Rapid detection of multidrug-resistant tuberculosis

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Rapid detection of multidrug-resistant tuberculosis. M. Goyal, R.J. Shaw, D.K. Banerjee, R.J. Coker, B.D. Robertson, D.B. Young. ©ERS Journals Ltd 1997.

ABSTRACT: Transmission of multidrug-resistant strains of *Mycobacterium tuberculosis* (MDR-TB) presents a serious problem for infection control in hospitals, particularly in the context of co-infection with the human immunodeficiency virus (HIV). We report on the use of molecular genetic tools to allow rapid assessment of samples from patients potentially infected with MDR-TB.

Sputum and bronchoalveolar lavage samples were obtained from two HIV-positive patients with suspected tuberculosis, who had previous contact with a known MDR-TB index case. Polymerase chain reaction (PCR) was used directly on clinical samples to amplify genetic loci associated with rifampicin resistance (*rpoB*), and strain-specific polymorphisms (the direct repeat (DR) region). Drug resistance was determined using a commercially available kit for detection of point mutations in the *rpoB* gene (Inno-Lipa RifTB; Innogenetics, Belgium), and confirmed by nucleotide sequencing. Strain variation was determined using the spoligotyping method, based on the presence or absence of variable linker sequences within the DR region.

In one patient, infection with a MDR strain identical to that of a known index case was demonstrated. A second patient, although positive for *M. tuberculosis*, was found to be infected with a rifampicin-sensitive strain. Results were obtained within 48 h, allowing appropriate treatment to be initiated and infection control measures to be implemented.

PCR-based tests for strain-typing and for identification of rifampicin resistance provide important tools for identifying patients with MDR-TB and for rapid monitoring of potential nosocomial spread of MDR-TB. Prompt confirmation or exclusion of possible transmission allows early clinical intervention to prevent future outbreaks of multidrug-resistant *M. tuberculosis*.

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Strains of *Mycobacterium tuberculosis* resistant to frontline drugs, including isoniazid and rifampicin, (multi-drug-resistant *M. tuberculosis* (MDR-TB)) have been responsible for a series of frequently fatal disease outbreaks [1–3]. Outbreaks have generally been associated with nosocomial transmission in hospital or prison settings, often including, though not restricted to, patients co-infected with human immunodeficiency virus (HIV) [3–7]. Following outbreaks in the United States in the early 1990s, an extensive research effort has been invested in the design of improved procedures for infection control, and in development of tools for rapid detection of drug-resistant isolates [8, 9].

Faced with the slow growth rate of *M. tuberculosis*, it takes weeks or even months to identify drug-resistant strains by conventional microbiology. With the prospect of developing rapid deoxyribonucleic (DNA)-based tests for resistance, investigators have focused on identification of the genetic changes responsible for resistance [8]. For each of the isolates analysed in detail, drug-resistance in *M. tuberculosis* has been mediated by changes affecting chromosomal genes, with no evidence of in-

volvement of any transmissible genetic elements. Multi-drug resistance is built-up by an accumulation of individual mutations conferring resistance to single drugs [10]. Mutations at several different loci can give rise to isoniazid resistance [11, 12], but resistance to rifampicin is almost always mediated by mutation of residues within a short stretch of the gene encoding the β -subunit of ribonucleic acid (RNA) polymerase (*rpoB*) [13, 14]. Rifampicin resistance is often associated with resistance to isoniazid, and is considered a useful surrogate marker for MDR-TB [8]. In the present study, we have used a commercially available kit (Inno-Lipa RifTB; Innogenetics, Belgium) for rapid detection of *rpoB* mutations associated with rifampicin resistance.

DNA-fingerprinting based on variations in the pattern of IS6110 insertions has been widely used as an epidemiological tool in monitoring patterns of transmission of *M. tuberculosis*, including analysis of MDR outbreaks [15, 16]. Bacterial cultures are required for this type of analysis, however, and results are essentially retrospective. In order to allow tracking of mycobacterial strains within a clinically useful timeframe, we have

used a polymerase chain reaction (PCR)-based typing system referred to as "spoligotyping". This technique is based on amplification of a region of the *M. tuberculosis* genome containing a series of direct repeat elements interspersed by variable spacer sequences [17]. PCR products are subsequently analysed by hybridization to membrane-bound oligonucleotides corresponding to the variable spacers, different isolates being characterized by their pattern of hybridization to differing sets of spacers. The level of strain discrimination provided by spoligotyping is slightly less than that of IS6110 fingerprinting, but its direct application to clinical samples represents a significant advantage when rapid results are required [18].

We report on the combined use of molecular tests for rifampicin resistance and strain-typing to facilitate rapid intervention and control of transmission of MDR-TB.

Materials and methods

Patients and clinical samples

Samples were obtained from three patients. The isolates from patient No. 1, the index case of tuberculosis, were resistant to rifampicin, isoniazid and pyrazinamide, as well as to cycloserine, rifabutin, clofazimine and ciprofloxacin. A sputum sample obtained from patient No. 2, an HIV-positive male and a contact of patient No. 1 was obtained on July 4, 1996 and stained weakly positive for acid-fast bacteria. Patient No. 3, an HIV-positive male, had also been exposed to MDR-TB from this outbreak. He subsequently developed a fever and unproductive cough, and bronchoalveolar lavage (BAL) was performed. No acid-fast bacteria were found in the BAL sample. A bacterial culture from an epidemiologically unrelated case of tuberculosis, patient No. 4, was also included in the study.

Clinical specimens (sputum and BAL) were digested and decontaminated by the N-acetyl-L-cysteine (NAC) 2% NaOH method. The samples were concentrated and the pellet resuspended in 100 µL of sample buffer (Tris-ethylenediamine tetra-acetic acid (EDTA) (TE buffer), pH 8.0), and then placed in a heat block at 80°C for 20 min. DNA was extracted from the treated clinical samples and bacterial cultures by the method described by VAN SOOLINGEN *et al.* [19]. Briefly, organisms were lysed using a cocktail of lysozyme (final concentration 1 mg·mL⁻¹), proteinase K (10 mg·mL⁻¹) and Sodium dodecyl sulphate (SDS) (10%), followed by treatment with N-acetyl-L-N,N,N-trimethyl ammonium bromide. DNA was prepared from the supernatants by phenol chloroform extraction and ethanol precipitation.

Detection of rifampicin resistance

A portion of the *rpoB* gene was amplified from DNA preparations by nested PCR, using primers provided as part of the Inno-Lipa RifTB kit according to the instructions provided by the manufacturers (Innogenetics, Belgium). Resulting biotinylated PCR products were hybridized with oligonucleotide probes immobilized as

parallel lines on membrane strips. The oligonucleotides included five probes corresponding to sequences present in the wild-type, rifampicin-sensitive, gene (S1–S5), together with four probes corresponding to the most frequently observed resistance mutations (R2, R4a, R4b and R5). After hybridization and washing according to the manufacturer's protocol, streptavidin labelled with alkaline phosphatase was added to detect bound biotinylated hybrids. Binding was visualized as a purple precipitate following incubation with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) chromogen. At the same time, the initial 450 base pair (bp) PCR products were also characterized by sequence analysis of both strands, using an ABI 310 Genetic Analyser (Perkin Elmer, Applied Biosystem Division, Warrington, Cheshire.). Sequencing reactions were performed with the ABI PRISM Dye Terminator cycle sequencing Ready Reaction Kit (Perkin Elmer), using the internal oligonucleotides as primers. Data were analysed using the SeqEd Software (Perkin Elmer).

Strain-typing (spoligotyping)

The direct repeat (DR) region from each sample was amplified by PCR, using the primers:

The conditions used for PCR were 3 min incubation at 96°C, followed by 20 cycles of 1 min at 96°C, 1 min at 55°C, and 30 s at 72°C. PCR products (20 µL in 150 µL of 2×saline sodium phosphate EDTA (SSPE), supplemented with 0.1% SDS) were heat denatured for 10 min at 100°C, and then hybridized for 60 min at 55°C to a Biotodyne C membrane with immobilized oligonucleotides corresponding to spacer sequences (provided by J. van Embden, Bilthoven, The Netherlands). Spacer oligonucleotide sequences were derived from the DR regions of *M. tuberculosis* H37Rv, and *Mycobacterium bovis* bacille Calmette-Guérin (BCG). The filter was washed with 2×SSPE (supplemented with 0.5% SDS) for 10 min at 55°C, and then incubated in streptavidin-peroxidase conjugate (Boehringer, Mannheim; 2.5 µL in 10 mL of 2×SSPE, supplemented with 0.5% SDS) for 45 min at 42°C. The membrane was washed and hybridizing DNA was detected using enhanced chemiluminescence (ECL) detection liquid (Amersham, UK) and exposure of the membrane to X-ray film for 5 min (Hyperfilm, Amersham).

Results

Five samples were analysed for the presence of mutations associated with rifampicin resistance. These included: two independent bacterial cultures from the index case (patient No. 1); a smear-positive sputum from patient No. 2; a smear-negative BAL from patient No. 3; and a bacterial culture from patient No. 4. Using the Inno-Lipa RifTB test, samples from patients Nos. 1 and 2 both showed loss of binding to oligonucleotide S5, along with

Fig. 1. — Analysis of rifampicin resistance using the Inno-Lipa RifTB test (Innogenetics, Belgium). A fragment of the *rpoB* gene was amplified from bacterial cultures (patients Nos. 1 and 4), or clinical samples (patients Nos. 2 and 3), and tested for mutations by hybridization to membrane-bound oligonucleotides corresponding to sensitive (S1–S5) and resistant alleles (R2, 4a, 4b and 5). The hybridization pattern of isolates from patients Nos. 1 and 2 (lanes 1–3) indicated mutations in the region corresponding to amino acids 529–533. Sequence analysis confirmed the presence of a point mutation in codon 531. Isolates from patients Nos. 3 and 4 (lanes 4 and 5) had wild-type *rpoB* sequences. Lane 6 shows the hybridization pattern obtained using the rifampicin-sensitive laboratory strain, *M. tuberculosis* H37Rv. Lane 7 is a control without deoxyribonucleic acid (DNA).

positive binding to R5 (fig. 1). These two probes cover the region of the *rpoB* gene encoding amino acids 529–533, with the binding pattern being consistent with that of a resistant allele. Sequence analysis confirmed the presence of an identical substitution of thymidine (T) for cytosine (C) within codon 531 in each case, resulting in exchange of leucine (TTG) for serine (TCG) in the amino acid sequence derived. The corresponding *rpoB* fragment was similarly amplified from the smear-negative BAL sample from patient No. 3. Results from the Inno-Lipa RifTB test (fig. 1, lane 4), and from DNA

sequence analysis, identified this as the wild-type rifampicin-sensitive allele. Similarly, the PCR product from an unrelated *M. tuberculosis* isolate (patient No. 4) was confirmed as rifampicin-sensitive (fig. 1, lane 5).

M. tuberculosis isolates from patients Nos. 1, 2 and 4 were also characterized by spoligotyping (fig. 2). In this assay, the single round of PCR amplification did not generate a product from the smear-negative BAL sample provided for patient No. 3. The results demonstrated an identical spoligotype for isolates from patients Nos. 1 and 2, again consistent with nosocomial transmission.

Fig. 2. — Spoligotype analysis of *M. tuberculosis* isolates. Isolates from patients Nos. 1 (lanes 5 and 6), 2 (lane 4) and 4 (lane 2) were compared by spoligotyping. Patients Nos. 1 and 2 were found to be infected with identical isolates; patient No. 4 was epidemiologically unrelated and was infected with a different strain type. Lane 1 shows the spoligotype pattern for *M. tuberculosis* H37Rv. Lane 3 is a control without deoxyribonucleic acid (DNA).

Table 1. — Characteristics of strains isolated from different patients

Pt no.	Contact with MDR-TB	Sample	<i>rpoB</i> sequence	Spoligotype pattern
1	Index case	Bacterial culture	Ser ⁵³¹ →Leu	A
2	Yes	Sputum (smear-positive)	Ser ⁵³¹ →Leu	A
3	Yes	BAL (smear-negative)	Wild-type	ND
4	No	Bacterial culture	Wild-type	B

ND: not determined; MDR-TB: multidrug-resistant tuberculosis; Pt: patient; BAL: bronchoalveolar lavage; Leu: leucine; Ser: serine.

Table 1 summarizes the results of the molecular analysis. Patient No. 2 was placed on a drug regimen consisting of a combination of amikacin, cycloserine, prothionamide, ofloxacin and ethambutol, which was changed on August 19, 1996 to amikacin, cycloserine, ethambutol, pyrazinamide and clarithromycin, and was accommodated in an isolation facility. The sputum sample from patient No. 2 subsequently grew *M. tuberculosis* after 6 weeks, which was found after another 4 weeks to be resistant to rifampicin, isoniazid, streptomycin and ethambutol, and sensitive to ethionamide, cycloserine, capreomycin, period-acid-Schiff (PAS), amikacin and pyrazinamide. Patient No. 3 was being treated and responded to conventional therapy (rifampicin, isoniazid and pyrazinamide) as an out-patient. No growth was observed from the BAL sample from patient No. 3 by 12 weeks of culture. However, the sample was found by an independent laboratory to be positive for *M. tuberculosis* using the Gen-Probe method.

Discussion

This study was conducted following requests from clinicians for a rapid assessment of the possibility of transmission from a known MDR-TB index case. Based on strain-typing and analysis of resistance-associated mutations, the present results strongly suggested that patient No. 2 had, in fact, been infected with the same strain as the index case, while patient No. 3 had probably acquired an unrelated infection with a rifampicin-sensitive strain. A key feature of the present study is that a sputum sample from patient No. 2 was collected on July 4, 1996 and transferred after the weekend to our laboratory, where it arrived on the evening of July 9, 1996, and we were able to relay detailed results to the clinician on July 12, 1996, thus allowing initiation of appropriate treatment, and implementation of infection control measures to limit any further spread. Furthermore, rapid detection of tuberculosis and clarification of sensitivity allowed conventional treatment to be initiated early for patient No. 3, with early discharge from hospital. The speed of this new approach contrasts with conventional culture and sensitivity testing, the results of which were still awaited at the time of submission of this manuscript.

The Inno-Lipa RifTB test provides a relatively simple assay for detection of mutations associated with rifampicin resistance and, as demonstrated here, the nested PCR format provides a sensitive detection system capable of generating a signal even in a smear-negative sample. Studies of the reproducibility of the Inno-Lipa RifTB on a large number of extracts from sputum have yet to be completed and, therefore, in the present study we have combined this method with automated DNA sequencing. A potential drawback of this approach is that it will only detect the set of mutations already defined in the *rpoB* gene, and may, therefore, fail to identify any novel resistant alleles. However, a series of studies have identified *rpoB* mutations in >97% of more than 200 rifampicin-resistant isolates of *M. tuberculosis*, with mutation of codon 531, as observed in the present study, the most frequent alteration [8]. Combination of

rpoB analysis with a PCR-based typing system provides a powerful approach to monitoring the transmission of MDR-TB. Detection of rifampicin resistance by molecular biology techniques is considerably more straightforward than detection of resistance to other drugs. Although the final choice of antimycobacterial agents used to treat the patient requires full knowledge of the drug sensitivity patterns of the organisms, rifampicin resistance rarely occurs in isolation and serves as a good surrogate marker for multidrug resistance.

The spoligotyping system for strain-typing is fast and reliable, generating data that can be readily analysed by eye, and stored using any simple word-processing programme. Strain-typing itself does not provide direct information about drug susceptibility, but a register of spoligotypes from known MDR isolates may be particularly useful in providing early warning of possible infection with these strains. Whilst previous MDR-TB outbreaks have been characterized using IS6110 fingerprinting [20], there are considerable clinical advantages in having a test which, when applied to smear-positive sputum, can provide strain information within one or two days. It is during this period, and in these potentially infectious patients, that crucial decisions have to be made in relation to treatment regimens and appropriate patient accommodation.

In the present study, spoligotyping was possible in the smear-positive samples but not in the smear-negative samples. Other studies on several hundred sputum samples (unpublished) suggest that spoligotyping can provide typing data on smear-positive sputum samples. As with any PCR-based technique, the test has lower sensitivity in smear-negative disease. Other PCR-based typing techniques which have been developed include: arbitrarily primed (AP) PCR [21], random amplified polymorphic DNA analysis (RAPD) [22, 23], and mixed linker [24]. A less complex PCR-based typing method was outlined by ROSS and DWYER [25]. They used primers that bind to the ends of IS6110 to amplify DNA between closely spaced copies of this element. PLIKAYTIS *et al.* [26] combined multiple polymorphic tandem repeat (MPTR) and IS6110 to develop the IS6110 amplifying typing method. Another method exploited the variability in *KatG* gene [27]. The method called "double repetitive element PCR" (DRE-PCR) utilized the amplification of *M. tuberculosis* DNA segments located between two copies of repetitive elements IS6110 and the polymorphic guanine-cytosine rich repetitive sequence (PGRS) [28]. Recently, another PCR-based method called "hemi-nested inverse PCR" for IS6110 has also been developed [29]. There is little evidence to date that these PCR-based techniques can be used routinely on sputum samples.

Initial outbreaks of MDR-TB in the United States were associated with high numbers of fatalities amongst patients and health care workers. Use of molecular tests for rifampicin resistance and strain-typing to detect transmission of multidrug-resistant tuberculosis will facilitate the rapid response required to limit the extent and severity of future outbreaks.

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