Rapid, amplification-based fingerprinting of *Mycobacterium tuberculosis*

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Insertion element IS6110 occurs in multiple copies throughout the *Mycobacterium tuberculosis* genome, and the variability of its insertion sites is the basis for the IS6110 restriction fragment length polymorphism (RFLP) method for typing. We describe a novel gene amplification method to assess the variability of the location of IS6110. A unilateral-nested polymerase chain reaction and hybridization procedure was used to measure the variability in the distances between IS6110 elements and copies of a major polymorphic tandem repeat sequence of *M. tuberculosis*. The pattern of amplicons produced could be used to cluster epidemiologically related strains of *M. tuberculosis* into groups which correlated with the groups formed using IS6110-RFLP typing. Reliable patterns can be generated directly from sputum specimens as well as from *M. tuberculosis* cultures. We designated the novel method as IS6110-ampliprinting.

Introduction

Tuberculosis remains a major source of morbidity and mortality throughout the world and is increasing in the United States today. The resurgence of tuberculosis in the United States is largely related to the human immunodeficiency virus (HIV) epidemic (Barnes *et al.*, 1991). Because of the reduction in cell-mediated immunity in HIV-infected persons, active disease may develop quickly after exposure to *Mycobacterium tuberculosis* (Barnes *et al.*, 1991). As the number of hospitalized patients infected with HIV and tuberculosis increases, the risk of nosocomial infection with *M. tuberculosis* increases not only among patients, but also among health care providers (Pearson *et al.*, 1992).

An important factor in the control of tuberculosis is the ability to identify outbreaks and track the transmission of a particular strain of *M. tuberculosis*. The standard procedure for distinguishing strains of *M. tuberculosis* isolates has been phage typing; however, a more sensitive molecular approach has been described recently (Hermans *et al.*, 1990; Cave *et al.*, 1991). An insertion element which has considerable homology to

the IS3 family of enterobacteria has been described and designated as IS6110 (Thierry *et al.*, 1990); it is virtually identical to the more recently described IS986 (McAdam *et al.*, 1990). This approach takes advantage of the facts that *M. tuberculosis* strains carry multiple copies of IS6110, and that the precise locations of the IS6110 elements in the *M. tuberculosis* genome vary significantly from strain to strain, providing a unique DNA fingerprint for each *M. tuberculosis* strain. The IS6110-restriction fragment length polymorphism (RFLP) technique has been shown to be a reliable and reproducible method for differentiating *M. tuberculosis* strains (Cave *et al.*, 1991; Otal *et al.*, 1991), and a recent investigation utilized this technique to study the epidemiology of multidrug-resistant tuberculosis among hospitalized HIV-infected patients (Edlin *et al.*, 1992).

The IS6110-RFLP procedure requires growth of the organism followed by purification of genomic DNA from the bacteria. The purified DNA is digested with a restriction enzyme which cleaves within the IS6110 sequence. The digested genomic DNA is then electrophoresed on agarose gels, transferred to a membrane, and hybridized with a portion of the IS6110 sequence. This is a time-consuming method that is useful for retrospective epidemiology but has limitations concerning disease management or a rapid response to outbreak situations.

We explored the possibility of using a polymerase

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Abbreviations: MPTR, major polymorphic tandem repeat; RFLP, restriction fragment length polymorphism.

chain reaction (PCR) (Mullis & Faloona, 1987) and hybridization procedure to measure the variability in the distances between IS6110 elements and a major polymorphic tandem repeat (MPTR) sequence of *M. tuberculosis* (Hermans *et al.*, 1992; Shinnick, 1987). The MPTR sequence is composed of tandem repeats of a 10 bp consensus sequence separated by 5 bp highly heterogeneous spacers. The consensus sequence of the 10 bp tandem repeat is (5')GCCGGTGTG, which has homology to the repetitive extragenic palindromic (REP) sequences of *Escherichia coli* (Hermans *et al.*, 1992). REP sequences have been used to produce PCR-based fingerprints from various bacterial genomes (Versalovic *et al.*, 1991). The MPTR sequences are found in the *M. tuberculosis* genome in as many as 80 different regions (Hermans *et al.*, 1992). Importantly, their distribution in the *M. tuberculosis* genome appears to be stable, in contrast to the variability in IS6110 location (Hermans *et al.*, 1992). Thus, by using IS6110 and MPTR primers in a unilateral-nested amplification procedure followed by hybridization with an IS6110-specific oligonucleotide, we were able to generate a pattern of amplification products that clustered strains of *M. tuberculosis* into groups matching those formed using the IS6110-RFLP technique. As a test of the procedure, we analysed strains from several recent multidrug-resistant tuberculosis outbreaks. We also tested the method on a limited number of sputum samples to determine the feasibility of fingerprinting *M. tuberculosis* directly from sputum specimens (i.e. without culturing). We refer to this method as ampliprinting.

Methods

Bacterial strains and preparation of DNA. The strains used in this study are listed in Table 1. DNA was prepared from *M. tuberculosis* isolates using the CTAB (cetyltrimethylammonium bromide) method as previously described (Wilson, 1990). Crude lysates of the non-*M. tuberculosis* strains listed in Table 1 were prepared by glass bead lysis as previously described (Plikaytis *et al.*, 1992).

Preparation of lysates from sputum specimens. Smear-positive sputum samples were obtained from the Arkansas State Health Department laboratory. The specimens were liquefied and decontaminated using the standard *n*-acetylcysteine/NaOH protocol (Kent & Kubica, 1985), and the sediments remaining after microscopy and inoculation on solid media were stored at -20 °C until they were processed for PCR. The sediment was centrifuged at 16000 *g* for 5 min and the supernatant was discarded. The pellet was resuspended in 200 µl of 10 mM-Tris/HCl (pH 8.0)-1 mM-EDTA-10 mM-NaCl and mixed with 200 µl siliconized 0.1 mm-diameter glass beads and 100 µl chloroform. The mixture was homogenized for 2 min at room temperature in a Mickel apparatus (Brinkman Instruments, Westbury, NY, USA) to disrupt the cells. The homogenized suspension was centrifuged at 16000 *g* for 5 min, and the aqueous supernatant was transferred to a fresh tube and boiled for 10 min.

Oligonucleotide primers. Primers corresponding to portions of the *M. tuberculosis* IS6110 and MPTR sequences were synthesized on a DNA

synthesizer (model 381A; Applied Biosystems) at the Biotechnology Core Facility, Centers for Disease Control. The sequences and locations of these primers are listed in Table 2, and the relative positions within the IS6110 sequences are depicted in Fig. 1. The MPTR-primer sequences were determined by combining the consensus sequence for the 10 bp repeat with five flanking bases, which were selected by comparing the reported sequences (Hermans *et al.*, 1992; Shinnick, 1987) and identifying 5 bp spacer sequences which appeared only once in each reported sequence. For example, the primer MPTR-6 is composed of the consensus sequence flanked by two bases on the 5'-end and a unique set of three bases on the 3'-end (Table 2).

Gene amplification. The amplification reaction contained 10 µl of template DNA and 90 µl of a reaction mix [200 µM (each) deoxy-nucleotide triphosphates, 1.0 µM (each) primers, 2.5 U *Taq* polymerase, 10 mM-Tris/HCl (pH 8.3), 50 mM-KCl, 1.5 mM-MgCl₂, 0.01 % gelatin] as recommended by the *Taq* polymerase manufacturer (Perkin-Elmer Cetus). The first round of amplification with an IS6110 primer and an MPTR primer consisted of 25-30 cycles in a programmable thermal cycler (Perkin-Elmer Cetus) with a three-step cycle of denaturation for 1.5 min at 94 °C, annealing for 1.75 min at 45 °C, and extension for 2.5 min at 72 °C. The second round of amplification was carried out for 15-25 cycles after transferring 10 % of the first-round amplification

Table 1. Strains used in this study

Species	Strain	Fingerprint type*
<i>M. tuberculosis</i>	91-8358, 91-8359, 91-8360	011-8114
<i>M. tuberculosis</i>	91-8361, 91-2627, 91-2742, 91-2743, 91-2744, 91-2745, 91-2746, 92-8081, 92-8082, 92-8083, 92-8088, 92-8089, 92-8090, 92-8091, 92-8092, 92-8093, 92-8095	021-2072
<i>M. tuberculosis</i>	91-8271, 92-8272, 91-8273	023-8271
<i>M. tuberculosis</i>	91-8275, 91-8276	024-8275
<i>M. tuberculosis</i>	92-8078, 82-8079	036-8078
<i>M. tuberculosis</i>	92-8084, 92-8085, 92-8086	036-8084
<i>M. tuberculosis</i>	91-3048	028-3048
<i>M. tuberculosis</i>	91-3054	028-3054
<i>M. tuberculosis</i>	91-8309	031-8309
<i>M. tuberculosis</i>	91-8310	031-8310
<i>M. tuberculosis</i>	91-8311	031-8311
<i>M. tuberculosis</i>	91-8312	031-8312
<i>M. tuberculosis</i>	91-8313	031-8313
<i>M. tuberculosis</i>	91-8270	023-8270
<i>M. tuberculosis</i>	91-8277	024-8277
<i>M. tuberculosis</i>	H37Rv	H37Rv
<i>M. bovis</i>	TMC 401, TMC 410	
<i>M. bovis</i> BCG	TMC 1024	
<i>M. africanum</i>	TMC 5122	
<i>M. avium</i>	TMC 1461	
<i>M. chelonae</i>	TMC 1524	
<i>M. fortuitum</i>	TMC 1530	
<i>M. gastri</i>	ATCC 25157	
<i>M. gordonae</i>	TMC 1325	
<i>M. intracellulare</i>	TMC 1469	
<i>M. kansasii</i>	ATCC 12478	
<i>M. scrofulaceum</i>	TMC 1312	
<i>M. smegmatis</i>	TMC 1533	
<i>M. szulgai</i>	91-698	

* The IS6110-RFLP fingerprint type consists of the outbreak or study number followed by the isolate number. All subsequent isolates having that identical fingerprint pattern are given the same type designation as the initial isolate having that pattern.

Table 2. Sequences of oligonucleotides used as primers or hybridization probes

Primer	Target	Sequence (5'-3')	Location*
IS49	IS6110	CATGTCAGGTGGTTCATCG	54-72
IS54	IS6110	TCGACTGGTTCAACCATCGCCG	1210-1231
IS55	IS6110	TCTGATCTGAGACCTCAGC	1330-1312c
IS56	IS6110	GCGACCTCACTGATCGCTGC	170-151c
IS61	IS6110	GACCGCGGATCTCTGCGACC	133-114c
IS62	IS6110	ACCAGTACTGCGGCGACGTC	1237-1256
MPTR-1	MPTR	GCCGGTGTGGTGTC	154-168†
MPTR-6	MPTR	GGCAACACCGGCCTC	255-241c†

* The residues of IS6110 are numbered according to the system of Thierry *et al.* (1990), and those of MPTR according to Hermans *et al.* (1992). A 'c' indicates that the listed sequence is the complement of the published sequence.

† The primer sequence for MPTR-1 and MPTR-6 includes the consensus sequence (GCCGGTGTGGT) instead of the actual sequences for these residues, which are CCCGGTGTGGTGTC (154-165) and GGCAGCACTGGGCTC (255-241c).

Fig. 1. Schematic representation of IS6110 and MPTR sequences and the location of oligonucleotides used in PCR and Southern blotting. * denotes the ECL fluorescein-labelled oligonucleotide used as a probe in the Southern blot.

mixture into a fresh tube containing reaction mixture with a nested IS6110 primer and the same MPTR primer as used in the first round. The second round of amplification used a three-step cycle similar to that used in the first round, except that the annealing temperature was 60 °C.

Hybridization. Fifteen microlitres of the second-round amplification mixture were electrophoresed on a 1.5% agarose gel, and the reaction products were visualized by ethidium bromide fluorescence. The DNA was then denatured, neutralized, and transferred by capillary blotting to Hybond-N⁺ membrane (Amersham) as recommended by the manufacturer of the membrane. DNA was bound to the membrane using a Stratalinker UV crosslinker (Stratagene). An IS6110 (IS49 or IS55) or MPTR (MPTR-6) specific oligonucleotide was labelled using the ECL 3'-oligolabelling and detection system (Amersham). The membrane was hybridized according to the manufacturer's recommendations at 42 °C for 3 h and washed stringently at 42 °C in 3 mM-sodium citrate, 30 mM-sodium chloride and 0.1% sodium dodecyl sulphate. After development with the ECL detection solutions, the signals were detected using X-OMAT AR autoradiography film (Eastman Kodak).

Results and Discussion

Evaluation of primers for *M. tuberculosis* amplified fingerprinting (ampliprinting)

Two sets of IS6110-specific primers, one at the left (IS56, IS61, IS49) and one at the right (IS54, IS62, IS55) end were evaluated. Each set contained two nested primers for amplification and a third for use as a hybridization probe (Fig. 1). In the first round of amplification a 45 °C annealing temperature was used to allow for potential

mismatches in the slightly heterogeneous MPTR consensus sequence. The second round, which is a unilateral-nested amplification since the same primer is used for the MPTR sequence, employs a more stringent annealing temperature of 60 °C. The use of two IS6110-specific primers and the increased annealing temperature improves the efficiency of amplification of IS6110-containing sequences. Initially six MPTR primers were tested with each of the two sets of IS6110 primers. MPTR-1 was found to produce the most informative patterns when paired with the IS6110 left set of primers, and MPTR-6 was found to be best for use with the IS6110 right set (data not shown). These primers and the IS6110 primers were then used individually and in combination with each other to characterize further the amplification patterns produced from *M. tuberculosis*. That is, strain 91-8361 DNA was amplified using IS56 alone, MPTR-1 alone, IS56 + MPTR-1 (first round), and IS56 + MPTR-1 followed by IS61 + MPTR-1 (second round), or using IS54 alone, MPTR-6 alone, IS54 + MPTR-6, and IS54 + MPTR-6 followed by IS62 + MPTR-6. DNA fragments ranging in size from approximately 250 to 2200 bp were amplified by each primer individually or by each combination of primers tested (Fig. 2a, c). To enhance the signal from the IS6110-containing amplicons, the samples were hybridised with an IS6110-specific oligonucleotide probe (Fig. 2b, d). The hybridization step eliminated the signals from the products amplified due to mispriming or due solely to the MPTR primer (lane 2, Fig. 2b, d) and produced a discrete pattern of 9 to 11 IS6110-containing amplicons (lane 4, Fig. 2b, d).

Comparison of 5'-end and 3'-end primer sets

To determine which set of primers was the most useful or if data from both sets would be necessary to distinguish different strains of *M. tuberculosis*, 16 strains were

Fig. 2. Amplification with *IS6110* and MPTR primers individually and in combinations. The oligonucleotide primer(s) used to amplify DNA from a multidrug-resistant strain of *M. tuberculosis*, 91-8361, were *IS56* only (lane 1), MPTR-1 only (lane 2), *IS56* + MPTR-1 (lane 3), *IS56* + MPTR-1 followed by *IS61* + MPTR-1 (lane 4), *IS54* only (lane 5), MPTR-6 only (lane 6), *IS54* + MPTR-6 (lane 7), *IS54* + MPTR-6 followed by *IS62* + MPTR-6 (lane 8). Panel (b) is the Southern blot analysis of the agarose gel shown in panel (a) hybridized with the *IS6110* 5'-end oligonucleotide *IS49*. Panel (d) is the Southern blot analysis of the agarose gel shown in panel (c) hybridized with the *IS6110* 3'-end oligonucleotide *IS55*. Lane S contains molecular size markers (ϕ X174RF DNA/*Hae*III fragments), with the sizes in bp indicated on the left.

Fig. 3. Evaluation of the 3'-end primer set. (a) Agarose gel electrophoresis and (b) Southern blot analysis showing the amplicants generated using primers *IS54* + MPTR-6 in the first round of amplification followed by *IS62* + MPTR-6 in the second round of amplification. DNA templates were *M. tuberculosis* isolates of 91-8309 (lane 1), 91-8313 (lane 2), 91-8310 (lane 3), 91-8312 (lane 4), 91-3054 (lane 5), 91-8270 (lane 6), 91-8271 (lane 7), 91-8272 (lane 8), 91-8273 (lane 9), 91-8358 (lane 10), 91-8359 (lane 11), 91-8360 (lane 12), 92-8082 (lane 13), 92-8093 (lane 14), 92-8095 (lane 15). Lane S contains the molecular size markers (ϕ X174RF DNA/*Hae*III fragments), with the sizes in bp indicated on the left.

amplified using primer sets *IS56* + MPTR-1 (5'-end) and *IS54* + MPTR-6 (3'-end) in the first round followed by a second round of amplification with *IS61* + MPTR-1 (5'-end) and *IS62* + MPTR-6 (3'-end) and then were hybrid-

ized with the corresponding probe. The 16 strains analysed represent six distinct *IS6110*-RFLP types (J. T. Crawford, unpublished data). Each set of primers generated a distinctive pattern of *IS6110*-containing

amplicons for each of the six IS6110-RFLP types (data not shown). The 3'-end primer set was chosen for further study because it appeared to give a slightly more reproducible pattern.

Evaluation of the 3'-end primer set

Purified DNA from *M. tuberculosis* H37Rv was titrated to determine the detection limit of the method using 30 cycles of amplification in the first round of amplification and 15 cycles in the second round of amplification. The pattern of IS6110-containing amplicons was maintained to 100 pg of DNA, which is equivalent to approximately 2×10^4 organisms (data not shown). At smaller amounts of DNA, the major PCR products were still amplified; however, several of the minor amplification products were not. This reduces the amount of information in the patterns, which, in turn, may lessen the ability of the method to differentiate strains.

The specificity of the method was evaluated by amplifying crude lysates of *M. africanum*, *M. avium*, *M. bovis*, *M. bovis* BCG, *M. chelonae*, *M. fortuitum*, *M. gastri*, *M. gordonae*, *M. intracellulare*, *M. kansasii*, *M. scrofulaceum*, *M. smegmatis* and *M. szulgai*. Various numbers of ethidium-bromide-stained fragments were amplified from each species. However, only the *M. africanum* sample contained amplicons that hybridized with the IS6110-specific IS55 oligonucleotide (data not shown). The positive hybridization results with *M. africanum* are expected since this strain contains 6 to 9 copies of the IS6110 sequence (Plikaytis *et al.*, 1991). The negative hybridization results with *M. bovis* and *M. bovis* BCG are not surprising, however, since there are only 1 to 3 copies of the IS6110 sequence in the *M. bovis* genome.

Many fragments in the amplified samples from each of the *Mycobacterium* species tested hybridized to MPTR-6 (data not shown). This suggests that sequences homologous to the MPTR consensus sequence are present in the genome of each of these species. However, these amplification results do not distinguish clusters of tandem repeats from fortuitous positioning of individual sequences homologous to the MPTR primer. The latter may be important because a Southern hybridization analysis of genomic DNA suggested that clusters of homologous tandem repeats were found in only *M. tuberculosis* complex species, *M. gastri*, *M. gordonae*, *M. kansasii* and *M. szulgai* (Hermans *et al.*, 1992).

Clinical isolates and sputum specimens

Type strain H37Rv and 39 *M. tuberculosis* clinical isolates from seven tuberculosis outbreak investigations representing 16 IS6110-RFLP types were analysed with

Fig. 4. Ampliprints obtained directly from sputum samples. (a) Agarose gel electrophoresis and (b) a Southern blot showing the ampliprints generated using primers IS54+MPTR-6 in the first round of amplification followed by IS62+MPTR-6 in the second round of amplification. DNA templates were crude lysates of organisms obtained directly from sputum samples from tuberculosis patient D: specimens 11923, 11925 and 13066 (lanes 1, 2 and 3); patient E: specimen 11770 (lane 4); and patient F: specimen 12907 (lane 5). The sizes in bp of the molecular size standards (ϕ X174RF DNA/*Hae*III fragments) are indicated on the left.

the 3'-end primer set. Fig. 3(a) shows the agarose gel containing the products from the second round of amplification of 15 of these *M. tuberculosis* strains representing nine IS6110-RFLP types, and Fig. 3(b) shows the hybridization of these products with the 3'-end probe, IS55. Each of the 16 IS6110-RFLP types produced a unique set of IS6110-containing amplicons. Furthermore, strains that had identical IS6110-RFLP patterns also had identical IS6110-ampliprint patterns (e.g. lanes 7 to 9, Fig. 3).

Six smear-positive (3 to 4+) sputum specimens from three tuberculosis patients were processed as described in Methods, and the crude lysates were amplified for 30 cycles in the first round and 25 cycles in the second round using the 3'-end primers. Fig. 4 shows the agarose gel and the results of the hybridization with the IS55 probe of five of these samples from the three patients. As would be expected for epidemiologically unrelated cases, the ampliprints generated from each patient's specimens were clearly different, while identical hybridization patterns were observed for each of the three serial specimens from one patient (e.g. lanes 1 to 3, Fig. 4b).

The ability to type strains directly from sputum specimens suggests that the method could be used to rapidly identify infections with a given strain. If so, this method may be useful in outbreak situations where a particular ampliprint has been identified and strains with this ampliprint are multidrug-resistant. In such cases the ampliprint generated by this amplification procedure might be used as a surrogate marker for drug resistance before the results of standard susceptibility tests become available. However, much work remains to be done to evaluate the predictive value of such an approach.

In summary, we have described a novel approach to differentiate *M. tuberculosis* strains by combining the variability of the IS6110 insertion sites with the conserved locations of the MPTR sequences in the *M. tuberculosis* genome. Primers corresponding to portions of these sequences were used to amplify a set of DNA fragments that can differentiate *M. tuberculosis* strains into groups that correlated well with those obtained by IS6110-RFLP typing. This method should be useful as a rapid screening method for fingerprinting *M. tuberculosis*. However, because of the limited number and sizes of amplicons produced, and the resulting limited information, ampliprinting may provide somewhat less information about strain relatedness than the IS6110-RFLP fingerprinting method.

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