Diagnostics of non-tuberculous mycobacteria

Bruijnesteijn van Coppenraet, L.E.S.

Chapter 6.

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

Lesla ES Bruijnesteijn van Coppenraet[1], Paul HM Savelkoul[2], Nico Buffing[2], Madelon W van der Bijl[2], Joyce Woudenberg[2], Jerome A Lindeboom[3], Timothy E Kiehn[4], Frank Haverkort[5], Zmira Samra[6] and Edward J Kuijper[1].

[1]Dept of Medical Microbiology / Center of Infectious Diseases, Leiden University Medical Center, Leiden; [2]Dept of Medical Microbiology and Infection Control, VU University Medical Center, Amsterdam; [3]Dept of orolangeal Surgery, Academic Medical Center, Amsterdam. Netherlands; [4]Department of Clinical Laboratories, Memorial Sloan-Kettering Cancer Center, New York, USA; [5]Division of Microbiology & Infectious Diseases Diagnostic Bacteriology & Mycology; Western Australian Centre for Pathology & Medical Research, Nedlands WA, Microbiology department, Australia; [6]Rabin Medical Center. Petach-Tiqva, Israel.

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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.

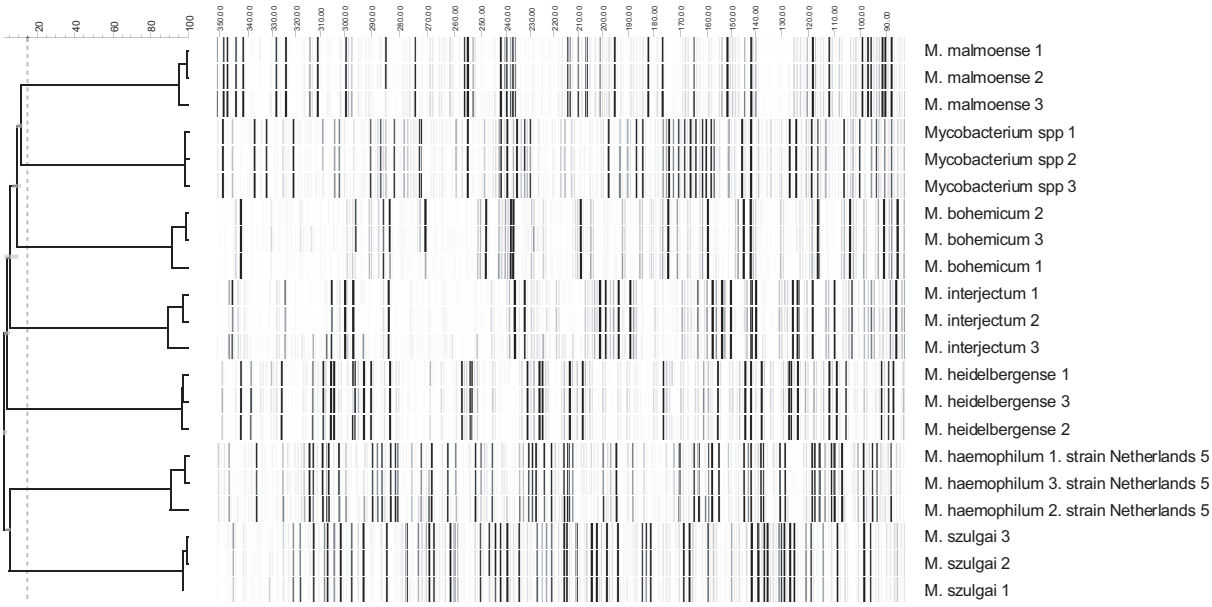
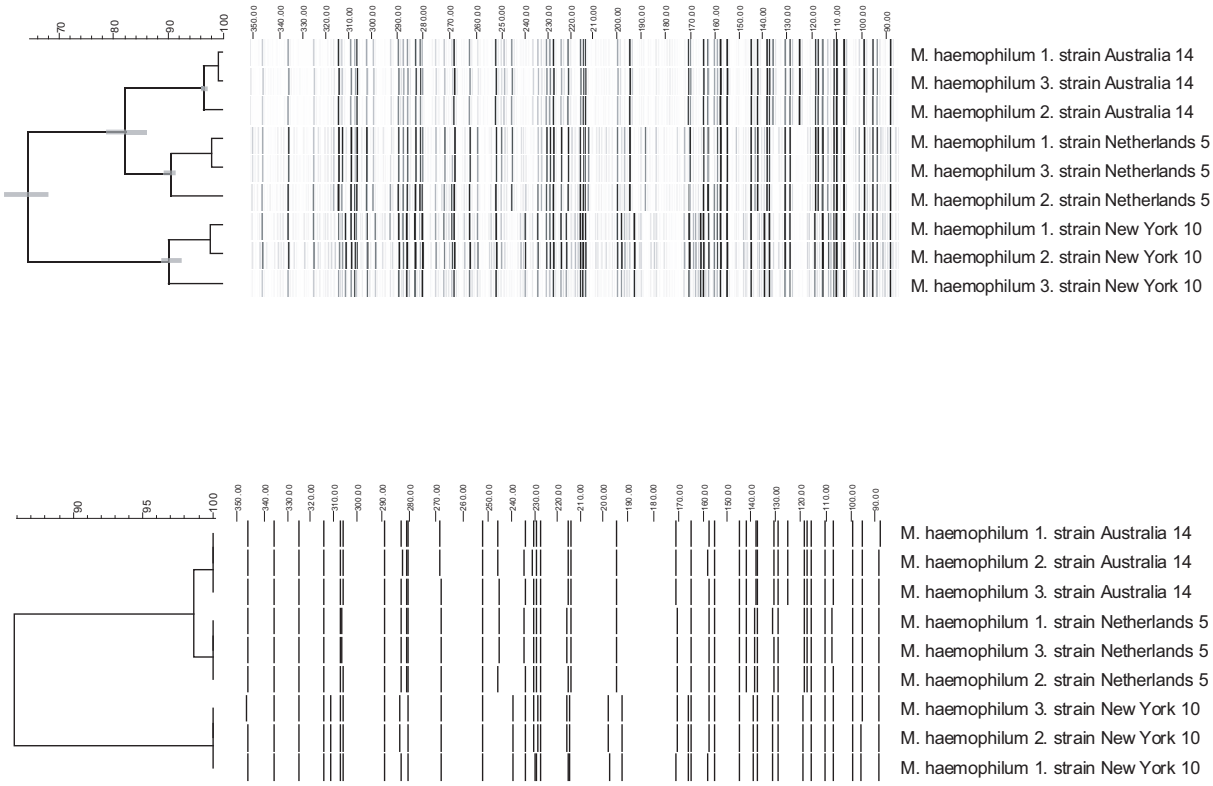


Figure 2. AFLP patterns of 3 *M. haemophilum* strains in triplicate. A) calculations with pearsons coefficient, B) with digitilised bands, calculated with DICE.



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)

References: Chapter 6.

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