Diagnostics of non-tuberculous mycobacteria

Bruijnesteijn van Coppenraet, L.E.S.

Chapter 7.

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

Lesla ES Bruijnesteijn van Coppenraet[1], Petra EW de Haas[3], Jerome A Lindeboom[2], Edward J Kuijper[1] and Dick van Soolingen[3].

[1]Dept of Medical Microbiology, Center of Infectious Diseases, Leiden University Medical Center, Leiden; [2]Dept of Oral and Maxillofacial Surgery, Academic Medical Center, Amsterdam; [3]Mycobacterial Reference Laboratory, National Institute for Public Health and the Environment, Bilthoven, The Netherlands.

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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.

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

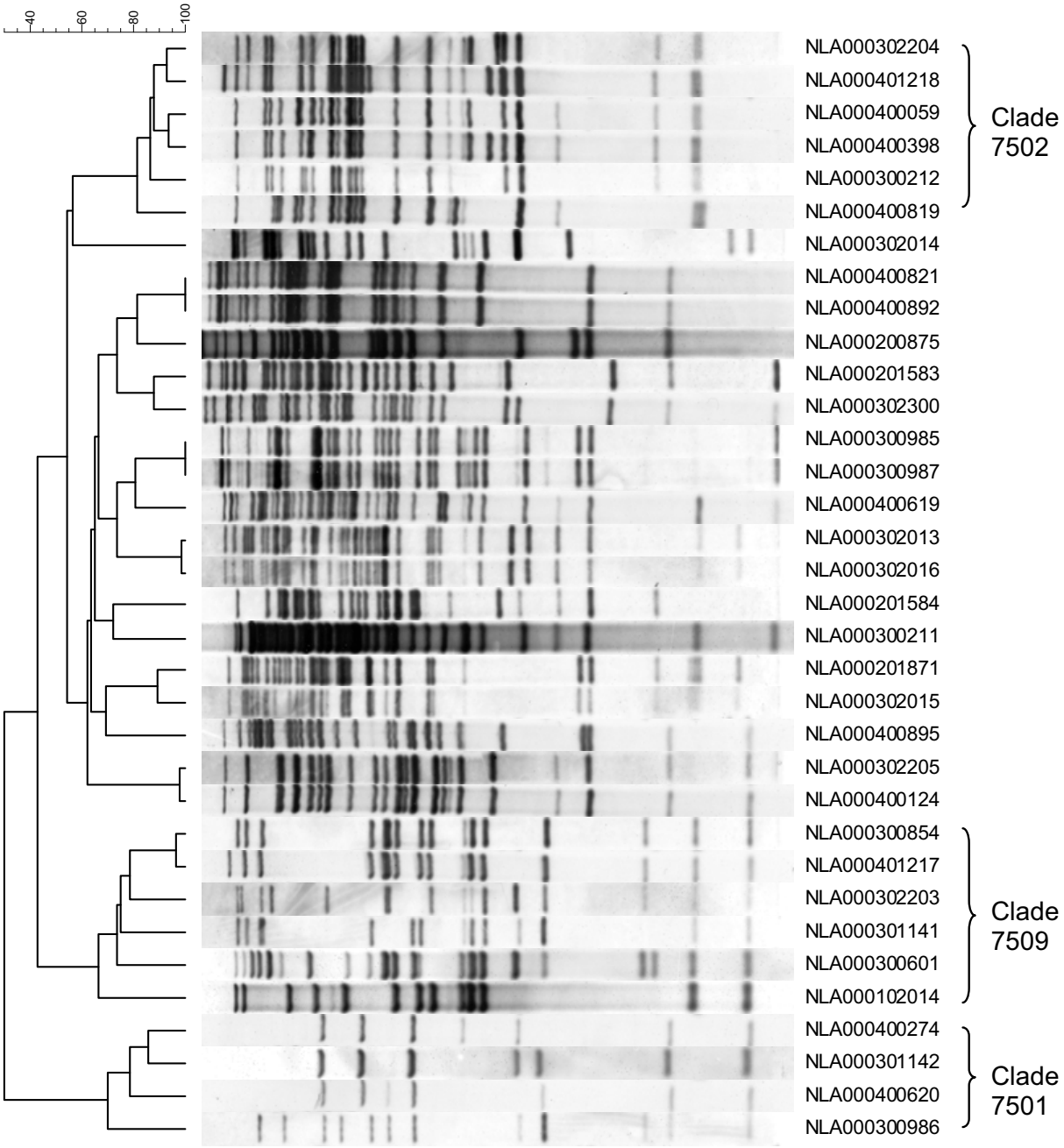

Table 1: characteristics of patient strains.

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.

References: Chapter 7.

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