JOURNAL OF CLINICAL MICROBIOLOGY, Sept. 1995, p. 2290–2293 Vol. 33, No. 9
0095-1137/95/$04.00+0

Demonstration of Homology between IS*6110* of *Mycobacterium tuberculosis* and DNAs of Other *Mycobacterium* spp.

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Received 27 February 1995/Returned for modification 7 April 1995/Accepted 17 May 1995

The insertion sequence IS*6110* has an important role in diagnostic PCR and typing of *Mycobacterium tuberculosis*. We have evaluated a one-tube nested PCR which detects IS*6110*. Positive results were obtained with DNAs from four of four *M. tuberculosis* isolates, seven of eight *M. fortuitum* isolates, four of seven *M. avium-M. intracellulare* complex isolates, four of five *M. kansasii* isolates, five of five *M. xenopi* isolates, two of four *M. malmoense* isolates, and two of two *M. chelonei* isolates. These results were confirmed by hybridization of genomic DNA from bp 505 to 685 of the IS*6110* from *M. tuberculosis* H37Rv. Dot blot hybridization of genomic DNAs from these isolates with the same probe confirmed the presence of a homologous sequence in these mycobacterial species. These data suggest that false-positive results may be obtained for clinical samples when some methods based on IS*6110* are used.

Molecular approaches to diagnosis are transforming the investigation of tuberculosis. Rapid diagnosis by PCR with a number of different targets (12), including the IS*6110* insertion sequence, has been described (1–3, 5, 6, 13–15, 17, 19, 21, 25).

The insertion sequence IS*6110*, also known as IS*986* and IS*987* (13, 28), is thought to be found only in members of the *Mycobacterium tuberculosis* complex. *M. tuberculosis* strains typically carry multiple copies of the element, although a minority of strains with only a single copy have been identified (8). In contrast, *M. bovis* isolates usually have a low copy number, and *M. bovis* BCG strains have either one or two copies (7). The presence of multiple copies in the majority of *M. tuberculosis* strains enhances the sensitivity of PCR tests, and several different protocols based on different portions of IS*6110* have been devised. Further development and simplification of the technique with a one-tube nested PCR protocol and adaptation for colorimetric detection have improved its suitability for the routine diagnosis of clinical specimens, including sputum (26, 27). However, the discovery that occasional strains of *M. tuberculosis* lack IS*6110* implies that caution has to be exercised in relying too heavily on IS*6110*-based PCR for the detection of *M. tuberculosis* (23).

IS*6110* is also used as the hybridization target in a restriction fragment length polymorphism method for typing *M. tuberculosis*. A standard methodology has now been agreed on internationally to enable results from different laboratories to be compared (22). This method has been applied to identifying cross-infection within the hospital environment (16) and cross-contamination in the laboratory (20). It has also been used to demonstrate routes of transmission to and from closed communities of intravenous-drug abusers and homeless men (4, 10) and to define epidemiological relationships between strains in a wider context (11).

In view of the serious clinical consequences of false-positive reactions in sputum containing this organism when IS*6110* PCR is used, we examined clinical isolates of mycobacteria other than *M. tuberculosis* for positive reactions.

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MATERIALS AND METHODS

Bacterial isolates. The mycobacteria studied were isolated from routine samples submitted to the clinical laboratory of the Department of Medical Microbiology, Royal Free Hospital, London, United Kingdom, for mycobacterial culture. In addition, *M. tuberculosis* H37Rv NCTC 7416 was studied. The species of all isolates were confirmed by the PHL Regional Tuberculosis Reference Laboratory, Dulwich, United Kingdom.

Genomic-DNA extraction. Genomic DNA was isolated by using a standard method (18). Mycobacterial isolates were cultivated in Middlebrook 7H9 broth, and the bacterial cells were harvested by centrifugation (3,750 × *g*, 4°C). The organisms were treated at 80°C for 20 min before incubation at 37°C for 45 min in a prelysis solution (0.05 M Tris, 30 mg of lipase [in 0.005 M $CaCl_2$] per ml, 0.03% [wt/vol] sodium taurocholate, 0.005 M $CaCl_2$, 20% [wt/vol] sucrose, 5 mg of lysozyme per ml). After centrifugation (3,750 × *g*, 4°C) in suspension buffer (0.1 M NaCl, 0.01 M Tris, 0.01 M EDTA, 20% [wt/vol] sucrose), the deposit was resuspended in lysis buffer (suspension buffer with 1% sodium dodecyl sulfate [SDS]) and incubated with 0.1 mg of proteinase K per ml at 37°C for 30 min. Polysaccharides and residual proteins were removed with a 10% cetyldimethylethyl-ammonium bromide–0.7 M NaCl solution. Genomic DNA was extracted by phenol-chloroform extraction and ethanol precipitation by standard techniques. The DNA was resuspended in Tris-EDTA buffer and treated with RNase A (0.1 mg/ml) by incubating at 37°C for 1 h. DNA was further purified with another phenol-chloroform extraction and precipitation step.

The concentration and purity of the extracted DNA were calculated from readings of A_{260} and A_{280} on a Unicam 8625 UV/VIS spectrophotometer. All reagents were supplied by Sigma (Poole, Dorset, United Kingdom).

PCR. The PCR target was the IS*6110* insertion sequence-like element currently used to identify members of the *M. tuberculosis* complex. A nested PCR protocol was used as described previously (26). DNA samples were adjusted to 100 ng for each reaction with *M. tuberculosis* H37Rv DNA as a positive control; unrelated bacterial DNA and reagent blanks served as negative controls. Reaction mixtures were prepared by using a master mix and a conventional three-room system to avoid contamination. Outer primers Tb294 (5′-GGACAACGC CGAATTGCGAAGGGC-3′) and Tb850 (5′-TAGGCGTCGGTGACAAAGG CCACG-3′) were used at concentrations of 10 nM; inner primers Tb505 (5′-ACGACCACATCAACC-3′) and Tb670 (5′-AGTTTGGTCATCAGCC-3′) were used at concentrations of 500 nM (26, 27). The outer primers Tb294 and Tb850 and the inner primers Tb505 (5′ digoxigenin labelled) and "Tb670" (5′ biotin labelled) were synthesized by R & D Systems Europe Ltd. (Abingdon, Oxon, United Kingdom).

A hot-start PCR protocol was used to reduce primer-dimer formation; primers were separated from the reaction components with paraffin wax (pastillated; congealing point, 60°C; BDH, Merck Ltd., Poole, Dorset, United Kingdom) and entered the reaction when the wax plug melted at 60°C. The first 30 cycles, with an annealing temperature 65°C, produced a 580-bp product from the outer primers. A second round of 20 cycles, with a 50°C annealing temperature, produced a 181-bp fragment from the inner primers. Denaturation (93°C) and extension (72°C) temperatures were maintained throughout the two-stage program, which was performed on a Hybaid Combi TR2 thermal reactor.

Colorimetric detection. Colorimetric detection of the PCR product was also as described previously (26). Briefly, the target PCR product incorporating the inner primers labelled with biotin and digoxigenin was detected on avidin-treated

microtiter plates (10 μg/ml in 50 mM carbonate buffer [pH 9.6]; plates were coated for 16 h at 4°C) (Sigma). The wells were washed twice with Tris-buffered saline (pH 7.5)–0.5% (vol/vol) Tween 20 (TBS-Tween). The PCR product was incubated for 30 min at room temperature, and the wells were washed again with TBS-Tween. Anti-digoxigenin–alkaline phosphatase conjugate (1:5,000 dilution; Boehringer Mannheim GmbH, Mannheim, Germany) was added to each well and incubated for 30 min at room temperature. Unbound conjugate was removed with three washes with TBS-Tween and one wash with TBS. *para*-Nitrophenol (Sigma) in 50 mM carbonate buffer (pH 9.6)–1 mM $MgCl_2$ was used as the substrate, which was incubated at room temperature for 30 min. The optical density at 405 nm was then read (Titretek; Flow England, Thame, Oxon, United Kingdom). Cutoff limits were three standard deviations above the mean of negative results, but in practice, the tests gave clear positive and negative values.

Southern blot hybridization. PCR products were electrophoretically separated on 1.8% agarose gels containing ethidium bromide (500 ng/ml). The DNA was denatured and transferred to Hybond-N nylon membranes (Amersham) by capillary action and cross-linked with UV light for 3 min (UV Transilluminator; UVP Inc.). Genomic DNAs (10 μg) from different mycobacterial isolates were also denatured and spotted onto Hybond-N nylon membranes to produce dot blots.

DNA from *M. tuberculosis* H37Rv was amplified to produce a 181-bp product of the inner primers, which was separated on low-melting-point agarose, cut out, and purified with the Wizard DNA purification system (Promega, Southampton, England) according to the manufacturer's instructions. The fragment was labelled with fluorescein II-dUTP by the ECL random prime labelling and detection system, version II (Amersham International, Little Chalfont, England), according to the manufacturer's instructions. Filters were incubated (16 h at 60°C) with labelled probe DNA (10 ng/ml) and washed (0.5× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% [wt/vol] SDS) under the highest-stringency conditions advised by the manufacturer. Hybridization was detected by incubating filters with anti-fluorescein–horseradish peroxidase conjugate with subsequent enzymatic reduction of peroxide and oxidation of luminol in the presence of enhancer molecules, according to the manufacturer's instructions (Amersham). Light emitted through the breakdown of luminol was detected on blue-light-sensitive film (Amersham). Previous hybridization studies (results not shown) with a digoxigenin-dUTP-labelled probe detected PCR products at the high-stringency temperature of 75°C and washing conditions of 0.1× SSC–0.1% (wt/vol) SDS, using a nonradioactive DIG DNA Labelling and Detection Kit (Boehringer Mannheim Biochemica, Lewes, England).

RESULTS

The nested PCR method was applied to extracted DNAs from clinical isolates of different mycobacterial species: four *M. tuberculosis*, eight *M. fortuitum*, seven *M. avium-M. intracellulare* complex, five *M. kansasii*, five *M. xenopi*, four *M. malmoense*, and two *M. chelonei* isolates. The strains studied represent the most common non-*M. tuberculosis* isolates encountered in our laboratory. The PCR products were evaluated by colorimetric detection and by electrophoresis in agarose. The specificity of the PCR product was confirmed by Southern hybridization in each instance. False-positive results were common with the colorimetric detection system, and the majority of non-*M. tuberculosis* isolates were considered positive by three techniques (Table 1) in our laboratory. Agarose gel electrophoresis enables additional discrimination on the basis of product size; Fig. 1 shows examples of PCR products from representative isolates considered to show a positive reaction, in which there is a band corresponding in size to the product expected from the inner pair of primers. The specificity of this product is confirmed by Southern hybridization with the IS*6110*-derived probe.

TABLE 1. Comparison of techniques used to evaluate IS*6110* one-tube nested PCR

Species	Total no. of strains	No. of strains positive by:		
		ELISA[a]	Gel electrophoresis	Hybridization
M. tuberculosis	4	4	4	4
M. avium-M. intracellulare	7	6	7	7
M. fortuitum	8	7	7	7
M. kansasii	5	4	4	4
M. xenopi	5	4	4	4
M. malmoense	4	2	2	2
M. cheloniae	2	1	1	1

[a] ELISA, enzyme-linked immunosorbent assay.

These results indicate that the nested PCR is capable of amplifying DNA from non-*M. tuberculosis* mycobacteria. In order to establish whether both pairs of primers are involved, a selection of strains were reexamined by using only the inner pair of primers under the same conditions. The results shown in Fig. 2 show that these primers alone are capable of producing the amplified product in a series of *M. avium-M. intracellulare* complex isolates. Figure 2 demonstrates PCR products of various sizes and additional bands in two isolates, the specificity of which was confirmed by Southern hybridization (data not shown).

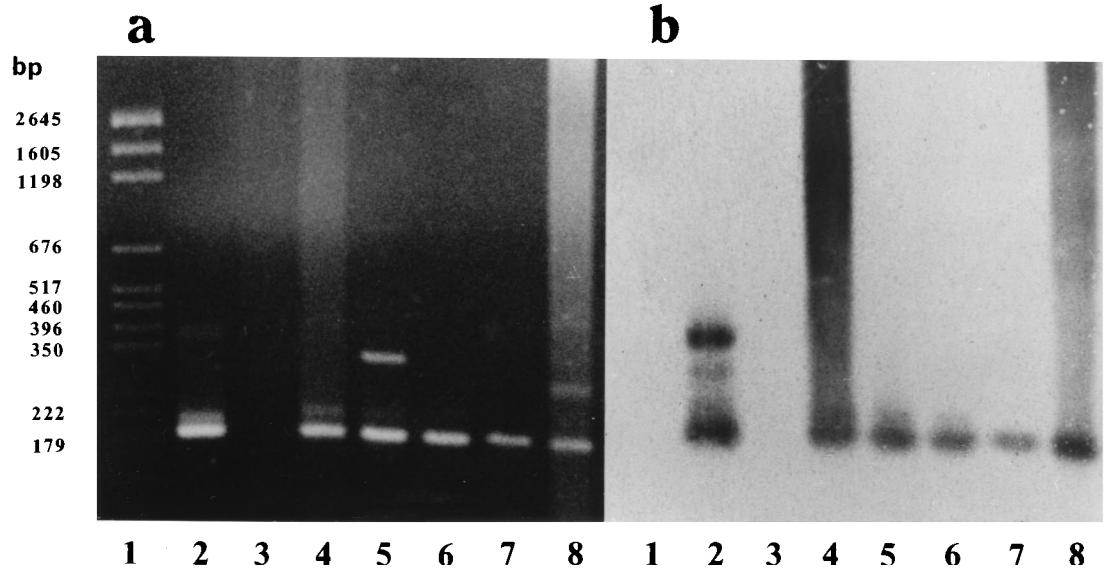

FIG. 1. Products obtained from various mycobacterial species by using IS*6110* one-tube nested PCR (a) and Southern blot hybridization of PCR products with an IS*6110*-derived probe (b). Lanes 1, pGem (Promega) molecular size marker; lanes 2, *M. tuberculosis* H37Rv DNA; lanes 3, *Toxoplasma gondii* DNA; lanes 4, *M. fortuitum* DNA; lanes 5, *M. avium-M. intracellulare* complex DNA; lanes 6, *M. kansasii* DNA; lanes 7, *M. malmoense* DNA.

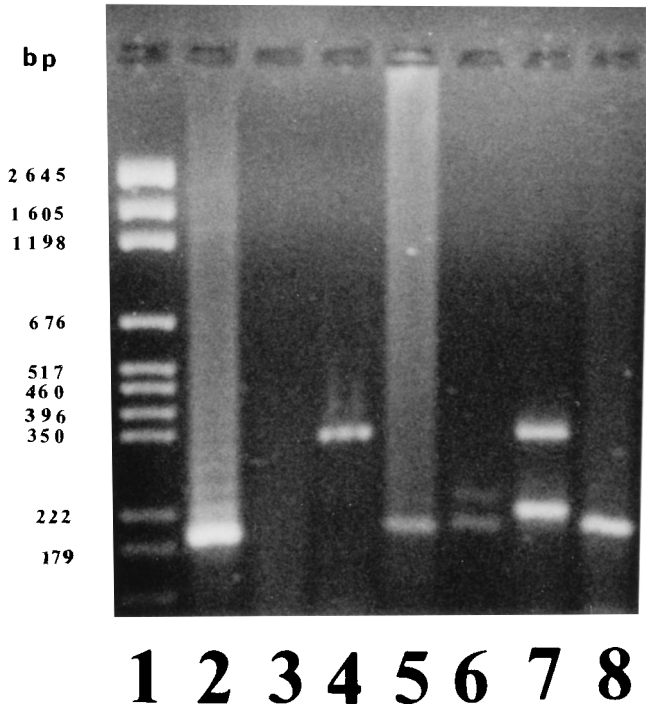

FIG. 2. PCR products derived from IS*6110* inner primers only (bp 505 to 685). Lane 1, pGem (Promega) molecular size marker; lane 2, *M. tuberculosis* H37Rv DNA; lane 3, *T. gondii* DNA; lanes 4 to 8, *M. avium-M. intracellulare* complex DNA.

Despite precautions to avoid contamination, it is conceivable that the PCR amplification arises from contamination with *M. tuberculosis* DNA or IS*6110*-derived PCR products. Genomic DNAs from selected isolates were therefore tested for hybridization to a probe derived from the central region of IS*6110*, which was amplified with the inner primers (Tb505 and Tb670). The results shown in Fig. 3 confirm that these strains carry a sequence homologous to this portion of IS*6110*.

DISCUSSION

Molecular methods have the most to offer in the detection of organisms which grow slowly or cannot be cultivated in vitro.

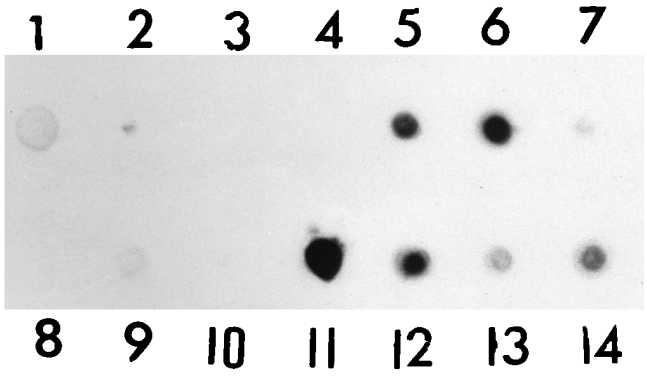

FIG. 3. Dot blot hybridization of genomic DNAs from representative isolates of *Mycobacterium* spp. with an IS*6110*-derived probe. Well 1, *M. tuberculosis* H37Rv DNA; well 2, *M. tuberculosis* clinical isolate; well 3, *T. gondii* DNA; well 4, Tris-EDTA buffer; wells 5 and 6, *M. fortuitum* DNA; well 7, *M. avium-M. intracellulare* complex DNA; wells 8 and 9, *M. kansasii* DNA; well 10, *M. xenopi* DNA; wells 11 and 12, *M. malmoense* DNA; wells 13 and 14, *M. chelonei* DNA.

They are particularly valuable when sensitivity testing is not required in the initial management, when organisms have predictable susceptibility patterns, or when initial therapy is by an agreed-upon protocol. The detection of *M. tuberculosis* fulfills all of these criteria.

We have shown that the majority of non-*M. tuberculosis* isolates tested possess a sequence which may be amplified by a one-tube nested PCR (25, 26). This gives rise to multiple discrete bands which vary between species but include a constant band corresponding to the 181-bp product of the inner primers. This pattern differs from that obtained with IS*6110* amplified from *M. tuberculosis* in such a way that a skilled observer would be able to distinguish them; however, had a colorimetric system been used alone, these false-positive results would not have been recognized. IS*6110* is a target for primer pairs designed by other authors (Fig. 4). False-positive results may also be obtained by techniques which use this central region. In one study, which utilized a PCR which generated a 317-bp fragment between bp 638 and 954, false-positive results were found with specimens from which *M. avium-M. intracellulare* complex, and *M. xenopi* were subsequently isolated. The majority of specimens from which non-*M. tuberculosis* mycobacteria were isolated were negative, however (2). PCRs based on other primers have not had false-positive results when tested against other species of mycobacteria (5, 6, 14). This may indicate that the homologous sequence is confined to the central region of IS*6110*.

The central region amplified by our primers hybridized with genomic DNAs from the majority of non-*M. tuberculosis* species, confirming the presence of a homologous sequence and excluding the possibility that our results have arisen through PCR contamination. This was further confirmed by the results of the PCR experiment utilizing only the internal primers. The product of this reaction was usually a 181-bp fragment. For one isolate of *M. avium-M. intracellulare* complex two bands are formed, and for another the size of the product differs from that obtained with *M. tuberculosis*. This raises the question of the nature of this sequence. IS*6110*/IS*986* is a member of the widespread family of insertion sequences related to IS*3* (27); other elements of this family have been identified in mycobacteria, including IS*1141* from *M. intracellulare* (24) and IS*1137* from *M. smegmatis* (9). In a search of GenBank with the 181-bp sequence, we found 63.3% homology in a 177-bp overlap with the insertion sequence IS*629* reported for *Shigella sonnei* and 63.8% homology in a 177-bp overlap with the *Escherichia coli* insertion sequence IS*3411*. These data suggest that this central region is a sequence common to many repetitive elements and that we may have identified such elements in other species. The nature of these products identified here will be characterized in later studies.

Since PCR for tuberculosis must be applied to respiratory specimens, which are naturally contaminated with bacteria of many different species, the specificities of PCR protocols must be confirmed with not only mycobacteria but also other respiratory commensals and pathogens. This may call into question the strategy of using repetitive elements as a PCR target, since they are likely to contain conserved sequences irrespective of their host bacterium. Other published protocols which use primers from the homologous region reported here require further study with non-*M. tuberculosis* mycobacteria, since these organisms are regular but uncommon contaminants of sputum samples and therefore may not register as common sources of false-positive reactions. This emphasizes the importance of checking for false-positive results from multiple isolates of a wide range of related species when developing a PCR for clinical use.

Typing of *M. tuberculosis* by IS*6110* restriction fragment

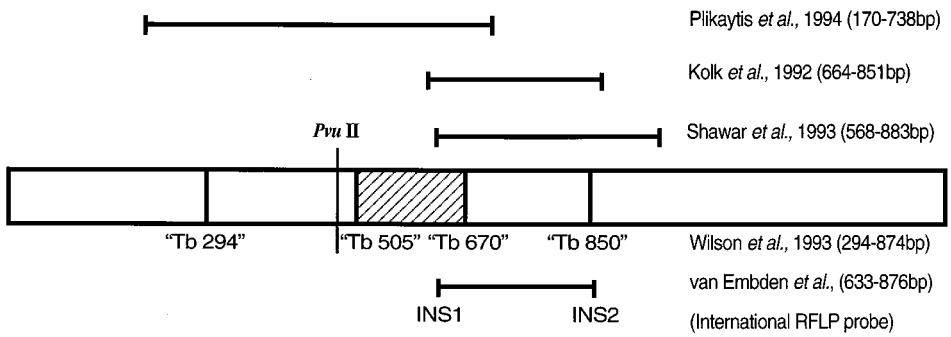

FIG. 4. Locations of sequences of IS*6110* amplified by published PCR protocols and the International restriction fragment length polymorphism (RFLP) probe.

length polymorphism is a powerful tool to unravel the epidemiology of this infection. Isolates which do not have a copy of IS*6110* have now been reported (23). The central sequence found to be homologous with the majority of non-*M. tuberculosis* mycobacteria studied overlaps the internationally agreed probe for the standard restriction fragment length polymorphism method (Fig. 4). This, in itself, should not pose a problem for strains which have been clearly identified as *M. tuberculosis* but does indicate that isolates must be fully identified before being submitted for restriction fragment length polymorphism analysis.

ACKNOWLEDGMENTS

We are grateful to M. Yates of the PHL Regional Tuberculosis Reference Laboratory, Dulwich Hospital, for identifying the isolates.

This work was supported by the Royal Free Hospital Special Trustee Funds held by S. H. Gillespie.

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