Characterization of a Mycobacterium tuberculosis Insertion Sequence, IS6110, and Its Application in Diagnosis

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An insertion sequence-like element, IS6110, was isolated from a Mycobacterium tuberculosis cosmid library as a repetitive sequence. IS6110 shows similarities with elements of the IS3 family. This insertion sequence was found to be specific to mycobacteria belonging to the M. tuberculosis complex. For detection and identification of M. tuberculosis bacilli in uncultured specimens, oligonucleotides derived from the IS6110 sequence were used as primers and probes in polymerase chain reaction studies. The results obtained were consistent with results of classical identification procedures, bacteriological data, and clinical criteria.

At present, diagnosis of tuberculosis and related diseases is dependent on isolation of the pathogenic bacteria and their subsequent identification by biochemical testing. Such procedures usually require 4 to 8 weeks. More rapid techniques such as the BACTEC detection system (18), which is based on measurement of the radioactive CO₂ released by metabolism of radioactive palmitate added to cultures, and the Gen-Probe identification system (8), which is based on hybridization of the 32P-labeled DNA fragments corresponding to 16S RNA sequences, have been described previously. Although these techniques reduce the identification time, they still require at least a 1-week culturing period.

The technique of in vitro amplification of specific DNA sequences is a sensitive method for the detection of viruses and bacteria in pathological samples (10, 16, 20). We have recently adapted this technique to the detection and identification of mycobacteria in uncultured clinical specimens (3, 12) by making use of polymorphism in the gene coding for the 65-kDa heat shock protein in bacteria.

In several cases it has been shown that repetitive DNA sequences display species specificity. For example, the insertion sequence IS900 was shown to be specific to Mycobacterium paratuberculosis (9). In this report, we describe the identification of a repetitive sequence from Mycobacterium tuberculosis. This element, IS6110, possesses similarities to insertion sequences of the IS3 family (15, 19, 22). IS6110 is 1,361 bp long and contains 28-bp, imperfect inverted repeats at its extremities with three mismatches and 3-bp direct repeats that probably result from repetition of the target sequence. Among the various mycobacterial species examined, IS6110 was detected only in species belonging to the M. tuberculosis complex. Oligonucleotides derived from this sequence were used to detect M. tuberculosis in clinical specimens following in vitro DNA amplification.

MATERIALS AND METHODS

Construction of an M. tuberculosis cosmid library. M. tuberculosis H37r v genomic DNA was partially digested for 1 h at 37°C with 0.03 U of Sall per μg of total DNA. DNA fragments were fractionated by electrophoresis on a 0.6% agarose gel in TAE (0.04 M Tris acetate, 0.001 M EDTA). The 30- to 40-kb fragments were electroeluted in TBE (0.089 M Tris borate, 0.089 M boric acid, 0.002 M EDTA) in dialysis membranes (4). Fragments (1.5 μg) were ligated with 100 ng of the pHC79 cosmid (13), digested with Sall, and depolymerized with alkaline phosphatase. Reaction was carried out at 14°C for 16 to 18 h with 2.5 U of T4 ligase in 0.06 M Tris hydrochloride (pH 7.5) buffer containing 5 mM MgCl₂, 5 mM dithiothreitol, and 1 mM ATP. Recombinant cosmids were packaged (14) and used to infect Escherichia coli HB101. Ampicillin-resistant, tetracycline-susceptible colonies were picked and grown in LB broth supplemented with ampicillin, and cosmid DNAs were extracted (2).

Library screening and isolation of a fragment hybridizing to M. tuberculosis DNA. Cosmid DNA samples were digested with restriction enzyme SalI, electrophoresed through a 0.6% agarose gel, and transferred onto Hybond N filters (Amersham) by the Southern blot method (17). Filters were probed with M. tuberculosis and/or Mycobacterium bovis BCG DNA labeled with [α-32P]dCTP by using the multiprime random-labeling system (7) (Amersham). Hybridizations were performed at 68°C for 16 to 18 h in 6× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–10% dextran sulfate–5× Denhardt–10 mM EDTA–0.5% sodium dodecyl sulfate (SDS)–100 μg of salmon sperm denatured DNA per ml–10⁶ cpm of probe.

Filters were washed twice with 2× SSC at 65°C for 10 min, once with 2× SSC–0.1% SDS at 65°C for 30 min, and once with 0.1× SSC at 65°C for 10 min. Filters were briefly air dried and exposed to Kodak XAR5 film with intensifying screen at ~80°C.

A SalI fragment which strongly hybridized with M. tuberculosis DNA was eluted from the agarose gel, purified, and sequenced by the chain termination method of Sanger et al. (20a) by using Taq polymerase and 7-deaza-dGTP instead of dGTP, to prevent band compression in the GC-rich regions (TaqTrack sequencing system from Promega Corp.). To complete the IS6110 sequence, a larger fragment (3.5 kb) was cloned after HindIII digestion of the original cosmid, I21.

Preparation of an IS6100 internal fragment as probe. From the 3.5-kb fragment described above, a 902-bp HindIII-BamHI fragment was subcloned into pUC18 (26); the result-
ing plasmid was named pMT02. The 902-bp fragment was labeled with [α-32P]dCTP by using the multiprime random-labeling system (7) (Amersham) and was used as a probe in Southern hybridization analysis.

Southern hybridization analysis of genomic DNAs. Total DNA (1.2 μg) from species of the M. tuberculosis complex (M. tuberculosis, M. bovis BCG, M. bovis, and M. microti) and DNAs from other mycobacteria (M. avium, M. paratuberculosis, M. intracellulare, and M. scrofulaceum) (see legend to Fig. 4) were digested with 70 U of the restriction enzymes PstI, EcoRI, BamHI, and SalI in appropriate buffers for 16 to 18 h at 37°C. DNA fragments were electrophoresed through a 0.6% agarose gel. Gels were incubated for 15 min in 0.25 M HCl, twice for 15 min each time in 1.5 M NaCl–0.5M NaOH, and twice for 15 min each time in 1 M CH3COONH4.

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PCR experiments. (i) Synthetic oligonucleotides. Oligonucleotides were synthesized on an Applied Biosystems synthesizer by the automated phosphoramidite coupling method (1). Oligonucleotides used as primers and probes for polymerase chain reaction (PCR) experiments are described in French patent application no. 90.02676 (A. Brisson-Noël). Oligonucleotides used in hybridization assays were radiolabeled at their 5′ ends with T4 polynucleotide kinase (Boehringer) and [32P]ATP (Amersham). Labeled oligonucleotides were separated from unincorporated [32P]ATP by chromatography on Sephadex G-50.

(ii) DNA preparation. For amplification experiments, mycobacteria from 1-ml cultures were pelleted, resuspended in 200 μl of lysis buffer (0.1 M NaOH, 2 M NaCl, 0.5% SDS), and incubated at 95°C for 15 min. DNAs from the resulting lysates were extracted twice with phenol-chloroform, precipitated with ethanol, and resuspended in 100 μl of sterile water.

DNA was extracted from 0.2 to 1 ml of crude or sodium hydroxide-decontaminated clinical specimens by the procedure described above. Ten-microliter aliquots were used for amplification.

(iii) DNA amplification. Amplification reactions were performed in a total volume of 100 μl in 10 mM Tris hydrochloride (pH 8.3)–1.5 mM MgCl2–50 mM KCl–12.5 pmol of each primer–125 μM (each) deoxynucleoside triphosphates (dATP, dGTP, dTTP, and dCTP)–2 U of Thermus aquaticus DNA polymerase (Perkin Elmer Corp., Norwalk, Conn.). The amplification mixture was overlaid with 50 μl of mineral oil and subjected to 40 cycles of amplification as follows. Samples were incubated for 2 min at 94°C to denature the DNA, for 2 min at 60°C to anneal the primers, and for 2 min at 72°C to extend the annealed primers. Thermal cycling was performed in a programmable heat block (Perkin Elmer). Samples were then electrophoresed through a 1.5% agarose gel; amplification products were visualized by ethidium bromide staining.

(iv) Hybridization with labeled oligonucleotides. Amplified DNAs were resolved on agarose gels and transferred onto Hybond N nylon filters (Amersham) by Southern blotting (17). After a 15-min prehybridization in Rapid Hybridization Buffer (Amersham) at 65°C, membranes were hybridized with 108 cpm of 32P-labeled probe in the same buffer for at least 2 h at 65°C. Filters were then washed twice in 2× SSC–0.1% SDS at 20°C for 10 min and once in 1× SSC–0.1% SDS at 65°C for 15 min. Autoradiography was performed for 18 h at −80°C with an intensifying screen.

FIG. 1. Southern hybridization of cosmids I21. I21 DNA (15 μl, corresponding to about 3 μg) was digested with 10 U of restriction enzyme SalI. After agarose electrophoresis, DNA was transferred to filters by Southern blotting and hybridized with radiolabeled probes: M. bovis BCG total DNA (A) and M. tuberculosis total DNA (B). The arrow indicates the 1-kb fragment of interest.

RESULTS AND DISCUSSION

Construction of an M. tuberculosis cosmids library and isolation of IS6110. With the aim of detecting M. tuberculosis-specific repeated genomic DNA sequences, we constructed a cosmids library. About 2 × 108 recombinant cosmids were obtained. Since in vitro packaging ensures selective pressure for insertion of large fragments (20- to 40-kb fragments), and since M. tuberculosis is believed to contain about 4 × 106 bp, this cosmid library was expected to be representative of the complete M. tuberculosis genome.

A set of 150 recombinant clones was screened by dot blot analysis (4) by using total M. tuberculosis DNA as a probe. Cosmids giving strong hybridization signals were further analyzed by Southern blotting by using M. tuberculosis, M. bovis BCG, or M. avium total DNA as probes. After restriction enzyme digestion, several clones were found to contain fragments that hybridized strongly with M. tuberculosis DNA. Typical results for clone I21 are shown in Fig. 1. Cosmid I21 contains a 1-kb fragment that gives a strong positive signal with M. tuberculosis DNA after a 24-h exposure and a weak signal with M. bovis BCG DNA after a 48-h exposure (see arrow in Fig. 1). This fragment was purified and cloned in phages M13mp18 and M13mp19. The nucleotide sequences of this fragment and its adjacent regions were determined and shown to be similar to insertion sequences of the IS3 family. The complete nucleotide sequence of this insertion sequence-like element IS6110 has been described previously (21).

Specificity of IS6110. Total DNA from mycobacteria of the M. tuberculosis complex and of closely related mycobacteria was digested with the restriction enzyme BamHI and analyzed in Southern blots by using the HindIII-BamHI 902-bp fragment of PAMT0 to be a probe (see Materials and Methods). Results are shown in Fig. 2. Significant hybridization was observed only in mycobacteria belonging to the M. tuberculosis complex, i.e., M. tuberculosis, M. bovis, and M. microti. No hybridization was observed with M. avium, M. paratuberculosis, or M. intracellulare. The weak signal observed with M. scrofulaceum DNA may have corresponded to the presence of sequences sharing similarities with IS6110. However, with IS6110-derived primers, no amplification was detected with M. scrofulaceum DNA (see Fig. 4), which suggests that M. scrofulaceum does not
contain IS6110 sequences. Similar results have already been described for IS900, an insertion sequence-like element from \textit{M. paratuberculosis}. Cross-hybridization was observed between IS900 and chromosomal fragments of several \textit{M. avium} strains. When IS900-derived oligonucleotides were used as primers for PCR, no amplification was obtained with DNA extracted from the \textit{M. avium} strains that cross-hybridized with IS900 DNA (24).

With IS6110, different hybridization patterns were observed for \textit{M. tuberculosis}, \textit{M. bovis}, and \textit{M. microti}, suggesting differences in copy number and genomic location of the element. We estimated that \textit{M. tuberculosis} and \textit{M. microti} harbor 10 to 20 copies of IS6110, while \textit{M. bovis} and \textit{M. bovis} BCG (Pasteur strain) appear to contain single copies. Southern blot experiments with \textit{M. bovis} DNA cleaved with different restriction enzymes (\textit{PstI}, \textit{EcoRI}, \textit{BamHI}, or \textit{SalI}) are in agreement with the presence of a single IS6110 sequence in this species (Fig. 3). Preliminary experiments with pulsed-field electrophoresis followed by Southern blotting showed that IS6110 sequences are scattered throughout the \textit{M. tuberculosis} chromosome (data not shown). A search for the presence of IS6110 in \textit{M. tuberculosis} strains isolated from various patients revealed a different arrangement of these elements in the genomes of these strains (6). These findings suggest that IS6110 could be used for epidemiologic studies of \textit{M. tuberculosis}. In addition, an analysis of restriction fragment length polymorphism with IS6110 used as a probe could differentiate \textit{M. bovis} from other members of the \textit{M. tuberculosis} complex.

**Specificity of IS6110-derived primers and probes in PCR experiments.** In order to detect mycobacteria of the \textit{M. tuberculosis} complex directly in uncultured specimens, oligonucleotides derived from IS6110 were used as primers and probes for in vitro amplification. Two 20-mer primers, ISTB2 and ISTB7, were selected for amplification experiments. A 30-mer designated IS-2 was chosen as a probe for the detection of the 325-bp fragment amplified with ISTB2 and ISTB7.

The amplification specificity was tested on 30 mycobacterial strains, including 19 reference mycobacterial species and 11 \textit{M. tuberculosis} strains isolated from patients. One nanogram of purified total DNA was amplified simultaneously with primers ISTB2 and ISTB7, corresponding to IS6110, and TB1 and TB2, corresponding to the 65-kDa antigen (11). TB1 and TB2 amplified a 383-bp fragment in all mycobacterial species tested. Amplification of both 325- and 383-bp fragments was observed for all species belonging to the \textit{M. tuberculosis} complex, i.e., \textit{M. tuberculosis}, \textit{M. africanum}, \textit{M. bovis}, \textit{M. bovis} BCG, and \textit{M. microti} reference strains (Fig. 4), and for the 11 clinical \textit{M. tuberculosis} strains (data not shown). In contrast, DNAs from all other mycobacterial species were amplified with TB1 and TB2 but not with ISTB2 and ISTB7 (Fig. 4). These results were confirmed by hybridization of the 325-bp amplified fragment with the IS-2 probe.
FIG. 4. Specificity of DNA amplification. Mycobacterial DNA (1 ng) was amplified simultaneously with ISTB2 and ISTB7 (325 bp) and TB1 and TB2 (383 bp) primers. (A) Ethidium bromide-stained agarose gel containing a 1/10th volume of amplification reactions; (B) DNAs of panel A transferred to a nylon membrane and hybridized with 32P-labeled IS-2 probe. Lanes 1, *M. avium* ATCC 25291; lanes 2, *M. avium* ATCC 25276 (American Type Culture Collection); lanes 3, *M. chelonae* ATCC 19977; lanes 4, *M. flavescens* ATCC 14474; lanes 5, *M. gordoniae* ATCC 12478; lanes 6, *M. kansasii* 890493 (clinical isolates, references of the Centre National de Reference des Mycobacteries, Paris, France); lanes 7, *M. malmoense* ATCC 29571; lanes 8, *M. marinum* ATCC 927; lanes 9, *M. tuberculosis* ATCC 27294 H37Rv; lanes 10, control without DNA; lanes 11, *M. paratuberculosis* 1120066; lanes 12, *M. scrofulaceum* 0220031; lanes 13, *M. simiae* ATCC 25275; lanes 14, *M. szulgai* NCTC 10831 (National Collection of Type Cultures); lanes 15, *M. terrae* ATCC 15755; lanes 16, *M. xenopi* NCTC 10042; lanes 17, *M. bovis* ATCC 19210; lanes 18, *M. bovis* BCG (Institut Pasteur); lanes 19, *M. africanum* ATCC 25420; lanes 20, *M. microti* NCTC 8710; lanes 21, *ΦX174* DNA hydrolyzed by *Hae*III.

probe. The amplification specificity was further verified by using DNA from other bacterial species, i.e., *Staphylococcus aureus*, *E. coli*, *Micromonospora chalcea*, *Nocardia asteroides*, and several streptomycetes (*S. lividans*, *S. hygroscopicus*, *S. viridochromogenes*, *S. antibioticus*, and *S. fradiae*). None of these species was amplified with the ISTB2 and ISTB7 set of primers (data not shown).

In order to test the sensitivity of in vitro amplification of mycobacterial DNA with ISTB2 and ISTB7 and TB1 and TB2 primers, different concentrations of *M. tuberculosis* DNA (10 ng to 1 fg) were amplified. The results presented in Fig. 5 show that the ISTB2 and ISTB7 primers enable detection of less than 10 fg of DNA. This DNA amount corresponds to about three genome equivalents (one genome being approximately 2.5 x 106 Da mol-1). For the lowest DNA amounts, the signals obtained with ISTB2 and ISTB7 were stronger than those obtained with TB1 and TB2. In fact, amplification with ISTB2 and ISTB7 primers allowed detection of DNA from mycobacteria at a 10-fold-lower level than amplification with TB1 and TB2 did. This result is in agreement with the presence of 10 to 20 copies of IS6110 in the *M. tuberculosis* genome. The gene coding for the 65-kDa protein was believed to be present as a single copy, while more recent studies with *E. coli* strains have suggested the presence of another gene similar to *groEL* encoding the 65-kDa heat shock protein (11). An IS6110-derived PCR appears to be more specific, since both amplification and hybridization are specific for bacilli of the *M. tuberculosis* complex, whereas the 65-kDa-based PCR uses nonspecific

FIG. 5. Sensitivity of *M. tuberculosis* detection by PCR. Dilutions of *M. tuberculosis* DNA were coamplified with ISTB2 and ISTB7 and TB1 and TB2. (A) A 1.5% Agarose gel stained with ethidium bromide; (B) hybridization with 32P-labeled IS-2 probe. Lanes 1 to 8, 10 ng, 1 ng, 100 pg, 10 pg, 1 pg, 100 fg, 10 fg, and 1 fg of *M. tuberculosis* DNA, respectively; lane 9, control without mycobacterial DNA; lane 10, *ΦX174* DNA hydrolyzed with *Hae*III.
primers for amplification followed by hybridization with specific probes. Another PCR based on IS6110 has also been described by Eisenach and colleagues (5, 21), who obtained comparable results with different primers.

Detection of *M. tuberculosis* strains in uncultured clinical specimens. In vitro amplification with IS6110 and the 65-kDa antigen-derived oligonucleotides used as primers and probes was applied to the detection of *M. tuberculosis* in 75 clinical specimens, including 42 gastric aspirates, 17 sputum, 6 cerebrospinal fluid, 2 serum, 3 cystotomy, 4 pleural liquid, and 1 urine specimen. These specimens were tested in parallel by classical bacteriological methods, direct microscopic examination after Ziehl-Neelsen staining, and culture on Loewenstein-Jensen medium. Total DNA from 0.2 to 1 ml of each sample was extracted and resuspended in 100 µl of water. Aliquots (10 µl) were amplified with the ISTB2 and ISTB7 primers, and the amplification products were analyzed by Southern blot hybridization by using IS-2 as a probe. The results obtained with several samples are shown in Fig. 6. Conclusions obtained by PCR analyses were based on results obtained from two or three independent experiments.

Results obtained by PCRs were consistent with bacteriological data and clinical criteria. All samples positive by direct examination and/or culture gave a positive result by DNA amplification (Table 1). Among 45 samples found negative by bacteriological analysis, 5 were positive by PCRs. These samples were not likely to represent false-positive results, since they were from patients in whom tuberculosis was highly suspected or already diagnosed. Three independent cystotomy specimens were from a patient for whom renal tuberculosis was diagnosed on the basis of positive culture results obtained with other specimens (ureteral samples). Two pleural liquid specimens came from a patient for whom tuberculosis was highly suspected and whose general state greatly improved under anti-tuberculous treatment. The presence of *M. tuberculosis* bacilli was also detected in two serum specimens, which were not tested by culture, from patients with tuberculosis meningitis and for whom cerebrospinal fluids were found to be positive both by culture and by PCRs. On the other hand, analysis of samples known to contain *M. avium* bacilli by bacteriological analysis were found to be negative by amplification with ISTB2 and ISTB7 primers derived from the IS6110 sequence.

Two samples found to be positive by direct examination were initially found to be negative by PCR methods. To test the efficiency of DNA amplification in these samples, 10 ng of pUC19 (26) corresponding to plasmid DNA and primers allowing the amplification of a 517-bp internal fragment of this plasmid (a part of the *bla* gene, which is responsible for ampicillin resistance) was added to the reaction. No amplification of the 517-bp fragment was observed when 10 µl of the sample mixture was added in the amplification reaction. However, amplification for both pUC19 and IS6110 sequences was obtained when 10-fold dilution of the sample preparation was added (Fig. 7). This result demonstrates that some samples may occasionally contain inhibitors of the amplification reaction. Consequently, all specimens should be systematically tested for their amplification capability.

The results presented here indicate that greatly improved tuberculosis diagnosis is possible by the PCR technique, which is more rapid and more sensitive than classical bacteriological techniques. The PCR might be especially useful in patients for whom no presumptive diagnosis is available, such as for disseminated forms of tuberculosis that are frequently encountered in patients with acquired immune deficiency syndrome (23, 25).

Technical improvements of the method, including the use of nonradioactive probes and hybridization format, are being investigated to simplify the technique for routine use in clinical laboratories.

![FIG. 6. Detection of *M. tuberculosis* in clinical specimens after amplification with ISTB2 and ISTB7 primers. (A) A 1.5% agarose gel containing 1/10th of the amplification reactions; (B) hybridization with 32P-labeled IS-2 probe. Lane 1, Control without mycobacterial DNA; lane 2, sputum which is smear negative and culture positive; lanes 3 and 4, gastric aspirates which are smear and culture positive; lane 5, sputum which is smear and culture negative; lane 6, cerebrospinal fluid which is smear negative and culture positive; lane 7, gastric aspirate which is smear negative and culture positive; lane 8, *M. tuberculosis* DNA-positive control; lane 9, ΦX174 hydrolyzed with *HaeIII*.

TABLE 1. Comparison of DNA amplification results with the data obtained by standard procedures

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* Direct exam, Microscopic examination after Ziehl-Neelsen staining; culture, culture on Loewenstein-Jensen solid medium.
FIG. 7. Presence of inhibitors of the amplification reaction in clinical specimens. Different volumes of sample DNA preparations were amplified either with TEMA and TEMB primers in the presence of pUC19 DNA (A) or with ISTR2 and ISTR7 primers (B). Lane 1, Control without mycobacterial DNA; lanes 2 to 4, 10, 1, and 0.1 μl of the 100-μl DNA preparation from sample 1, respectively; lanes 5 to 7, 10, 1, and 0.1 μl of the 10-μl DNA preparation from sample 2, respectively; lane 8, positive controls with pUC19 DNA (A) or M. tuberculosis DNA (B).

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LITERATURE CITED