Diagnosis of *Mycobacterium microti* Infections among Humans by Using Novel Genetic Markers

DICK van SOOLINGEN,[1]* ADRI G. M. van der ZANDEN,[2] PETRA E. W. de HAAS,[1]
GERDA T. NOORDHOEK,[3] ALBERT KIERS,[4] NORBERT A. FOUDRAINE,[5]
FRANCOISE PORTAELS,[6] AREND H. J. KOLK,[7] KRISTIN KREMER,[1]
and JAN D. A. van EMBDEN[8]

*Diagnostic Laboratory for Infectious Diseases and Perinatal Screening[1] and Research Laboratory for Infectious Diseases,[8]
National Institute of Public Health and the Environment, 3720 BA Bilthoven, Regional Public Health Laboratory
Deventer, 7400 GD Deventer,[2] Regional Public Health Laboratory Friesland, 8900 JA Leeuwarden,[3] Municipal
Health Service Noord Friesland, 8901 BK Leeuwarden,[4] National AIDS Therapy Evaluation Centre,
Academical Medical Centre,[5] and Department of Biomedical Research, Royal Tropical
Institute,[7] 1105 AZ Amsterdam, The Netherlands, and Mycobacteriology Unit,
Institute of Tropical Medicine Prince Leopold, 2000 Antwerp, Belgium[6]*

As a result of DNA typing of *Mycobacterium microti* isolates from animals in the United Kingdom and The Netherlands, we diagnosed four human *M. microti* infections. These are the first *M. microti* infections among humans to be reported. Three of the patients were immunocompromised and suffered from generalized forms of tuberculosis. The fourth patient was a 34-year-old immunocompetent male with a persistent cough and undefined X-ray abnormalities. Two of the *M. microti* infections were recognized by their IS*6110* restriction fragment length polymorphism (RFLP) patterns, which showed a high degree of similarity with those of *M. microti* strains isolated from a pig and a ferret in The Netherlands. The two other human *M. microti* infections were recognized by using the recently developed DNA fingerprinting method, "spoligotyping," directly on clinical material. All *M. microti* isolates from the United Kingdom and The Netherlands were found to contain an exceptionally short genomic direct repeat region, resulting in identical two-spacer sequence reactions in spoligotyping. In contrast, the highly similar IS*6110* RFLP patterns of the vole strains from the United Kingdom differed considerably from the RFLPs of all *M. microti* strains isolated in The Netherlands, suggesting that geographic isolation led to divergent strains in the United Kingdom and on the continent.

Tuberculosis in the wild vole, or field mouse (*Microtus agrestis*), was discovered by Wells in 1937 (27, 28), and this epizootic disease was found to be rather common among these animals in the United Kingdom, with a prevalence ranging from 9 to 31% (27). The causative agent was named *Mycobacterium tuberculosis* subsp. *muris* (2), and later this species was designated *Mycobacterium microti* and classified as a member of the *M. tuberculosis* complex (26). *M. microti* differs from other *M. tuberculosis* complex strains in its S-shaped cell morphology, its slow growth in vitro, and its distinct host-specific pathogenicity for laboratory animals (13, 17, 27). Based on biochemical properties, this bacterium is difficult to distinguish from *M. tuberculosis*, *Mycobacterium africanum*, or *Mycobacterium bovis* (26).

In subsequent studies, *M. microti* was also detected in a limited number of other mammalian species, i.e., the bank vole (*Clethrionomys glareolus*) (27), the wood mouse (*Apodemus sylvaticus*) (27), the shrew (*Sorex araneus*) (27), cats and pigs (8, 9), and a zoo llama (*Lama vicugna molina*) (12). A morphologically similar organism, the "dassie bacillus," was isolated in South Africa from the Cape hyrax, or dassie (*Procavia capensis*) (3, 15, 25), but this isolate differed from the vole bacillus in not being virulent for mice.

Recently, various repetitive genetic elements have been iden-

tified in *M. tuberculosis* complex bacteria, and these have been used to differentiate clinical isolates of *M. tuberculosis*, *M. africanum*, and *M. bovis* (14). The most frequently used repetitive elements in the molecular epidemiology of tuberculosis are the insertion sequence IS*6110*, the polymorphic GC-rich sequence (PGRS), and the direct repeat (DR), which are present in all members of the *M. tuberculosis* complex group, including *M. microti* (4, 14). However, there are no reported data on the DNA polymorphism associated with these repetitive DNA elements among human isolates of *M. microti*. The DNA polymorphism associated with these genetic markers is usually detected by restriction fragment length polymorphism (RFLP). Some DNA fingerprints characteristic of *M. tuberculosis* complex (sub-)species (14, 19) have been found, such as the characteristic combination of the IS*1081* and IS*6110* RFLP patterns of *M. bovis* BCG vaccine strains (22) and the specific IS*1081* RFLP pattern, consisting of a single band, of the recently recognized subspecies *Mycobacterium tuberculosis* subsp. *canetti* (also called *Mycobacterium canetti*) (23). Furthermore, spacer oligonucleotide typing (spoligotyping) permits recognition of *M. bovis* by the characteristic absence of five spacer sequences in the 3′-terminal part of the DR cluster, and the absence of spacers 2, 9, and 16 (1, 10).

The present study was undertaken to determine the DNA polymorphism among *M. microti* isolates by using IS*6110* fingerprinting and spoligotyping. We show that *M. microti* strains display characteristic IS*6110* banding patterns and spoligotypes, distinct from types previously observed in other *M. tuberculosis* complex strains. Unexpectedly, these characteristic
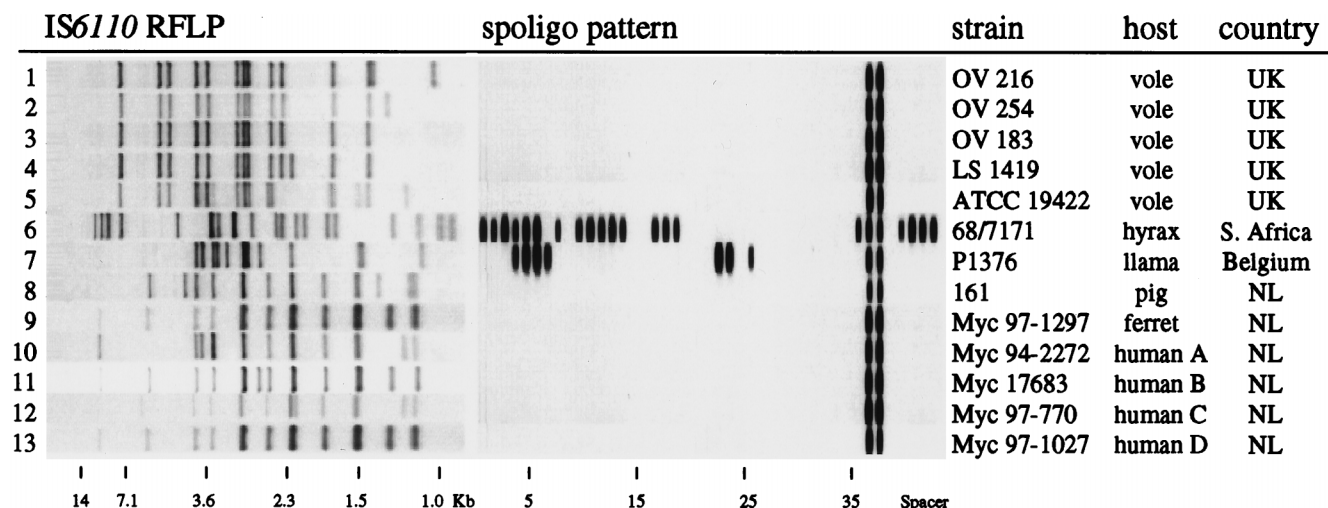
FIG. 1. IS*6110* RFLP patterns and spoligotyping (spoligo) patterns of all described mycobacterial isolates.

fingerprints and spoligotypes allowed us to identify four human tuberculous infections caused by *M. microti*.

MATERIALS AND METHODS

Mycobacterial strains. *M. microti* strains isolated from voles in the 1930s in the United Kingdom (OV 254, OV 216, OV 183, and LS 1419) and the strain isolated from a Cape hyrax in 1958 in South Africa (dassie bacillus, 68/7171) were supplied by T. Jenkins and P. Draper, National Institute for Medical Research, The Ridgeway, Mill Hill, London, United Kingdom. The *M. microti* pig isolate, strain 161, isolated in 1965 in the southern part of The Netherlands, and strain 97-1297, isolated in 1993 from a ferret kept as a household pet in the western part of The Netherlands, were obtained from J. Haagsma, Central Veterinary Institute, 8200 AB Lelystad, The Netherlands. Strain P1376, isolated in 1968 from a llama born in the Antwerp Zoo, Antwerp, Belgium, was supplied by L. Rigouts, Mycobacteriology Unit, Institute of Tropical Medicine Prince Leopold, Antwerp 2000, Belgium. The *M. microti* type strain, ATCC 19422, a vole isolate, was obtained from the American Type Culture Collection, Manassas, Va.

Patient isolates. Strain myc 17683 was isolated in 1993 from alveolar lavage fluid of a 12-year-old kidney transplantation patient, and strain myc 94-2272 was isolated in 1988 from the perfusion fluid of a 41-year-old dialysis patient. Strain 97-770 was isolated in 1997 from a lung biopsy of an immune-competent 34-year-old male with a persistent cough and undefined abnormalities on the chest X ray. Strain 97-1027 was isolated in 1997 from sputum of a 39-year-old human immunodeficiency virus (HIV)-positive male.

Bacteriological identification. All strains were subjected to the Accuprobe culture confirmation test for the *M. tuberculosis* complex (Genprobe, San Diego, Calif.) and standard bacteriological analysis for *M. tuberculosis* complex bacteria (26).

Molecular typing. Typing by RFLP using IS*6110*, PGRS, and DR as DNA probes was performed as described previously (14, 20, 21). The recently developed spoligotyping technique is based on the strain-dependent presence or absence of short nonrepetitive spacer sequences, which interspace the repetitive DR sequences (10, 24). Comparison of IS*6110* fingerprints and spoligotypes by computer-assisted analysis was performed as described previously (7, 24). The IS*6110* fingerprint database contained approximately 4,000 patterns of *M. tuberculosis* complex isolates from patients in The Netherlands in the period from 1990 through 1997 and 2,000 patterns from patients' isolates originating in 30 different countries (18). The spoligotype database was established in 1995 through 1996 and contained 800 *M. tuberculosis* strains, mainly from patients in The Netherlands, and 800 *M. bovis* strains from humans and animals in The Netherlands and 15 other countries.

The presence of the *mtp40* sequence, alleged *M. tuberculosis* specific, was determined by the PCR-based method described by Del Portillo et al. (5).

RESULTS

Genetic characterization of *M. microti* isolates. Nine *M. microti* strains from animal sources, including the dassie isolate, were subjected to IS*6110* RFLP typing. The results are shown in Fig. 1, lanes 1 to 9. The five vole strains isolated in the United Kingdom in the 1930s (27, 28) all displayed distinct, but highly similar, IS*6110* banding patterns. The fingerprints of these strains shared at least 11 of the 13 IS*6110*-containing *Pvu*II restriction fragments (Fig. 1, lanes 1 to 5), suggesting that these strains derived relatively recently from a common ancestor. This close genetic relatedness was confirmed by the virtually identical PGRS fingerprints (data not shown). Two other strains, isolated from a Cape hyrax in South Africa and a zoo llama in Antwerp, displayed very different IS*6110* and PGRS fingerprints, and these were also unrelated to the banding patterns found among the vole strains from the United Kingdom (Fig. 1, lanes 6 and 7). The remaining two strains, isolated from a ferret and a pig in The Netherlands, showed highly similar IS*6110* RFLPs, and these were unrelated to those of the other seven animal isolates (Fig. 1, lanes 8 and 9).

By spoligotyping, only three different patterns were obtained. The five vole strains and the isolates from the ferret and the pig displayed reactions with only 2 of the 43 spacer oligonucleotides (spacers 37 and 38 [Fig. 1]). This indicates the presence in the DR region of only 2 of the 43 spacer DNA sequences currently used in spoligotyping and suggests that these seven *M. microti* strains may have an unusually small DR cluster. In order to confirm this observation, we subjected *Pvu*II-digested DNA from these strains to Southern blotting using synthetic DR DNA as a probe. Only a single DR-hybridizing fragment was found among these strains, and the hybridization signal was weak compared to that in other strains such as *M. tuberculosis* H37Rv and *M. bovis* BCG strain P3, which harbor more than 40 DRs and spacers in the DR region (data not shown). Therefore, the DR region in these seven *M. microti* strains may be relatively short, presumably containing fewer DRs than have been observed previously among other *M. tuberculosis* complex strains (1, 6, 9).

The spoligotypes of the dassie strain and the zoo llama isolate were very different from the vole, ferret, and pig strains. The spoligotyping-PCR products of these strains hybridized with 22 and 9 spacer sequences, respectively (Fig. 1, lanes 6 and 7).

The spoligotypes of all eight *M. microti* isolates and the dassie strain (Fig. 1, lanes 1 to 9) were compared with those in the spoligotype database. No matching spoligotypes were found. Therefore, the spoligotype found in seven of the nine animal isolates seems to be characteristic of *M. microti*.

Recognition of *M. microti* infections in humans. The IS*6110* banding patterns of the *M. microti* strains and the dassie isolate were compared with the patterns in the database of fingerprints of human isolates. Only two patient isolates showed at least 80% similarity with the *M. microti* strains, a level which allows three to four IS*6110* fragment mismatches. The patterns of these two strains were highly similar to the IS*6110* fingerprints of the *M. microti* strains isolated from the ferret and the pig (Fig. 1, lanes 10 and 11). Spoligotyping of these two isolates revealed *M. microti*-characteristic hybridization patterns, identical to those of the ferret isolate, the pig isolate, and the five vole strains (Fig. 1, lanes 10 and 11). Because of its extremely slow growth during the primary isolation and the circumstantial evidence for transmission from mice, as discussed in the next paragraph, one of the two isolates, myc 94-2272, had previously been suspected to be *M. microti* by the regional public health laboratory where the strain was isolated. The other mycobacterial isolate, myc 17683, had previously been identified by the National Reference Laboratory as an "aberrant *M. tuberculosis* complex isolate" on the basis of doubtful biochemical criteria and slow growth (data not shown).

Two additional putative *M. microti* infections among humans were found in two different regional public health laboratories by performing spoligotyping directly on clinical material to detect the presence of *M. tuberculosis* complex-specific DNA. In both cases, the *M. microti*-characteristic spoligotype pattern was observed, as described above for the isolates from the first two patients, the pig, and the ferret and the five vole strains. Subsequent IS*6110* DNA fingerprinting of these isolates showed a high degree of similarity with those of the isolates from the other humans, the ferret, and the pig (Fig. 1, lanes 12 and 13).

The geographic origins of the human and animal cases in The Netherlands are depicted in Fig. 2. Two human cases of *M. microti* infections, diagnosed in 1988 and 1997, were found in the same city. The houses of these patients were positioned only 1 km apart.

Bacteriological identification. All four human *M. microti* isolates were found to grow extremely slowly on Löwenstein-Jensen medium during the primary isolation. Therefore, it was impossible to perform biochemical identification on these isolates. The remaining strains, which had been recultured more frequently and therefore grew more rapidly in vitro, were subjected to biochemical analysis, and the results are shown in Table 1. By biochemical identification, the strains shared properties of both *M. tuberculosis* and *M. bovis*, which is characteristic of *M. microti* (17, 26). However, the characteristic curved morphology of *M. microti* was not observed in any of the Ziehl-Neelsen-stained microscopic preparations of the bacteriological cultures. Because reexamination of the original microscopic slides of the peritoneal biopsy (patient A), the bronchial biopsy (patient B), the biopsy of the right apical segment of the upper lobe (patient C), and sputum (patient D) showed the presence of curved acid-fast bacilli (Fig. 3), we conclude that all four human isolates belong to the species *M. microti*. We presume that the characteristic property of S-curved cell morphology was lost during repeated in vitro culturing, as has been described previously (13).

Clinical backgrounds of human cases. Patient A, a 41-year-old male, had undergone kidney transplantation in 1982. Because of insufficient kidney transplant function, continuous ambulatory peritoneal dialysis was started in 1986. In 1988 the patient had fever, lost weight, suffered from progressive anemia, and was found to have esophagitis, gastritis, bulbitis, and colitis. No pathogenic bacteria were isolated. The patient was hospitalized, and the peritoneal fluid was found to be positive
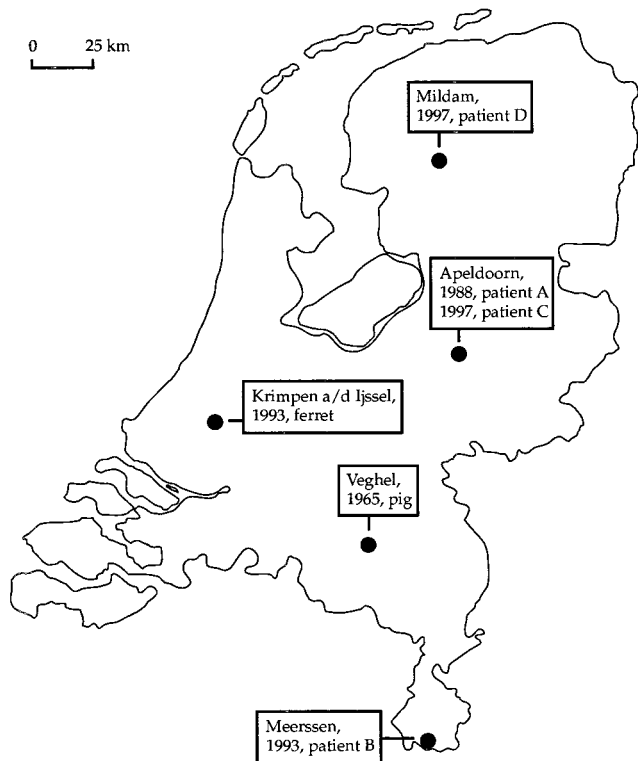

FIG. 2. Map of The Netherlands indicating the geographic origins of the animal and human tuberculous infections caused by *M. microti* and the times of diagnosis.

in Ziehl-Neelsen staining. The patient died shortly after the diagnosis of tuberculous peritonitis was made. After 4 months acid-fast bacteria were cultured, whereas no other bacteria were found. A small laparotomy revealed extensive tuberculous peritonitis. Histologic examination of peritoneal biopsy confirmed this diagnosis. The patient died shortly after the tuberculous peritonitis had been diagnosed. Because spoligotyping allows the simultaneous detection and typing of *M. tuberculosis* complex bacteria in clinical specimens (10), we subjected the 8-year-old tissue specimen, embedded in paraffin, to spoligotyping in 1996. The sample was positive, and the amplified DNA hybridized with spacers 37 and 38 only. This spoligotype pattern was identical to that of the cultured strain from the same patient (data not shown). This result confirms that the patient had indeed been infected with *M. microti* and excluded the possibility that only the dialysis fluid had been contaminated with *M. microti*. It should be noted that in 1988, when the strain was isolated, contact tracing did not lead to possible human sources of infection. However, during a visit of the social nurse, it was found that the patient kept his dialysis fluid bags in an unclean shed behind his house and that mouse droppings were present on the surface of the bags. This suggests that murine fecal contamination was the source of infection.

Patient B was a 12-year-old boy who had undergone kidney transplantation and was therefore treated with immunosuppressives (prednisone plus azathioprine). He was suffering from coughing. From the results of a chest X ray, tuberculosis was suspected. Acid-fast bacilli, later identified as *M. microti*, were isolated from both the bronchoalveolar lavage fluid and a bronchial biopsy. Besides the presence of the acid-fast bacilli, the histologic examination of the biopsy revealed extensive

TABLE 1. Results of biochemical tests of some of the isolates included in this study[a]

Strain	Growth[b] at the following temp (°C):			Growth on Löwenstein-Jensen medium with:		Niacin	Nitratase	Urease	Catalase	PO$_4$	Pyrazinamidase	Pigment	Colony morphology
	24	36	45	Pyruvate	Thiophene								
OV 216	−	−	−	++	−	+	−	+	−	−	−	No	Smooth
OV 254	−	++	−	++	−	+	−	+	−	−	−	No	Rough
OV 183	−	+	−	+	−	+	−	+	−	−	−	No	Rough
LS1419	−	−	−	++	−	+	−	+	−	−	+	No	Smooth
ATCC 19422	−	++	−	++	−	+	−	+	−	−	−	No	Rough
68/7171	−	−	−	++	−	+	−	−	−	−	+	No	Smooth
P1376	−	+	−	+	−	−	−	+	−	−	−	No	Rough
161	−	−	−	+	−	−	−	−	−	−	−	No	Smooth

[a] −, negative; +, positive; ++, strongly positive.
[b] On Löwenstein-Jensen medium.

caseation and necrosis. A computed tomography (CT) scan of the head visualized a cerebral tumor suggestive of a tuberculoma, although no biopsy was taken. The family of the patient kept no pets, and contact tracing did not offer any clue about a possible source of infection. The patient was set on triple therapy with antituberculosis drugs, resulting in regression of the roentgenographic abnormalities and disappearance of the acid-fast bacilli.

Patient C, a 34-year-old male living in a caravan camp, presented with persistent coughing. On the chest X ray, a cavernous abnormality in the right apical segment of the upper lobe was seen. The Mantoux reaction was negative, as were the Ziehl-Neelsen staining and the sputum culture. A CT scan-guided lung biopsy was taken and revealed the presence of acid-fast bacilli. Spoligotyping performed directly on biopsied lung tissue was positive for *M. microti*. Mycobacterial culturing was successful after 3 months of incubation. After 6 months of triple antituberculosis therapy, the abnormalities had disappeared completely.

Patient D was a 39-year-old HIV-positive X-ray assistant, working in a hospital. This patient presented with coughing and an infiltrate in the left lower lobe. Microscopic examination of his sputum yielded high numbers of acid-fast bacilli (Fig. 3). Treatment was started after the multiplex PCR (11) directly on sputum had been found positive for *M. tuberculosis* complex (11). The patient was successfully treated (regression of the roentgenographic abnormalities and disappearance of the acid-fast bacilli) with antituberculosis drugs.

A possible indication for zoonotic transmission was found in the fact that an infestation of mice afflicted the house of the patient 5 months prior to the manifestation of the *M. microti* infection. Contact tracing, using routinely applied *M. tuberculosis* complex purified protein derivative (PPD), resulted in three positive skin tests, two from colleagues 33 and 35 years old and one from a 13-year-old cousin, none of whom were immunocompromised. These PPD-positive contacts are highly suggestive of human-to-human transmission of *M. microti*, since in The Netherlands the current annual risk of infection, and consequently the percentage of positive skin tests in the population, is very low.

Presence of the genomic *mtp40* sequence. The *mtp40* DNA sequence has been described as species specific for *M. tuberculosis* and therefore is useful for differential diagnosis of infections due to *M. tuberculosis* (13). The PCR to detect this sequence was negative for all vole isolates, as well as for the hyrax strain. The PCR was positive for the llama, ferret, and pig strains and for all four human isolates.

DISCUSSION

In this study for the first time, *M. microti* infections among humans are described. This might indicate that such infections occur only rarely. On the other hand, human infections with *M. microti* are difficult to detect because of the extremely slow growth of this organism and because of the difficulty in recognizing this species by traditional bacteriological methods. Furthermore, as discussed by Pattyn et al. (13), phenotypic traits of *M. microti* strains, like the curved cell morphology and growth characteristics, may change after a few passages in vitro, a phenomenon which we also observed in this study. Because of these difficulties in diagnosing *M. microti* infections by traditional bacteriological methods, we presume that *M. microti* infections among humans have simply been overlooked so far. This makes it impossible to estimate the frequency of human *M. microti* infection.

This study shows that genetic markers generally used in the epidemiology of tuberculosis can be used to recognize *M. microti* infections among humans. Although IS*6110*, PGRS, and the DR have previously been identified in *M. microti* (4), no data have been available on the DNA polymorphism associ-
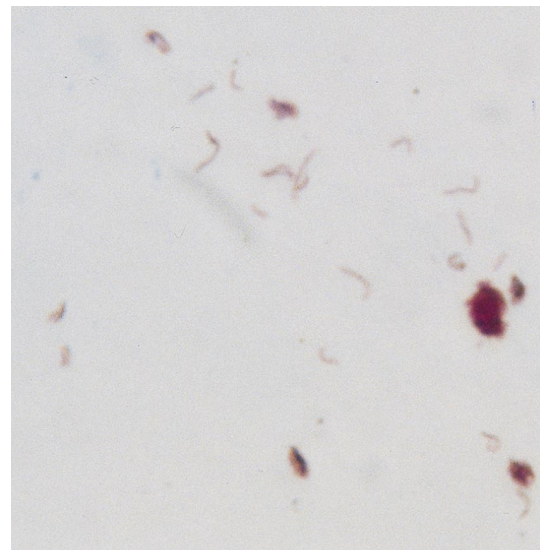

FIG. 3. Ziehl-Neelsen-stained sputum of patient D. Note the characteristic curved acid-fast bacilli.

ated with these repetitive DNA elements in this species of the *M. tuberculosis* complex. The IS*6110* fingerprints of the strains analyzed in this study indicate that there are at least two genetically distinct groups of *M. microti* in Europe. These two IS*6110* genotypes have an unusually short chromosomal DR cluster in common, in which only two DR spacer sequences, derived from *M. tuberculosis* H37Rv and *M. bovis* BCG, are present. The IS*6110* fingerprints and the spoligotypes of these European strains were unrelated to the two *M. microti* strains originating from the exotic Cape hyrax from South Africa and the llama from a zoo in Belgium.

The *M. microti* infections among humans were recognized because of their characteristic spoligotype and their uncommon IS*6110* fingerprint patterns, which were similar to those of two animal *M. microti* strains, one isolated from a pig in 1965 in the southern part of The Netherlands (9) and the other isolated from a ferret kept as a household pet in the western part of The Netherlands in 1993. The former strain caused localized lesions in the laryngeal lymph nodes of the pig. During the same period Huitema and colleagues described the occurrence of similarly, characteristically curved mycobacteria in other pigs and also in cats (8, 9). Unfortunately, these strains were no longer available for DNA typing, and there are no reports on the isolation of *M. microti* from rodents in The Netherlands. However, it seems plausible to assume that the *M. microti* infection of the pig was acquired by contamination of soil with feces of mice or voles. The ferret *M. microti* strain was isolated in 1993 after a clinical diagnosis of mycobacterial infection was made, following illness featuring anorexia, weight loss, and malaise. After euthanasia, numerous acid-fast bacteria were found in all organ tissues. It is conceivable that the ferret contracted the *M. microti* infection through consumption of an infected rodent.

The limited IS*6110*-associated DNA polymorphism among the vole strains isolated in the 1930s in the United Kingdom indicates that they belong to a genetically closely related group of *M. microti* strains. This genotype family is distinct from the other IS*6110* grouping of *M. microti* comprising two animal isolates and four human strains isolated in The Netherlands. Additional support for the existence of two genogroups is the observation that these groups differ in the presence or absence of the *mtp40* sequence.

For one of the human isolates, we found circumstantial evidence for transmission from rodents to a human, because this strain was isolated from a patient who stored his dialysis fluid bags under nonhygienic conditions, where contamination with fecal material from mice or voles was likely to occur. For one of the other patients zoonotic transmission is also conceivable, as an infestation of mice afflicted his house a few months prior to the manifestation of tuberculous symptoms. For the two remaining patients no direct indications for involvement of zoonotic transmission were found, although one of the patients, a 41-year-old immunocompetent male, was living in a caravan camp, where the chances of contact with the habitat of mice may be greater than in an ordinary household.

Prewar studies on the prevalence of *M. microti* infections in Scotland, Wales, and England showed a prevalence ranging from 9 to 31% depending on the place and season of capture (27, 28). Unfortunately, no data on the prevalence of *M. microti* among voles in The Netherlands or other parts of Europe are available, but there is no reason to suspect that the prevalence in these areas differs from that previously observed by Wells and Oxon in the United Kingdom (27, 28). Taking into account the extended period during which *M. microti* strains were isolated in The Netherlands (from 1965 to 1997) and the wide geographical distribution of *M. microti* infections among

humans and animals in The Netherlands, it is likely that *M. microti* is endemic among rodents in The Netherlands.

The dissimilarity in IS*6110* banding patterns between the Dutch strains and the vole strains from the United Kingdom is probably due to their geographic isolation for extended periods. A similar effect of geographic isolation has been found for *M. bovis* strains isolated in Europe and South America (19).

Although the number of Dutch *M. microti* strains examined is small, they show little IS*6110*-associated polymorphism. This is in contrast with the genetic heterogeneity observed among *M. bovis* and *M. tuberculosis* isolates in this region (7, 19–21).

In the 1950s and 1960s, *M. microti* was considered nonpathogenic for humans, and for this reason, *M. microti* has been used as an experimental tuberculosis vaccine in trials in the United Kingdom and in the Czech Republic (16). One of the *M. microti* strains analyzed in this study (strain LS1419) was used in the United Kingdom as a vaccine strain in the Medical Research Council vaccine trial in the 1950s (5a). Our present study indicates that the use of live *M. microti* for vaccination may constitute a health hazard of a similar magnitude as the use of BCG for immunocompromised individuals.

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