"*Mycobacterium avium* subsp. *hominissuis*" in Neck Lymph Nodes of Children and their Environment Examined by Culture and Triplex Quantitative Real-Time PCR[▽]

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"*Mycobacterium avium* subsp. *hominissuis*" often causes cervical lymphadenitis in children; its prompt and accurate identification enables adequate therapy, tracing, and prevention. The aims of this study were to determine the causative agent of lymphadenitis using culture, PCR, and triplex quantitative real-time PCR (qPCR) methods with DNA directly isolated from tissue, as well as to identify possible sources of infection from the environment. We confirmed the diagnoses by detecting *M. avium* subsp. *hominissuis* using qPCR with DNA directly isolated from lymph node biopsy specimens of two patients. In order to trace the source of infection from the environment, a method of DNA isolation from soil and other environmental samples, such as dust, cobwebs, and compost, was developed. The triplex qPCR examination revealed the presence of *M. avium* subsp. *hominissuis* in a high proportion of the environmental samples (42.8% in the first patient's house and 47.6% in the second patient's house). Both patients were also exposed to *M. avium* subsp. *avium*, which was present due to the breeding of infected domestic hens. The high infectious dose of *M. avium* subsp. *hominissuis* or the increased susceptibility of humans to *M. avium* subsp. *hominissuis* compared to *M. avium* subsp. *avium* could be the reason why the children were infected with *M. avium* subsp. *hominissuis*.

Lymphadenitis in children occurs as a result of inflammation of the cervical, submaxillary, or submandibular lymph nodes. Cases usually present as unilateral enlargement of the lymph nodes. Awareness of the condition has increased in recent years due to the consistent upward trend in the incidence of the disease (13, 27). The identification of the causative agent of cervical lymphadenitis is essential for the administration of correct treatment and subsequent follow-up. Differentiation between enlargement caused by the *Mycobacterium tuberculosis* complex and that caused by nontuberculous mycobacteria (NTM) is crucial for determining the appropriate therapy.

The most prevalent causative agent in children between the ages of 1 and 5 in developed countries with a low incidence of human tuberculosis is NTM or potentially pathogenic mycobacteria (PPM) (3, 5, 22, 53), especially "*Mycobacterium avium* subsp. *hominissuis*" (50). The routes of *M. avium* subsp. *hominissuis* infection have not yet been clearly identified, but bearing in mind that human-to-human transmission has not been demonstrated, infection from an environmental source is considered to be most likely. *M. avium* subsp. *hominissuis* is ubiquitous in the environment and has been isolated from different types of samples such as soil, water, aerosols, dust, protozoa, and even small invertebrates (6, 12, 19, 22, 25).

The diagnosis of PPM causing cervical lymphadenitis is difficult and is often delayed due to the long incubation period required to grow the mycobacteria *in vitro*. Other methods of diagnosing the etiological agent include microscopic examination after Ziehl-Neelsen (ZN) staining, cytopathological analysis, DNA isolation from fine-needle aspirates (FNA), and subsequent PCR identification (37, 55). So far, culture-independent methods lack the specificity required to identify members of the *M. avium* complex (MAC), which consists of 28 different serotypes (54).

The most widely used tool for molecular identification of the members of the MAC is the detection of the insertion sequences IS*1245* and IS*901* (1, 10, 32). *M. avium* subsp. *avium* strains of serotypes 1, 2, and 3, and *M. avium* subsp. *hominissuis* strains of serotypes 4 to 6, 8 to 11, and 21 are closely related but differ in the presence and number of copies of these insertion sequences. *M. avium* subsp. *hominissuis* is characterized by multiple copies of IS*1245* and the absence of IS*901* (9, 20, 31, 51), whereas *M. avium* subsp. *avium* possesses multiple IS*901* copies and a single copy of IS*1245* (23). The principal source of human infection is infected birds, especially domestic hens (25, 35).

The aims of this study were to determine the causative agent of lymphadenitis through the use of conventional culture and triplex quantitative real-time PCR (qPCR) methods with DNA directly isolated from lymph node tissue. In order to identify the source of the infection from the environment, a method for DNA isolation from soil and other environmental samples, such as dust, cobwebs, and compost, was introduced, and its DNA yield based upon the model of *M. avium* subsp. *paratuberculosis* was determined. PCR results were compared to results of conventional microbiological culture.

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TABLE 1. Distribution of mycobacteria in patients' environmental samples as determined by microscopy with Ziehl-Neelsen staining, culture, and triplex qPCR

| Patient | Area | Sample | | Hen contact | ZN staining | qPCR (copies/g) | | Isolate identification |
		Location	Origin			IS*901*	IS*1245*	
1	Residence	Kitchen	Potting soil from supermarket	−	−	−	5.88×10^3	−
	Hennery	Coop with young (6-wk-old) chickens	Cobwebs by window	−	−	−	−	−
		Coop with old hens	Dust and cobwebs	+	−	1.00×10^0	−[a]	−
			Drinking water from vessel	+	−	−	−	−
	Greenhouse	Soil	Soil without hen and chicken droppings	−	−	−	5.64×10^2	−
		Iceberg lettuce	Leaf without soil contamination	−	−	−	−	−
	Garden	Compost	Material without hen and chicken droppings	−	−	−	1.42×10^4	−
2	Residence	Children's room	Potting soil fertilized with chicken droppings	+	+	7.14×10^1	2.25×10^7	*M. colombiense*
		Living room	Soil from store-bought cactus	−	−	−	4.42×10^4	
		Bedroom	Dust from a vacuum cleaner	−	−	−	6.21×10^3	*M. engbaekii*
	Hennery	Coop with old hens	Cobwebs above nests	+	−	1.00×10^0	2.60×10^4	*Mycobacterium* sp.
			Dry droppings from hens	+	+	1.00×10^0	3.93×10^3	*Mycobacterium* sp.
			Sand from pen	+	+	−	4.47×10^5	
	Greenhouse	Patch with vegetable	Soil fertilized with chicken droppings	+	+	3.28×10^1	2.67×10^7	
		Tagetes patula	Soil fertilized with chicken droppings	+	+	1.52×10^1	7.20×10^6	
		Thuja	Soil fertilized with chicken droppings	+	+	5.46×10^0	2.06×10^7	
	Garden	Pen for dog	Soil with dog feces and urine	−	+	−	3.11×10^1	
		Grass	Soil	+	+	−	1.50×10^7	
		Pelargonium	Soil fertilized with chicken droppings	+	+	−	−	*M. kumamotonense*
			Cobwebs with dust on plants	−	−	−	7.19×10^4	
		Hedge plants	Soil	+	−	4.14×10^1	1.34×10^6	*M. kumamotonense*
	Street	Near residence	Soil next to road	+	+	−	1.57×10^5	*Mycobacterium* sp.
	Garden of neighboring farm I	Shaded corner	Moss	−	−	−	2.22×10^1	*M. chelonae*
		Compost	Material without chicken droppings	−	+	−	1.00×10^0	
		Spruce tree	Soil under tree	+	+	−	2.17×10^5	
		Grass	Soil	+	+	1.05×10^2	1.59×10^4	
	Hennery of neighboring farm II	Coop with old hens	Dry chicken droppings from pen	+	+	−	−	*Mycobacterium* sp.
			Cobwebs	+	+	2.60×10^2	9.68×10^3	

[a] IS*1245* qPCR was negative due to the low number of *M. avium* subsp. *avium* cells in the examined matrix.

MATERIALS AND METHODS

Sample origin. Two patients, ages 7 and 2 years, were included in this study. Neither of them had any history of health problems. The time taken to develop an enlarged neck lymph node was several months; both were diagnosed with cervical lymphadenitis. A typical symptom that both of the patients shared was unilateral enlargement of the lymph node. Normal X-ray scans and no history of tuberculosis were also noted. Before the samples were obtained, informed consent was obtained from the parents of the children, and the samples were used according to the regulations of the respective hospital.

(i) Patient 1. A 7-year-old boy from the Czech Republic with no history of health problems developed an enlarged lymph node in the neck area on the left side in a period of 6 months. The isolate was cultured from a tissue sample after surgical excision of the enlarged lymph node. To identify possible sources of infection, seven samples from the patient's environment, i.e., the patient's house and garden, were collected (Table 1). An additional 32 soil samples were examined only by microscopy and mycobacterial culture. These samples were collected from the garden where the patient spent most of his time in the few months before the enlargement of the lymph node.

(ii) Patient 2. A girl from Slovakia, admitted to the Faculty Hospital in Bratislava, was 2 1/2 years old at the time of diagnosis. Samples for culture and qPCR examination were taken directly from the enlarged neck lymph node, which was situated on the left side. Sampling was done immediately after surgical

TABLE 2. Examined tissue samples from patients with neck lymphadenitis

Patient	Country	Age (yr)/sex	Specimen	AFB[a]	Culture	PCR, IS6110	qPCR IS901	qPCR IS1245
1	Czech Republic	7/male	Extirpated tissue with caseous lesions	−	+	−	−	+[b]
2	Slovakia	2/female	Extirpated tissue with caseous lesion, periphery	−	−	−	−	−
			Tissue without caseous lesions	−	−	−	−	−
			Extirpated tissue with caseous lesion, caseated center of 1 cm in diam	−	−	−	−	+[c]

[a] AFB, acid-fast bacilli visible after Ziehl-Neelsen staining.
[b] Identification was carried out with isolated DNA from the mycobacterial isolate.
[c] Quantification was not possible.

excision. Altogether, samples from three parts (peripheral, internal, and caseous center) were collected. Approximately 2 g of each tissue was collected in a sterile container, immediately chilled, and transported to the laboratory. To assess the prevalence of mycobacteria in the environment as a potential source of infection, a total of 21 environmental samples from the patient's immediate environment were examined, including samples from their home, garden, greenhouse, and hen house, as well as the backyards of their closest neighbors (Table 1). Most of the samples examined were from places in the house and garden where the child played. Environmental samples were taken using a sterile scraper and placed into sterile plastic bags. After transportation to the laboratory, the tissue and environmental samples were aliquoted into separate boxes to avoid possible cross contamination between samples.

Microscopy and culture examinations. (i) Microscopy. Smears were prepared from each sample after homogenization and decontamination and were stained using the ZN technique for the presence of acid-fast bacilli (AFB). At least 200 fields of view were examined for each sample using a light microscope at a magnification of ×1,000 under oil immersion (B17; Olympus, Tokyo, Japan).

(ii) Examination by culture. The homogenization and decontamination of the samples were performed as previously described (18). Briefly, approximately 1 g of each sample was suspended in 10 ml of sterile water, shaken for 30 min at 1,400 rpm, and then left standing at room temperature for 30 min. After decontamination of the centrifuged pellet, 80 μl of the suspension was inoculated onto two solid media and one liquid medium: Herrold's egg yolk medium, Stonebrink's egg-based medium, and Sula's liquid serum medium (26). Incubation of the cultures was carried out at 25 and 37°C. The cultures were monitored for the first week in order to rule out contamination and then every second week for up to 3 months.

(iii) Identification of isolates. AFB-positive isolates were further identified to species level by multiplex conventional PCR as described previously (32). If they did not belong to the MAC, they were sequenced (16S rRNA gene) as described by Harmsen et al. (21). The sequences obtained were compared to the available sequences deposited in the RIDOM (21) and NCBI databases.

DNA isolation from lymph nodes. DNA isolation from 50 mg of tissue samples obtained from the patient after surgery was based on a slightly modified DNeasy blood and tissue kit protocol (Qiagen, Hilden, Germany) as described by Slana et al. (45). Every isolation run of up to 12 samples included a negative isolation control, which contained corresponding amount of water.

PCR methods. In order to classify the bacterial species in the extracted lymph node samples, several PCR methods were used. Isolated DNA was tested for the presence of mycobacterial DNA using the conventional multiplex PCR system (32). To exclude the possibility of infection by *M. tuberculosis*, an IS6110 PCR detection method (*Mycobacterium tuberculosis* PCR kit; Geneproof, Brno, Czech Republic) was used. Triplex qPCR with an internal amplification control was used for the sensitive detection of *M. avium* subsp. *hominissuis* and *M. avium* subsp. *avium* in all tissue and environmental samples (45). All samples were analyzed with experimental replicates. Each PCR run included both positive (plasmid standard) and negative (water instead of DNA) PCR controls.

DNA isolation from environmental samples. The DNA isolation protocol was based on the MoBio PowerSoil DNA isolation kit (MoBio, Carlsbad, CA) with slight modifications. The principle of extraction is based on mechanical and chemical cell lyses followed by immobilization of the DNA on a silica spin column and subsequent washing and elution. Briefly, 0.25 g of soil was added to the PowerSoil bead beating tubes containing cell lysis solution, followed by the addition of 60 μl of the C1 solution and 6.25 μg of fish sperm DNA (Amresco,

Solon, OH). The samples were homogenized using a MagNA Lyser instrument (Roche Molecular Diagnostics, Mannheim, Germany) at 6,400 rpm for 60 s.

The following steps were performed in accordance with the manufacturer's instructions. After the DNA binding solution was added to the cleared supernatant, the mixture was loaded onto the spin column. Washing of the spin column was performed according to the manufacturer's instructions with an additional centrifugation step to remove any remaining ethanol from the column. The DNA was eluted in 100 μl of preheated Tris-EDTA (TE) buffer (Amresco) after a 3-min incubation period on the column. After centrifugation, the filtrate was reapplied onto the spin column in order to improve the yield. As for DNA isolation from tissue, the negative isolation control was included in all isolation runs.

Efficiency of DNA isolation from soil and limit of detection. The efficiency of the described protocol for DNA isolation from soil was tested by artificial contamination of four different types of soil: field soil, sand, peat, and pot soil. A model described previously for *M. avium* subsp. *paratuberculosis* was used (46). Briefly, a 1-ml aliquot of liquid culture of *M. avium* subsp. *paratuberculosis* was washed twice in TE buffer. Twenty 1-mm zirconia silica beads (Biospec, Bartlesville, OK) were added before mechanical disruption using the MagNA Lyser instrument (Roche). The suspension was centrifuged at $0.1 \times g$ for 30 s to remove the largest clumps. The supernatant was transferred to a new tube and diluted to six different concentrations, which were quantified and used to spike negative soil samples. After DNA isolation, *M. avium* subsp. *paratuberculosis* was quantified using the *F57* qPCR assay (46). Each *M. avium* subsp. *paratuberculosis* concentration was analyzed with eight physical replicates. The limit of detection was determined as the lowest cell concentration that could be detected in all replicates of the isolated samples. The DNA isolation yield was determined by calculating the median of all replicates (within the respective *M. avium* subsp. *paratuberculosis* artificial contamination concentrations).

IS1245 RFLP analysis. Tissue and soil isolates from patient 2 were subjected to restriction fragment length polymorphism (RFLP) analysis according to the standardized method described previously (29, 51). DNA was isolated according to the method described by van Soolingen et al. (52) and subsequently digested with the restriction endonuclease PvuII. DNA fragments were separated by agarose gel electrophoresis and transferred to a nylon membrane by vacuum blotting. DNA was hybridized with a labeled probe, and IS1245 profiles were analyzed according to the number and position of the bands.

RESULTS

Patient diagnosis. (i) Patient 1. The tissue sample was negative under microscopy with ZN staining but positive through culture examination for mycobacteria. The isolate *M. avium* subsp. *hominissuis* was identified through the presence of the specific 16S rRNA gene for mycobacteria and the IS1245 sequence as well as the absence of IS901, as analyzed by triplex qPCR (Table 2).

(ii) Patient 2. The tissue samples were negative under microscopy and culture examinations. However, after direct DNA isolation from the tissue, the presence of IS1245 was confirmed by triplex qPCR testing, which revealed that the

TABLE 3. DNA isolation efficiency and limit of detection of *Mycobacterium avium* subsp. *paratuberculosis* in different types of soil tested

Theoretical no. of *MAP* cells	Field soil		Sand		Peat		Potting soil	
	Signal ratio[a]	DNA recovery (%)	Signal ratio	DNA recovery (%)	Signal ratio	DNA recovery (%)	Signal ratio	DNA recovery (%)
5.5×10^4	8/8	54.9	8/8	52.8	NT[b]	NT	NT	NT
1.3×10^4	8/8	57.8	8/8	44.3	NT	NT	NT	NT
6.1×10^3	8/8	60.5	8/8	54.9	8/8	26.2	8/8	31.5
4.2×10^3	8/8	44.0	8/8	49.6	NT	NT	NT	NT
2.5×10^3	7/8	148.0	8/8	65.3	7/8	30.2	6/8	36.9
7.9×10^2	5/8	92.1	7/8	75.0	3/8	56.7	1/8	48.9

[a] Number of positive samples/total number of samples tested.
[b] NT, not tested.

lymphadenopathy was in fact due to *M. avium* subsp. *hominissuis* infection (Table 2).

Limit of detection and DNA isolation efficiency. The detection limit varied for the soil types tested in the range of 2.5×10^3 to 8.9×10^3 copies per g of soil. Specifically, detection limits of 4.2×10^3 copies per 1 g of field soil, 2.5×10^3 copies per 1 g of sand, and 8.9×10^3 copies per 1 g of peat and potting soil were established. At these concentrations in all eight replicates, positive results using qPCR was obtained. Samples with lower numbers of mycobacteria were still considered to be positive, but the probability of the detection in repeated experiments is lower. The median DNA isolation efficiency was 59.1% for field soil, 53.8% for sand, 30.2% for peat, and 36.9% for pot soil samples (Table 3).

Environmental samples. (i) Residence of patient 1. None of the seven environmental samples examined by microscopy, culture, and triplex qPCR were positive for AFB by ZN staining. The samples were also negative under culture examination. Three of the samples examined by triplex qPCR were positive for *M. avium* subsp. *hominissuis* DNA (two samples of soil and one sample of dunghill), and one sample was positive for *M. avium* subsp. *avium* DNA (dust and cobwebs from the hen house; Table 1).

There were 32 additional samples of pot soil or soil from the garden examined using only the culture method, 5 of which resulted in *M. avium* subsp. *hominissuis* isolates. They were all subjected to RFLP analysis and showed the same RFLP profile as the isolate from the lymph node of the patient (Fig. 1).

Residence of patient 2 and neighboring houses. The ZN microscopy examination of the decontaminated samples resulted in 16 (69.5%) out of 21 samples being positive for AFB. Fourteen of these samples were also positive for either *M. avium* subsp. *avium* or *M. avium* subsp. *hominissuis* by triplex qPCR, of which two were positive by culture; these were identified by 16S rRNA sequencing as *M. kumamotonense* and a *Mycobacterium* strain that could not be further identified. Culture examination resulted in seven positive isolates, none of which belonged to the *M. avium* species as confirmed by conventional multiplex PCR (32). The 16S rRNA gene was sequenced, and the following strains were obtained: *M. colombiense*, *M. engbaekii*, *M. chelonae*, two isolates of *M. kumamotonense*, and two isolates of a *Mycobacterium* species that could not be identified using the available deposited sequences. From the 21 environmental samples examined by triplex qPCR, 10 (47.6%) were positive for *M. avium* subsp. *hominissuis* and 9 (42.8%) were positive for *M. avium* subsp. *avium*. The *M. avium* subsp. *hominissuis*-positive samples were present in the house of the patient (potting soil and dust) as well as in the soil from the garden and greenhouse. *M. avium* subsp. *avium* was found predominantly in the hennery and neighbor's garden (Table 1).

DISCUSSION

Although NTM isolates have been differentiated using real-time qPCR (28, 39, 44, 47), the majority of research is still devoted to detecting and identifying members of the *M. tuberculosis* complex (14). Real-time PCR for the detection of MAC and *M. tuberculosis* complex in fine-needle aspirates and tissue biopsy samples has been confirmed as a useful tool for the rapid diagnosis of mycobacterial lymphadenitis (49). Recently, a triplex qPCR system for the simultaneous detection of *M. avium* subsp. *avium* and *M. avium* subsp. *hominissuis* in pig tissues has been described (45). We have used the same triplex qPCR system, and it has proven to be an efficient and rapid tool for the identification of *M. avium* subsp. *hominissuis* from tissue samples of two patients (Table 2) and their environments (Table 1).

The recovery of mycobacteria by culture is a standard method, although due to limitations encountered (i.e., their slow growth), in recent times culture-independent methods have been preferred (25). Our results show that qPCR is a much more sensitive method for the detection of mycobacteria, as shown with patient 2, whose diagnosis was not confirmed by culture. The diagnosis of *M. avium* subsp. *hominissuis* as a causative agent in neck lymphadenitis in children by using qPCR has been performed also with patients in Germany, where the amount of IS*1245* ranged between 10^1 and 10^2 copies (data not shown). The reliability of the culture method was evaluated in a study analyzing diagnostic methods for detecting mycobacteria in pig tissue samples. A high number of tuberculous lesions present in lymph nodes were ZN positive but not confirmed by culture (43). This and other studies suggest that correlation among different diagnostic methods would be complicated (24, 45).

The infectious route of mycobacteria (other than in tuberculosis) has been studied on several occasions. Some studies implicate water as the source of infection, especially in HIV-infected individuals (16, 17, 36). Van Coppenraet et al. (50) could not find a link between pet birds and the incidence of mycobacterial lymphadenitis in children, concluding that infection can occur from any environmental source, while other
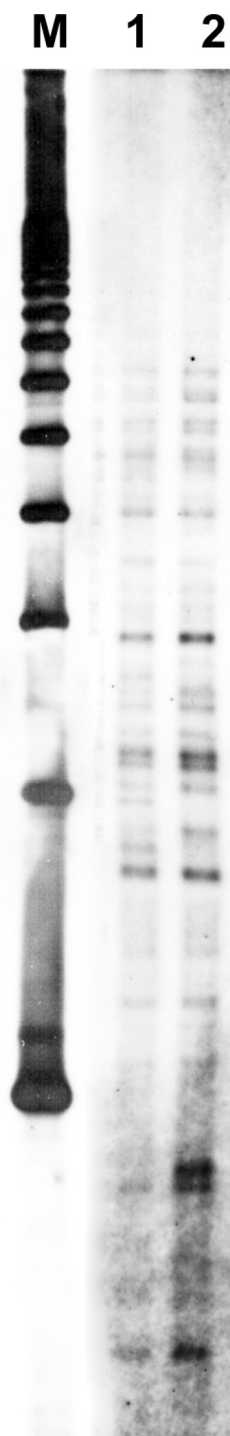
FIG. 1. Comparison of IS*1245* RFLP types of the isolate obtained from patient 1 (lane 1) and an isolate obtained from pot soil in his house (lane 2). A 1-kb ladder was used as a marker (lane M).

authors have found a strong correlation between soil and mycobacterial infection (7, 38, 56). Our results suggest that soil contaminated with *M. avium* subsp. *hominissuis* was the main source of infection for both children. Surprisingly, both children were in close contact with soil and dust contaminated with *M. avium* subsp. *avium* in connection with flocks of infected hens reared near their residences (Table 1). Using the triplex qPCR system, we are not able to differentiate between *M. avium* subsp. *avium* and mixed *M. avium* subsp. *avium*/*M. avium* subsp. *hominissuis* infections. In some samples we have detected a larger amount of IS*1245* than of IS*901*; however, we cannot conclude that there was a mixed infection solely on the basis of this result. These results suggest a higher sensitivity of humans to *M. avium* subsp. *hominissuis* infection than to infections caused by *M. avium* subsp. *avium* (25, 35, 50).

Although *M. avium* subsp. *hominissuis* has often been isolated from soil using the culture method, studies of microbial soil ecology suggest that a large percentage of microbial cells present in the environment are not able to be isolated *in vitro*; i.e., they could be viable but noncultivable, making their detection using molecular techniques necessary (2, 48). Furthermore, mycobacteria present in soil samples are easily outgrown by other, faster-growing microorganisms (15). Their strong adherence to soil particles and their viability can also present difficulties in their isolation by culture (4, 8). Due to these obstacles, the use of molecular methods for detection and identification of mycobacteria in the environment is favorable. Mycobacterial DNA has been isolated from soil in previous studies for the purpose of an ecological study or genus-specific identification, although no further species identification was done (30, 33, 34).

Previous studies indicate that *M. avium* subsp. *hominissuis* is the most common etiological agent of NTM lymphadenitis in children (50). The patients in our study were exposed to an infectious environment containing *M. avium* subsp. *avium* due to the breeding of domestic hens. In such small flocks, old hens (3 years or older) could be present. The number of positive *M. avium* subsp. *avium* DNA samples was similar to that of positive *M. avium* subsp. *hominissuis* DNA samples (Table 1). The reason why the children were infected with *M. avium* subsp. *hominissuis* and not with *M. avium* subsp. *avium* is not clear. It has been described that *M. avium* subsp. *hominissuis* is pathogenic mainly to humans (31, 35), while infections with *M. avium* subsp. *avium* are more typical for birds (11, 35, 40–42), perhaps indicating why there was an increased susceptibility to *M. avium* subsp. *hominissuis* in these patients. Moreover, *M. avium* subsp. *hominissuis* DNA was found in a higher quantity in the environment, indicating an exposure of the patients to a higher infectious dose.

In conclusion, the source of infection was confirmed as soil for one of the patients by RFLP of the IS*1245* sequence, and this has also been suggested for the second patient, using direct triplex qPCR and culture methods.

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