with tissue biopsy specimens. The *M. avium*-specific PCR also performed better with FNA but was positive for six tissue samples for which the genus-specific PCR remained negative. An NCBI BLAST search of the probe sequences in the human sequence database showed a higher homology of the genus-specific probe than of the *M. avium*-specific probe with human chromosomal sequences. As a result, less genus-specific probe will be available, resulting in reduced efficiency of the real-time PCR for tissue samples or FNA. This situation will mainly affect weak positive samples, as demonstrated by high threshold cycle values (between 38 and 45) for the 6 *M. avium*-positive samples with negative results in the genus-specific PCR, in contrast to low cycle threshold values (between 30 and 37) for 15 *M. avium*- and genus *Mycobacterium*-positive samples.

Traditionally, culturing has been regarded as the most appropriate "gold standard." A definite diagnosis of mycobacterial lymphadenitis is made by recovery of the bacterium from lymph node material, but some causative pathogens may require a long incubation period (e.g., *M. malmoense*) or special culture conditions (*M. haemophilum*) (28). Skin tests may prove beneficial for the evaluation of mycobacterial lymphadenitis (29, 30). All children included in this study were investigated by one of the authors (J.A.L.), who also performed a skin test. Only children with a clinical syndrome suspected of being mycobacterial lymphadenitis and a positive skin test were further investigated. Since no other pathogens besides mycobacteria were detected in this study, a positive skin test can be considered indicative of mycobacterial infection. The skin test that we used is a well-standardized preparation from *M. avium*, *M. scrofulaceum*, and *M. kansasii*. Intradermal skin testing for the diagnosis of NTM lymphadenitis has been reported to have a sensitivity of 87%, but the specificity has not been well investigated (20). For instance, studies of asymptomatic healthy children in Sweden and Denmark with the same skin test that we used demonstrated that 6 to 32% had positive reactions (9, 16). NTM are common environmental isolates present in soil and water, and extensive cross-reactivity may exist among these mycobacteria. Therefore, a species-specific skin test is difficult to interpret, and more information on the incidence of a positive skin test in

healthy Dutch children is needed to determine the diagnostic value of the skin test more precisely.

For staining- and culture-negative samples from eight patients, the genus-specific PCR results were positive, whereas the real-time PCR results for *M. avium* and *M. tuberculosis* were negative. In two cases, sufficient PCR product was obtained for sequence analysis, and *M. haemophilum* was identified in both cases. This species requires special culture conditions and was therefore not detected in routine mycobacterial cultures. Our next objective is to expand the real-time assay with an *M. haemophilum*-specific probe. Approximately 70% of NTM lymphadenitis cases are due to *M. avium*. The remaining species identified in this study were *M. malmoense*, *M. kansasii*, *M. haemophilum*, and *M. tuberculosis*. The real-time PCR assay developed in this study can be expanded with probes specific for other species because of the large sequence variations in the ITS region. These variations are much greater than those found in any other known region, including the 16S rRNA gene. This finding has also been demonstrated by the ability of the real-time PCR to distinguish *M. avium* from *M. intracellulare*, whereas several other molecular methods do not distinguish this difference. The *M. avium*-specific real-time PCR detects only *M. avium* subsp. *avium*, *M. avium* subsp. *silvaticum*, and *M. avium* subsp. *paratuberculosis*.

Mycobacterial culturing is not optimal. When DNA was isolated from 12 negative MGIT cultures of *M. avium*-specific real-time PCR-positive samples, 5 samples gave a weak positive signal in the PCR. This result indicates that mycobacteria were present in the MGIT cultures. In two MGIT cultures, contamination with other bacteria was apparent and could have caused this growth inhibition, but in the remaining three MGIT cultures, no growth was detected at all. For these cultures, it is possible that the bacteria were dead or the conditions were not optimal.

In summary, the 71.6% sensitivity and the 100% specificity of the real-time PCR assay for the detection of atypical mycobacteria in patients with lymphadenitis suggest that the real-time PCR is an important diagnostic test and is more sensitive than conventional acid-fast staining techniques and mycobacterial culturing. The development of a rapid diagnostic test for the identification of mycobacteria as the cause of lymphadenitis is important, since an increasing number of reports suggest that rapid initiation of drug therapy may be of benefit and may be able to replace surgical excision of the involved lymph node (2, 10, 17).

A

```
                                1                                                  50
            M. ulcerans         GGGGTGTGGTGTTTGAGAATTGGATAGTGGTTGCGAGCATC.AA.TTGGA
            M. gordonae         GGGGTGTGGTGTTTGAGAATTGGATAGTGGTTGCGAGCATCAAA.ATGTA
         M. scrofulaceum        GGGGTGTGGTGTTTGAGTATTGGATAGTGGTTGCGAGCATCTAA.ACGGA
            M. malmoense        GGGGTGTGGTGTTTGAGTATTGGATAGTGGTTGCGAGCATCTAA.ACGGA
               M. xenopi        GGGGTGTGGTGTTTGAGTGTTGGATAGTGGTTGCGAGCATC.....TG..
         M. tuberculosis        GGGGTGTGGTGTTTGAGAACTGGATAGTGGTTGCGAGCATC..A.ATGGA
            M. kansasii         GGGGTGTGGTGTTTGAGAATTGGATAGTGGTTGCGAGCATC.AA.ATGGA
      M. intracell MinA         GGGGTGTGGTGTTTGAGTATTGGATAGTGGTTGCGAGCATCTAG.ATGAG
M. avium subsp avium MavB       GGGGTGTGGTGTTTGAGTATTGGATAGTGGTTGCGAGCATCTAG.ATGAG
            M. fortuitum        GGGGTGTGGTGTTTGATTTGTGGATAGTGGTTGCGAGCATCTAGCACGCA
     primerF/genusprobe         GGGGTGTGGTGTTTGAG    TGGATAGTGGTTGCGAGCATC

                                51                                                 100
            M. ulcerans         ....TGCGCTGCC.........TTTTGGTGAGCGTGTTCTGTT.......
            M. gordonae         ....TGCGTTGTCG........TTCTCGGCAACGTGTTCTTTT......T
         M. scrofulaceum        ....TGCGTTGCCG........GCAACGGTGGCGTGTTCGTTGAA...AT
            M. malmoense        ....TGCGCTGCC.........CGTAGGGACGCGTATTCGTTT......T
               M. xenopi        .....GCAAAGAC............TGTGGTAAGCGGTTTTT........
         M. tuberculosis        ....TACGCTGCCGG.......CTAGCGGTGGCGTGTTCTTT........
            M. kansasii         ....TGCGTTGCC.........CTACGGGTAGCGTGTTCTTTT.......
      M. intracell MinA         ....CGCATAGTCC........TTAGGGCTGATGCGTTCGTCGAA...AT
M. avium subsp avium MavB       ....CGCATGGTC.........TTGGTGGCCGGCGTTCATCGAA...AT
            M. fortuitum        GAATCGTGTGGTCTCACTCCTTGTGGGTGGGGCTGGTTTTGTGTGTTGAT

                                101                                                150
            M. ulcerans         GTGCAATTTTAT.........TCTTTGGTTTTTGT...GTTTATAAG.T
            M. gordonae         GTGCAATTT.AT.........TCTTTGGTTTTTGTAGTGTTTGTAAG.T
         M. scrofulaceum        GTGTAATTT.CT.........TCTTTGGTTTTTGT.GTGTAAGTAAG.T
            M. malmoense        GTGTAATTT.CT.........TCTTTGGTTTTTGT...GTTAGTAAG.T
               M. xenopi        GTTGAGTGT.............TTTCTGGTGTTTGT.........AAG.T
         M. tuberculosis        GTGCAATAT.............TCTTTGGTTTTTGTTGTGTTTGTAAG.T
            M. kansasii         GTGCAATTTTAT.........TCTTTGGTTTTTGT...GTTTGTAAGGT
      M. intracell MinA         GTGTAATTT.CT.........TCTTTGGTTTTTGT.GTGTAAGTAAG.T
M. avium subsp avium MavB       GTGTAATTT.CT.........TTTTTAACTCTTGT.GTGTAAGTAAG.T
            M. fortuitum        GTGCAATTTCTTTTGAAACTCATTTTTGGTTTTTGT...GTT.GTAAG.T

                                151                                                200
            M. ulcerans         GTCTAAGGGCGCATGGTGGATGCCTTGGC.ATCGAGAGCCGATGAAGGAC
            M. gordonae         GTCTAAGGGCGCATGGTGGATGCCTTGGC.ATCGAGAGCCGATGAAGGAC
         M. scrofulaceum        GTTTAAGGGCGCATGGTGGATGCCTTGGCGATCGAGAGCCGATGAAGGAC
            M. malmoense        GCTTAAGGGCGCATGGTGGATGCCTTGGC.ATCGAGAGCCGATGAAGGAC
               M. xenopi        GTGTAAGGGCGCATGGTGGATGCCTTGGC.ATCGAGAGCCGATGAAGGAC
         M. tuberculosis        GTCTAAGGGCGCATGGTGGATGCCTTGGC.ATCGAGAGCCGATGAAGGAC
            M. kansasii         GTCTAAGGGCGCATGGTGGATGCCTTGGC.ATCGAGAGCCGATGAAGGAC
      M. intracell MinA         GTTTAAGGGCGCATGGTGGATGCCTTGGC.ATCGAGAGCCGATGAAGGAC
M. avium subsp avium MavB       GTTTAAGGGCGCATGGTGGATGCCTTGGC.ATCGAGAGCCGATGAAGGAC
            M. fortuitum        GTTTAAGGGCGCATGGTGGATGCCTTGGCAATCGAGAGCCGATGAAGGAC
           PrimerR                                                  GATGAAGGAC

                                201
            M. ulcerans         GTGGGAG
            M. gordonae         GTGGGAG
         M. scrofulaceum        GTGGGAG
            M. malmoense        GTGGGAG
               M. xenopi        GTGGGAG
         M. tuberculosis        GTGGGAG
            M. kansasii         GTGGGAG
      M. intracell MinA         GTGGGAG
M. avium subsp avium MavB       GTGGGAG
            M. fortuitum        GTGGGAG
           PrimerR              GTGGGAG
```

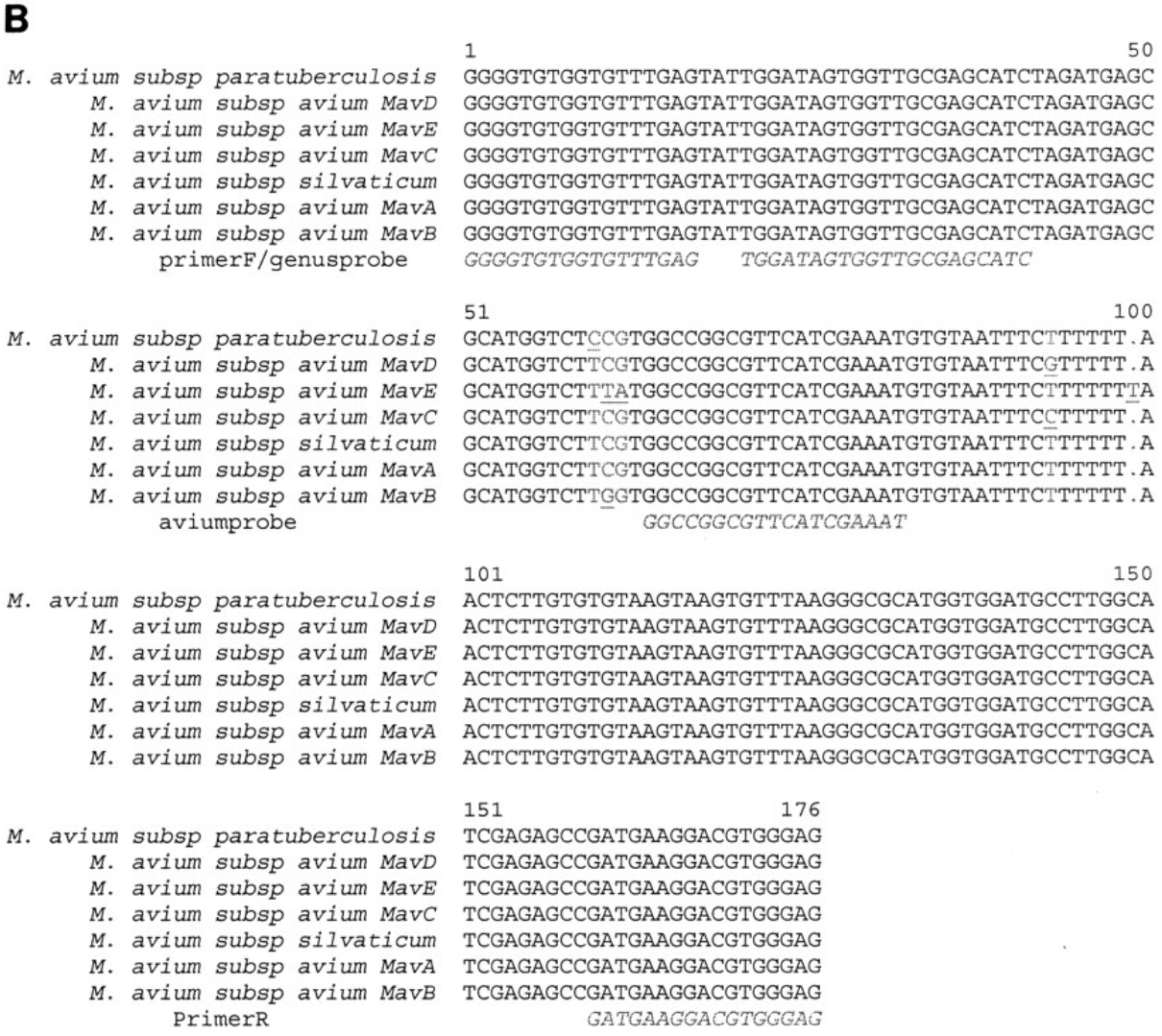

FIG. 1. (A) Alignment of ITS fragments used in the real-time PCR assay of various mycobacterial species. *M. intracell*, *M. intracellulare*. (B) Alignment of ITS fragments used in the real-time PCR assay of *M. avium* strains.

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Chapter 4.

Mycobacterium haemophilum and lymphadenitis in children.

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Abstract.

Infections associated with *Mycobacterium haemophilum* are underdiagnosed because specific culture methods required for its recovery are not applied routinely. Using polymerase chain reaction (PCR) technology on fine needle aspirates and biopsied specimens from 89 children with cervicofacial lymphadenitis, we assessed the importance of *M. haemophilum*. Application of a *Mycobacterium* genus–specific real-time PCR in combination with amplicon sequencing and a *M. haemophilum*–specific PCR resulted in the recognition of *M. haemophilum* as the causative agent in 16 (18%) children with cervicofacial lymphadenitis. *M. avium* was the most frequently found species (56%), and *M. haemophilum* was the second most commonly recognized pathogen. Real-time PCR results were superior to culture because only 9 (56%) of the 16 diagnosed *M. haemophilum* infections were positive by culture.

Introduction.

Cervicofacial lymphadenitis is the most frequently encountered manifestation of nontuberculous mycobacterial (NTM) disease in children. In previous studies, *Mycobacterium avium* has been identified as the cause in >80% of the patients (1). Other mycobacterial species isolated from patients with lymphadenitis are *M. tuberculosis*, *M. malmoense*, *M. kansasii*, *M. scrofulaceum*, *M. intracellulare*, and *M. xenopi*. *M. haemophilum* has been described as the causative agent of lymphadenitis as well (2–7).

In an ongoing multicenter study in the Netherlands, the optimal treatment for NTM lymphadenitis is investigated. Diagnosis of mycobacterial infection is performed by using mycobacterial differential skin tests and fine needle aspiration biopsy. Biopsied specimens are subjected to acid-fast staining, mycobacterial culturing, and *Mycobacterium* genus–specific real-time PCR. Of 89 patients included in the study so far, mycobacterial species were identified in 55 cases, of which *M. avium* had been found in 50 patients (8). In addition, a mycobacterial infection without further identification was detected in 16 patients. An atypical mycobacterial infection was diagnosed in these patients because either acid-fast staining results were positive or the *Mycobacterium* genus–specific real-time polymerase chain reaction (PCR) was positive. Cultures or species-specific real-time PCR for *M. avium* and *M. tuberculosis* remained negative. Previously, an attempt to characterize these mycobacteria by sequence analysis of the genus-specific PCR fragment was successful in only 2 cases and showed *M. haemophilum* (8). In the current study, we further analyzed these uncharacterized mycobacteria.

M. haemophilum requires special growth conditions (9), and most of the diagnostic laboratories do not use these culture conditions. Furthermore, no molecular test is available to detect *M. haemophilum* directly in clinical materials. Therefore, *M. haemophilum* infection could be seriously underdiagnosed (4,10–12). In this study, we developed a species-specific real-time PCR to detect *M. haemophilum* directly in patient materials. This assay can show

the actual prevalence of *M. haemophilum* in patients with mycobacterial lymphadenitis, but it could also be applied in other diseases and help elucidate the incidence and distribution of this species.

Materials and Methods.

Bacterial strains.

Five *M. haemophilum* reference strains (all clinical isolates) were available for 16S and internal transcribed spacer (ITS) sequencing. Three strains were provided by the National Institute for Public Health and the Environment and 2 were provided by the Institute for Tropical Medicine (Antwerp, Belgium). The 25 mycobacterial strains used for specificity testing included *M. tuberculosis* complex, *M. kansasii*, *M. xenopi*, *M. avium*, *M. intracellulare*, *M. gordonae*, *M. chelonae*, *M. fortuitum*, *M. marinum*, *M. scrofulaceum*, and *M. malmoense.* A complete list of all strains (species and subspecies) has been published in Bruijnesteijn et al. (8). The strains were cultured in liquid Dubos medium at 35°C. The *M. haemophilum* strains were cultured at 30°C on solid Löwenstein-Jensen (LJ) medium with added iron citrate or in liquid Mycobacteria Growth Index Tube (MGIT) medium with X-factor-strip added (Becton-Dickinson, Alphen a/d Rijn, the Netherlands).

Patients and samples.

Clinical materials were obtained from patients included in the CHIMED-study. In CHIMED (a multicenter nationwide study on the optimal treatment for children with lymphadenitis), treatment is randomized between surgical and medical treatment. Pediatric patients were included on the basis of clinical appearance of atypical mycobacterial lymphadenitis and a positive skin test (13,14). Fine needle aspirates were taken from affected lymph nodes. In patients who underwent surgical treatment, the removed lymph nodes were also submitted for investigation. A control group to assess the specificity of the assay was assembled from 50 patients with lymphadenitis caused by *Bartonella henselae*.

Mycobacterial diagnostics.

Clinical materials were decontaminated with a Nalc-NaOH decontamination protocol (15). Auramine staining was performed on the decontaminated materials for detection of acid-fast rods. Standard mycobacterial culturing was performed at 35°C in liquid MGIT medium and on solid LJ medium. *M. haemophilum*–specific culturing was performed at 30°C on LJ medium with added iron citrate and in MGIT medium with X-factor-strip added. Mycobacterial species were identified by using the Inno-Lipa and more recently using the Inno-Lipa V2 assay (InnoGenetics, Gent, Belgium) (16). When no growth was detected after 12 weeks of incubation, the culture results were listed as negative. Samples were also investigated for the presence of other bacterial pathogens by conventional bacterial cultures and by PCR for *B. henselae* (17).

Nucleic Acid Isolation.

All clinical materials were processed as described in Bruijnesteijn et al. (8). DNA was extracted from bacterial strains and clinical materials according to the method of Boom et al. (18) with an overnight incubation with proteinase K.

Primers and probes.

Genus-specific primers for sequencing the total ITS region of mycobacteria were described by Frothingham et al. (19). Primers described previously for a genus-specific real-time PCR (8) were also used for sequencing a part of the ITS region directly from clinical materials. Using these primer sets combined, we applied a seminested PCR approach to increase the amount of amplicon. The part of the ITS region used in this real-time PCR shows considerable variation between mycobacterial species (20) (Figure). The primers used in the real-time PCR are genus-specific, and for the design of the *M. haemophilum*–specific minor groove binding (MGB) probe, the intraspecies and interspecies variation in the amplified ITS region was investigated. Alignments were made of the sequences of the *M. haemophilum* strains and of different mycobacterial species. The *Mycobacterium* genus–specific probe is described in Bruijnesteijn et al. (8).

The *M. haemophilum*–specific probe sequence was checked by using the primer 3 program (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi/) (21) and oligo-analyzer 3.0 (http://biotools.idtdna.com/analyzer/) (IDT Biotools, Coralville, IA), to ensure minimal self-complementary binding and to prevent the presence of secondary structures. By using the unique features of the MGB group (22), a short and highly specific probe could be designed. The probe was designed on the antisense strand to ensure an A/T rich MGB-site. An NCBI BLAST search was performed to check the specificity of the probe. The primers were prepared by Biolegio (Biolegio, Malden, the Netherlands), and the MGB probe was generated by ABI (Applied Biosystems Inc, Nieuwekerk a/d IJssel, The Netherlands). The broad range primers P1 and P4 were used for 16S sequencing. Primers and probes are listed in Table 1, and their positioning in the genome is illustrated in the Figure.

Sensitivity testing.

A plasmid with the ITS sequence of *M. haemophilum* was prepared by cloning the PCR product in a vector and was subsequently quantified (IQ corporation, Groningen, the Netherlands). Dilution series of this plasmid were tested in duplicate in the genus-specific and the *M. haemophilum*–specific real-time PCR.

Sequence analysis.

After amplification, PCR products were subjected to a cycle sequencing reaction with the Big Dye Terminator Cycle Sequencing ready reaction kit (Applied Biosystems). Samples underwent electrophoresis and sequences were detected and analyzed on ABI model 310 DNA sequencer (Applied Biosystems).

Real-time PCR.

Real-time PCR was performed in 50 µL of reaction mixture consisting of 25 µL of 2x IQ supermix (Bio-Rad, Veenendaal, the Netherlands), 20 pmol of each primer, 12.5 pmol of the genus-specific probe or 10 pmol of the *M. haemophilum*–specific probe, and 10 µL template. The PCR thermal profile consisted of an initial incubation of 3 min at 95°C for activation of the enzyme, followed by 50 cycles of 30 s at 95°C, 40 s at 55°C, and 30 s at 72°C. Amplification, detection, and data analysis were performed with an iCycler IQ real-time detection system (Bio-Rad). The reaction mix and PCR profile were similar for both the genus-specific probe and the *M. haemophilum* probe.

Each DNA extract was tested by real-time PCR for the detection of the genus *Mycobacterium* and species *M. haemophilum*. As positive control for the genus-specific real-time PCR and extraction protocol, a dilution of *M. bovis* was used.

Table 1. Sequences of oligonucleotides used in this study*

primer	probe sequence (5´–3´)	target sequence
ITS [a] forward primer real-time PCR	GGGGTGTGGTGTTTGAG	partial ITS
ITS reverse primer real-time PCR	CTCCCACGTCCTTCATC	partial ITS
Forward primer Ec16S.1390p [b]	TTGTACACACCGCCCGTCA	total ITS
Reverse primer Mb23S.44n [b]	TCTCGATGCCAAGGCATCCACC	total ITS
16S forward primer P1 [c]	TAACACATGCAAGTCGAACG	16S
16S reverse primer P4 [c]	TCGTTGCGGGACTTAACCCAAC	16S
Mycobacterium genus–specific TaqMan probe	Fam-TGGATAGTGGTTGCGAGCATC-Tamra	ITS
Mycobacterium haemophilum–specific MGB-probe	VIC–ACGCCACCATTACT-MGB	ITS

[a] ITS, internal transcribed spacer. [b] Primers published in (19). [c] Primers published in (23).

Results.

Identification of *M. haemophilum* in patient material.

In 16 (18%) of 89 patients from the CHIMED study, a mycobacterial infection was suspected, but initially no species identification could be established. After a positive signal was generated by the genus-specific real-time PCR and negative results from the cultures, the amplicons generated in real-time PCR were sequenced to determine the species. Sequencing of the ITS fragment formed in the real-time PCR was difficult, owing to the small amount of amplicon, but eventually the sequences of 4 patient samples were successfully derived. On 4 more samples, a seminested PCR was performed to increase the amount of specific amplicon. This enhancement of PCR resulted in the successful amplification and sequencing of all fragments. No variation was encountered between the ITS sequences of all 8 strains analyzed here. Because no *M. haemophilum* ITS sequences were available in the public databases, 3 complete ITS sequences from *M. haemophilum* strains isolated from different CHIMED patients were determined and submitted to the NCBI database (accession

no. AY579398, AY579399, and AY579400). After specific culturing, the identity of the strains was confirmed by comparing partial 16S-gene sequences to the sequences in the NCBI (http://www.ncbi.nlm.nih.gov/) and the RIDOM database (http://www.ridom.com/). A variable part of the 16S gene of 330 base pairs was analyzed, and a 100% agreement was obtained with 16S sequences of 7 available *M. haemophilum* strains, including ATCC 29548. The *M. haemophilum* strains had at least 4 mismatches in the analyzed 16S PCR fragment in comparison to other mycobacterial species; therefore, all these strains were *M. haemophilum*. The identity was also confirmed because of a minimum of 4 mismatches in the 16S fragment between the *M. haemophilum* sequence and other mycobacterial species.

Application of real-time PCR for the recognition of *M. haemophilum.*

The real-time PCR was designed to the ITS region. The same conserved primers were used as described previously. The obtained ITS sequences were used to select an *M. haemophilum*–specific probe.

The detection limit of the *M. haemophilum*–specific real-time PCR was assessed at 1 copy per reaction by using a dilution series of the plasmid standard. The mycobacterial genus–specific PCR was tested simultaneously with the *M. haemophilum*–specific PCR and resulted in the same analytical sensitivity. As determined previously, the sensitivity of the primer set in clinical materials was estimated to be 1,100 CFU in pus (8). Specificity testing of the *M. haemophilum*–specific real-time PCR with 25 other species and subspecies showed no aspecific reactions. All 50 *Bartonella*-positive samples from the control group remained negative in the real-time PCR assay as well.

Of 16 patients with evidence for *M. haemophilum* infection, 9 (56%) were positive on auramine staining, and 9 (56%) were positive in *M. haemophilum*–specific cultures. In addition, in 1 patient (6%), the pathogen was able to grow on and in normal mycobacterial cultures. Thirteen patients (81%) had positive specimens in mycobacterial genus–specific real-time PCR, 11 of which were also positive in the *M. haemophilum*–specific real-time PCR (Table 2). In contrast, 2 genus-specific *M. haemophilum*–negative specimens were positive in the *M. haemophilum*–specific real-time PCR. Thus, the 2 PCRs combined yielded 15 positive (94%) patients. These 4 samples with inconsistent results all had high threshold cycle values, indicating that the amount of bacterial DNA present was close to the detection limit of the assays. This finding was confirmed by retesting the samples 5 times in both PCRs, which yielded 2 or 3 positive reactions in the genus-specific PCR and in the *M. haemophilum*–specific PCR. No correlation was found between the threshold cycle values in the real-time PCR assay and the culture or auramine-staining results. All 9 patients with positive specimens by auramine staining also had positive results in the real-time PCR assay. Three patients' conditions were diagnosed by real-time PCR only. Only 1 patient had a positive culture while results of the real-time PCR or auramine staining remained negative. Real-time PCR on the isolate cultured from this patient resulted in a positive signal.

The *M. haemophilum*–specific culturing method was less sensitive than the real-time PCR assay. The materials from the first 6 patients were cultured specifically for *M. haemophilum* after negative results were obtained from conventional culturing methods. The stored

decontaminated materials were thawed and incubated at 30°C on enriched media. From these 6 patients, 2 samples (33%) yielded positive cultures. The materials from the 10 other patients were cultured directly and yielded positive results from 7 (70%) patients. *M. haemophilum*–specific real-time PCR was performed additionally on all positive cultures to confirm the specific growth of *M. haemophilum*.

From the 16 patients positive for *M. haemophilum*, 22 samples were collected: 9 tissue biopsy specimens and 13 fine needle aspirates. Of these samples, 19 (86%) were positive in the real-time PCR assay, while 11 (50%) samples yielded positive results in auramine staining and 9 (36%) were positive in culture. No discrepancies were encountered in the real-time PCR assay when all samples instead of patients were considered. Application of the real-time PCR assay increased the diagnostic yield by 23%.

Table 2. Results of diagnostics tests of 16 *Mycobacterium haemophilum*–positive patients.

***M. haemophilum*–positive patient**	**acid-fast staining**	**culture 30°C[a]**	**genus-specific real-time PCR**	***M. haemophilum* real-time PCR**
1	+	–	+	+
2	–	+	–	–
3	+	–	+	+
4	–	–	+	+
5	–	–	+	+
6	+	+	+	+
7	+	–	+	+
8	+	+[b]	+	+
9	+	+	+[c]	–[c]
10	+	+	–[c]	+[c]
11	+	–	+[c]	–[c]
12	–	+	–[c]	+[c]
13	–	+	+	+
14	–	–	+	+
15	–	+	+	+
16	+	+	+	+
Total positive patients	9	9	13	13

[a] Löwenstein-Jensen (LJ) medium with added iron citrate or liquid MGIT medium with X-factor-strip added. Cultures at 30°C were performed after storage for patients 1 to 6.

[b] Patient material was also culture positive at 35°C.

[c] Because of discrepant polymerase chain reaction (PCR) results with high threshold cycle values, the PCR was performed 5 times on these samples, which resulted in at least 2 specific positive signals for both PCRs on every sample. Therefore. the amount of mycobacterial DNA is estimated at the detection limit of the assay. The first obtained PCR result is described in the table.

Discussion.

M. haemophilum was found to be the causative agent of lymphadenitis in 16 (18%) of the children included in this study. Despite the use of specific enriched culture mediums, only 9 (56%) of the 16 *M. haemophilum* infections were culture-positive. In contrast, the real-time PCR assay was positive in 15 (94%) patients.

M. haemophilum infection is not diagnosed frequently and is therefore not considered a common cause of lymphadenitis. However, most studies on children with mycobacterial lymphadenitis have not used optimized cultures for *M. haemophilum*, and infection with this species is therefore likely underdiagnosed. Nevertheless, differences in geographic distribution may also contribute to the variable prevalence of *M. haemophilum*. For instance, no *M. haemophilum* was found in children with atypical mycobacterial lymphadenitis in a study in Ohio (24), whereas in a study in Israel, *M. haemophilum* was found in 12 of 29 patients (5). Both studies used appropriate culture conditions for *M. haemophilum*. Another reason for an underestimation of the occurrence of *M. haemophilum* infections is the misleading positive skin test. *M. haemophilum* can induce similar reactions in the Mantoux test as *M. tuberculosis* and could be misdiagnosed when no positive cultures are obtained (4, 5).

The natural source of *M. haemophilum* infection is unknown. Its geographic distribution appears to be related to the occurrence of large bodies of water (6). A few natural reservoirs have been suggested (25–27), but studies focusing on the environmental reservoirs of NTM tend to culture without optimized conditions for *M. haemophilum*, which may be the reason the organism is rarely found. The temperature for culture is often too high (28, 29), cultures do not contain hemin or iron citrate, or the incubation time is too short (30). Only 1 study detected *M. haemophilum* in water distribution systems, although the culture method was not optimal (26). Therefore, *M. haemophilum* may be widely distributed and present in several natural reservoirs; water is the most likely one (12).

M. haemophilum is slow growing, iron dependent, and has an optimal growth temperature from 30°C to 32°C. It is unable to grow on routine media such as LJ Middlebrook 7H9 and 7H10, or BACTEC broth. Media used to recover *M. haemophilum* on primary isolation include commercially available solid media or broth enriched with ferric ammonium citrate or hemin (31). Chocolate agar and lysed blood agar are mentioned as inexpensive and suitable alternatives (32,33). Little is known about the sensitivities of direct culturing of clinical materials for the recovery of *M. haemophilum*, and not all media have equal capacity for stimulating the growth (34).

Because application of culture conditions specific for *M. haemophilum* are not likely to become standard in clinical microbiologic laboratories, including this specific diagnosis might be useful for molecular methods. A species-specific real-time PCR was developed to identify *M. haemophilum* directly in patient materials. Because *M. haemophilum* was not expected to be an important pathogen, no specific culturing was applied initially. After the recognition of *M. haemophilum* by molecular methods, the culture methods were optimized, which resulted in 70% positive cultures. Additionally, all stored decontaminated materials from culture-

negative specimens were recultured under *M. haemophilum*–specific conditions. Most likely because of these additional freezing and thawing steps, cultures were less sensitive for these materials and resulted in 33% positive cultures.

Identification of *M. haemophilum* in patient materials was performed by 16S sequencing (of cultures) and ITS sequencing. Two versions of a commercial reverse line hybridization assay (the Inno-Lipa assay and the V2 Inno-Lipa assay) were also used for the recognition of *M. haemophilum*, but these tests can only be applied on cultured isolates. The V2 Inno-Lipa can identify *M. haemophilum* by a specific probe, which was absent in the previous version of the Inno-Lipa assay. The reactions were uniformly positive only for *M. haemophilum* in the V2 Inno-Lipa.

The design of the real-time PCR MGB probe was based on the ITS sequences that were obtained from the patient materials and reference strains. An MGB probe enables specific detection of the target by using a shorter sequence than that of a TaqMan probe or a molecular beacon.

Sequencing of the ITS amplicons from the genus-specific real-time PCR on patient samples was difficult because of the small amounts of target sequence. To enhance the specific amplification, a seminested PCR was applied. Of the 8 clinical samples from which sequences were obtained, 4 samples also yielded positive cultures once the culture protocol was optimized. The ITS and 16S sequences derived from the cultured isolates confirmed the authenticity of the identified pathogen.

In this study, both fine needle aspiration and excisional biopsy were not applied as treatment options but as diagnostic procedures. Complete surgical excision of the affected lymph nodes is considered as the treatment of choice for atypical mycobacterial lymphadenitis (1, 35, 36). However, surgical excision leaves scarring and carries the risk of damaging branches of the peripheral facial nerves (37, 38). Antimicrobial therapy as a conservative treatment is currently the topic of our study. Incision and drainage increase the risk for sinus tract formation or recurrence of infection (33,35). This risk also applies to fine needle aspiration, but the usage of fine needle aspirate for PCR will provide a rapid diagnosis and thereby allow treatment to begin earlier and thus lower the risk for complications.

In conclusion, for detecting and identifying *M. haemophilum*, real-time PCR is a sensitive and specific assay suitable for direct application on clinical materials. In this study, by using the real-time PCR, *M. haemophilum* was shown to be an important pathogen involved in lymphadenitis. Because of special growth requirements, the clinical spectrum of diseases associated with *M. haemophilum* is largely unknown. Real-time PCR may be particularly useful for testing clinical samples such as sputum, cerebrospinal fluid, and synovial fluid for *M. haemophilum* to determine the role of *M. haemophilum* in more detail.

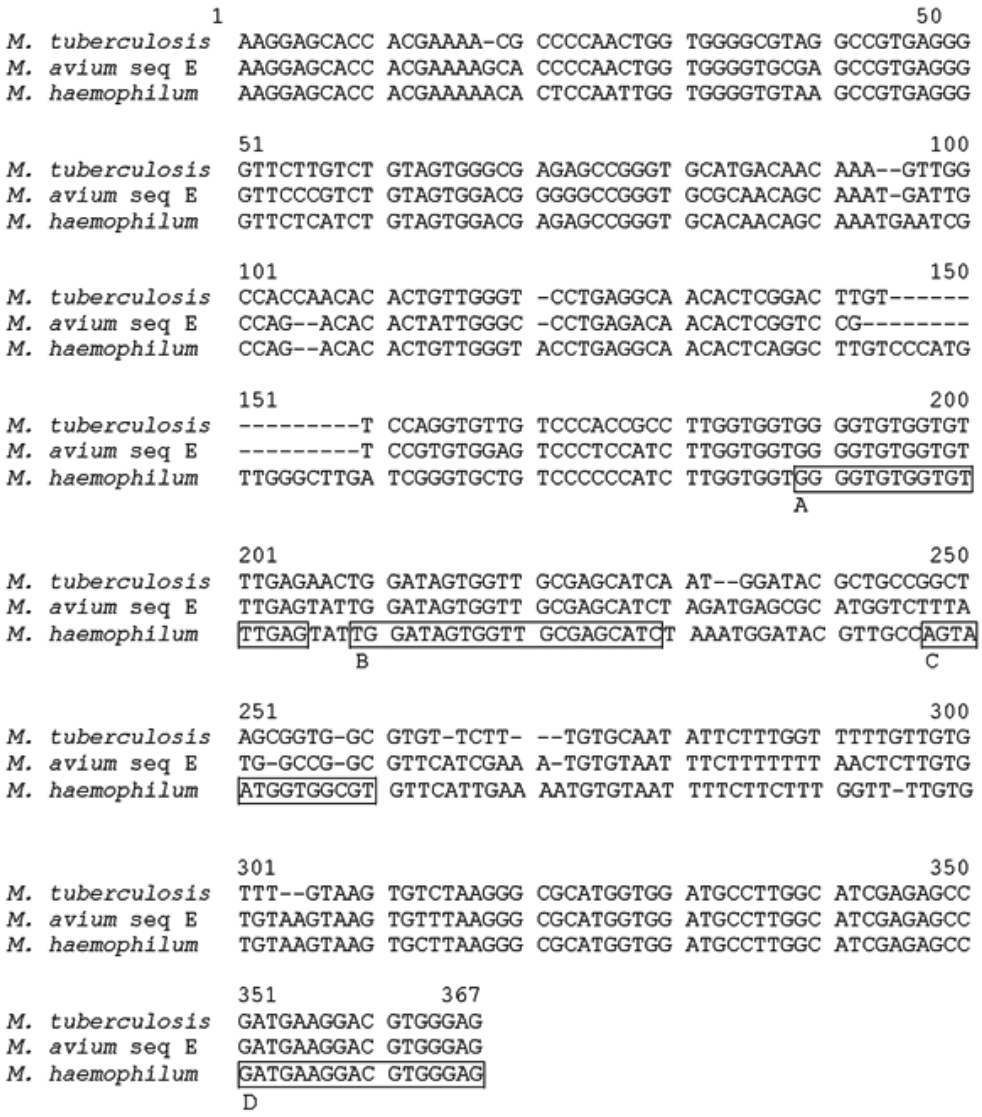

Figure 1. Alignment of internal transcribed spacers (ITS) and partial 23S sequences with primers and probes used for real-time polymerase chain (PCR) reaction. (nucleotides [nt] 1 to 301 make up the total ITS region; nt 302 to 367 are coding for partial 23S gene). The *Mycobacterium haemophilum* sequence was derived from 3 different patients, but no variation was found. A, forward primer for real-time PCR; B, *Mycobacterium* genus–specific probe; C, *M. haemophilum*–specific probe; D, reverse primer for real time–polymerase chain reaction.

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Chapter 5.

Application of real-time PCR to recognize atypical mycobacteria in archival skin biopsies: High prevalence of *Mycobacterium haemophilum*.

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Abstract.

Atypical mycobacterial skin infections are difficult to diagnose owing to their aspecific histopathologic presentations and to the presence of few bacteria. Therefore, these infections are often not recognized. Molecular detection of mycobacterial DNA has proven to be useful in clinical samples. The aim of this study was to investigate the incidence of mycobacterial involvement in skin biopsies showing granulomatous inflammation, using real-time polymerase chain reaction (PCR).
Real-time PCR specific for the genus *Mycobacterium* and the species *Mycobacterium avium* and *Mycobacterium haemophilum* was performed on formalin-fixed/paraffin-embedded biopsies from patients with granulomatous inflammation of the skin, from the period 1984 to 2004. A control group was assembled from patients with proven basal cell carcinoma. Amplicons of all positive reactions were sequenced to confirm or identify the mycobacterial species.
Of 30 patients, 13 (43%) were found to be positive for mycobacterial infection, of whom only 5 patients had been previously diagnosed with a mycobacterial disease. *M. haemophilum* was identified as the most common species (n=7). The other identified species were *Mycobacterium malmoense, Mycobacterium gordonae*, and *Mycobacterium marinum*. The results show that real-time PCR is useful in detecting mycobacterial infections in undiagnosed formalin-fixed / paraffin-embedded skin samples and that the application of molecular approaches would improve the diagnoses of mycobacterial skin infections.

Introduction.

Nontuberculous mycobacteria (NTM) cause a variety of diseases mostly in immunocompromised patients in lungs, skin, lymph nodes, bone, joints, and intestines. The incidence of NTM-infections has increased over the last few years, mainly in immunocompromised patients but also in immunocompetent. The increase may be related to the development of new diagnostic technologies (1). However, the true incidence is still probably many times higher than that clinically diagnosed.
Mycobacterium leprae (leprosy) and *Mycobacterium* ulcerans (Buruli ulcer) are the most notorious pathogens causing specific cutaneous infections, but other NTM like *Mycobacterium marinum*, *Mycobacterium avium*, and *Mycobacterium haemophilum* have highly variable clinical presentations. They can manifest as pustuls, hyperkeratotic papels, suppurative noduli, ulcerations, cellulitis, or erythematous plaques. The histologic picture is often a granulomatous lesion that can show variable forms of cellular infiltration (2,3). Owing to the aspecific presentation, clinical and histologic recognition of mycobacterial involvement is difficult. The conventional diagnostics for a mycobacterial infection in formalin-fixed/paraffin-embedded (FF/PE) materials is mostly restricted to acid-fast staining but this has an unsatisfying sensitivity. Therefore, a molecular approach is considered to be a valuable alternative method.

Recently, a real-time polymerase chain reaction (PCR) assay, specific for the genus *Mycobacterium*, which could detect the slow-growing species and the species *M. avium* and *M. haemophilum*, was developed in our hospital. Application of this test on cervical lymph node biopsies in comparison with acid-fast stain and culture revealed that the molecular approach had a significantly higher sensitivity (4). This assay helped to elucidate the incidence of slow-growing mycobacterial species-associated lymphadenitis in The Netherlands (5).
The aim of this study was to retrospectively apply the real-time PCR assay on FF/PE skin biopsies with histologic evidences of granulomatous inflammation and to compare the results with the earlier diagnostic examinations and clinical information.

Materials and Methods.

Patient materials.

A digital search was performed in the database of the department of pathology from our hospital. The database was searched from 1984 to 2004 for cases that consisted of a histologic diagnosis of granulomatous inflammation of the skin. Forty-two cases were identified; 12 of the 42 were excluded as either there was insufficient material or they could not be found in the archives. The clinical and histologic specifications of the remaining 30 patients who were included are listed in Table 2. The collection included materials from 14 male and 16 female patients. The patients' ages ranged from 14 to 81 with a medium age of 47 years. A control group was assembled from archival skin biopsies of 30 patients with diagnosed basal cell carcinoma (BCC). The patients were matched for age and the biopsies were matched for year of investigation and body location.

Conventional mycobacterial diagnostics.

In 5 cases, mycobacterial diagnostics had been requested at the time of the case presentations (Table 2). On the original biopsy materials, Ziehl-Neelsen (Zn) staining was performed and a culture at 35°C on solid Löwenstein-Jensen (LJ) medium, and, after the year 2000, also in liquid MGIT medium (Mycobacterial Growth Indicator Tube, Becton Dickinson). No *M. haemophilum* specific culture conditions were applied until 2004.

DNA-extraction.

The FF/PE biopsies were at least 1 mm^2 in size. Five to 10 sections of 10 µm were collected in sterile tubes for DNA extraction. The sections were cut after removal of the outer parts of the paraffin blocks. To avoid cross contamination, the microtome block was wiped with xylene and the blade was shifted or discarded with each new block. With every 8 samples, a negative control was added, which consisted of a paraffin block without tissue. Deparaffinization and tissue digestion was performed according to an in-house method using an overnight incubation at 56°C with proteinase K (Invitrogen, Breda, The Netherlands) (add per sample: 0.5 mg proteinase K, dissolved in 400 µL buffer solution with 0.01M Tris-HCl and

0.45% Tween 20). Then the paraffin was separated from the tissue cells by increasing the temperature to 95°C for 5 minutes, centrifuging, cooling at 4°C for 5 minutes. This allows the paraffin to coagulate and the transfer of the approximately 350 µL of the liquid volume to a clean tube. Subsequently, a second incubation with fresh proteinase K (add per sample: 0.2 mg proteinase K, dissolved in 50 µL buffer solution with 0.01M Tris-HCl and 0.45% Tween 20) was performed for 1 hour at 60°C for an extra rigorous digestion of the acid-fast wall of the intracellular bacilli. The total volume of 400 µL tissue cells and buffer was then used for DNA extraction. For 1 patient (patient 30), only sections on glass slides were available. These were scraped off the glass slide and were processed similarly for DNA extraction. The DNA-extraction protocol described by Boom et al (6) was followed. DNA was eluted in 50 µL TE and stored at 4°C before using in the PCR.

Table 1: sequences of primers and probes.

oligo	**sequence (5'-3') with attached fluorophore and quencher for probes**	**product size**
16S forward primer [a]	TAACACATGCAAGTCGAACG	ca 323 bp
16S reverse primer [a]	CCCATTGTGCAATATTCCCC	
ITS forward primer [b]	GGGGTGTGGTGTTTGAG	ca 170 bp
ITS reverse primer [b]	CTCCCACGTCCTTCATC	
genus-specific Taqman-probe [c]	FAM -TGGATAGTGGTTGCGAGCATC- Tamra	
M. avium specific molecular beacon [c]	FAM-CCCACCGGCCGGCGTTCATCGAAATGGTGGG-DabCyl	
M. haemophilum specific MinorGrooveBinder-probe [c]	VIC –ACGCCACCATTACT- MGB	

[a] 16S PCR used in early molecular identification of culture in patient 10.

[b] Genus and species specific real-time PCR retrospectively used for all specimens.

[c] Probes applied in combination with ITS forward and reverse primer.

Real-time PCR and nucleotide sequence analysis.

Real-time PCR was performed as described previously (4,7). Oligos used for real-time PCRs and for the sequencing of the internal transcribed spacer (ITS) and 16S rRNA gene are described in Table 1. The genus-specific PCR was performed twice for each sample, the *M. avium* and *M. haemophilum*-specific PCRs only once because of the limited amount of DNA extract.

When a positive signal was found in the real-time PCR, the amplicon was sequenced to determine the involved species or to confirm a species-specific signal. All positive PCR reactions were analyzed with gel-electrophoresis. Bands between 150 and 200 bp were subsequently cut out and purified with the Qiagen gel-extraction kit (Qiagen, Venlo, The Netherlands). These bands represent the amplified region of the mycobacterial ITS. Sequence reactions have been performed with the PCR primers using the Qiagen Big Dye terminator ready reaction kit (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and have been analyzed on an ABI 3100 automatic sequencer (Applied Biosystems).

Results.

Archival FF-PE materials from 30 patients were collected from the archives of the pathology department. All the available clinical and histologic information on these patients was collected and the data revealed 14/30 immunocompromised patients (Table 1). Overall, the materials from 13 of the 30 (43%) patients contained mycobacteria (or mycobacterial DNA), diagnosed by conventional methods or by real-time PCR. Table 3 describes the diagnostic results of 15 samples from the 13 positive patients. Fourteen samples from 12 patients showed a positive signal in the genus-specific PCR.

Amplicons formed in real-time PCR were sequenced to confirm or determine the species. All the amplicons but 1 (patient 3) were successfully sequenced and were recognizable as mycobacteria. The materials of 7 patients were positive for *M. haemophilum*, of which 3 were also directly identified in the *M. haemophilum*-specific PCR. No variation was found between the *M. haemophilum* sequences and 100% concordance was found with *M. haemophilum*-specific sequences in the NCBI BLAST database. The latter differentiated this species clearly from others. Other identified species were *Mycobacterium malmoense* (n=1), *Mycobacterium gordonae* (n=2), and *M. marinum* (n=1). Two sequences did not match any known sequences.

For several samples only 1 of the duplicate genus-specific reactions was positive. All of these samples had high Threshold cycle (Ct) values (Ct between 40 and 47). For the samples for which duplicate positive reactions were obtained, the Ct-values were lower (Ct between 32 and 41). The sequences of the positive duplicate reactions were identical.

Conventional mycobacterial diagnostics was requested for only 5 patient materials. All the 5 patients were subsequently diagnosed with a mycobacterial infection. Two were only positive in Zn staining (patients 8 and 28) and 3 were both Zn stain and culture positive (patients 7, 9, and 10). Discrepancies to the current molecular identification were found in the latter 3 patients: 2 patients (patients 7 and 10) were previously diagnosed with a *M. marinum* infection, but the real-time PCR assay identified *M. haemophilum* in both cases. One patient (patient 9) previously had a positive culture for *M. marinum* but yielded no positive signal in real-time PCR. Fresh sections were cut from the materials from these 3 patients and processed again. The new PCR results confirmed our initial PCR results. From 1 patient, 3 different materials were collected. The first sample dated from 1987 and 2 dated from 1993 but were from different locations of the body. All the 3 samples yielded positive signals in PCR but all with different sequences. The sample from 1987 yielded a *M. haemophilum*, 1 from 1993 resembled an *M. gordonae* and 1 sequence did not match any known sequence (Table 3).

A control group was assembled from the biopsies of 30 patients with histologic proven BCC. Of these, 2 materials yielded positive signals for the genus *Mycobacterium* (Ct values: 42.1 and 39.0) in one of the duplicate tests. From these 2 materials paraffin sections were prepared again and tested a second time in the real-time PCRs. Now only 1 sample yielded positive signals, twice in the genus-PCR and once in the *M. haemophilum*-specific PCR (Ct values: genus-specific PCR: 38.9 and 39.3, *M. haemophilum*-specific PCR: 41.1). The other

sample now remained consequently negative in all PCRs performed and could not be confirmed. However, it was only weakly positive the first time round (Ct value: 42.1). No positive reactions were obtained in the *M. avium*-specific PCR. Negative controls (paraffin blocks) remained negative in all reactions.

Discussion.

In the last few years, several molecular assays for the detection of mycobacteria in clinical materials have been developed. Especially, the diagnosis of *Mycobacterium tuberculosis* infection has improved considerably, mainly because of the ability of PCR to deliver results in a clinically relevant timeframe and the resulting improved sensitivity (8–12). For the detection of atypical mycobacteria, several PCR assays have been developed (4,13–17). Also for the detection of mycobacteria in FF/PE materials, molecular assays have been applied to improve the sensitivity of the diagnostics. However, a real-time PCR assay for the direct recognition of nontuberculous mycobacteria in FF/PE specimens has not been evaluated, as described previously.

From the 14 materials that yielded positive real-time PCR signals, 13 were successfully identified as mycobacteria by sequencing of the PCR amplicon. The obtained sequences of *M. haemophilum*, *M. malmoense*, and *M. marinum* were 100% identical to sequences in the NCBI database and were, therefore, clearly identifiable. The sequences of *M. gordonae* showed 99% and 97% homology to known sequences. Because the variability of the ITS sequences in this species is considerable, the obtained sequences can be considered representative for *M. gordonae*. For 2 patient materials, however, the obtained ITS sequences did not yield species identification. Both were recognizable as mycobacteria but could not be ascribed to 1 species. They both resembled the species *M. tuberculosis* and *Mycobacterium asiaticum* with 90% to 94% homology. Probably, as there were few bacteria available to be detected in the samples, several of the cases were on the detection limit of the assay (shown by the high Ct-values) and hence only positive in 1 PCR. However, comparison with the results from the control group shows that these results are associated with granulomatous inflammation. Moreover, a more sensitive assay would require a multi-copy target, which is not available for the detection and differentiation of slow-growing mycobacteria.

The risk of PCR inhibition has not been completely excluded. An internal control could be of benefit, but it could also affect the sensitivity of the assay and has, therefore, not been applied here. The sensitivity of the assay (both genus and species specific) has been determined previously to be 27 CFU per reaction in spiked pus (4). This explains the high Ct values encountered in several samples for which a more sensitive assay would provide stronger PCR reactions.

As the diagnosis of rapid-growers is less difficult, the assay applied here is mainly designed for slow-growers. The detection limit for several rapid-growing species like *Mycobacterium chelonae* can be completely different owing to sequence variability.

Several discrepancies were examined in more detail. In 2 patients *M. haemophilum* was identified by ITS sequencing; whereas, previously *M. marinum* infection had been diagnosed using conventional cultures not suitable for the recovery of *M. haemophilum*. In the first patient (patient 7), the species *M. marinum* was identified biochemically. For the second patient (patient 10) 16S sequencing of the cultured isolate revealed *M. marinum*. Both 16S and ITS are sufficiently discriminative. A possible explanation for these discrepancies is the presence of a mixed infection. Dual infections have been described before and it is suggested that superinfections of previously established granulomas can occur (18–21). For another patient a positive culture for *M. marinum* had originally been obtained but real-time PCR remained repeatedly negative, although it was suitable for the detection of this species. This could be caused by the degrading of DNA, a sensitivity problem or nonhomogenous distribution of the bacteria in the sample.

The prevention of laboratory contamination with mycobacterial bacilli or DNA has been carefully taken into account and precautionary measures have been applied as much as possible. Although it cannot be excluded, it is not expected that positive reactions, obtained during this study, are the result of previous contaminating handling of the materials because the sections that were cut out of the paraffin blocks had all been cleared from the outer parts. However, as no other diagnostic tool can distinguish between contaminant and true positive sample, this assay cannot either.

The control group was assembled from patients with diagnosed BCC. Because mycobacterial infections are not linked to specific histopathologic patterns (22), it is very difficult to exclude mycobacterial involvement in samples of a control group. Our aim of testing this control group was to confirm that the mycobacteria are not a contamination owing to the handling of the sample but are indeed present within the tissue. Two materials from the control group gave positive real-time PCR signals. This could indicate carriership or colonization. However, these patients have conclusive diagnoses of BCC, but seemed to have severe and variable skin disease. In the histologic description of the biopsies inflammatory infiltrates were found besides evidence of BCC. This could be indicative for an additional infection by atypical mycobacteria.

Mycobacterial infection has often been described in immunocompetent patients although the pathogenic properties of most atypical mycobacteria are unclear. In this test group 6 patients were found positive for mycobacterial skin involvement, which had no known underlying disease at the time of diagnosing the skin infection, but it remains possible that they had a genetic disorder predisposing for mycobacterial infections (23, 24) or a yet unrecognized underlying disease. Interestingly, such an immunocompromising condition was recognized in 2 patients after the onset of the skin inflammation.

Striking is the high prevalence of *M. haemophilum* in these patient materials. In 7 of 14 positive biopsies *M. haemophilum* was identified. This species is an important pathogen in cervicofacial lymphadenitis (5,7), has been described as cause of pneumonia (25,26), arthritis (25,26), and bacteremia (27) and is probably underestimated in skin infections as well. *M. haemophilum* was first described as the cause of skin inflammation in 1978 (28). Since then approximately 100 cases of *M. haemophilum* associated skin infection have been

described worldwide. In The Netherlands the first skin infection owing to *M. haemophilum* was described in 1997 (29). Other *M. haemophilum* infections occurred only sporadically until a series of *M. haemophilum*-associated lymphadenitis was recently discovered (7). The histologic manifestation of *M. haemophilum* is generally granulomas with giant cells, lymphocytes, and histiocytes. Necrosis can be absent. The number of bacteria is mostly low. Several publications suggest this species to be underdiagnosed (25,30,31), which is confirmed by the results described here. Although increasing number of laboratories are applying specific culturing methods suitable for the recovery of *M. haemophilum*, some laboratories still do not. In some cases when the culture becomes positive at 30 °C, it is assumed that *M. marinum* is involved without proper species identification (32). It is shown here that *M. haemophilum*-infections were present in The Netherlands years before the first diagnosis.

In conclusion, the application of molecular diagnostics can be useful to diagnose atypical mycobacterial skin infections in FF/PE patient materials. A higher awareness about mycobacterial involvement and the diagnostic possibilities should be raised by both clinicians and pathologists.

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Table 2: description of archival tissue samples and the available diagnostic data.

patient no.	year of biopsy	location biopsy	clinical diagnosis (previous) [a]	diagnosis pathology [b]	underlying disease [c]
1	1998	skin eyelid	furuncle	Zn-negative granulomatous inflammation	-
2	1987/1993	skin calf	recurrent nodular nonsuppurative panniculitis	Zn negative gran inflamm, dd TB or sarcoidosis	Weber-Christians Disease
3	1995	skin cervix	cystadenoma multiplex, -mucinose	granulomatous inflammation	-
4	1995	skin leg	vasculitis?	Zn-negative granulomatous inflammation	RA / Methrotrexaat
5	1995	skin arm	gran annulare, BCC, atyp myc?	Zn-negative granulomatous inflammation	osteoporose
6	1988	skin biopsy	exanthema, viral reaction.	Zn-negative granulomatous inflammation	-
7	1994	skin elbow	granuloma	granulomatous inflammation	-
8	1993	skin hand	chronic inflammation of the skin	gran inflamm, ZN positive fitting TB	-
9	2002	skin hand	swimming pool granuloma	swimming pool granuloma	DM II + kindeyfailure
10	2001	skin hand	mycosis or swimming pool granuloma	Zn-neg atyp gran inflamm, swimming pool granuloma?	-
11	1997	skin face	BCC	BCC, one lesion granulomatous inflamm	-
12	1997	skin cheek	dermatomycosis, CDLE??	Zn-neg, sarcoidosis/ leprosy/ gran inflamm	-
13	1999	skin back	leukemicskin infiltration	gran dermatitis	CLL
14	2000	skin cheek	naevus	Zn-neg, granulomatous dermatitis	-
15	1984	skin leg	vasculitis, panniculitis	mycosis	AML in 1993
16	1987	skin biopsy	epithial cyst	granulomatous inflammation	endometrium carcinoma in 1995
17	1994	skin cheek	actinic keratosis, eczema	granulomatous inflammation	-
18	1991	skin arm	kerato acanthoma	ulcerating granulomatous inflammation	kidney transplant
19	1988	skin arm	pseudolymphoma	gran inflammation reactive but secundary?	-
20	2001	skin wrist	chronic inflammation of the skin	Zn-neg, gran inflamm/ sarcoidosis	-
21	1996	skin side	vasculitis	chronic inflammation, persistent insect-bite	SLE
22	1986	skin biopsy	lichen nitidus/striatus	chronic inflammation	-
23	1992	skin eyelid	chalazion	chalazion	-
24	1989	skin eyelid	granuloma	chalazion (zn-neg)	-
25	1999	skin eyelid	granuloma	granulomatous inflammation (corpus alienum)	-
26	1998	skin eyelid	-	granulomatous inflammation (chalazion?)	-
27	1996	skin eyelid	chalazion	chalazion	RA
28	2002	skin arm/leg	chronic inflammation, sarcoidosis/ mycobacterial skin infection	Zn positive granulomatous inflammation	RA + lymphoedema / Methrotrex + prednisone
29	2004	skin arm/leg	vasculitis, sarcoidosis, infectious ??	ulcerating chronic and granulomatous inflammation	DM I
30	2004	skin hand	cellulitis + arthritis		RA / Methrotrexaat

[a] clinical diagnosis is given by a dermatologist and confirmation is requested from the pathologist. [b] diagnosis is determined by a pathologist by means of microscopical examination of the biopsy. [c] any given information about possible immune-influencing disease or treatment.

Table 3: description of results.

previously performed conventional diagnostics [a]			retrospectively performed real-time PCR and sequencing of the obtained amplicons [b].			
patient no.	**Zn-stain**	**mycobacterial culture (and species identified)**	**PCR genus 1**	**PCR genus 2**	**PCR *M. haemophilum***	**sequencing of PCR product and BLAST results.**
2	-	n.r.	-	38,3	-	*M. haemophilum:* 178/178 (100%) AY579400.1
(3 biopsies)	-	n.r.	38,9	38,7	-	*M. gordonae:* 134/138 (97%) AB026692.1
	-	n.r.	-	37,9	-	143/155 (92%) AY722098.1 *M. asiaticum*; 103/106 (97%) Z17211.1 *M. gastri*; 156/173 (90%) X58890.1 *M. tuberculosis*
3	n.r.	n.r.	47	-	-	no recognisable *M. spp*
7	+	+ (*M.marinum*)	41,9	40	-	*M. haemophilum:* 141/141 (100%) AY579400.1
8	+	-	-	45,5	-	*M. marinum:* 168/168 (100%) AJ315573.1
9	+	+ (*M.marinum*)	-	-	-	-
10	+	+ *(M.marinum*)	38,1	-	36,6	*M. haemophilum:* 110/110 (100%) AY579400.1
11	n.r.	n.r.	-	41	-	146/155 (94%) AY722098.1 *M. asiaticum*; 162/175 (92%) AE000516.2 *M. tuberculosis*
15	n.r.	n.r.	41,5	-	-	*M. haemophilum:* 178/178 (100%) AY579400.1
16	n.r.	n.r.	45,0	39,6	-	*M. malmoense:* 171/171 (100%) AF367025.1
25	n.r.	n.r.	-	36,8	-	*M. gordonae:* 161/162 (99%) AY604571.1
28	+	-	37,1	36	35,4	100% *M. haemophilum:* 130/130 (100%) AY579400.1
29	n.r.	n.r.	41,2	39,2	-	100% *M. haemophilum:* 144/144 (100%) AY579400.1
30	n.r.	n.r.	-	39,5	36,6	100% *M. haemophilum:* 140/140 (100%) AY579400.1

[a] conventional mycobacterial diagnostics performed by the department of pathology or medical microbiology. n.r. = no mycobacterial diagnostics requested.

[b] ITS directed genus-specific (performed twice) or *M. haemophilum*-specific real-time PCR result. Either negative or noted in Ct-value (the higher the Ct-value, the weaker the PCR reaction). Blast results are noted in length of successfully obtained bi-directional sequence and percentage of homology with the most likely Blast hits.

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Chapter 6.

Amplified Fragment Length Polymorphism (AFLP) analysis of human clinical isolates of *Mycobacterium haemophilum* from different continents.

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Abstract.

The role of the species *Mycobacterium haemophilum* as a pathogenic non-tuberculous microorganism is becoming more well-defined with the use of specific detection methods. However, epidemiological investigations of this species are still scarce. We analyzed the genetic diversity of *M. haemophilum* by amplified fragment length polymorphism (AFLP) typing and compared strains from different parts of the world. A total of 128 strains including 41 from the United States, 51 from Australia, 28 from Europe and 8 strains from Israel were compared using AFLP methodology. Two restriction enzymes (*Mse*I and *Eco*RI) and one selective primer were applied and provided a high discriminatory power. Clusters of strains with identical AFLP patterns were observed from the Netherlands, New York and Australia which could indicate a possible common source. No clear clustering based on continental origin was determined. However, types were restricted to geographic areas and not found on other continents. A high genetic stability within the species was demonstrated by the long term existence of a single type.

Introduction.

Mycobacterium haemophilum was first described in 1978 following isolation from cutaneous lesions of a woman with Hodgkin's disease [1]. The organism is unusual in that it has an optimal temperature for in vitro growth between 30° and 32° C, and a requirement for supplementation of growth media with iron containing compounds, including ferric ammonium citrate or hemin. *M. haemophilum* most often causes joint, cutaneous and pulmonary infections in immunocompromised patients and lymphadenitis in immunocompetent children, although infections in immunocompetent adults do occur [2, 3]. In recent studies, *M. haemophilum* was identified as the second most common mycobacterial pathogen causing cervicofacial lymphadenitis in children in The Netherlands [4, 5]. There is no evidence of patient-to-patient spread of the organism and *M. haemophilum* infections appear to be acquired from the environment, most likely water and bio-films [6-8]. Clusters of cases representing a variety of clinical manifestations from Australia [9], Israel [10], New York City [11] and The Netherlands [4] have been reported. Molecular epidemiology studies have suggested clonal geographical clustering [12, 13]. To analyze the molecular relatedness and global diversity of *M. haemophilum*, a collection of isolates from Australia, Europe, Israel and the United States were typed by amplified fragment length polymorphism (AFLP). The AFLP method is based on the selective amplification of genomic fragments after digestion by one or more restriction enzymes and visualized by band patterns. The genomic mutations that cause a restriction site to emerge or disappear or that change the length of the fragment between the restriction sites, give rise to differences in band patterns [14].
Compared with the PFGE as a gold standard for typing, AFLP has been shown to be as discriminative or even more discriminative for certain mycobacteria [15, 16].

Materials and Methods.

Mycobacterial strains.

Isolates of *M. haemophilum* were obtained from Leiden University Medical Center, Leiden, The Netherlands; Memorial Sloan-Kettering Cancer Center, New York, USA; Western Australian Centre for Pathology & Medical Research, Nedlands WA, Australia; Rabin Medical Center, Petach-Tiqva, Israel; Institute of Microbiology, University of Oslo, Oslo, Norway; and the Laboratory for Microbiology and Virology, Ospedale Careggi, Firenze, Italy. Isolates were shipped either freeze-dried or as a culture, and upon arrival in Leiden cultured on Lowenstein-Jensen medium with added FAC (ferric ammonium citrate) and incubated at 30 °C. Other mycobacterial species were cultured on Lowenstein-Jensen medium with added PACT (polymyxin B, amphotericin B, carbenicillin, and trimethoprim) and incubated at 35 °C. (Bectondickinson, Alphen a/d Rijn, The Netherlands). After sufficient growth, aliquots of the organisms were stored at -20°C pending DNA-extraction. In total, 182 *M. haemophilum* strains were collected of which 54 failed to grow. The remaining 128 *M. haemophilum* strains (including two strains tested in duplicate) were subjected to genotyping and are described in Table 1.

Seven mycobacterial species, encompassing *M. haemophilum* and six other mycobacterial species were tested for species differentiation based on the AFLP protocol. Closely related species to *M. haemophilum*, based on combined phylogenetic analysis of different genomic targets, were *Mycobacterium malmoense* and *Mycobacterium heidelbergense* [17]. Species that appeared closely related based on only 16S rRNA gene phylogeny were *Mycobacterium szulgai* and *Mycobacterium bohemicum* [18, 19]. Mycobacterial species with less phylogenetic relations to *M. haemophilum* were *Mycobacterium interjectum* and one unidentified species, OMS011. All of these strains were clinical isolates from the Leiden University Medical Center collection or provided by the National Tuberculosis Reference Center (RIVM) and all were identified by the RIVM by 16S rRNA gene sequencing [20].

AFLP procedure optimization.

Optimization of the procedure included 1) Different methods of DNA-extraction: QIAamp DNA mini kit (Qiagen Benelux, Venlo, Netherlands), MoBio® Ultraclean Microbial DNA isolation Kit (Sanbio, Uden, The Netherlands), ZR Genomic DNA kit and DNA II kit (Zymo research, Orange, CA, USA) and the Boom extraction method [21]. All DNA extraction methods were tested according to the manufacturers protocols, with or without sonication and proteinase pre-steps. 2) Variations in the AFLP protocol: incubation time and cycling conditions. Strains were re-cultured several times to provide fresh isolates for the different trials during the optimization process. During optimization of the typing procedure, DNA extraction was performed in triplicate.

DNA-extraction.

For DNA extraction, one or more colonies were suspended in molecular grade water and sonicated 3 times 5 seconds at 8 microns (amplitude) prior to an overnight incubation with

proteinase K (2 mg/ml) at 50 °C (Invitrogen, Breda, The Netherlands). DNA was extracted using the MoBio Ultraclean Microbial DNA isolation Kit (Sanbio) and eluted in 100 µl (50 µl elutionbuffer with 50 µl molecular grade water). Final concentration and purity of DNA was confirmed with the Nanodrop ND 1000 (Nanodrop technologies, Wilmington, Delaware USA), and for successful patterns a concentration range of 5-20 ng/µl (5 µl/reaction) and a purity OD range of 1.50-2.30 (260/280 coefficient) was used.

Genotyping.

The AFLP procedure was as follows: 3 hr 37 °C (digestion and simultaneous ligation of the adaptors). The restriction/ligation mix contained 10x T4-ligation buffer (Westburg, Leusden, The Netherlands), 0.5 M NaCl, 0.5 µg BSA (Westburg), 2 pmol *Eco*RI adaptor (Eurogentec, Maastricht, The Netherlands), 20 pmol *Mse*I adaptor (Eurogentec), 80 U T4-ligase (Westburg), 1 U *Mse*I (Westburg) and 1 U *Eco*RI (Westburg) in a 5 µl volume per reaction and 5 µl DNA. Subsequently, the restriction/ligation product mixture was diluted 20x in 0.1 Tris-EDTA-buffer before amplification. PCR was carried out in 10x PCR buffer (Applied Biosystems, Foster City CA, USA), 15 nmol MgCl2, 2 nmol DNTP's, 20 ng primer Eco-0 (5'-GACTGCGTACCAATTC-3') (Applied Biosystems), 60 ng primer Mse-C (5'-GATGAGTCCTGAGTAAC-3') (Eurogentec) and 1 U Ampli-taq polymerase (Applied Biosystems). 5 µl PCR mix was added to 5 µl of diluted restriction/ligation product mixture. The PCR program started with an activation of 2 min at 72 °C, 35 cycles with a touchdown principle: 30 sec 94°C, 10 sec 65-56 °C, 1 min 72 °C (annealing first cycle at 65 °C, 12 cycles 0.7 degrees down per cycle, followed by 23 cycles at 56 °C), and a final extension of 10 min at 72 °C. 2.5 µl of PCR product was added directly in 22.5 µl HiDi formamide (Applied Biosystems) with ROX500 (Applied Biosystems) as internal marker and analyzed in an ABI3100 genetic analyzer (Applied Biosystems).

Analyzing patterns.

AFLP patterns were analyzed and calculations were performed with the Bionumerics software (Applied Maths, Sint-Martens-Latem, Belgium). During optimization of the protocol, the AFLP patterns (PCR fragments length distribution) were analyzed with Pearsons coefficient and the unweighted pair group method with mathematical averaging (UPGMA) cluster analysis. Application of the Pearsons coefficient resulted in intensity differences of bands which affected the similarity calculations for strains with identical and highly similar band patterns. Therefore, another approach was chosen for the study of the *M. haemophilum* strain collection. Digitized bands were assigned to the AFLP fragments and the DICE coefficient and UPGMA cluster analysis were applied to the band patterns. For a subset of three *M. haemophilum* strains, Pearson analysis was compared with DICE analysis (Fig 2a and 2b). In order to exclude subjective interpretation duplicate AFLP reactions were included of each strain of the *M. haemophilum* strain collection. The average amount of bands per pattern is 50. Bands between 85 and 350 nucleotides have been included in the analysis. A strain was considered different from another when at least 1 band is different in the DICE pattern, since one band difference is associated with a detectable mutational event [22]. One

band difference was designated a “variant type”, two or more bands difference was considered a “type”. Designation of types was given by continent of origin and did not yield information concerning the genetic distance (e.g. type-designation USA A has no correlation with designation Aus A).

Results.

Optimization of the molecular typing procedure.

The optimization process utilized the method as described in Materials and Methods. A panel of strains was used to validate the different levels of discrimination. Seven different species, including *M. haemophilum* (strain Netherlands 5) were tested in triplicate and compared (Figure 1). All patterns were calculated with the Pearsons coefficient. The similarity between species was well below 15% with this AFLP protocol. Therefore, the applied method was clearly capable of discriminating different mycobacterial species. The pattern homology between different DNA extracts of the same strain was 90% or more when calculations were made with Pearsons coefficient (Figure 2a). The variability between these DNA extracts after assignment of digitized bands for DICE calculation showed a similarity of 100% (Figure 2b).

Figure 1. AFLP patterns of different species in triplicate, calculation with Pearsons coefficient
Mycobacterium spp is the unidentified species OMS011, which clearly not belongs to one the species included in the analysis. The similarity between different species is well below 15%, including the standard deviation.

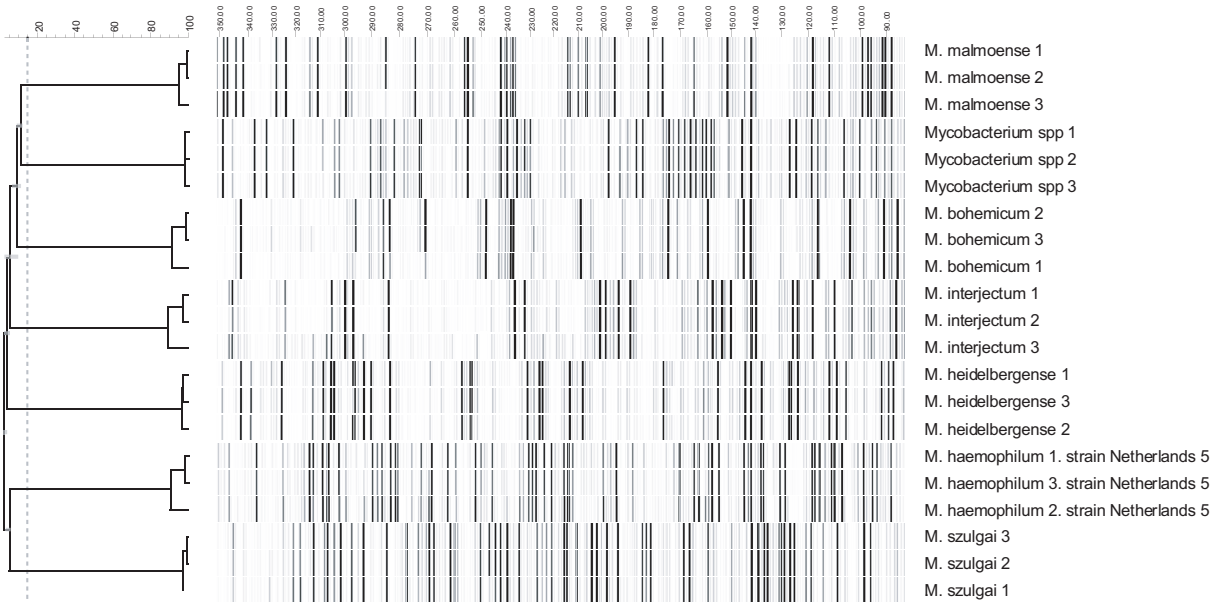
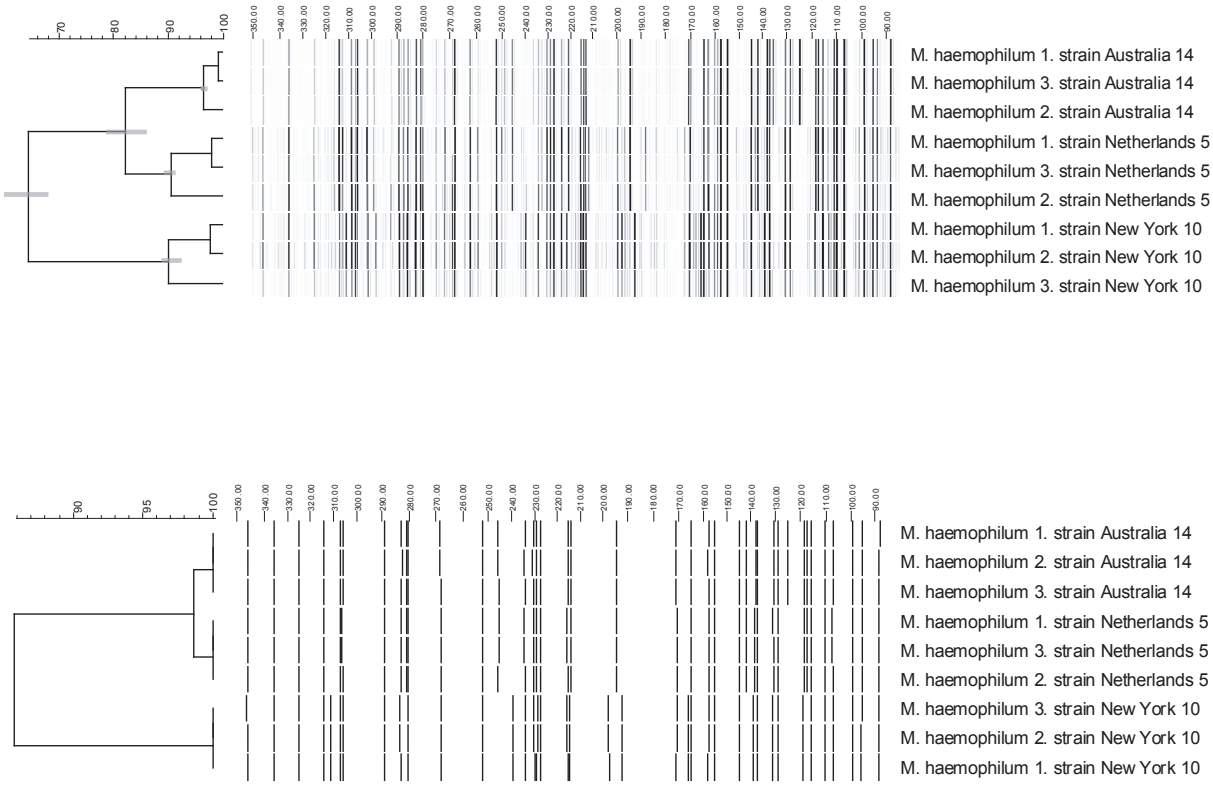


Figure 2. AFLP patterns of 3 *M. haemophilum* strains in triplicate. A) calculations with pearsons coefficient, B) with digitilised bands, calculated with DICE.



Molecular typing.

The AFLP procedure (including Dice analysis) was used for typing 128 clinical isolates of *M. haemophilum* representing four geographic areas; 8 were from Israel, 41 from the United Sates, 28 from Europe and 51 from Australia (Table 1).

Figure 3 (supplemental data) depicts pattern comparisons of the collection of 128 *M. haemophilum* strains. The lowest similarity between all strains was 78%. No clear clustering based on continental origin was observed. All types were unique to their continent of origin and not found in other continents. However, the types from each continent were not necessarily the most similar to each other. For instance: Type USA A was restricted to New York but other USA types could be located in other clusters of the dendrogram. Only one band difference was observed between types Eur A and Aus A, and are therefore considered variant strains of one another.

Table 1: Clinical isolates of *Mycobacterium haemophilum.*

country or continent	number of strains	year of isolation	AFLP types (no of strains)	AFLP type variants included
USA	41	1991-2001	USA A (29)	4
		1990-1991	USA B (5)	
		1998	USA C (1)	
		1991-1993	USA D (3)	
		2000	USA E (2)	
		1984	USA F (1)	
Israel	8	1996-2003	East A (2)	1
		2003	East B (1)	
		1971	East C (1)	
		2003	East D (1)	
		2003-2004	East E (2)	1
		1996	East F (1)	
Australia	51	1987-2003	Aus A (23)	3
		1987	Aus B (1)	
		1986-2004	Aus C (16)	
		1990-1996	Aus D (2)	
		2003	Aus E (1)	
		2004	Aus F (1)	
		2000	Aus G (1)	
		1977	Aus H (1)	
		unknown	Aus I (1)	
		unknown	Aus J (1)	
		unknown	Aus K (1)	
		unknown	Aus L (2)	
Europe	28	2003-2005	Eur A (24)	1
		2004	Eur B (1)	
		2004	Eur C (1)	
		2003	Eur D (1)	
		2000	Eur E (1)	

Forty-one strains were from the USA; 38 from 7 New York City hospitals (1989-2001), 2 from Seattle (2000) and 1 from Phoenix (1984). All of the patients except one were adults, and most had AIDS or cancer and/or were transplant patients. There were 6 AFLP types (USA A-F). USA A, including a type variant, was the most prevalent type (29 isolates) and was only found at New York City hospitals. Among the New York isolates, 15 were from patients at one hospital. Type USA B was found in 4 patients at two New York hospitals, and all 4 patients possibly acquired the infection outside New York City, in the late 1980's (according to their physicians). Type USA C was isolated from a lung nodule of a patient at a New York hospital, but the immunocompetent patient probably acquired the infection while at home in the Philippines [23]. Type USA D was only found in 3 AIDS patients at one New York City hospital early in the 1990's. Type USA E was isolated from two patients at a hospital in Seattle; one patient with AIDS and the other with diabetes, in 2000. Type USA F was isolated from a renal transplant patient in Phoenix in 1984.

Of the 8 strains from Israel, 7 were isolated from lymph node specimens between 1996 and 2003 and the other represented the first known isolate of *M. haemophilum* (ATCC 29548), from 1971. The ATCC strain from Israel clustered with other strains from Israel (94%), but identical patterns were not observed. The 8 isolates could be divided in two clusters of which one cluster forms the most distinct cluster of strains of this collection; 77% similarity with all others.

Within the collection of 51 strains from Australia, two large type clusters of identical patterns, Aus A and Aus C and 12 strains representing 10 other types could be recognized. Type Aus A consisted of 23 strains, including 3 strains with type Aus A var, a type that remained unchanged from 1987 thru 2002. The type Aus C included 16 strains and remained unchanged from 1986 thru 2004. The minimum similarity between the other Australian types, Aus B and Aus D-L, was 85% and appears scattered throughout the dendrogram. About half of the Australian isolates were from immune compromised patients and isolates were recovered from variable sites.

The European collection of 28 strains consisted of 26 from the Netherlands of which 22 from the Amsterdam region and 4 from other regions of the Netherlands, one from Norway and one from Italy. Twenty-three Netherlands strains yielded type Eur A of which 21 strains were from the Amsterdam region and all from children with cervicofacial lymphadenitis. The Norwegian strain differed in only one band with the Amsterdam type and is therefore designated type Eur A var. Four other types, Eur B-E, were identified once for each type, of which one (Eur B) was from the Amsterdam region and one (Eur E) was from Italy.

Discussion.

Molecular methods play an increasingly important role in our understanding of epidemiological strain characteristics and patient related significance of infectious micro-organisms. Although several species of mycobacteria have been examined extensively by molecular methods, there is very limited information regarding the diversity of this organism. Previously, two molecular methods, a Restriction Fragment Length Polymorphism (RFLP) assay [12] and a Pulse Field Gel Electrophoresis (PFGE) assay [13], were used to type USA isolates, most from the New York City area, of *M. haemophilum*. These two studies demonstrated genetic clustering within the tested strain collections which could indicate a common source. The RFLP showed similar discriminatory power compared to the PFGE [12, 13]. We compared the results of 15 isolates typed by RFLP in New York in 1994 with our results of AFLP and found that 5 different RFLP types corresponded with 5 different AFLP types (data not shown) [12]. Therefore, the discriminatory power of all three methods seem similar.

The main objective of the present study was to investigate the level of genetic diversity within and between isolates of *M. haemophilum* collected from several part of the world and typed by AFLP. Prior to comparing the different isolates of *M. haemophilum*, a validation procedure was conducted to determine the optimal discriminatory power of the AFLP method for *M.*

haemophilum at the strain and species level. The enzyme combination with one selective primer showed a suitable fragment distribution for this species. Because *M. haemophilum* has a strong tendency to clump, a stringent protocol was necessary to obtain high quality and quantity of DNA from bacterial colonies for reproducible AFLP patterns: A combination of sonication, enzymatic and mechanical disruption resulted in pure DNA and subsequent reproducible AFLP patterns. Although Pearsons analogue analysis of the AFLP patterns revealed similar clustering, DICE analysis enabled a clearer visualization of the true differential patterns of a large panel of *M. haemophilum* isolates. Among the 128 strains typed in this study, there were indications of clustering. Strains from the Amsterdam region (Netherlands 1-21) resulted in one identical AFLP pattern, type Eur A, while patterns of strains from other regions of the country varied, thus confirming the epidemiological relatedness of the Amsterdam strains. Most strains from the New York area yielded an identical pattern as well, type USA A, which was observed from 1989 to 2000. Among Australian strains, two clusters were observed. One of these clusters remained unchanged for 18 years (type Aus C), and the other for 15 years (Aus A), suggesting an extremely low evolutionary rate for this mycobacterium, and perhaps the ability of the organism to create a stable niche. Previously, the persistent colonization of the species M. avium was demonstrated in water distribution-systems, which illustrates the ability of mycobacteria to prevail long-term in a stable niche [24]. A similar water borne niche for *M. haemophilum* might exist.

The variety between strains is relatively high between different types (minimal similarity of 78%), while the types Aus A and Eur A are variant strains of each other, suggesting an evolutionary link between these two types. Also, type Aus A appears common in immune competent patients ($p<0.001$) and this could indicate differences in virulence between types. The immuno status of patients was equally distributed among the 51 isolates from Australia, in contrast to the isolates from other continents. Of 23 type “Aus A” and type “Aus A variant” strains, 19 (83%) were isolated from immuno competent patients, while only 4 strains from immuno competent patients yielded another type. In addition, the type “Eur A” is solely found in immune competent children (no background information is available for the Norwegian strain with type “Eur A variant”). Two Netherlands strains with other types (201: type “Eur C” and 200: type “Eur D”) had both been isolated from the only two Netherlands patients with immune compromising conditions.

It is difficult to define the exact origin of a clinical isolate due to a lack of information regarding patient travel history, out-patient or in-patient history, precise clinical history, etc. Also, the incubation period for the infections is probably, in some cases, extensive, thus the exact origins of the strain might vary from the documented patient history. For example, among the Australian strains two large clusters of similar strains have been determined of which a few isolates have been encountered in geographically distant locations from the rest of the strains. Therefore, a common source or geographical linkage to type distribution cannot be excluded. The source and spread of *M. haemophilum* infections is still in question.

It has been difficult to recover the organism from the environment, although the scant literature suggests a water and/or biofilm source [6, 8]. This study and the New York studies suggest a clustering of cases both by geography, patient types, clinical presentations and time period of the cluster. For example, the New York City cases began, rather abruptly, in 1990, were mostly found in adult, immunocompromised adults, and most with AIDS or who had received bone marrow transplants. The methods of diagnostics in New York had not been adjusted prior to this abrupt rise of *M. haemophilum* isolates. Investigators of the *M. haemophilum* infections in the Amsterdam region suggested a public indoor swimming pool or home water supplies as possible sources of the infection. The home water supply of one child with lymphadenitis was examined for the presence of *M. haemophilum*, but real-time PCR and culturing methods yielded only the species *M. kansasii*. That investigation was performed several months after onset of the disease and the home water supply can therefore not be excluded as infection source.

As reported cases of *M. haemophilum* increase, additional epidemiologic information will become available, and we now have the molecular tools to further define the global diversity of this organism.

Figure 3. AFLP typing results of clinical *Mycobacterium haemophilum* isolates in dendrogram calculated with DICE coefficient. Submitted as supplement to this article. (included as double page in thesis, separate file)

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Chapter 7.

Lymphadenitis in children is caused by *Mycobacterium avium hominissuis* and not related to 'bird-tuberculosis'.

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Abstract.

Mycobacterium avium is the most commonly encountered *mycobacterium* species among non-*Mycobacterium tuberculosis* complex (NTM) isolates worldwide and frequently causes lymphadenitis in children. During a multi-center study in the Netherlands that was performed to determine the optimal treatment for mycobacterial lymphadenitis, concern was expressed in the media about the possible role of birds as sources of these *M. avium* infections, referred to as 'bird-tuberculosis'. To examine the involvement of birds in mycobacterial lymphadenitis, 34 *M. avium* isolates from lymphadenitis cases were subjected to IS*1245* restriction fragment length polymorphism (RFLP) typing. This genotyping method enables the distinction of the sub-species *M. avium* subsp. *hominissuis* and the "bird-type" *M. avium* spp. *avium*.
Highly variable RFLP patterns were found among the lymphadenitis *M. avium* isolates and all belonged to the *M. avium hominissuis* subspecies. A relation to pet-birds in the etiology of mycobacterial lymphadenitis could not be established and the source of the infections may be environmetal.

Introduction.

While the absolute number of clinical isolates of non-tuberculous mycobacteria (NTM) has worldwide increased gradually with the upcoming of AIDS, the number of isolates in The Netherlands remained stable in the last decade. In the period from 1996 to 2005, 4640 NTM isolates were collected at the national tuberculosis reference laboratory (RIVM), of which 1484 (32 %) belonged to the *M. avium* complex. *Mycobacterium avium* is not only a well-known cause of pulmonary disease, especially in immunocompromised patients, but is also the most commonly encountered species in mycobacterial lymphadenitis in children. The exact incidence of *M. avium* lymphadenitis is difficult to assess but is estimated at 1.15 per 100,000 children (1-18 years) in the Netherlands *[1]*.
From 2001 to 2004, a nation-wide multi-center to examine the optimal treatment of NTM mycobacterial cervicofacial lymphadenitis (surgery vs medical therapy) was performed *[2]*. In this study it was found that 94 (70%) of 135 diagnosed *mycobacterium* infections were caused by *M. avium [3]*. As a consequence of this study, numerous articles appeared in regional and national newspapers in the Netherlands in which the concern was expressed that mycobacterial lymphadenitis in children is a form of 'bird-tuberculosis'. This is based on the conventional, but still widely accepted dogma that *M. avium* infections in humans are derived from birds. Moreover, many patients included in the treatment study and diagnosed with *M. avium*-associated lymphadenitis, appeared to keep pet-birds in the household or had an otherwise frequent contact with birds, as was demonstrated in a questionnaire for epidemiological purposes *[3]*. However, no demographic information on the presence of pet-birds in the general population in The Netherlands is available and it therefore remains unclear whether there is any correlation between household-pets and lymphadenitis in children.

With the improved possibilities for the diagnosis of mycobacterial infections, new (sub-) species have been identified and new insights in taxonomy are being developed. Previously, in the *M. avium- M. intracellulare* complex (MAC), 28 serotypes were distinguished *[4].* The species *M. avium* consists of the subspecies *M. avium* ssp*. avium*, *M. avium* ssp*. silvaticum* and *M. avium ssp. paratuberculosis [5]*. Restriction Fragment Length Polymorphism (RFLP) typing, using insertion sequence IS*1245* as a probe is a standardised epidemiological tool for the molecular typing of *M. avium [6, 7]*. The targeted insertion element IS*1245*, is specific for the species *M. avium*, and is present in a highly variable number and location in the genome of these bacteria.

Application of this typing method led to the recognition that *M. avium* isolates from birds of a wide variety of species exhibited one identical pattern *[7, 8, 9, 10]*. This three band pattern was designated the "bird-type" RFLP pattern. To exclude the raised confusion in the epidemiology of *M. avium* infections, the "bird-type" *M. avium* strains were further characterized and this sub-species was named *M. avium avium [9]*. The *M. avium* isolates from human- and porcine sources showed highly polymorphic IS*1245* RFLP patterns and were designated "*M. avium hominissuis*" *[9]*.

In this study the contribution of the bird-type *M. avium avium* to lymphadenitis in children in The Netherlands is examined to confirm or exclude the involvement of birds in the aetiology of this disease.

Materials and Methods.

Strains and culturing methods.

M. avium strains were isolated from children included in the lymphadenitis treatment study. Inclusion criteria for this study were an enlarged cervicofacial lymphadenitis for a period longer than 3 weeks, with negative serology for other infectious causes of chronic lymphadenitis. Excluded were patients with an immunosuppressive therapy or disease *[3]*. In addition, skin testing with *M. tuberculosis* complex- and 3 NTM sensitins was performed *[11]*. Diagnosis was confirmed by culture and/or specific real-time PCR, performed on lymph node aspirates or surgically obtained tissue biopsies *[3, 12]*. Culturing was performed at 35 °C in liquid MGIT medium enriched with PANTA and OADC or on solid Löwenstein Jensen medium (Becton Dickinson, Alphen a/d Rijn, The Netherlands). Strains were sent to the RIVM in Bilthoven and processed in IS*1245* RFLP typing (Table1).

Species identification.

Real-time PCR was performed as described previously *[12]*. A genus-specific and a *M. avium*-specific PCR were performed on all isolated strains as well as on DNA extracts of bird materials. When a positive signal was found in exclusively the genus-specific real-time PCR, the amplicon was purified using a gel-extraction kit (Qiagen, Venlo, The Netherlands) and the PCR product was sequenced to determine the species the respective bacteria belonged to.

Sequencing was performed on basis of the PCR primers using the Big Dye Terminator ready reaction mix (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands) and analysed on an ABI 3100 automatic Sequencer (Applied Biosystems). A second identification was performed at the RIVM; either by 16S-sequencing *[13]* or by a reverse line blot (InnoLipa V2, Innogenetics, Gent, Belgium) to confirm the identity of the *M. avium* strains.

Molecular typing.

A database has previously been assembled by the RIVM of RFLP patterns from *M. avium* isolates from humans in the Netherlands in the period 1996-1997, or from animals and soil in a period of decades. IS*1245* RFLP typing was performed as described previously *[6, 10]*. RFLP patterns of 34 *M. avium* isolates from lymphadenitis cases were analysed and compared with known patterns in the database using Bionumerics software (Applied Maths BVBA, Sint-Martens-Latem, Belgium).

Bird materials.

Materials of pet-birds from two patients diagnosed with *M. avium*-associated lymphadenitis were collected. One patient owned two parakeets and kept them inside the house. Another owned 8 parakeets and they were kept in an aviary in the yard. Considering the age of the included children it is not expected that the children were the direct care-takers of the birds. The parakeets had different ages, gender and breed. The birds did not show any sign of illness. Swabs were collected from the cloacae of the parakeets and faeces were scraped from the bottom of the cages (as fresh as possible). All samples were divided equally in two portions and examined by two different institutes (CIDC Lelystad and LUMC, Leiden, The Netherlands). Swabs were processed as clinical materials *[12]* and decontaminated once with the Nalc-NaOH method prior to culture. Faeces were decontaminated twice prior to culture, but otherwise processed identically, including the culturing. One institute also performed real-time PCR for the direct detection of mycobacterial DNA in the bird materials.

Results.

Human materials.

In the lymphadenitis treatment study, 210 lymph node biopsies of cervicofacial lymphadenitis patients were examined for the presence of mycobacteria by culture and real-time PCR *[2, 12]*. Mycobacterial infection was diagnosed in 138 children, of which 94 (70%) were caused by *M. avium*. Sixty positive cultures were obtained in the four years period of the study. All positive *M. avium* cultures from between November 2001 and June 2004 (n=34) were sent to the RIVM and subjected to RFLP typing.

Analysis of the background information of the 34 children demonstrated only one child to have ducks in the immediate vicinity of its house and none of the 34 children had been into direct contact with horses, chickens, geese, pigs, goats and pigeons. Twenty-one (62%) children, however, played in a sandpit, 13 (38%) children swam in a closed swimming pool

(none in open water) and a total of 18 (53%) children had pet animals of which in 33% of the cases this concerned birds (Table1).

Molecular typing.

In total 34 *M. avium* isolates from the patients included in the lymphadenitis treatment study were subjected to IS*1245* RFLP typing and the results are depicted in figure 1. Among the *M. avium* isolates of the lymphadenitis patients a high variety of multi-banded IS*1245* RFLP types was found and all belonged to the ssp. *avium hominissuis* and not to *M. avium* ssp. *avium*. The obtained IS*1245* RFLP patterns were compared with the Dutch IS*1245* RFLP database and showed similarity with the patterns of previously described genotype families in The Netherlands (clades 7501-7509) *[14]*. No association was found between the clades -to which the isolates were assigned to- and pet-birds or other pet-animals, time of onset of the disease, playing in sandpits, visiting the children's farm, swimming or location of housing.
Also, no geographical clustering could be found for these strains, the residence of the respective patients appeared to be completely random in The Netherlands. In fact, the RFLP patterns of several *M. avium* strains were 95-100% similar to that of another strain while no correlation was observed with any patient factor (Table 1: designation α-ε).

Bird materials.

Culture and real-time PCR performed on the bird materials revealed two faeces specimens positive for mycobacteria. One was only detected by culture and was identified as *M. terrae*. The other one was concordantly detected by culture and real-time PCR and identified as *M. malmoense*. Both identifications were done by sequencing the Internal Transcribed Spacer (ITS) region and comparison in the NCBI database (http://www.ncbi.nlm.nih.gov).

Discussion.

Atypical mycobacteria are ubiquitous present in our environment, but few clinical cases have so far been directly linked to their natural reservoirs *[15, 16, 17]*. Traditionally, lymphadenitis in children caused by *M. avium* is indicated as 'bird tuberculosis', because historically *M. avium* bacteria were thought to have an association with birds. Because a striking percentage of the lymphadenitis patients in the Netherlands were in contact with birds, the concern was expressed in the media that birds were the source of infection for these lymphadenitis cases. This was not only driven by the fact that children naturally play outside in sand and soil, but also by the fact that 8 of 34 children were in households where pet birds were present. Therefore, in this study, *M. avium* isolates from the lymph nodes of 34 children with lymphadenitis were subjected to IS*1245* RFLP typing and compared. The main objective in this comparison was to verify the occurrence of the bird-type *M. avium* within this group of patients. Although the patient strains revealed a high degree of variation in the IS*1245* RFLP patterns, the bird-type was not found in any of the 34 *M. avium* isolates which excludes the

possibility that bird-type strains are the source of infection. Moreover, no *M. avium* could be isolated from bird materials collected from the patients' pet birds.
In several studies this DNA typing method has been applied for epidemiological purposes *[7, 8, 14, 18, 19, 20]* and almost all *M. avium* isolates from birds revealed a characteristic bird-type 3-banded IS*1245* RFLP pattern, while multi-banded RFLP types were very rarely encountered in bird isolates. The method has been investigated for its stability of patterns *[21]* and its specificity for the IS*1245* has been discussed in several papers *[7, 22]*.

Earlier studies [*partially published: 14]* conducted by the RIVM demonstrated a high degree of similarity between human and porcine *M. avium* isolates. IS*1245* RFLP typing of 146 isolates from humans in The Netherlands- the total number of *M. avium* strains collected in the year 1996 and 1997- yielded highly variable DNA fingerprint patterns and a large part of the isolates could be assigned to nine different clades with a high degree of similarity among the RFLP patterns. A large part of the isolates both from human and porcine sources were grouped in the same clades.
The *M. avium* isolates of this study were scattered over several clades as was similarly found for the lymphadenitis isolates from the period 1996-1997. Six strains from this study belonged to clade 7502 in which in previous studies the majority of strains from HIV-infected patients clustered (unpublished: Thesis Schneider, Bilthoven, the Netherlands). The high variability among the RFLP patterns may demonstrate the presence of high numbers of *M. avium hominissuis* bacteria in environmental sources and/or variability in source of infection.
While a high degree of variation was generally observed between the typed *M. avium* strains of the lymphadenitis cases, several patient strains were identical to one-other strain. Comparison of the strains to the IS*1245* RFLP database showed one strain from 1996 to be identical to one strain from 2004. This indicates the long-term genomic stability of the representative strains and the ability of these bacteria to prevail in a stable niche.
The bird-type *M. avium* is pathogenic to birds *[7, 23]*. However, the birds kept in the patients' home showed no signs of illness. It is, however, still possible that pet-birds are carriers of *M. avium* subsp. *hominissuis* strains and regularly transmit these *M. avium* bacteria to children causing lymphadenitis in a significant number of them. In this study, we were not able to grow *M. avium* from bird faeces and cloaca swabs. Therefore, this hypothetical carriership of birds could not be confirmed. Because the pilot experiment with bird materials was very small in size and the materials were not collected at time of onset of the disease, the possibility that non-diseased pet-birds are carriers of *M. avium hominissuis* cannot be completely excluded. The pet-birds can also be an indirect cause; because *M. avium* is ubiquitous in soil, water, animals, food and sawdust *[14, 15, 19, 24]*, these bacteria may well spread directly from such sources.
No other contact with farm animals than an occasional trip to the children's farm (33%) was apparent in these patients. Therefore, pigs or other farm animals are not likely to have served as sources of infection *[19]*.
Reed and colleagues described in a random household survey conducted in Florida, the only apparent risk factor for *M. avium* infection is prolonged exposure to soil *[25]*. Von Reyn and

colleagues described that while some clinical *M. avium* cases could be linked to the household water supply by molecular typing, colonization of the water supply with *M. avium* does not increase the risk of infection *[26]*.
No correlation could be found in strain distribution with any kind of behaviour or geographical residence of the patient. We therefore conclude that the sources of *M. avium* causing lymphadenitis in the Netherlands are variable and ubiquitous and no relation to pet-birds has been determined.

Acknowledgement.

We gratefully acknowledge the National Institute for Animal Disease Control (CIDC) Lelystadt, The Netherlands, for advice on sampling bird materials and performing mycobacterial cultures from bird materials.

Figure 1: Comparison of IS1245 RFLP patterns of all *M. avium* strains. Analysis in Bionumerics. Clade designation according to Komijn et al *[14]*.

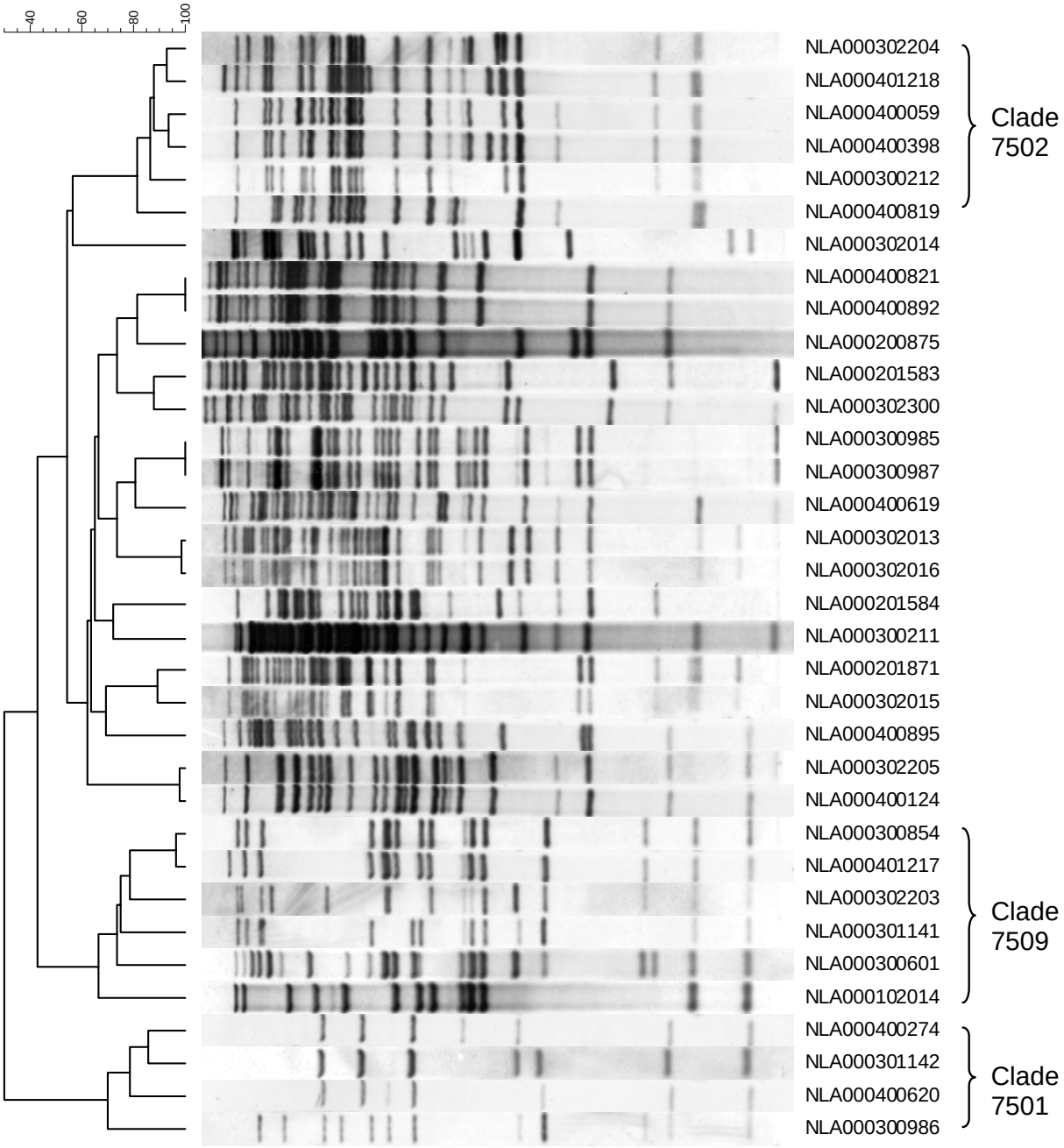

Table 1: characteristics of patient strains.

strain no.	onset of disease	county /region	age month	pets	swimming	sandpit	children's farm	season	etnicity	Typing details [a]
102014	june '01	FL	35	-	-	+	+	summer	NE	
200875	feb '02	OI	20	+	-	-	-	spring	NE	
201583	jun '02	NH	25	-	-	+	+	summer	NE	
201584	jul '02	NH	19	-	-	-	-	summer	NE	
201871	jul '02	NH	21	-	-	-	-	autumn	NE	
300211	sep '02	GL	27	-	-	+	+	autumn	NE	
300212	oct '02	FL	34	-	-	+	-	autumn	Afghan	Clade 7502
300987	oct '02	ZH	148	+	+	-	-	winter	NE	β
300601	jan '03	FL	38	+	-	+	+	winter	NE	Clade 7509
400124	may '03	ZH	31	+	-	-	-	winter	NE	δ
300985	feb '03	GL	67	+	+	-	-	spring	NE	β
300986	feb '03	NH	18	+	-	-	-	winter	NE	Clade 7501
302015	jul '03	NH	45	-	-	+	-	summer	NE	
301141	mar '03	LB	57	+	+	+	-	spring	NE	Clade 7509
301142	mar '03	Dr	17	+	-	+	+	spring	NE	Clade 7501
302014	jul '03	NB	15	+	-	-	-	spring	NE	Clade 7509
302016	may '03	NH	50	-	+	+	+	spring	NE	γ
302013	jun '03	FL	73	-	+	-	+	summer	Maroccan	γ
400892	may '03	NB	47	+	+	+	-	autumn	NE	α
302203	jul '03	NB	21	-	-	-	-	autumn	NE	Clade 7509
302205	sep '03	ZL	58	+	+	+	-	autumn	NE	δ
302204	may '03	ZL	36	+	+	+	-	spring	NE	Clade 7502
302300	aug '03	UR	25	+	-	+	-	autumn	NE	
400059	sep '03	GL	47	-	-	+	+	autumn	NE	Clade 7502
400274	dec '03	GL	20	-	-	+	-	winter	NE	Clade 7501
400819	feb '04	NB	104	+	+	-	-	autumn	NE	Clade 7502
400398	nov '03	ZH	44	-	+	+	+	autumn	NE	Clade 7502
400620	dec '03	NH	27	+	-	+	-	winter	NE	Clade 7501
400619	feb '04	NH	26	-	-	-	-	winter	NE	
400821	feb '04	NH	52	+	+	+	+	winter	NE	α
400895	mar '04	NH	18	-	-	-	-	spring	NE	
401217	feb '04	NB	56	-	+	+	-	spring	NE	Clade II / ε
401218	feb '04	LB	19	+	-	+	-	spring	NE	Clade I
300854	feb '04	FL	32	+	+	+	+	spring	NE	Clade II / ε

[a] typing details include clade designation according to Komijn et al [14] and similarity (95-100%) designation between strains of this collection: α, β, γ, δ or ε.

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Chapter 8.

Comparison of gene targets for the differentiation of mycobacterial species.

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The work presented in this chapter is part of a more extensive analysis on the diagnostic value of gene targets for the identification of mycobacterial species, which is currently performed by the same group.

Abstract.

Sequencing of specific genomic targets like 16S and the 16S-23S spacer for the identification of *Mycobacterium* isolates is a widely used approach. However, it is not known whether the currently used targets are the most optimal for speciation of mycobacteria. Hence, the most widely used gene; the 16S rRNA, automatically remains the favourite target of choice for this purpose, while no independent study has been conducted to compare the utility of the other candidate genes.
In this study, the performance of Important for the functionality of a target is the quality of sequences (practicability), availability of sequences in public databases and inter- and intra-species variation.
six gene targets (16S, ITS, *sodA*, *secA1*, *hsp65* and *rpoB*) were compared regarding the differentiation of 3 or 4 isolates of each of the five most frequently encountered *Mycobacterium* species; *M. avium, M. kansasii, M. gordonae, M. chelonae/abscessus, and M. malmoense* and 1 isolate of *M. tuberculosis.*
The results demonstrate that all targets can be used to adequately identify all mentioned species groups. Furthermore, 16S sequence information provided one intra-species distinction; between *M. gordonea* I and II. All five other targets revealed different levels of intra-species variation. Most different intra-species types among the 18 strains tested were provided by the *secA1* (17), followed by *sodA* (16), *hsp65* (13) and ITS (13). We conclude that apart from the currently used genomic targets for identification of mycobacteria, other loci contain genetic information to subdivide the currently recognised species. This should lead to a more detailed identification and eventually taxonomy, if there is a difference in the clinical relevance of isolation of bacteria of subgroupings.

Introduction.

To date, 128 species of the genus *Mycobacterium* have been registered in the approved list of bacterial species (DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, april 2006). In fact, the correct designation of Atypical mycobacteria is 'non-*Mycobacterium tuberculosis* complex' (NTM).
Infections by NTM have been increasingly recognized in the last decades, mainly due to the high prevalence of these infections in immune-compromised patients. However, the clinical significance of the isolation of these bacteria is often not clear. A part of the microbiological laboratories in The Netherlands are equipped to perform cultures and to identify of mycobacterial species by molecular tests. While several molecular assays *(1, 2, 3, 4)* have been designed for the direct detection of the genus *Mycobacterium* in clinical materials, culturing of the mycobacteria is still widely propagated to confirm the result of the molecular detection and to determine its susceptibility pattern.
Commercially available molecular assays for the identification of cultured mycobacteria are restricted to the Accuprobe hybridization assay (Gen-Probe Inc, San Diego, CA, USA) for the

species *M. gordonae*, *M. avium-complex*, *M. kansasii* and *M. tuberculosis*, and and to reverse line hybridization assays: the GenoType Mycobacterium for a wide variety of *M. tuberculosis* complex and NTM species (Hain Lifesciences, Nehren, Germany), and the Inno-Lipa V2 Mycobacteria for NTM species (Innogenetics, Gent, Belgium). The latter two assays identify respectively 16 and 13 different species and both target the Internal Transcribed Spacer (ITS) region between the 16S and the 23S rRNA genes. For the identification of all other NTM species DNA sequencing is currently the golden standard and this is mostly based on the analysis of the 16S rRNA. However, in principle there are multiple semi-conserved genes suitable for identification procedures. The *hsp65* gene (encoding the 65 kDa heat shock protein) and the *rpoB* gene (encoding the ß subunit of RNA polymerase) are often used as sequence target for the identification of mycobacterial isolates. Other targets like the gyrase genes (*gyrA/B), dnaJ, sodA, secA1, recA* have also been reported to contain sufficient genetic variation for identification. Important for the utility of a target for identification at species level is the rate of DNA polymorphism among *Mycobacterium* species and between strains of the respective species. Suitable genes offer sufficient polymorphism to distinguish between species, but are so conserved in the evolution that strains of the same species have little to no variation in sequences.

Genes which reveal too much intra-species variation may not be suitable for the identification within the currently used taxonomy, but sub-division of groupings within species may be important in the light of studies on the clinical relevance of the isolation of certain NTM. Furthermore, the performance of the sequence analysis and the interpretability of the results is of importance.

The National Reference Laboratory for Mycobacteriology (RIVM) collects all *M. tuberculosis* complex, as well as the largest part of the clinical NTM isolates. In the last decade the most common mycobacterial species were *M. tuberculosis*-complex (n= 11777), *M. avium*-complex (n=1480), *M. gordonae* (n=468), *M. kansasii* (n=353), *M. malmoense* (n=185) and *M. chelonae/abscessus* (n=131).

We compared 6 gene targets (16S, ITS, *sodA*, *secA1*, *hsp65* and *rpoB*), all applied as gene targets for the identification of NTM isolates in diagnostic laboratories in the Netherlands, for their usefulness in the identification of isolates of the most commonly encountered species.

Strains & Methods.

Of each NTM species, 3 or 4 strains were included in this study. The strains were all clinical isolates from the Netherlands collected by the RIVM in 2003: *M. chelonae/abscessus* (n=4), *M.avium*-complex (n=4), *M. malmoense* (n=3), *M. kansasii* (n=3), *M. gordonae* (n=3) and ATCC control strain *M. tuberculosis* H37RV. The isolates were randomly selected and no background information was available on clinical relevance of isolation of these mycobacteria or the clinical picture of the respective patients. Strains were identified at the RIVM by 16S sequencing (Table 1). This method is the current standard and has previously been described by Bottger et al. *(5)*.

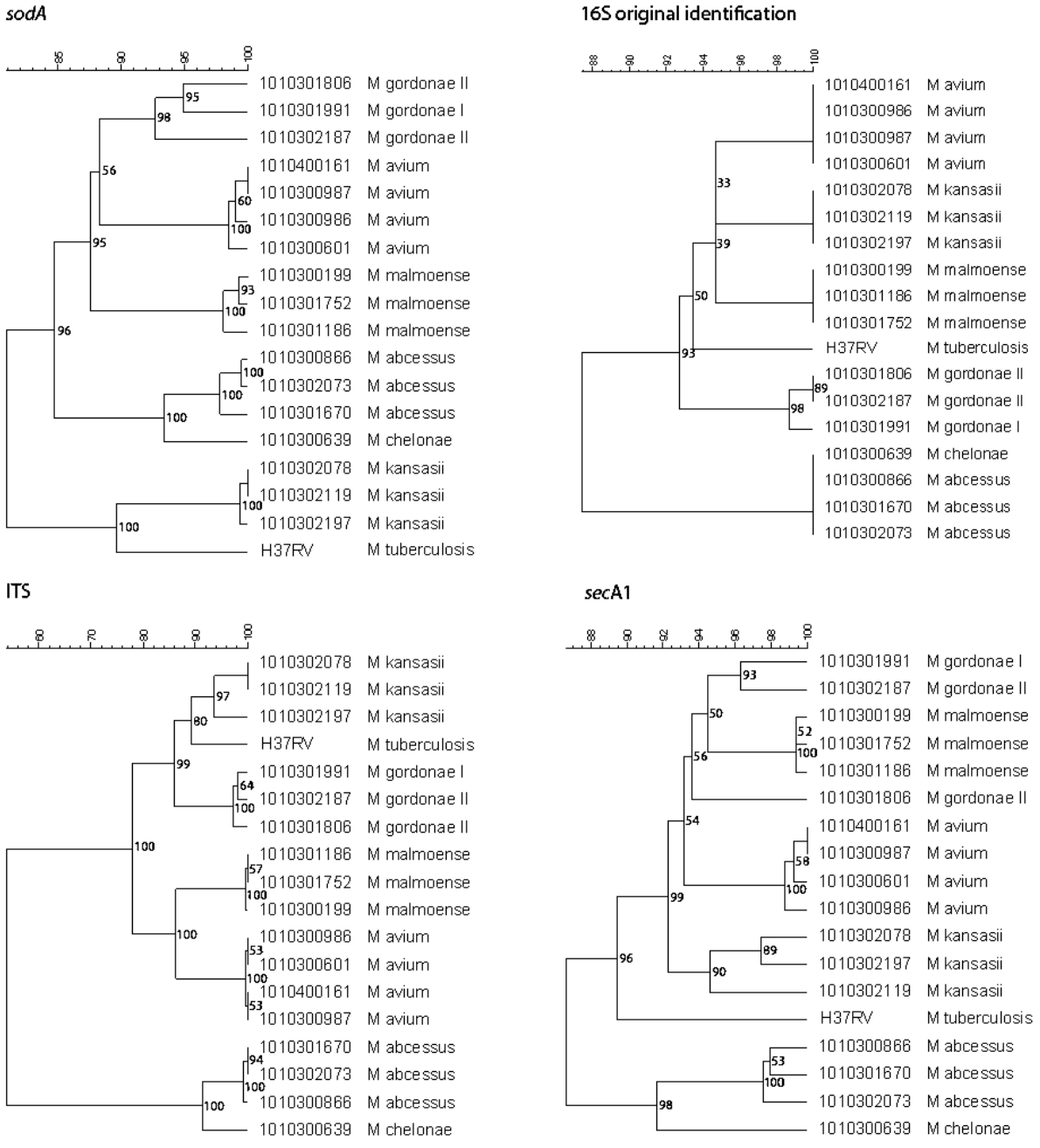
DNA was extracted using the QiaAmp DNA extraction kit (Qiagen, Venlo, The Netherlands) and diluted to a final concentration of 250 ng/µl.
The DNA extract was divided into aliquots and send to all participating institutes which performed their method(s) of species identification as described below.
For the targets 16S, ITS, *sodA*, *hsp65* and *rpoB*, standard 3-step PCR protocols were used using the primers depicted in Table 1. The same primers were used in the PCR as for direct sequencing. For *secA1,* the protocol has been described by Zelazny et al *(6)*.
Both forward and reverse sequences were obtained and assembled. All sequences were sent to one institute for further analysing. All targets were subjected to a BLAST recognition in the NCBI database (http://www.ncbi.nlm.nih.gov/) and the 16S and ITS sequences in the RIDOM database (http://www.ridom.com/) as well.
A phylogenetic tree was assembled for each target to visualise the variability between the strains using Bionumerics software 3.5 with UPMGA applied as clustering method (Applied-Maths, Belgium). The lengths of all sequences were shortened to the shortest product per target to apply in phylogenetic tree calculations (table 1 / fig 1-6).

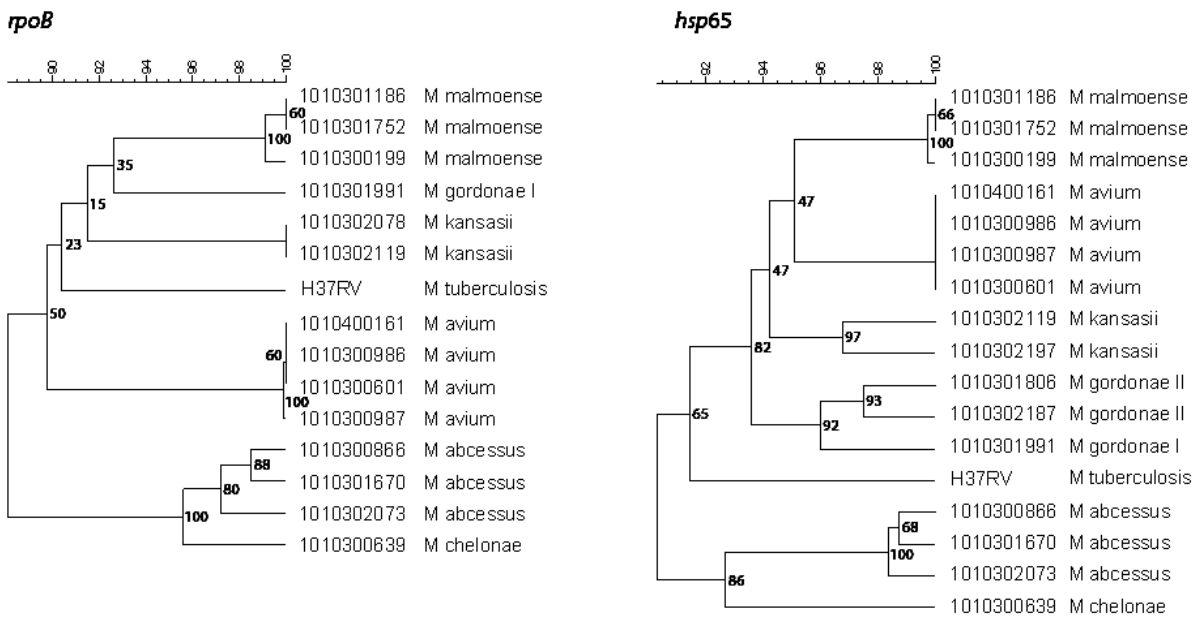
Results.

The analysis of the *sodA*, 16S, ITS and *secA1* sequences resulted in readable sequences for all strains. For the target *hsp65* one *M. kansasii* strain repeatedly yielded no PCR product as was the case as well for the *rpoB* target with several *M. malmoense* and *M. gordonae* strains (table 2). Only a part of the *rpoB* sequences were successful for *M. malmoense* isolates. For both targets, alignments of known target-sequences revealed mismatches in the primer regions of several species.
Inter- and intraspecies diversity of the analysed NTM isolates per target is shown in figure 1.
ITS and *sodA* sequences show less than 90% sequence homology for strains in different species groups. *RpoB* sequences show less than 93% homology for strains of different species groups and 16S, *hsp65* and *secA1* sequences show less than 95% homology. All targets except for the 16S, yielded a clear difference between *M. abscessus* and *M. chelonae*.
Table 2 describes the amount of different sequences (subtypes) found within species groups and the amount of mismatches between strains of the same species group. Variability between isolates of the same species was significant for the ITS, SecA1, hsp65 and *sodA* targets. The *sodA* and *secA1* targets reveal the most subtypes over all species and the largest amount of mismatches within species. No variability between strains of the same species group or species is observed for the 16S target. However, a clear differentiation between species groups is observed.
As shown in table 3, the RIDOM database does not provide more information on the identity of the strains than given by the NCBi database. In fact, no ITS sequences of *M. malmoense* are present in the RIDOM database and are therefore not recognised. *M. massiliensis* and *M. bolletii* sequences are not present in the RIDOM database either. The 16S sequence of *M. malmoense* was present in both databases. The NCBI database provides identifications of all

strains of all targets to the species group level. However, discrepanties were seen between the strains belonging to the *M. avium*-complex. Both the *rpoB* and the *secA*1 targets identify *M. paratuberculosis* on multiple occasions while the other targets either identify *M. avium* or *M. avium*-complex. *M. gastri* is recognised twice by secA1 while all other targets yield *M. kansasii*. None of the discrepant results have a 100% concordance with the database sequences which indicates that the matching sequences are not available.

Fig 1. Phylogenetic trees per target.





Discussion.

Molecular identification of mycobacterial species provides two primary advantages to phenotypic (conventional) identification: it is more rapid and accurate in identification. Although fast and easy to perform, "sequence based" methods, like species specific real-time PCR and PCR-RFLP have their limitations, since they only recognise the evaluated species. Most laboratories are equipped with a sequencer and are therefore able to perform sequence identifications. Six different sequence targets have been analysed for their ability to differentiate the most common clinical mycobacterial species in the Netherlands. Considerations for a preferable protocol are: practicability (high success rate of creating identifiable sequences and easy to use protocol) , availability of sequences in public databases and suitable levels of discrimination within the target. The level of resolution for routine diagnostic application of species differentiation is difficult to ascertain; clinical relevance of determining subspecies or only group levels is not known. Trial information is lacking on this subject.

The 16S rRNA gene has an advantage in its presence in all prokaryotic organisms, therefore a broadrange PCR would be able to detect sequences of all genera and differentiate based on sequence variation. Still, because this gene does not contain a high degree of variation compared to some of the other targets, the use of this target for the differentiation of mycobacteria is limited. Several species have identical 16S sequences, among which the species belonging to the *M. tuberculosis* complex, "*M. kansasii* and *M. gastri"*, and the subspecies of *M. avium*. Using only the 5'part of the gene, species as "*M. chelonae* and *M. abscessus"* and "*M. ulcerans* and *M. marinum"* cannot be differentiated either. In other words: 16S differentiation does not exceed the group level, as confirmed with the strains used here. An important advantage of the 16S gene is the abundance of sequences in the public databases. However, the reliability of sequence information can be questionable *(11)*.

Both *rpoB* and *hsp65* contain a higher degree of divergence between species than the 16S target. The additional advantage of the *rpoB* target is that it contains species-specific sequences as well as polymorphisms responsible for rifamycin resistance and can therefore be used for the simultaneously detection of susceptibility and species identification. *rpoB* and *hsp65* protocols lack in reliability: sufficient amounts of sequences are available, but primer sequences are often not completely genus specific due to too much variation, which explains the unsuccessful PCR of several strains.
In 1993 the Internal Transcribed Spacer (ITS) region was added as a possible target in species differentiation and proven useful as such *(12, 13)* for the recognition of virtually all known mycobacterial species . It is used in the commercial assays InnoLipa V2 and GenoType Mycobacterium. The ITS-protocol has a similar performance but with more discriminative power than the 16S. However, while many sequences are available for the ITS target, differentiation between *M. kansasii* and *M. gastri* is not possible and no clear differentiation between species belonging to the *M. avium* group can be made. Also, many short and incomplete sequences are a pollution to the database and might cause false identifications when analysed incautiously.

In 1994 the *sodA* gene was proposed as possible target for differentiation *(14)* and since then sporadically applied *(10, 15) SecA1* is the most recent evaluated target gene used here for species identification *(16)*. Correct identifications can only be made with an extended database. S*odA* appears superior in species differentiation to *secA1*, probably due to the availability of more than 130 sequences (less species) while for *secA1* 42 sequences of 30 different species are available. Considering there are over 150 species known to date, a second sequence target would be necessary for the identification of uncommon (sub)species for both targets. Also, when the amount of interspecies variation within a target is too small, species differentiation will be compromised. However, if there is too much variation, the risk of mismatches in the primer sequences will increase as well as the occurrence of unidentifiable new sequences. In either way a second target will be necessary for the correct differentiation of uncommon species or difficult taxonomical groups.
None of the tested targets is capable of differentiation within the *M. tuberculosis*-complex, but all targets are 100% reliable in identifying the correct species group.
M. gordonae and *M. malmoense* are both clearly separated from other species by all targets and no complicated taxonomy intricate differentiation of these species.
SecA1 is the only target to identify *M. gastri* in two cases while no other targets confirm these results. Both sequences are 99% similar to an ATCC strain of *M. gastri*, and therefore a 100% concordance with a *M. kansasii* would be optional if more sequences were available. However, ITS and 16S are not able to differentiate the two species, while *rpoB*, *hsp65* and *sodA* have no 100% assurance either for the identification of *M. kansasii*.
The *M. abscessus/chelonae* group is recently divided into more species. *M. massiliensis* and *M. bolletii* belong to this group. They are an example of the increasing complexity of mycobacterial taxonomy. Not for all targets the sequences of these species are known yet, but for *sodA* and the ITS the sequence variability within this species group is probably sufficient enough to harbour specific differences.

The *M. avium*-complex appears to be the most difficult group to differentiate. This species-group is known to harbour extensive sequence variation. Still, 16S and *hsp65* show no variability between the (sub)species of this complex. All strains yield discrepant results for the other targets. While one target shows two strains to have similar sequences, another target shows another combination of strains to be similar. Not one strain is identified consistently by different targets. While by two targets identified as such, the recognition of *M. paratuberculosis* is unlikely to be correct, due to the difficult growth kinetics of the species. The identification of this species group in particular would benefit by an extensive and quality controlled database.

Concluding: When is chosen for identification to the group level, then 16S sequencing is the most reliable. When identification to the (sub)species level is preferred, no target will be sufficient on its own and a combination of two targets will be necessary to give an accurate identification.

Table 1: Oligo's used in PCR and nucleotide sequence analysis.

target gene	name and sequence forward primer	name and sequence reverse primer	product length (including primers) and location in gene	reference
rpoB	rpoB-for1: 5'-TGGTCCGCTTGCACGAGGGTCAGA -3'	rpoB-rev1: 5'- CTCAGGGGTTTCGATCGGGCACAT-3'	437 bp: bp 1033-1496 (Includes rifamycin resistance hotspot)	7
hsp65	TB11: 5'-ACCAACGATGGTGTGTCCAT-3'	TB12: 5'-CTTGTCGAACCGCATACCCT-3'	440 bp 5'end: (bp 270- 710)	8
ITS	MycoITSfwd: 5'-ACCTCCTTTCTAAGGAGCACC-3'	MycoITSrev2: 5'-TCTCGATGCCAAGGCATCCACC-3'	Approx. 350 bp: (includes total ITS region)	8 (only forward primer)
16S rRNA	8F: 5'-AGTTTGATCMGGYCAG-3'	M259: 5'-TTCACGAACAACGC GACAA-3'	5'end: 1st 590 bp (analysed bp 120-273)	5
sodA	SodF: 5'-ACATCTCGGGTCAGATCAACGAGC-3'	SodR: 5'-GACGTTCTTGTACTGCAGGTA-3'	463 bp: bp 55-518 (gene is 620 bp long)	10
secA1	Mtu forward1: 5'-GACAGYGAGTGGATGGGYCGSGTGCACCG-3' M13 forward tail: 5'-GTAAAACGACGGCCAG-3'	Mtu.reverse3: 5'-ACCACGCCCAGCTTGTAGATCTCGTGCAGCTC-3' M13 reverse tail: 5'-CAGGAAACAGCTATGAC-3'	761 bp 3'end: bp 1030 through 1790 ** (open reading frame is antisense)	6

* M. malmoense is only 84 bp long for rpoB.

Table 2. Intraspecies difference in basepairs resulting in amount of subtypes per species.

species	no. of isolates	16S (155 bp)		ITS (280 bp)		*Hsp*65 (400 bp)		*rpo*B (340 bp)*		*sec*A1 (450 bp)		*sod*A (400 bp)	
		subtypes	bp diff	subtypes	bp diff	subtypes	bp diff	subtypes	bp diff	subtypes	bp diff	subtypes	bp diff
M. abscessus	4	1	-	3	-, 2, 27	4	-, 5, 6, 31	4	-, 5, 9, 15	4	-, 13, 16, 38	4	-, 2, 9, 26
M. avium	4	1	-	2	-, 1	1	-	2	-, 1	3	-, 4, 5	3	-, 4, 6
M. gordonae	3	2	-, 2	3	-, 6, 8	3	-, 10, 16	nvt	nvt	3	-, 17, 30	3	-, 20, 30
M. kansasii	3	1	-	2	-, 17	2	-, 13	2	-, 12	3	-, 12, 25	2	-, 3
M. malmoense	3	1	-	2	-, 1	2	-, 1	2	-, 1	3	-, 3, 3	3	-, 3, 8

Table 3: Comparison of sequences with the public databases. The 16S sequencing protocol used for the original identifications of the strains resulted in the sequences described in the 16S column.

Analysed length of products can vary for some targets: only two-directional fragments compared to database. 16S = 153 bp, *secA1*= 459-761 bp, *rpoB* = 101-437 bp, *hsp65* = 338-440 bp, ITS = 269-(approx)350 bp, *sodA* = 356-463 bp.

strain:	**16S (BLAST)**	**16S (RIDOM)**	**ITS (BLAST)**	**ITS (RIDOM)**	***hsp65***	***rpoB***	***sec1A***	***sodA***
300866	absces/chel 100%	absces/chel 100%	massiliense 99%	abscessus 98%	absces/mass 99%	massiliense 100%	abscessus 98%	massiliense 100%
301670	absces/chel 100%	absces/chel 100%	abscessus 99%	abscessus 100%	absc/bolletii 99%	bolletii 99%	abscessus 97%	bolletii 99%
302073	absces/chel 100%	absces/chel 100%	abscessus 99%	abscessus 100%	absc/chel 100%	abscessus 100%	abscessus 98%	abscessus 100%
300601	avium (MAC) 100%	avium (MAC) 100%	avium (MAC) 100%	avium (MAC) 100%	avium (MAC) 100%	avium ptb 99%	avium ptb 98%	avium st 2 98%
300986	avium (MAC) 100%	avium (MAC) 100%	avium (MAC) 100%	avium (MAC) 100%	avium (MAC) 100%	avium ptb 99%	avium 99%	avium st 2 99%
300987	avium (MAC) 100%	avium (MAC) 100%	avium 99%	avium avium** 99%	avium (MAC) 100%	avium ptb 99%	avium ptb 99%	avium st 2 100%
400161	avium (MAC) 100%	avium (MAC) 100%	avium 100%	avium avium** 100%	avium (MAC) 100%	avium ptb 99%	avium ptb 99%	avium st 2 99%
300639	absces/chel 100%	absces/chel 100%	chelonae 98%	chelonae 99%	chelonae 100%	chelonae 100%	chelonae 97%	chelonae 99%
301991	gordonae 100%	gordonae 100%	gordonae 100%	gordonae 100%	gordonae 100%	gordonae 99%	gordonae 98%	gordonae 99%
301806	gordonae 100%	gordonae 100%	gordonae 96% + gap 17 bp	gordonae 96%	gordonae 99%	*PCR unsuccessful*	gordonae 95%	gordonae 96%
302187	gordonae 100%	gordonae 100%	gordonae 100%	gordonae 100%	gordonae 97%	*PCR unsuccessful*	gordonae 96%	gordonae 93%
302078	kansasii 100%	kansasii 100%	kansasii 100%	kansasii 100%	*PCR unsuccessful*	kansasii 100%	gastri 99%	kansasii 99%
302119	kansasii 100%	kansasii 100%	kansasii 100%	kansasii 100%	kansasii 100%	kansasii 100%	kansasii 99%	kansasii 99%
302197	kansasii 100%	kansasii 100%	kansasii 100%	kansasii 100%	kansasii 99%	*PCR unsuccessful*	gastri 99%	kansasii 99%
300199	malmoense 100%	malmoense 100%	malmoense 99%	*not recognised*	malmoense 99%	malmoense 99% *	malmoense 99%	malmoense 99%
301186	malmoense 100%	malmoense 100%	malmoense 99%	*not recognised*	malmoense 100%	malmoense 100% *	malmoense 99%	malmoense 100%
301752	malmoense 100%	malmoense 100%	malmoense 98% + insert 5bp	*not recognised*	malmoense 100%	malmoense 100% *	malmoense 99%	malmoense 99%
H37Rv	tb-complex 100%	tb-complex 100%	tb-complex 100%	tb-complex 100%	tb-complex 100%	tb-complex 100%	tb-complex 100%	tb-complex 100%

avium ptb = M. paratuberculosis of the M. avium-complex, MAC = M. avium-complex, aviu st 2 = M. avium serotype 2, tb-complex = M. tuberculosis-complex, absces = M. abscessus, chel = M. chelonae, mass = M. massiliensis.

* only a short sequence (84 bp) of the *M. malmoense* isolates was successful for the rpoB target.

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9 Discussion: diagnostics of non-tuberculous mycobacteria.

Molecular identification of species and recognition of subspecies.

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

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

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

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

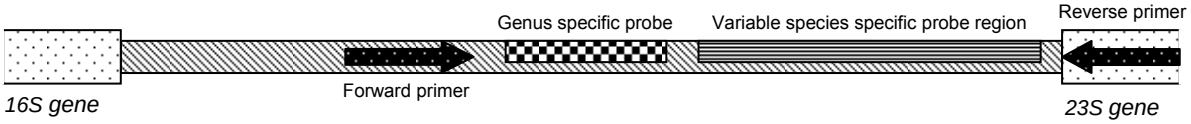
Using these results the next step is to evaluate the targets in their relation to clinical significance of isolates. It might be possible that these or other sequence targets harbour mutations which correlate to virulence or correlate to the same evolutionary lineages.

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

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

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

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



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

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

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

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

Suggestions to improve molecular detection of NTM in clinical materials.

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

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

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

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

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

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

Prevention of contamination.

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

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

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

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

Susceptibility.

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

General conclusion.

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

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

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

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Samenvatting: Diagnostiek van niet-tuberculeuze mycobacteriën.

Het genus *Mycobacterium* bestond in november 2007 uit 127 species waarvan 121 species behoren tot de niet-tuberculeuze mycobacteriën (NTM). Van 1990 tot 2000 werden 28 nieuwe species geïdentificeerd, van 2000 tot september 2007 kwamen er 41 bij. De ontdekking en identificatie van nieuwe species heeft nog steeds een continu voortgaande snelheid. Ongeveer tweederde van het aantal NTM species is initieel gevonden als infectieus agens bij mensen en al deze, met uitzondering van *Mycobacterium leprae* en *M. ulcerans*, worden beschouwd als opportunistisch pathogeen.
Klinisch kunnen deze NTM verantwoordelijk zijn voor ondermeer huidinfecties, longinfecties, gedissemineerde infecties, lymphadenitis, osteoperose en keratitis. NTM infecties worden voornamelijk aangetoond in immuun gecompromitteerde patiënten, maar zijn ook niet zelden gevonden in immuun competente patiënten. Vooral lymphadenitis is een veel voorkomende klinische NTM manifestatie bij “gezonde” kinderen.

De grote aantallen species en de grote verscheidenheid in klinisch beeld maken van de mycobacteriële diagnostiek een ingewikkeld stukje microbiologie.
Conventionele mycobacteriologie bestaat voornamelijk uit langdurige kweek, microscopische kleuringen en resistentiebepalingen in een aanvullende kweekperiode, eventueel aangevuld met of vervangen door immunologische of histopathologische methoden. Vanwege veiligheidsvoorschriften is er voor de conventionele mycobacteriologische diagnostiek een gespecialiseerd laboratorium vereist dat voldoet aan de normen voor bio-safety-level 3. De afwezigheid van een specialistisch laboratorium, alsmede de aspecificiteit van kleuringen èn de langdurige kweekperiode, resulteren in een groeiende behoefte aan moleculaire detectie- en identificatiemethoden.

Moleculaire technieken worden inmiddels als gouden standaard beschouwd bij de identificatie van positieve kweken, maar worden voor de detectie van NTM in klinische materialen nog maar mondjesmaat toegepast. Meestal bestaan deze methoden dan uit conventionele PCR, soms multiplex- of nested-, met additionele analysestappen, welke niet als optimaal gelden voor het hedendaagse microbiologische laboratorium. Vanwege de verschillende stappen zijn ze vaak contaminatiegevoelig, vereisen een variatie aan apparatuur en over het algemeen missen ze een hoge sensitiviteit. Real-time PCR is in theorie hiervoor een goede vervanging, maar vooralsnog was een genus-specifieke test moeilijk te realiseren wegens de grote variatie binnen de NTM groep.

Hoofdstuk 3 beschrijft het ontwerp van een genus-specifieke real-time PCR, die bij toepassing op lymfeklier-biopties, een NTM infectie kon aantonen binnen slechts 2 dagen. Bij vergelijking van de real-time PCR met conventionele diagnostische methoden, bereikte de PCR een sensitiviteit van 72% vergeleken met 42% voor de kweek. Door de toevoeging van een *M. avium*-specifieke probe werd de aanwezigheid van deze species direct herkend bij 38

van de 67 NTM positieve lymphadenitis-patiënten. De toepassing van deze diagnostische test resulteerde in de mogelijkheid binnen enkele dagen een gerichte behandeling te starten zonder de kweekresultaten af te wachten.

In *Hoofdstuk 4* is dezelfde real-time PCR uitgebreid met een *M. haemophilum*-specifieke probe. Deze species werd bij diverse patiënten aangetoond na sequencing van PCR producten met enkel een positief signaal in de genus-specifieke real-time PCR zoals beschreven in hoofdstuk 3. De toevoeging van de *M. haemophilum*-specifieke probe resulteerde in de ontdekking van 16 *M. haemophilum*-positieve lymphadenitis-patiënten. Omdat initieel niet aan de specifieke kweekvoorwaarden voor deze species werd voldaan, waren deze diagnoses niet gesteld zonder de toepassing van de real-time PCR. Hieruit volgde de vraag hoeveel mogelijke NTM infecties in het verleden gemist zouden zijn (!)

Hoofdstuk 5 beschrijft daarom de toepassing van de genus- en species-specifieke real-time PCR detectie in paraffine-ingebedde huidbiopties. Van de 30 biopties, verzameld tussen 1982 en 2004, bleken 13 materialen positief voor mycobacteriën. In 7 materialen werd *M. haemophilum* geïdentificeerd. Ten tijde van de afname van de klinische materialen waren maar in 5 van de 13 NTM-positieve biopties mycobacteriën aangetoond.

Hoofdstuk 6 omvat de genotypering van een collectie *M. haemophilum* stammen. Van de in totaal 32 *M. haemophilum* stammen, die aangetoond werden bij kinderen met lymphadenitis, bleken er 30 uit de regio Amsterdam afkomstig te zijn. Om hiervan aan te tonen dat deze stammen aan elkaar verwant waren, is een genotypering uitgevoerd. Slechts twee keer eerder waren genotyperings-studies uitgevoerd, beide op een kleine collectie stammen. 128 stammen werden verzameld vanuit verschillende continenten en klinische manifestaties om te vergelijken in AFLP. De methode had een goed onderscheidend vermogen en was in staat grote variabiliteit in genotypen te herkennen. Niet alleen de stammen uit Amsterdam bleken identieke patronen te vormen (en bewezen daarmee verwant te zijn), maar infecties met de species kwamen vaker geclusterd voor. Twee clusters uit Australië en een cluster uit New-York werden aangetoond. De identieke patronen bleven langdurig stabiel; ze bleven tot 18 jaar lang onveranderd.

In *hoofdstuk 7* is de genotypering van *M. avium* stammen beschreven. Lymphadenitis bij kinderen wordt vaak “vogeltjes-tbc” genoemd, naar de species *M. avium*. Tegenwoordig is een typeringstechniek beschikbaar die het onderscheid kan maken tussen het vogel-type *M. avium*, en humane typen. Deze methode is in hoofdstuk 7 toegepast om aan te tonen welke subspecies *M. avium* lymphadenitis veroorzaakt en hoe groot de genetische variabiliteit tussen stammen is. In tegenstelling tot de clustering van *M. haemophilum* stammen in hoofdstuk 6, werd tussen de *M. avium* stammen een grote variatie aangetoond. Geen enkele stam was van het “vogel-type” maar ze behoorden allen tot de sub-species *M. avium hominissuis*.

In *hoofdstuk 8* van dit proefschrift worden zes gen-targets vergeleken voor de identificatie van zes verschillende NTM species. De meest gebruikte identificatiemethode voor NTM species is direct sequencing van gen-targets na een positieve kweek. Er zijn verschillende sequentie targets bekend die geschikt zouden zijn voor dit doel, maar een vergelijking van de functionaliteit is nauwelijks uitgevoerd. Een geschikte target dient voldoende variabel te zijn, maar evengoed geconserveerd voor de toepassing van genus-specifieke primers. In de vergelijking beschreven in hoofdstuk 8 bleek dat de verschillende gen-targets een verschillend niveau van differentiatie bieden. De bekendste, het 16S rRNA-gen, biedt enkel differentiatie tot op het niveau van speciesgroepen, maar is zeer betrouwbaar. Daarentegen zijn de ITS, en het *rpo*B- en het *hsp*65-gen in staat tot op sub-species niveau te differentiëren. Het nadeel van deze drie genoemde targets is dat de variatie zo groot kan zijn, dat de genus-specifieke amplificatie niet gegarandeerd kan worden. De nieuwste targets: *sod*A en *sec*A1 lijken een gemiddeld differentiatieniveau te bieden. Helaas zijn van beide genen te weinig sequenties bekend om alle species te herkennen. Waarschijnlijk is de beste wijze van identificatie het gebruik van twee gentargets tegelijk, zoals bijvoorbeeld de combinatie van 16S en ITS sequenties.

Doordat het genus *Mycobacterium* zeer gevarieerd is en er onvoldoende kennis bestaat over de genetische opbouw van dit genus, is het tot nu toe niet geheel gelukt een moleculaire assay te ontwikkelen die zonder beperkingen toegepast kan worden ter vervanging van de huidige kweekmethoden. De algemene aanwezigheid van vervuilend mycobacterieel DNA in reagentia en het ontbreken van een “Multi-copy” target voor detectie, bemoeilijken het gebruik van real-time PCR in de directe detectie van NTM. Mogelijk kunnen RNA-detectiemethoden hier een oplossing vormen.
In toekomstig onderzoek naar de verbetering van de NTM diagnostiek zou de verdere ontwikkeling van een sensitieve en specifieke moleculaire test voor de detectie van NTM in klinische materialen centraal moeten staan. Voor meer kennis over de epidemiologische eigenschappen en klinische relevantie van NTM infecties, zal het gebruik van identificatie- en typeringstechnieken een belangrijke rol kunnen spelen en zouden toepassingen hiervan, zoals bijvoorbeeld van de MALDI-TOF techniek, onderzocht moeten worden.

Curriculum Vitae.

Lesla Elisabeth Sofia Bruijnesteijn van Coppenraet werd op 18 juli 1976 geboren in Arnhem. Na de eerste twee jaar aan het Stedelijk Gymnasium te Arnhem gevolgd te hebben, rondde ze in 1995 de HAVO af op het Rhedens Lyceum te Rozendaal.

Ze volgde de studie HTS Biotechnologie aan de Noordelijke Hogeschool in Leeuwarden en behaalde het diploma in 2001. In september 2001 begon ze als researchanalist in het LUMC op de afdeling Medische Microbiologie waar ze zich bezig hield met het ontwerpen van moleculair diagnostische testen en typeringen. In 2002 werd haar functie omgezet naar die van junior onderzoeker en startte het promotieonderzoek. Het dienstverband was op zijn eind in september 2006 met een proefschrift in wording.

Ze werd aangenomen voor de functie van moleculair bioloog door de afdeling Medische Microbiologie en Infectiepreventie van de Isala Klinieken in Zwolle terwijl ook het proefschrift een feit werd.

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