Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* complex in paraffin wax embedded tissues and in stained microscopic preparations

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Abstract

***Aims*—To detect and differentiate *Mycobacterium tuberculosis* simultaneously by polymerase chain reaction (PCR) in clinical samples prepared for histopathological analysis and for microscopic detection of acid fast bacteria.**

***Methods*—Paraffin wax embedded tissue samples and Ziehl-Neelsen (ZN) and auramine stained microscopic preparations from culture positive tuberculosis patients were subjected to DNA extraction and amplification by PCR. PCR was performed with primers specific for direct repeats and the product was detected by hybridisation to a set of 43 different oligonucleotides, a procedure designated as "spoligotyping".**

***Results*—*Mycobacterium tuberculosis* complex DNA was detected in all of the 23 paraffin wax embedded tissues analysed. Strain differentiation was possible in 20 of the 23 paraffin wax embedded tissues. *Mycobacterium tuberculosis* complex DNA was also detected and typed in eight of 10 ZN stained microscopic preparations. The hybridisation patterns obtained from virtually all of these samples were identical to those obtained from DNA extracted from cultures.**

***Conclusion*—Simultaneous detection and strain differentiation of *M. tuberculosis* complex bacteria is possible in clinical samples prepared by current methods for microscopic and histopathological analysis, without the need to culture. The methodology described opens the way to rapid disclosure of outbreaks in high risk settings, such as hospitals and prisons, where dissemination of tuberculosis might be very fast as a result of a high prevalence of human immunodeficiency virus infected patients.**

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Tuberculosis continues to be a major cause of morbidity and mortality throughout the world.[1 2] Rapid detection, adequate treatment, and contact tracing to arrest further transmission[3] are the key factors in the control of this infectious disease.

Laboratory diagnosis of tuberculosis relies on culture and direct examination of smears by the Ziehl-Neelsen (ZN) or auramine stain. Culture is sensitive, but time consuming, because of the slow growth rate of *Mycobacterium tuberculosis* complex.[3] Microscopic detection of acid fast bacilli in stained ZN smears is rapid, but not very sensitive (at least 10^4 bacterial cells are needed), and is not specific for pathogenic mycobacteria.[4] Therefore, in vitro nucleic acid amplification techniques have been introduced recently for rapid and sensitive diagnosis of *M tuberculosis* in clinical specimens.[5 6] However, a limitation of the polymerase chain reaction (PCR) detection of *M tuberculosis* complex is the inability to differentiate strains by polymorphic genetic markers for outbreak investigations and other epidemiological purposes. Recently, we described a novel method that enables the simultaneous detection and typing of *M tuberculosis* to be carried out in clinical specimens.[7] In this method, designated "spoligotyping" (from spacer oligotyping), we exploited the previously observed DNA polymorphism within the direct repeat (DR) locus of *M tuberculosis* complex. The DR locus contains multiple, well conserved 36 base pair (bp) direct repeats interspersed with non-repetitive spacer sequences, 34–41 bp in length.[8 9] Spoligotyping involves the amplification by PCR of the whole DR region using the DR as a target for *M tuberculosis* complex specific DNA, followed by hybridisation of the amplified DNA to a set of 43 spacer oligonucleotides covalently linked to a membrane. Because clinical isolates vary in the presence or absence of particular spacers,[7] the spoligo patterns obtained are strain specific. The direct repeats are extremely well conserved among *M tuberculosis* complex strains and because they are present as a multiple target for DNA amplification, spoligotyping is a specific method for the detection of *M tuberculosis* complex.[7] Spoligotyping has been used successfully to detect and type *M tuberculosis* simultaneously in clinical materials such as sputum, bronchial lavages, and tissues.[7] Application of spoligotyping to detect and type *M tuberculosis* complex on microscopic preparations, stained for the detection of acid fast bacilli, and on sections of paraffin wax embedded tissues would make it possible to determine the presence of pathogenic mycobacteria and to disclose outbreaks rapidly. This would be of particular value in institutions where

immunocompromised patients are found (such as hospitals housing human immunodeficiency virus (HIV) co-infected patients) because of the rapid progression of the disease and the spread of multidrug resistant strains.[4] In our study we describe the use of spoligotyping to detect and type *M tuberculosis* complex on stained microscopic preparations and in sections of paraffin wax embedded tissues.

Methods

SECTIONS AND MICROSCOPIC PREPARATIONS

Sections were cut from 23 formalin fixed, paraffin wax embedded tissues, originating from 21 different tuberculosis patients. The samples included materials from lung, lymph nodes, bone marrow, testis, and penis. The tissues had been embedded between one and 10 years ago and *M tuberculosis* complex strains had been isolated successfully from all 21 patients.

Before cutting each paraffin wax embedded sample, a 14 μm section of a negative tissue was cut to monitor for cross contamination during sectioning of the tissues. From each tissue we used two sections of 3 μm for microscopic examination and three sections of 14 μm for DNA extraction for spoligotyping in duplicate or triplicate.

For microscopic examination, one section was stained with ZN and screened for acid fast bacilli, and the other one was stained with haematoxylin and eosin for histopathogical analysis.

ZN and auramine positive smears from *M tuberculosis* culture positive sputum samples were used. The smears were made before the decontamination step of the samples and after decontamination with 0.5% *N*-acetyl-L-cysteine/2% NaOH (NaLC/NaOH)[10] or lauryl sulphate.[11]

BACTERIAL CULTURES

Mycobacterium tuberculosis complex cultures isolated from the 23 patients had been stored for one to 15 years at −70°C. The strains were regrown on coletsos slants (Diagnostics Pasteur, Marnes-la-Coquette, France)[12] or in modified Dubos broth (MDB: Middlebroek 7H9 broth (Difco), 4.7 g; Bactocasitone (Difco Laboratories, Detroit, Michigan, USA), 0.5 g; L-asparagine 4.0 g; L-glutamic acid 1.0 g; sodium pyruvate, 1.6 g; yeast extract, 1.0 g; biotin, 5.0 mg; glycerol, 50 ml; distilled water, 950 ml; pH 6.6 at 25°C; after sterilisation at 121°C for 15 minutes 50 ml inactivated new born calf serum (Gibco BRL, Gaithersburg, Maryland, USA) was added. All chemicals were from Sigma, St Louis, Missouri, USA unless otherwise stated. All strains were identified in our laboratory by using AccuProbe (Gen-Probe Inc, San Diego, California, USA), as described previously.[13–15]

DNA EXTRACTION FROM PARAFFIN WAX EMBEDDED SECTIONS

To prevent contamination of samples with previously amplified DNA, the PCRs were performed according to the recommendations of Kwok,[16] using three separate locations for the preparation of the PCR reaction mixture, DNA extraction, and the analysis of PCR products. Positive displacement pipettes or filter tips were used throughout the procedure. All working surfaces were cleaned with 10% bleach solution (Lever Nederland BV, Capelle a/d Ijssel, The Netherlands). dUTP and uracil DNA glycosylase (UDG) (Gibco BRL) were used to prevent contamination with amplicons from previous reactions.[17]

The Chelex method was used for the isolation of DNA from 14 μm sections of paraffin wax embedded tissues.[18 19] Briefly, a single section was mixed with 150 μl of a Chelex suspension containing 5% Chelex-100, (Bio-Rad Laboratories, Hercules, California, USA), 0.01% lauryl sulphate (Sigma, Brunschwig Chemie, Amsterdam, The Netherlands), 1% Nonidet P40 (Sigma), and 1% Tween 20 (Sigma). After thorough mixing, the samples were incubated for 30 minutes at 100°C. The samples were centrifuged for 10 minutes at 13 000 ×*g* and the solution in between the paraffin upper layer and the Chelex particles was transferred to a fresh microcentrifuge tube and used for PCR.

DNA EXTRACTION FROM BACTERIAL CELLS

DNA extracts were prepared by suspending ~ 10 mg of wet bacterial cells in 100 μl of sterile distilled water (Mallinckrodt, Baker, Deventer, The Netherlands) and heating in a water bath at 100°C for 30 minutes to kill the cells and to induce cell lysis.[20] The cell debris was removed by centrifugation at 13 000 ×*g* for two minutes. The samples were stored at −20°C until used for DNA amplification.

Purified DNA extracts were prepared according to the Boom method.[21]

DNA EXTRACTION FROM ZN AND AURAMINE POSITIVE PREPARATIONS

Spoligotyping of microscopic preparations was performed on sputum smears from 20 patients. The microscopic preparations were ZN or auramine stained. Before DNA extraction, all microscopic preparations were examined for acid fast bacilli and all were positive. The microscopic preparations were examined at 700× magnification with a visual field of 0.18 mm in diameter. All ZN stained preparations were rated as III on the Bronkhorst scale, which corresponds with an average of six mycobacteria in each visual field (40 visual fields were examined).[22]

After microscopic examination, the mineral oil was removed with xylene (Merck, Darmstadt, Germany). Stained microscopic preparations were scraped off the microscope slides after adding 25 μl of sterile distilled water. Chelex suspension (75 μl) was added and after thorough mixing the samples were incubated for 30 minutes at 100°C. The samples were centrifuged for 10 minutes at 13 000 ×*g*. The supernatant was transferred to a fresh microcentrifuge tube and used for PCR directly.

SPOLIGOTYPING

An aliquot of 10 μl of the extracted DNA from the cultured *M tuberculosis* complex, 10 μl and 1 μl from the sections of paraffin wax embedded tissues, and 10 μl and 2.5 μl from

the stained microscopic preparations were used in the PCR. PCR was performed as described by Kamerbeek *et al*,[7] except that 3.0 mM $MgCl_2$, 50 pmol of each primer, and 15 mM Tris, pH 9.0, were used in the PCR mixture.

The extracted DNA was added to 50 µl of PCR mixture. The mixture was overlaid with one drop of mineral oil (Sigma) and incubated for 60 minutes at 37°C for uracil DNA glycosylase incubation; three minutes at 95°C for uracil DNA glycosylase inactivation[17] and DNA denaturation; one minute at 57°C for primer annealing; and one minute at 72°C for primer extension. Cycling conditions were as follows: one minute at 95°C, one minute at 57°C, and 30 seconds at 72°C. Clinical samples were subjected to 45 cycles and DNA isolated from bacterial cultures was subjected to 25 cycles. PCR products were kept at −20°C until further analysis.

Table 1 Spoligo patterns from DNA obtained from cultures and paraffin wax embedded tissues of patients with culture proven tuberculosis

	Culture positive		*Paraffin embedded tissue*					
Patient	*Tissue*	*Date*	*Tissue*	*Date*	*Pathology*	*ZN staining*	*Spoligo pattern*	
1	Lymph node	26 Mar 86	Lymph node	26 Mar 86	g/cn	Positive		a
								b
								c
	Sputum	12 Feb 86	Oesophagus ulcer	12 Feb 86	nsi	Negative		a
								b
								c
2	Lung	5 Aug 86	Lung	8 Aug 86	nsi	Positive		a
								b
								c
3	Lung	13 Jul 88	Lung	13 Jul 88	g/cn	Positive		a
								b
								c
4	Urine	14 Sep 90	Testicle	10 Sep 90	g/cn	Positive		a
								b
								c
5	Sputum	22 Oct 86	Lung biopsy	20 Oct 86	g/cn	Positive		a
								b
								c
6	Sputum	26 Jun 90	Sputum	26 Jun 90	nsi	Positive		a
								b
								c
			Sputum	7 Oct 88	nsi	Positive		a
								b
								c
7	Lung	12 Jun 91	Lung	14 Jun 91	g	Positive		a
								b
								c
8	Lung	21 Nov 90	Lung	21 Nov 90	g/cn	Positive		a
								b
								c
9	Bronchial washing	11 Jun 85	Lung	11 Jun 85	g	Negative		a
								b
								c
10	Sputum	3 Oct 94	Lung	17 Nov 94	g	Negative		a
								b
								c
11	Bronchial washing	14 Sep 89	Bronchial biopsy	14 Sep 89	g	Negative		a
								b
								c
12	Abscess injection site	11 Aug 92	Lymph node (armpit)	11 Aug 92	g	Positive		a
								b
								c
13	Lymph node (neck)	23 Mar 83	Lymph node (armpit)	29 Mar 83	g	Negative		a
								b
								c
14	Lymph node	1 May 86	Lymph node	1 May 86	g	Positive		a
								b
								c
15	Bronchial washing	12 Aug 91	Bronchial biopsy	12 Aug 91	g/cn	Positive		a
								b
								c
16	Pus (origin unknown)	17 Apr 90	Lymph node (neck)	17 Apr 90	g/cn	Positive		a
								b
								c
17	Pus vertebra	6 May 93	Bone marrow	6 May 93	n	Negative		a
								b
								c
18	Lymph node	12 Aug 87	Lymph node	12 Aug 87	g/cn	Positive		a
								b
								c
19	Lymph node	1 Aug 90	Lymph node	1 Aug 90	g	Negative		a
								b
								c
20	Urine	1 Nov 90	Biopsy penis	14 Oct 87	g	Negative		a
								b
								c
21	Pus lymph node	4 Jun 93	Lymph node	4 Jun 93	g	Negative		a
								b
								c

Spoligo patterns obtained from DNA from cultures (a) and from paraffin wax embedded tissues (b, 10 µl; c, 1 µl used for spoligotyping). Pathology: cn, caseous necrosis; g, granulomas; ne, necrosis; nsi, non-specific inflammation. The spoligo patterns from the strains from patient 12 and 20 were characteristic for *Mycobacterium bovis*, consistent with the bacteriological determination of the corresponding culture.

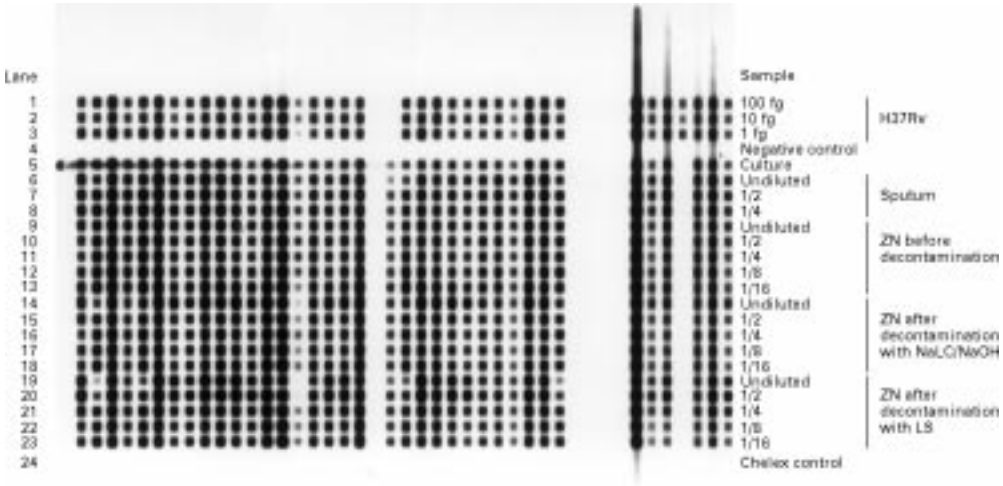

Figure 1 Sensitivity of detection of Mycobacterium tuberculosis DNA by spoligotyping and the effect of sample preparation on spoligo patterns. Lanes 1–3, 100 fg, 10 fg, and 1 fg purified M tuberculosis H37Rv (positive control), respectively; lane 4, negative control (sterile distilled water); lane 5, DNA extracted from the culture; lanes 6–8, DNA extracted from sputum; lanes 9–23, ZN stained microscopic preparations of sputum; lanes 9–13, sputum smears before decontamination; lanes 14–18, sputum smears after decontamination with NaLC-NaOH; lanes 19–23, sputum smears after decontamination with lauryl sulphate (LS); lane 24, negative Chelex control . The DNA extracted from the cultured mycobacterium strain (lane 5) was isolated from the same patient as the sputum samples used for spoligotyping (lanes 6–24).

PCR products were analysed by hybridisation using the reverse line blotting technique.[23] After hybridisation, washing of the membrane and detection of hybridised DNA with peroxidase labelled streptavidin was performed as described previously,[7] except that hybridisation was carried out in a hybridisation bag (Boehringer Mannheim, Mannheim, Germany) to keep the volume of the ECL detection reagent (Amersham International, Amersham, Buckinhamshire, UK) as small as possible.

Results

To spoligotype *M tuberculosis* from cultured cells we tested a simplified DNA extraction procedure, which involves only a heating and a centrifugation step. DNA obtained by this quick procedure was compared with DNA extracted according to the method described by Boom.[21] DNA was extracted from 10 cultures using both methods. Spoligotyping using both types of extracts resulted in identical hybridisation patterns (data not shown).

SENSITIVITY OF SPOLIGOTYPING IN PARAFFIN WAX EMBEDDED TISSUES

Twenty three paraffin wax embedded tissue samples from 21 tuberculosis patients were investigated by spoligotyping. All patients had been diagnosed as having tuberculosis and the diagnosis had been confirmed by the culture of *M tuberculosis* complex. In 14 of these tissue samples, acid fast bacteria had been demonstrated by ZN staining (table 1). Each DNA extract of the 23 clinical samples was subjected to amplification using 10 µl and 1 µl tissue DNA extract. The resulting spoligo patterns were compared with those obtained by using DNA from the strains of the corresponding patients. Table 1 shows the results. We detected *M tuberculosis* complex DNA by spoligotyping in all 23 samples. The spoligo patterns of 20 of the 23 clinical samples were identical to those of the corresponding cultured bacterial cells using either 10 µl or 1 µl of DNA for the PCR. Truncated spoligo patterns were obtained from three samples using 10 µl of DNA extract and from nine samples using 1 µl of DNA extract. In addition, one sample was negative using 1 µl of DNA. The use of 1 µl of DNA resulted in a more complete spoligo pattern compared with the use of 10 µl of DNA extract in one sample only (patient 16; pus).

Two samples were collected from the same patient (patient 6) and the time that elapsed between these two samples being taken was two years. The spoligo patterns obtained from both samples were identical, indicating that the patient remained infected with the same *M tuberculosis* strain during this period of time. From all patients, except one, the clinical samples for culturing and the paraffin wax embedded tissues originated from the same body site. The exception was patient 20, from whom the culture was obtained three years after the tissue biopsy (table 1). Again, the spoligo patterns were identical, showing that the patient had remained infected with the same *M tuberculosis* complex strain.

The spoligo patterns obtained from patients 12 and 20 showed no hybridisation with any of the five terminal 3' spacers, which is characteristic for *M bovis*.[7] Patient 12 had been vaccinated previously with *M bovis* BCG, and the abscess corresponded to the site of BCG injection. The spoligotype of the culture obtained from patient 12 is identical to that of *M bovis* BCG except for the presence of spacer 16 in the patient sample.

SPOLIGOTYPING USING STAINED MICROSCOPIC PREPARATIONS

DNA extracts obtained from sputum samples and ZN and auramine stained microscopic preparations were subjected to spoligotyping.

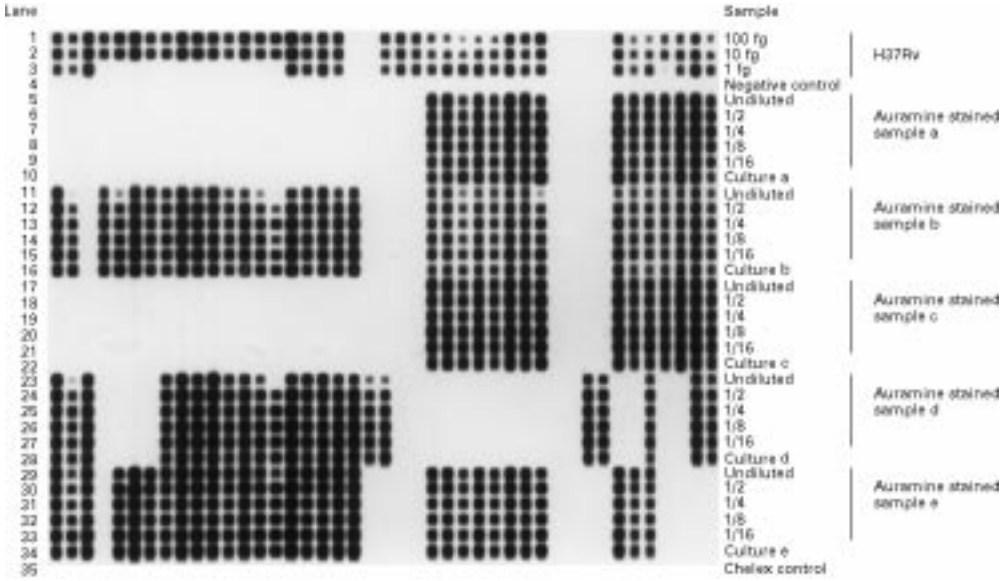

Figure 2 Spoligo patterns obtained from extracted DNA from auramine stained microscopic preparations and from the DNA isolated from strains of the corresponding patients. All preparations were tested in five dilutions: undiluted, 1/2, 1/4, 1/8, and 1/16 dilutions. Lanes 1–3, 100 fg, 10 fg, and 1 fg purified Mycobacterium tuberculosis H37Rv (positive control), respectively; lane 4, negative control; lanes 5–9, DNA extracted from auramine stained sample from patient a; lane 10, DNA extracted from the culture isolated from patient a; lanes 11–15, DNA extracted from auramine stained sample from patient b; lane 16, DNA extracted from the culture isolated from patient b; lanes 17–21, DNA extracted from auramine stained sample from patient c; lane 22, DNA extracted from the culture isolated from patient c; lanes 23–27, DNA extracted from auramine stained sample from patient d; lane 28, DNA extracted from the culture isolated from patient d; lanes 29–33, DNA extracted from auramine stained sample from patient e; lane 34, DNA extracted from the culture isolated from patient e; lane 35, negative Chelex control.

Figures 1 and 2 show the results. Both stained and unstained preparations, and DNA from the corresponding cultured bacterial cells resulted in the same spoligo patterns. Furthermore, these spoligo patterns were identical, irrespective of decontamination of the samples either by NaLC/NaOH or lauryl sulphate. Only a single sample resulted in a partial spoligo pattern. In the latter sample (fig 1; lane 19) one of the hybridisation reactions was very weak.

Spoligotyping on extracts obtained from 10 ZN preparations recorded as III in the Bronkhorst scale resulted in eight complete spoligo patterns and in two partial spoligo patterns (data not shown). Spoligotyping of five auramine stained preparations resulted in complete spoligo patterns (fig 2).

Discussion

Our study shows for the first time the feasibility of simultaneous detection and typing of *M tuberculosis* retrospectively in tissues prepared for histopathological analysis and in stained microscopic preparations. We showed that the spoligotypes obtained from such materials are identical to those obtained with cultures from the corresponding patients.

The simultaneous detection and typing of *M tuberculosis* complex in clinical samples offers the possibility of rapidly disclosing outbreaks of tuberculosis, without the need to change the methods traditionally used in the laboratory for the handling and preparation of clinical samples. Rapid disclosure of transmission is of particular use in cases where transmission within institutions, such as hospitals and prisons, is suspected.

Spoligotyping also enables the retrospective analysis of stored clinical samples and has shown that the Beijing genotype of *M tuberculosis*, which is prevalent in South East Asia, was also the prevalent type in China 40 years ago.[24]

The use of stained microscopic preparations as a target for DNA amplification of *M tuberculosis* DNA might also be exploited to monitor drug resistance in the framework of tuberculosis control programmes. For such a purpose, stained microscopic preparations could be shipped to a central facility where mutations can be determined in drug target genes, such as rpoB and katG, which lead to (multi)drug resistant tuberculosis.[25] Experiments using the technique of reverse line blotting are in progress to disclose drug resistance tuberculosis from DNA amplified from stained microscopic preparations.

Previously, we showed that spoligotyping using low DNA concentrations close to the detection limit often results in incomplete hybridisation patterns.[7] This might be a result of the presence of single copies of fragments of the partial DR region or because of shearing of the DNA.[26–28] In our present study the same phenomenon was observed and truncated spoligo patterns were obtained when the amount of DNA in the PCR was below 10 fg, which is equivalent to the amount of DNA from two mycobacterial cells. This quantity corresponds to the sensitivity observed in other PCR studies for reliable detection of *M tuberculosis*.[29 30]

False positive reactivity is one of the major drawbacks in PCR methods for detection. To prevent contamination with PCR products from previous amplifications, we included dUTP and uracil DNA glycosylase in our

assay. As expected, the use of dUTP and uracil DNA glycosylase did not interfere with spoligotyping. In addition to this method to destroy PCR amplified DNA, we took other commonly used precautions[16] to prevent cross contamination with previously amplified DNA. We did not find positive reactivity in negative control samples, indicating that such measures are also effective in the spoligotyping procedure.

Spoligotyping allows one to distinguish the various members of the *M tuberculosis* complex: *M bovis*, *M microti*,[31] and *M canetti* all have their own characteristic spoligotypes.[32] In our study there were two patients infected by strains with a spoligo pattern characteristic for *M bovis*, one of which was likely to be *M bovis* BCG.

This technique might be of practical use for the rapid disclosure of infections caused by multidrug resistant *M tuberculosis* complex strains. Recently, we demonstrated the international transmission of a multidrug resistant *M bovis* strain and spoligotyping played an essential role in the characterisation of this strain.[33 34]

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