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Simultaneous Detection and Strain Differentiation of *Mycobacterium tuberculosis* for Diagnosis and Epidemiology

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Widespread use of DNA restriction fragment length polymorphism (RFLP) to differentiate strains of *Mycobacterium tuberculosis* to monitor the transmission of tuberculosis has been hampered by the need to culture this slow-growing organism and by the level of technical sophistication needed for RFLP typing. We have developed a simple method which allows simultaneous detection and typing of *M. tuberculosis* in clinical specimens and reduces the time between suspicion of the disease and typing from 1 or several months to 1 or 2 days. The method is based on polymorphism of the chromosomal DR locus, which contains a variable number of short direct repeats interspersed with nonrepetitive spacers. The method is referred to as spacer oligotyping or "spoligotyping" because it is based on strain-dependent hybridization patterns of in vitro-amplified DNA with multiple spacer oligonucleotides. Most of the clinical isolates tested showed unique hybridization patterns, whereas outbreak strains shared the same spoligotype. The types obtained from direct examination of clinical samples were identical to those obtained by using DNA from cultured *M. tuberculosis*. This novel preliminary study shows that the novel method may be a useful tool for rapid disclosure of linked outbreak cases in a community, in hospitals, or in other institutions and for monitoring of transmission of multidrug-resistant *M. tuberculosis*. Unexpectedly, spoligotyping was found to differentiate *M. bovis* from *M. tuberculosis*, a distinction which is often difficult to make by traditional methods.

Key factors in the control of tuberculosis are rapid detection, adequate therapy, and contact tracing to arrest further transmission (4). Recent developments in DNA technology and molecular biology have led to methods for rapid detection of mycobacterial DNA or RNA in clinical specimens by in vitro nucleic acid amplification (19, 33). Furthermore, methods have been developed to trace tuberculosis transmission routes by the differentiation of clinical isolates based on polymorphism in genomic DNA of *Mycobacterium tuberculosis* (25). However, no methods have been described that allow simultaneous detection and strain differentiation of *M. tuberculosis* bacteria present in clinical specimens without the need to culture this slowly growing organism. This study was undertaken to develop such a method.

The method most widely used to differentiate *M. tuberculosis* complex strains is DNA fingerprinting by Southern blotting of genomic DNA by using the mobile element IS*6110* as a probe (27). Other repetitive elements used for strain differentiation are the polymorphic GC-rich sequence PGRS (22) and the triplet multimer $(GTG)_5$ (32). Although PCR-based methods have been described to visualize DNA polymorphism in *M. tuberculosis* complex strains associated with these repetitive DNA elements, the described methods are not suitable for routine simultaneous detection and typing of *M. tuberculosis* in clinical specimens because of polymorphism in the target for the PCR primers or because of too much variation in the size of the DNA target to be bridged by the primers (11, 20, 21).

We exploited the previously observed DNA polymorphism within the direct repeat (DR) locus of *M. tuberculosis* to develop a method to simultaneously detect and type *M. tuberculosis*. This locus contains multiple, well-conserved 36-bp DRs interspersed with nonrepetitive spacer sequences 34 to 41 bp long (15) (Fig. 1A). Strains vary in the number of DRs and in the presence or absence of particular spacers (14). This polymorphism is probably due to homologous recombination between neighboring or distant DRs and to rearrangements driven by the insertion element IS*6110*, which is present in the DR region of most *M. tuberculosis* strains (14). Since the DRs are extremely well conserved among *M. tuberculosis* strains, each DR copy within the DR locus is a potential target for in vitro PCR amplification. The variation of spacers within the conserved DRs has been used by Groenen et al. (14) to differentiate *M. tuberculosis* strains by using a PCR-based method; however, this method is not suitable for routine use in a clinical laboratory because the method is technically demanding and it is difficult to obtain a high throughput of samples. Here we describe a novel method, "spoligotyping" (spacer oligotyping), in which the DRs are used as a target for in vitro DNA amplification and in which the variation in the spacers is exploited to obtain different hybridization patterns of the amplified DNA with multiple synthetic spacer oligonucleotides, which are covalently bound to a membrane. We will show that spoligotyping is a simple, rapid, and robust method for simultaneous detection and typing of *M. tuberculosis* in clinical spec-

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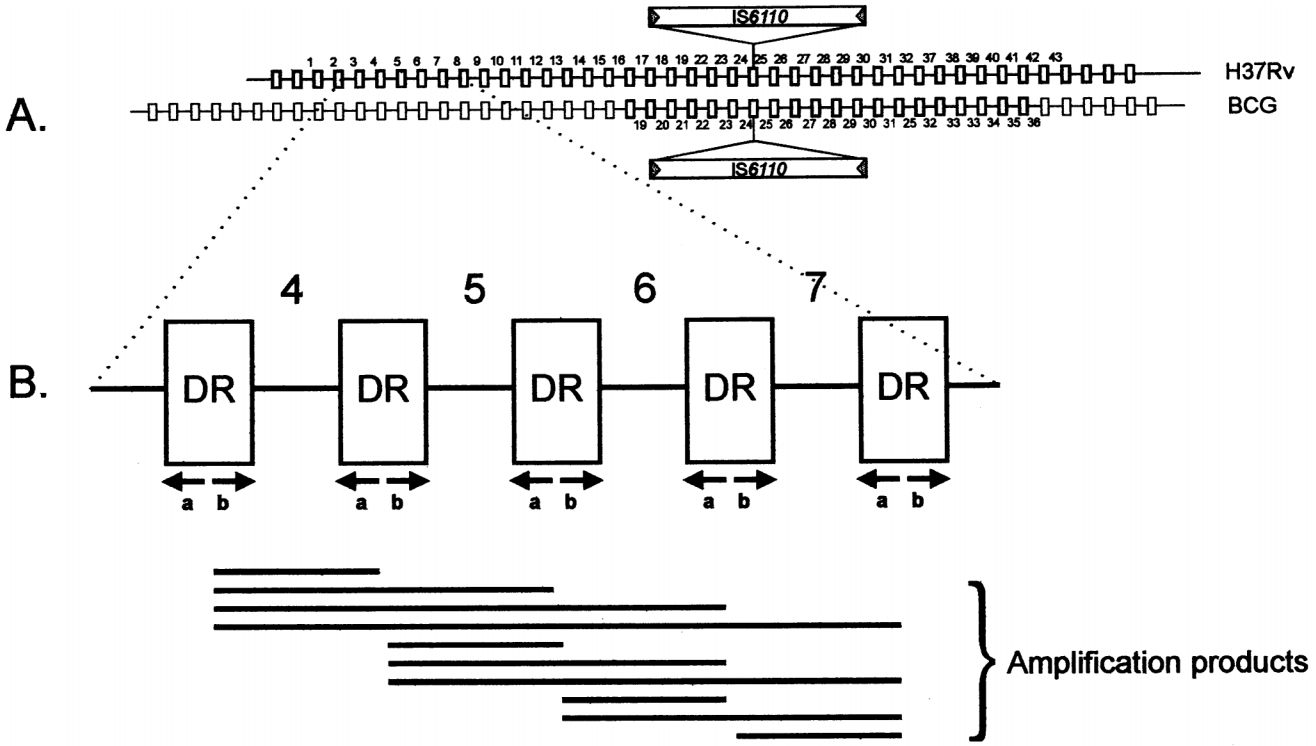
A.
IS6110
H37Rv
BCG
IS6110
B.
4
5
6
7
DR
DR
DR
DR
DR
a b
Amplification products


C.
H37Rv
BCG
1 H37Rv
2 Control
3 BCG
M.bovis

FIG. 1. (A) Structure of the DR locus in the mycobacterial genome. The chromosomes of *M. tuberculosis* H37Rv and *M. bovis* BCG contain 48 and 41 DRs, respectively (depicted as rectangles), which are interspersed with unique spacers varying in length from 35 to 41 bp. The (numbered) spacers used correspond to 37 spacers from *M. tuberculosis* H37Rv and 6 from *M. bovis* BCG. The site of integration of insertion element IS*6110* is depicted. (B) Principle of in vitro amplification of the DR region by PCR. Any DR in the DR region may serve as a target for these primers; therefore, the amplified DNA is composed of a mixture of a large number of different-size fragments. Shown is the combination of fragments that would be produced by in vitro amplification of a DR target containing only five contiguous DRs. (C) Hybridization patterns (spoligotypes) of amplified mycobacterial DNAs of 35 *M. tuberculosis* and 5 *M. bovis* strains. The order of the spacers on the filter corresponds to their order in the genome. Note that the spoligotype of strains 6, 12, and 37 corresponds to that of strains from the Beijing genotypic group described previously (30).

imens without the need to culture *M. tuberculosis* or purify DNA.

MATERIALS AND METHODS

Strains and clinical specimens. The laboratory strain *M. tuberculosis* H37Rv was used to sequence the whole DR region. H37Rv and the Pasteur strain of *M. bovis* BCG P3 were used as reference strains for spoligotyping. Most of the *M. tuberculosis* complex strains were obtained from the collection in Bilthoven, where all of the *M. tuberculosis* complex strains isolated in The Netherlands since 1993 have been routinely typed by IS*6110* fingerprinting (27) and tested for drug sensitivity. The *M. bovis* strains used were also from the collection in Bilthoven and originated from cattle. Sixteen *M. tuberculosis* strains were from patients involved in three suspected outbreaks in England. One was a community outbreak that comprised five patients from whom *M. tuberculosis* was isolated between October 1991 and November 1992; four isolates were resistant to isoniazid and streptomycin, and one was resistant to isoniazid alone. Another suspected outbreak involved five patients from a renal unit of a United Kingdom hospital. Contacts among three of the five patients had been demonstrated. The third suspected outbreak in a small town in northern England comprised two staff members of a training center who presented with pulmonary tuberculosis in March 1992 and March 1993, respectively, and a member of the family of one of these staff members who developed tuberculosis 2 years and 4 months after the first patient. Two isolates from patients in the same town served as controls.

The clinical specimens, which were directly subjected to spoligotyping, were obtained from six tuberculosis patients in different hospitals in The Netherlands and from eight patients in St. Mary's Hospital in London.

Fingerprint analysis and spoligotyping. The whole DR region of *M. tuberculosis* H37Rv was sequenced as part of this study (EMBL accession number Z48304). This was done by using the cosmid T211 (kindly provided by S. Cole, Institut Pasteur, Paris, France), a recombinant plasmid from a cosmid library of *M. tuberculosis* H37Rv. Only part of the sequence of the DR region of *M. bovis* BCG is known (15). The sequenced regions of the DR locus in *M. tuberculosis* H37Rv and *M. bovis* BCG are depicted in Fig. 1. The oligonucleotides DRa and DRb (see Table 1) were used as primers to amplify the whole DR region by PCR. Unless otherwise indicated, approximately 10 ng of genomic mycobacterial DNA purified from cultured cells (28) was used as a target for spoligotyping. DNA extraction from clinical material was done as previously described (17, 18). Fifty microliters of the following reaction mixture were used for the PCR: *Tth* buffer (5 mM Tris · HCl, 5 mM KCl, 0.7 mM $MgCl_2$, pH 9.0), each deoxynucleoside triphosphate at 200 mM, 20 pmol each of primers DRa and DRb, 10 ng of DNA, and 0.5 U of *Tth* polymerase (HT Biotechnology Ltd., Cambridge, United Kingdom). The mixture was heated for 3 min at 96°C and subjected to 20 cycles of 1 min at 96°C, 1 min at 55°C, and 30 s at 72°C. For direct amplification of mycobacterial DNA in clinical samples, dTTP was replaced with dUTP and 0.5 U of uracil DNA glycosylase was included in the reaction mixture to avoid contamination with previously amplified DNA (18). Thermal cycling of the latter samples included 10 min at 40°C, 3 min at 95°C, and 40 cycles of 1.5 min at 93°C, 2 min at 57°C, and 3 min at 72°C. The amplified product was hybridized to a set of 43 immobilized oligonucleotides, each corresponding to one of the unique spacer DNA sequences within the DR locus. The sequences of the oligonucleotides used are given in Table 1. These oligonucleotides were covalently bound to a membrane as previously described (16). In short, a membrane (Biodyne C; Pall Biosupport, Portsmouth, United Kingdom) was activated by using 16% (wt/vol) 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (Sigma). The oligonucleotides were applied to the membrane in parallel by using a miniblotter system (MN45; Immunetics, Cambridge, Mass.). After a short incubation, the membrane was inactivated by using 100 mM NaOH and washed in 2× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4, and 1 mM EDTA [pH 7.7]; Gibco BRL Life Technologies, Inc.) supplemented with 0.1% sodium dodecyl sulfate (SDS; Sigma). For hybridization, 20 μl of the amplified PCR product was diluted in 150 μl of 2× SSPE–0.1% SDS and heat denatured. The diluted samples (130 μl) were pipetted into the parallel channels in such a way that the channels of the miniblotter apparatus were perpendicular to the rows of oligonucleotides deposited previously. Hybridization was done for 60 min at 60°C. After hybridization, the membrane was washed twice in 250 ml of 2× SSPE–0.5% SDS for 10 min each time at 60°C and then incubated in 1:4,000-diluted streptavidin-peroxidase conjugate (Boehringer) for 45 to 60 min at 42°C. The membrane was washed twice, for 10 min each time, in 250 ml of 2× SSPE–0.5% SDS at 42°C and rinsed with 250 ml of 2× SSPE for 5 min at room temperature. Detection of hybridizing

TABLE 1. Sequences of the oligonucleotides used in this study[a]

Space no.	Oligonucleotide sequence	Spacer no.	Oligonucleotide sequence
1	ATAGAGGGTCGCCGGTTCTGGATCA	23	AGCATCGCTGATGCGGTCCAGCTCG
2	CCTCATAATTGGGCGACAGCTTTTG	24	CCGCCTGCTGGGTGAGACGTGCTCG
3	CCGTGCTTCCAGTGATCGCCTTCTA	25	GATCAGCGACCACCGCACCCTGTCA
4	ACGTCATACGCCGACCAATCATCAG	26	CTTCAGCACCACCATCATCCGGCGC
5	TTTTCTGACCACTTGTGCGGGATTA	27	GGATTCGTGATCTCTTCCCGCGGAT
6	CGTCGTCATTTCCGGCTTCAATTTC	28	TGCCCCGGCGTTTAGCGATCACAAC
7	GAGGAGAGCGAGTACTCGGGGCTGC	29	AAATACAGGCTCCACGACACGACCA
8	CGTGAAACCGCCCCCAGCCTCGCCG	30	GGTTGCCCCGCGCCCTTTTCCAGCC
9	ACTCGGAATCCCATGTGCTGACAGC	31	TCAGACAGGTTCGCGTCGATCAAGT
10	TCGACACCCGCTCTAGTTGACTTCC	32	GACCAAATAGGTATCGGCGTGTTCA
11	GTGAGCAACGGCGGCGGCAACCTGG	33	GACATGACGGCGGTGCCGCACTTGA
12	ATATCTGCTGCCCGCCCGGGGAGAT	34	AAGTCACCTCGCCCACACCGTCGAA
13	GACCATCATTGCCATTCCCTCTCCC	35	TCCGTACGCTCGAAACGCTTCCAAC
14	GGTGTGATGCGGATGGTCGGCTCGG	36	CGAAATCCAGCACCACATCCGCAGC
15	CTTGAATAACGCGCAGTGAATTTCG	37	CGCGAACTCGTCCACAGTCCCCCTT
16	CGAGTTCCCGTCAGCGTCGTAAATC	38	CGTGGATGGCGGATGCGTTGTGCGC
17	GCGCCGGCCCGCGCGGATGACTCCG	39	GACGATGGCCAGTAAATCGGCGTGG
18	CATGGACCCGGGCGAGCTGCAGATG	40	CGCCATCTGTGCCTCATACAGGTCC
19	TAACTGGCTTGGCGCTGATCCTGGT	41	GGAGCTTTCCGGCTTCTATCAGGTA
20	TTGACCTCGCCAGGAGAGAAGATCA	42	ATGGTGGGACATGGACGAGCGCGAC
21	TCGATGTCGATGTCCCAATCGTCGA	43	CGCAGAATCGCACCGGGTGCGGGAG
22	ACCGCAGACGGCACGATTGAGACAA		

[a] The primers used were DRa (GGTTTTGGGTCTGACGAC, 5′ biotinylated) and DRb (CCGAGAGGGGACGGAAAC). The numbers of spacer oligonucleotides 1 to 43 correspond to the spacer numbers in Fig. 1.

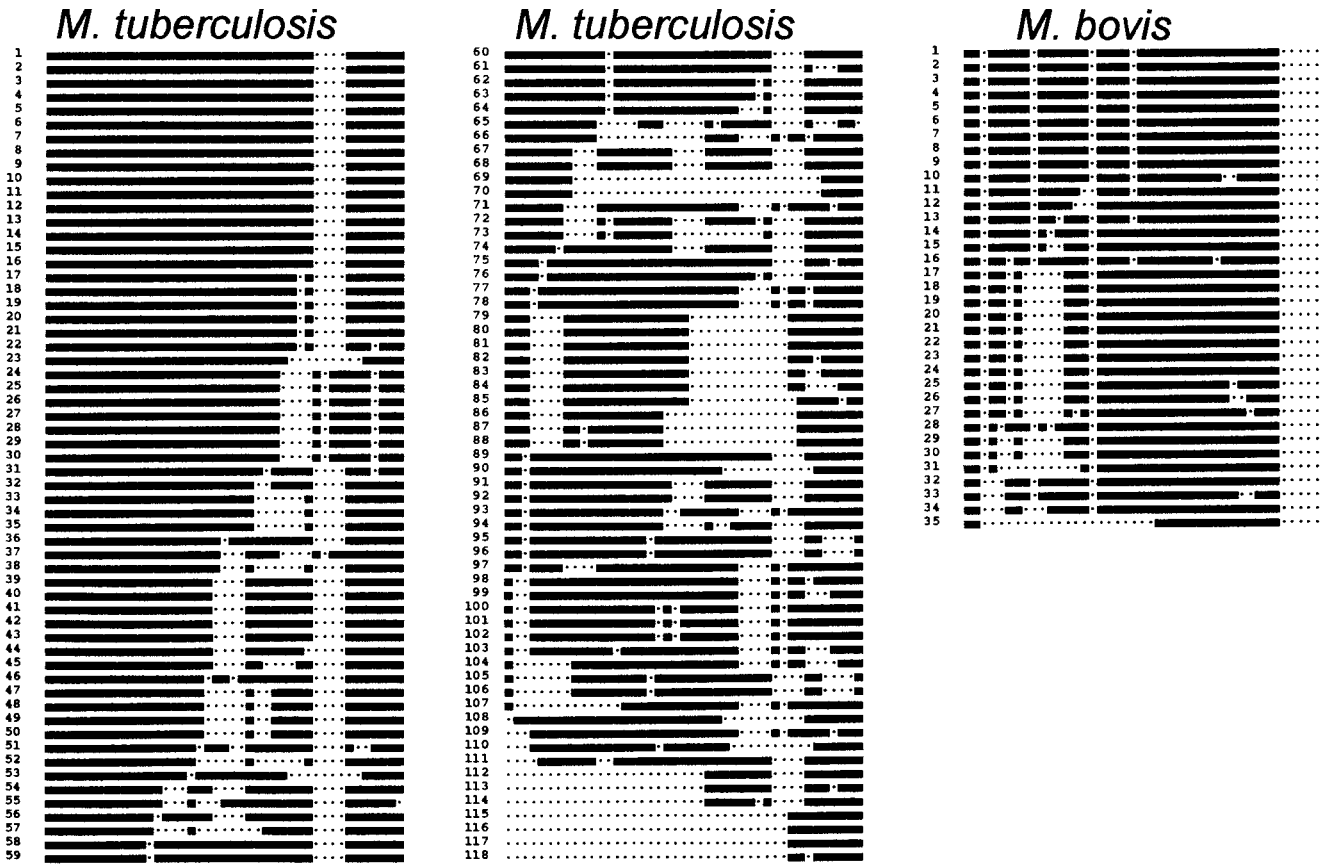

FIG. 2. Schematic representation of the spoligotypes of 118 *M. tuberculosis* and 35 *M. bovis* strains. Black rectangles depict positive hybridization signals, and dots represent lack of hybridization. The hybridization patterns (spoligotypes) were ordered by using the sort command of a word processor (Word Perfect, version 5.1).

DNA was done by using chemiluminescent ECL (Amersham) detection liquid (28, 29), followed by exposure to X-ray film (Hyperfilm ECL; Amersham) in accordance with the instructions of the manufacturer. For repeated use of membranes, the membranes were stripped by being washed two times for 30 min each time in 1% SDS at 80°C and then incubated for 15 min in 20 nM EDTA (pH 8) at room temperature. Membranes were sealed in plastic and stored at 4°C until further use.

RESULTS

Simultaneous detection and differentiation of *M. tuberculosis*. To amplify the whole DR region of *M. tuberculosis* complex strains by PCR, we used primers DRa and DRb, which allow amplification of the whole DR region, including the interspersed spacers (Fig. 1B). Because DRa is labeled with biotin, the amplified DNA could be used directly for hybridization to the 43 spacer oligonucleotides, which were covalently bound to a membrane. These spacer oligonucleotides were derived from sequences of the DR regions of *M. tuberculosis* H37Rv and *M. bovis* BCG. Because clinical isolates differ in the presence of such spacer sequences, mycobacterial strains can be differentiated by their different hybridization patterns or spoligotypes.

Figure 1C illustrates the variability in the hybridization patterns obtained with 34 randomly selected human *M. tuberculosis* strains isolated in the first half of 1993 and four *M. bovis* isolates from animals. Most of the strains show different spoligotypes, suggesting that spoligotyping allows good differentiation of the *M. tuberculosis* complex. To determine the potential of spoligotyping for strain differentiation of *M. tuberculosis* complex strains, we analyzed 118 randomly selected *M. tuberculosis* strains isolated in the first half of 1993 by spoligotyping and compared the results with those obtained by IS*6110*-associated restriction fragment length polymorphism (RFLP), which is the most widely used method of strain differentiation (27). The different spoligotypes were sorted on the basis of similarity by using a word processor, and the types obtained are schematically depicted in Fig. 2. Sixty-eight different spoligotypes were distinguished among the 118 *M. tuberculosis* isolates investigated, whereas 88 IS*6110* fingerprint types were found. Compared with IS*6110* fingerprinting, strain differentiation by spoligotyping was less successful for strains carrying a high number of IS*6110* copies but better for strains with a single or a few IS*6110* elements (Table 2). This feature of spoligotyping may be valuable for the analysis of strains from geographical regions where strains with a low IS*6110* copy number are prevalent and for differentiation of *M. bovis*, which often contains a single IS*6110* element (5, 7).

We tested 35 *M. bovis* strains by spoligotyping. Nineteen spoligotypes were distinguished, whereas only seven types could be distinguished by IS*6110*-associated RFLP (Fig. 2 and Table 2). Unexpectedly, the absence of the five 3′ spacers (spacers 39 to 43) in the DR region was characteristic of all of the *M. bovis* strains, and this feature distinguished *M. bovis* from all of the *M. tuberculosis* strains tested. Thus, spoligotyping may not only be useful for differentiation of *M. bovis* strains but may also be used to distinguish these from *M. tuberculosis*, a distinction which is often difficult to make by using conventional bacteriological techniques (31).

TABLE 2. Differentiation of *M. tuberculosis* and *M. bovis* by IS*6110* fingerprinting and spoligotyping

Organism and no. of strains		No. of IS*6110* copies	No. of IS*6110* types	No. of spoligotypes
M. tuberculosis[a]	19	1	1	10
	14	2–5	7	8
	85	>5	80	52
Total	118		88	68[b]
M. bovis[c]	32	1	4	18
	3	2–6	3	3
Total	35		7	19[b]

[a] Clinical human isolates, selected on the basis of IS*6110* copy number. Strains from suspected cases of transmission were excluded.
[b] Two strains had the same spoligotype.
[c] Isolated from either cattle or humans. Strains from suspected cases of transmission were excluded.

Sensitivity and specificity of spoligotyping. The sensitivity of the spoligotyping method to detect *M. tuberculosis* DNA was determined. Ten femtograms of chromosomal *M. tuberculosis* DNA, an amount corresponding to about two mycobacterial genomes, was reproducibly detectable. Although the average molecular weight of the amplified DNA was found to be dependent on the concentration of target DNA, the hybridization patterns were independent of the target DNA concentration, even when the target corresponded to only two mycobacterial genomes. When the quantity of target DNA for PCR was further decreased, truncated hybridization patterns were often obtained, suggesting that the DR region was broken into two or more separate parts and that single broken molecules were present in the separate vials containing the highly diluted DNA (data not shown).

Preliminary studies (14, 15) have shown that the DR cluster is present only in bacteria belonging to the *M. tuberculosis* complex group, which includes *M. tuberculosis*, *M. africanum*, *M. bovis*, and *M. microti*. We have tested the presence of the DR sequence in a variety of other mycobacteria. No amplification or hybridization to spacer oligonucleotides was found by using DNAs from 33 different mycobacterial species not belonging to the *M. tuberculosis* complex. Furthermore, no amplification or hybridization was found by spoligotyping of 10 gram-positive and 10 gram-negative nonmycobacterial species, confirming the specificity of the DR cluster for *M. tuberculosis* complex bacteria.

Detection and typing of *M. tuberculosis* in clinical specimens. The utility of spoligotyping in clinical practice was evaluated when DNA was extracted from sputum and other clinical specimens from five patients whose samples had been found to be smear positive and were later also found to be culture positive. All of these samples were positive by spoligotyping (Fig. 3). Furthermore, the spoligotypes obtained from the clinical samples were identical to those obtained from the corresponding cultured mycobacteria, indicating that neither the DNA extraction procedure nor contaminants in the DNA extracts of the clinical samples affected the spoligotyping results. Samples from each of these patients showed different spoligotypes, which is consistent with the absence of data supporting an epidemiological link between these patients from the Academic Hospital in Amsterdam. In the same hospital, a presumptive case of BCG-itis was disclosed by spoligotyping. A 3-year-old Turkish child developed severe lymphadenitis 1 year after BCG vaccination. The lymph node biopsy contained *M. tuberculosis* complex DNA as determined by PCR with IS*6110* DNA as the target (17, 18). Analysis of this sample by spoligotyping showed a hybridization pattern identical to that of *M. bovis* BCG. Because all of the *M. bovis* strains isolated from animals and humans have exhibited spoligotypes different from that of *M. bovis* BCG 4a), these data suggest infection of the child with *M. bovis* BCG. In St. Mary's Hospital, the utility of spoligotyping in clinical practice was evaluated by testing sputa of eight patients whose samples were smear and culture positive. Six of the eight samples exhibited distinct patterns (Fig. 4, lanes 1 to 4, 6, and 8). Two of the samples showed virtually identical patterns which differed only slightly in the intensity of a few weak hybridization signals (lane 5 and 7). This result prompted further epidemiological investigations, but no obvious link was found.

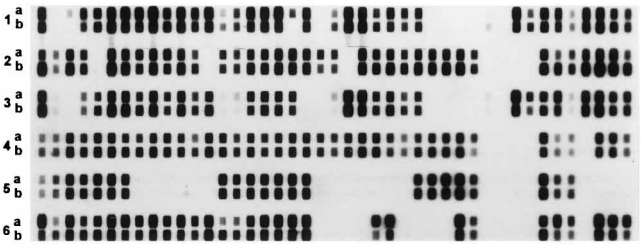

FIG. 3. Spoligotyping of *M. tuberculosis* DNAs isolated from clinical samples from extrapulmonary sites (lanes 1a, 3a, and 4a contained samples from patients with retropharyngeal, neck, and peritoneal abcesses, respectively) and pulmonary tuberculosis patients (lanes 2a, 5a, and 6a) and of DNAs obtained from bacterial cultures of samples of the corresponding patients (the b lanes). The clinical materials were pus (lanes 1a, 3a, and 4a), sputum (lane 2a), bronchoalveolar lavage (lane 5a), and a lung biopsy (lane 6a). DNA was extracted as previously described (16, 17).

Tracing of epidemiologically related tuberculosis infections. To evaluate the usefulness of spoligotyping to trace tuberculosis outbreaks, we typed strains from outbreaks in The Netherlands and the United Kingdom. Strains from four community outbreaks in The Netherlands caused by drug-sensitive *M. tuberculosis* were investigated. These strains, isolated from epidemiologically related cases, had previously been shown to

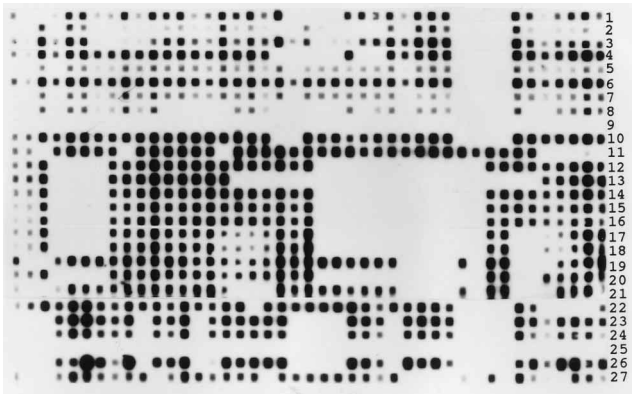

FIG. 4. Detection and simultaneous typing of *M. tuberculosis* by use of DNAs extracted from sputa (lanes 1 to 8) and clinical isolates from three outbreaks of tuberculosis in London (lanes 12 to 27). Lanes: 9, negative control; 10, H37Rv; 11, *M. bovis* BCG; 12 to 16, isolates from a community outbreak of multidrug-resistant tuberculosis indicating that the isolates in lanes 12, 14, 15, and 16 are related; 17 to 21, isolates from a hospital outbreak of isoniazid-resistant tuberculosis indicating that isolates 17, 18, and 20 are related; 22 to 27, isolates from a hospital outbreak of drug-sensitive tuberculosis affecting patients and staff indicating that isolates 23, 24, and 26 are related. The presence of a spacer in the *M. tuberculosis* DNA was inferred when the autoradiograph signal was as dark as that for the equivalent region in one of the controls.

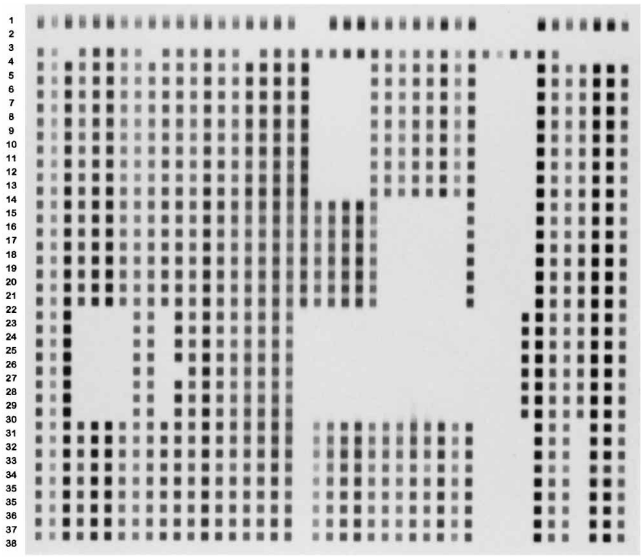

FIG. 5. Spoligotypes of 35 *M. tuberculosis* strains from four different outbreaks in The Netherlands. Strains belonging to the same outbreak have identical hybridization patterns. Lanes: 1, *M. tuberculosis* H37Rv; 2, negative control; 3, *M. bovis* BCG; 4 to 13, 14 to 21, 22 to 29, and 30 to 38, *M. tuberculosis* isolates from four different outbreaks.

have identical IS*6110* fingerprints. As shown in Fig. 5, strains belonging to the same outbreak had identical hybridization patterns. The spoligotype of a single isolate (lane 26) differed from those of the other strains in the same outbreak by the absence of a single spacer. Similarly, slight variations in IS*6110* fingerprints have occasionally been found among outbreak strains (6, 26, 28).

Furthermore, we analyzed strains from three suspected small outbreaks in the United Kingdom. One was a community outbreak caused by isoniazid- and streptomycin-resistant tuberculosis in patients presenting over a 2-year period (Fig. 4, lanes 12 to 16). The spoligotypes of the cultured bacteria were identical in four cases (Fig. 4, lanes 12 and 14 to 16). A fifth isolate from a patient from the community was isoniazid resistant only and showed a different pattern (lane 13). These data confirmed the previously reported identical RFLP patterns of the four identical strains (13).

Furthermore, suspected transmission of isoniazid-resistant *M. tuberculosis* among patients in a renal unit of a United Kingdom hospital was investigated (lanes 17 to 21). These patients had been treated prophylactically with isoniazid. Three male patients, one of whom was human immunodeficiency virus (HIV) positive, presented within a 2-month period with pulmonary tuberculosis. The spoligotypes of the strains isolated from these patients revealed similar patterns (Fig. 4, lanes 17, 18, and 20). Identical patterns were also observed by RFLP (data not shown). *M. tuberculosis* isolates from two other patients from the same unit which were not resistant to isoniazid showed different spoligotypes (Fig. 4, lanes 19 and 21). The last United Kingdom outbreak investigated involved three isolates from two hospital staff members and a member of the family of one of the staff members. These three strains exhibited similar spoligotyping patterns (Fig. 4, lanes 23, 24, and 26). Two isolates from the same town which served as controls were also typed and showed different spoligotype patterns (Fig. 4, lanes 22 and 25).

The hybridization signals shown in Fig. 4 are weaker than those shown in the other figures because the membrane used for hybridization had already been used 16 times for spoligotyping. Usually, the hybridization signals become too weak after the membrane has been used 20 times.

DISCUSSION

We have developed a method which brings routine detection and DNA typing of *M. tuberculosis* complex strains within the reach of clinical and public health laboratories. The method is rapid, reproducible, and easy to perform. As a target for in vitro amplification, we used the short, repetitive DR sequence shown to be present uniquely in bacteria of the *M. tuberculosis* complex. Over 1,000 *M. tuberculosis* strains have been tested (unpublished data), and no isolate not carrying this multicopy target has been encountered. This is in contrast to the genetic element IS*6110*, which is absent in certain strains of *M. tuberculosis* (7, 26), and therefore DNA from such strains in clinical material may be overlooked by PCR detection with IS*6110* as the target.

Compared to traditional IS*6110* fingerprinting, the degree of strain differentiation of *M. tuberculosis* by spoligotyping was lower for strains carrying five or more IS*6110* copies and higher for *M. tuberculosis* complex strains harboring fewer than five IS*6110* copies. Work is in progress to sequence the DR region of more strains, and we expect that novel spacers will become available to improve the degree of strain differentiation. However, given the high diversity of *M. tuberculosis* types observed in this study, we expect that even with the present state of the technique, routine use of spoligotyping may be of great value for the rapid detection and typing of *M. tuberculosis*. We showed that strains in a recognized outbreak exhibit identical spoligotypes, whereas other isolates are generally different.

Furthermore, we demonstrated that the method can be used to simultaneously detect and type *M. tuberculosis* present in clinical specimens, such as sputum, tissue, or bronchoalveolar lavages, a procedure that can be performed in less than 2 days. A large variety of clinical materials can be subjected to spoligotyping, and we have even used the method successfully to type *M. tuberculosis* in sections of 40-year-old paraffin-embedded tissue (21a). Furthermore, the method can be used to detect and type *M. tuberculosis* present on Ziehl-Neelsen- or auramine-stained microscopy slides (26a). In this study, spoligotyping was applied to only a limited number of clinical specimens and cultured strains; therefore, further studies are needed to validate the method for use in the diagnosis and epidemiology of tuberculosis. By the use of DNA from clinical material for spoligotyping instead of mycobacterial DNA from cultured cells, one may expect competition of excess foreign DNA with the primers and this may result in less amplified *M. tuberculosis* DNA. However, because a single primer set is used to amplify a large number of different spacers, all hybridization reactions are expected to be affected equally. Indeed, we have never observed the specific attenuation of any particular hybridization reaction when subjecting clinical samples directly to spoligotyping.

To identify the routes of infection and risk factors for transmission of tuberculosis, all of the *M. tuberculosis* strains isolated in The Netherlands since 1993 have been subjected to traditional IS*6110*-based fingerprinting. The preliminary data confirm previous reports (1, 12, 23) that most of the recently acquired infections are not detected by traditional contact tracing. Therefore, DNA typing could be helpful to identify nonsuspected sources of transmission. However, we have found that the delay between suspicion of tuberculosis and generation of a DNA fingerprint (usually about 3 months) is a major obstacle to effective exploitation of the predicted links between patients by using RFLP information. Usually, contact tracing is

started as soon as a case of infectious tuberculosis is suspected and it is therefore difficult to restart contact tracing months later, when RFLP data become available. The drawback of the delay caused by the currently available typing methods may be overcome by using spoligotyping, which can be performed within 1 or 2 days.

The resurgence of tuberculosis in the United States has been accompanied by alarming outbreaks of *M. tuberculosis* strains resistant to multiple antimicrobial drugs, mainly among HIV-infected patients (1–3, 6, 8–10, 24). Due to the increased susceptibility of HIV-infected patients to *M. tuberculosis* infection and the accelerated progression of the disease (6), rapid diagnosis and typing of the causative mycobacteria may help to effectively control multidrug-resistant tuberculosis. Furthermore, rapid detection and typing of *M. tuberculosis* are often desirable to distinguish reinfection from reactivation in individual patients who relapse during or after chemotherapy.

An unexpected finding was the ability to discriminate *M. bovis* from *M. tuberculosis* by spoligotyping, a distinction which is often difficult to make by traditional bacteriology. All of the 35 *M. bovis* strains investigated in this study were found to lack the five 3′ outermost spacers present in the DR locus of *M. tuberculosis* H37Rv. This characteristic difference has been confirmed for all of about 500 *M. bovis* and 1,000 *M. tuberculosis* strains investigated (4a). By using spoligotyping, we identified *M. bovis* BCG in a clinical specimen from a 3-year-old patient whose infection apparently reactivated 1 year after vaccination. Because *M. bovis* generally carries only a single IS*6110* element, isolates of this species are difficult to differentiate by IS*6110* fingerprinting (5, 29). The method developed during this study may help to define the animal reservoirs of *M. bovis* from which transmission to domesticated animals and to humans takes place.

In addition to the speed and simplicity of spoligotyping, the method is time saving and economical because in the "reverse line blot hybridization" technique (16) used here, filters with multiple covalently linked spacer oligonucleotides are used to simultaneously detect and type 43 clinical specimens and these filters can be reused at least 20 times. Furthermore, in contrast to IS*6110*-based typing, no sophisticated computer software is needed for storage of hybridization images and comparison of large numbers of strains. Because the spoligotype can be expressed as a single word of 43 letters (with an alphabet of three symbols, positive, negative, and indeterminate), a search for matching strains can be done simply by using a word processor. In this study, we occasionally encountered weak hybridization signals, probably due to sequence variation in spacers. Such weak signals may result in ambiguities in interpretation. By using wild cards for weak spots and assignment of a category of indeterminate reactions for weak hybridization signals, one may avoid overlooking strains having identical spoligotypes. In many laboratories, spoligotyping is used either for epidemiologic study of *M. bovis* infections in animals or for rapid typing of *M. tuberculosis* isolated in hospitals and other institutions with a high risk of tuberculosis transmission. We are currently building up a database of spoligotypes of strains from different countries. Although the database is still in its infancy, we have already discovered epidemiological links between multiple-drug-resistant strains that were unsuspectingly transmitted between various hospitals in different countries (18a).

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