Absence of the genetic marker IS*6110* from a strain of *Mycobacterium tuberculosis* isolated in Ontario

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ST Howard, MT Oughton, A Haddad, WM Johnson. Absence of the genetic marker IS*6110* from a strain of *Mycobacterium tuberculosis* isolated in Ontario. Can J Infect Dis 1998;9(1):48-53. A 35-year-old female patient from Waterloo, Ontario was diagnosed with pulmonary tuberculosis in June 1995. Records indicated that the patient had emigrated from Laos circa 1990. A culture grown from a bronchoalveolar lavage specimen was identified as *Mycobacterium tuberculosis* by standard biochemical methods. Drug-susceptibility testing indicated the strain was resistant to pyrazinamide (PZA), and a mutation was detected within *pncA*, a gene associated with PZA resistance. Sequence data from the 16S rRNA gene and the 16S/23S rRNA gene spacer confirmed that the strain was a member of the *M tuberculosis* complex, and analysis of the *mpcA* and *pncA* genes supported the identification of the strain as *M tuberculosis* rather than *Mycobacterium bovis*. However, the insertion element IS*6110*, which is used for epidemiological tracing of *M tuberculosis*, was not detected in this strain by either restriction fragment length polymorphism analysis or by polymerase chain reaction. Two other genetic markers associated with the *M tuberculosis* complex, IS*1081* and the direct repeat element, were present. The arrival of immigrants with tuberculosis from southeast Asia, where most strains of *M tuberculosis* lacking IS*6110* have been traced, has important implications for epidemiological studies of tuberculosis in North America.

Key Words: *Epidemiology, IS*6110, Mycobacterium tuberculosis*, Pyrazinamide resistance*

Absence du marqueur génétique IS*6110* dans une souche de *Mycobacterium tuberculosis* isolée en Ontario

RÉSUMÉ : Une patiente de 35 ans, de Waterloo, en Ontario, a reçu un diagnostic de tuberculose pulmonaire en juin 1995. Les dossiers indiquaient que la patiente avait émigré du Laos en 1990. La mise en culture d'un spécimen prélevé par lavage bronchique a permis d'identifier *Mycobacterium tuberculosis* au moyen de méthodes biochimiques standard. Les antibiogrammes ont pour leur part indiqué que la souche était résistante au pyrazinamide (PZA) et une mutation a été décelée dans le *pncA*, gène associé à la résistance au pyrazinamide. Les données de séquençage provenant de l'ARNr du gène 16S et l'espaceur de l'ARNr des gènes 16S/23S ont appuyé l'identification de la souche *M tuberculosis* plutôt que de *Mycobacterium bovis*. Toutefois, l'élément d'insertion IS*6110*, utilisé pour le dépistage épidémiologique de *M tuberculosis*, n'a pas été décelé dans cette souche soit par l'analyse du polymorphisme de la longueur du fragment de restriction ou par amplification génique. Deux autres marqueurs génétiques associés au complexe *M turberculosis*, IS*1081* et la séquence répétée directe, étaient présents. L'arrivée d'immigrants tuberculeux en provenance de l'Asie du Sud-Est, où la plupart des souches de *M tuberculosis* dépourvues d'IS*1081* ont été identifiées, a d'importantes répercussions sur les résultats d'études épidémiologiques sur la tuberculose en Amérique du Nord.

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IS*6110* is a mobile genetic element found in the genome of members of the *Mycobacterium tuberculosis* complex (1,2). In restriction fragment length polymorphism (RFLP) analysis, also known as DNA fingerprinting, it has been a valuable marker for studying the spread of *M tuberculosis* strains in communities and institutional settings (3,4), and on a broader geographic basis (5,6). Although other genetic markers have been used (7,8), RFLP analysis based on IS*6110* is the most widely used approach because the element varies sufficiently in copy number and chromosomal location to permit strains to be easily differentiated. In addition, a standardized method exists for IS*6110* analysis that allows more accurate comparison of RFLP patterns within and between laboratories (9). Amplification of IS*6110* from clinical samples by the polymerase chain reaction (PCR) has also been investigated as a method for diagnosing tuberculosis (10,11).

While the majority of strains have multiple copies of IS*6110*, isolates of *M tuberculosis* have been identified that lack this element (8,12,13). These strains were obtained from patients who were either living in India or Vietnam or who had emigrated from there. An awareness of the presence of these strains is important for investigators involved in epidemiological studies on tuberculosis as well as for those using amplification-based diagnostic methods that target IS*6110*. During a study involving several isolates of *M tuberculosis* from Ontario, we identified a strain that was not detectable by IS*6110*-based RFLP analysis and proceeded to characterize it further.

METHODS

Specimen source and bacteriological analysis: In June 1995, a bronchoalveolar lavage (BAL) specimen was submitted to the Laboratory Services Branch at the Ontario Ministry of Health Central Laboratory. The specimen was taken from a 35-year-old female patient who was diagnosed with pulmonary tuberculosis. Patient records indicated that she was of Chinese ancestry and had spent most of her life in Laos before emigrating to Canada. The exact date of her entry into Canada was not given but was indicated to be 'around 1990'. Acid-fast bacilli were detected in the BAL specimen, and the organism was cultured on Löwenstein-Jensen medium and using the Bactec 460 system (Becton and Dickinson, Maryland). It was identified as *M tuberculosis* complex by Gen-Probe (Gen-Probe Incorp, California) and was designated strain S384. The strain was determined to be *M tuberculosis* by standard biochemical tests including a positive niacin test, resistance to thiophene-2-carboxylic acid hydrazide (TCH) at 10 g/mL, and growth on glycerol-containing medium. Drug susceptibility testing using the Bactec 460 system method showed that S384 was sensitive to isoniazid, rifampin, streptomycin and ethambutol but resistant to pyrazinamide (PZA) at 100 g/mL. S384 was submitted to the Laboratory Centre for Disease Control for RFLP analysis.

Extraction of DNA: To prepare DNA for genetic analysis, strains of *M tuberculosis* were cultured on Löwenstein-Jensen medium and bacterial cells were harvested after two weeks' growth. Two loopful of bacteria were resuspended in 1 mL 7H9 media (Difco, Michigan) in 1.5 mL microcentrifuge tubes, heat-killed by incubating at 80°C for 20 mins, and then pelleted by centrifugation at 10,000 *g* for 5 mins. Cells were resuspended in 1 mL chloroform and vortexed briefly to remove lipids. After a second centrifugation, cells were resuspended in 0.5 mL Tris-EDTA (TE) (10 mM Tris, pH 8.0, 1 mM EDTA, pH 8.0). For the rest of the extraction procedure, the method of van Soolingen et al (14) was followed with the exception that the length of the cell lysis step with proteinase K and SDS was increased to an overnight incubation at 37°C. Extracted DNA was resuspended in TE buffer and stored at 4°C.

RFLP analysis: The protocol of van Embden et al (9) was followed with some modifications. Briefly, 2 g of genomic DNA were digested with 5 units of the restriction enzyme *Pvu*II (Stratagene, California) for 4 h at 37°C. Digested DNA samples were electrophoresed on a 0.8% agarose gel in TAE buffer and then transferred onto nylon membrane by Southern blotting using a Stratagene Posiblot pressure blotter. DNA was cross-linked to the membrane by exposure to ultraviolet light in a Stratagene Stratalinker.

To prepare a probe for IS*6110*, a 523 basepair (bp) amplicon mapping to the right of the single *Pvu*II site in IS*6110* was synthesized by PCR using primers IS43 and IS53 (Table 1). PCR products were electrophoresed on an agarose gel, the 523 bp fragment was excised from the gel and extracted from the agarose using a Wizard DNA purification column (Promega, Wisconsin). Next 500 ng of the purified amplicon were labelled with horseradish peroxidase using the Chemiluminescent Direct Labelling Kit (Amersham). After prehybridization, the Southern blot was incubated with labelled probe in a roller bottle apparatus (Bellco, New Jersey); the Amersham protocol was followed for the hybridization and washing steps and for detection on autoradiographic film.

To prepare probe for IS*1081*, a 514 bp amplicon was synthesized using primers 1081C and 1081D (Table 1), and the labelling, hybridization and detection steps were performed as described for the analysis of IS*6110*. For the detection of the direct repeat, labelling of the oligonucleotide DR-r (15) and subsequent hybridization and washing steps were carried out using the Amersham 3 -oligolabelling system according to the manufacturer's instructions.

Autoradiographic imaging: Autoradiographic films from Southern blot analyses were scanned with a Sharp JX-610 high resolution scanner and BioImage DNA Sequence Film reader software (BioImage, Michigan). Images were labelled using Corel Photo-Paint software and were printed using a Kodak Digital Science 8650 PS Printer (Eastman Kodak Co, Connecticut). The images shown were not altered and are accurate representations of the original autoradiographs.

Oligonucleotides: PCR primers and the 36-base oligonucleotide DR-r were synthesized on an Applied Biosystems 392 DNA synthesizer (Perkin-Elmer, Applied Biosystems Division).

PCR: For the multiplex PCR, 500 ng (approximately 75 pmol) of each primer were used in a 100 μL reaction mixture which consisted of 0.2 mM each dNTP, 2.5 units *Taq* DNA polymerase (Boehringer Mannheim), 1× PCR reaction buffer (Boehringer Mannheim) with 2.0 mM magnesium chloride, and 10 ng of genomic DNA as template. Reactions were run in a thermocycler (Gene Amp PCR System 9600, Perkin-Elmer-Cetus) under the

TABLE 1
Olignucleotide primers used in analysis of *Mycobacterium tuberculosis* strain lacking IS*6110*

Primer	Nucleotide sequence (5′ to 3′)	Target of primer pairs	Position of primers*	Reference
IS43	TCA GCC GCG TCC ACG CCG CCA	IS*6110*	568-588	2
IS53	CCG ACC GCT CCG ACC GAC GGT		1090-1070	
1081C	TGG CTG ACC AAC TCG CAC AG	IS*1081*	422-441	16
1081D	GTG TGC ACT CCG ACG ACG CG		935-916	
ISAm	CCT CCA TGG TCC TCG ACG C	IS*6110*	919-937	2
ISB	TGA GCG TAG TAG CGA GCC TC		1291-1272	
TB1m†	CAA CGC GCC GTC GGT GGT TC	mtp40 (*mpcA*)‡	158-177	24
TB3m	TCC ATG GTG AAC GCG CTG C		657-639	
ITSm1	CTT TCT AAG GAG CAC CAC GAA	16S/23S rDNA ITS	1510-1530	19
ITSm2	ATG CTC GCA ACC ACT ATC CA		1746-1727	
ISI	AGG ATG GGG TCA TGT CAG GTG	IS*6110*	44-64	2
ISII	CTG TGT GCA GAT CGA CTC GAC AC		464-442	
PN-1	CGG GCG TTG ATC ATC GTC GAC	*pncA*	3-18	25
PN-2	GGA GCT GCA AAC CAA CTC GAC		558-538	

**Primer positions are based on nucleotide numbering in the given references; †Primer sequence is based on that of primer PT1 (23); ‡Note that the nucleotide sequence given for mtp40 in references 23,24 is in the reverse orientation to the nucleotide sequence of* mpcA *(21)*

following conditions: an initial denaturation at 94°C for 2 mins, followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 65°C for 30 sec, extension at 72°C for 30 s and then a final 10 mins extension at 72°C. Products of the PCR reactions were electrophoresed in TAE buffer on a 1.5% agarose gel stained with ethidium bromide.

For the synthesis of amplicons for sequence analysis or for use as probes, the conditions used for the multiplex PCR were used with the following exceptions: the number of cycles was extended to 40; the annealing temperature was reduced to 60°C and the concentration of magnesium chloride was reduced to 1.5 mM. For the synthesis of the IS*6110* and IS*1081* probes, 100 ng of *Mycobacterium bovis* bacillus Calmete-Guérin (BCG) DNA served as template. For the preparation of the PN-1/PN-2 amplicon for sequence analysis, 10 ng of S384 were used as template, and the reaction was carried out in a PTC-200 DNA Engine thermocycler (MJ Research Inc, Maryland) under the following conditions: an initial denaturation at 93°C for 2 mins, followed by 40 cycles of denaturation at 94°C for 10 s, annealing at 60°C for 20 s, extension at 72°C for 90 s and then a final 10 mins extension at 72°C.

Sequence analysis: Sequence reactions were carried out in a Gene Amp PCR System 9600 thermocycler using an ABI Dye Terminator Cycle Sequencing Kit (Perkin-Elmer) according to the manufacturer's instructions with 150 ng of amplicon as template. Reaction products were run on an ABI 373 or 377 Sequencer (Perkin-Elmer), and data were analyzed on a Power Mac 7200/120 (Apple Computer Inc, California) using GeneWorks (IntelliGenetics Inc, California) and Sequencher 3.0 (Gene Codes Corp, Michigan) sequence analysis software.

RESULTS

Genomic DNA samples from strain S384, two other clinical isolates of *M tuberculosis* and a control strain were examined by RFLP analysis using a probe to the insertion element IS*6110*. An autoradiograph of the results is shown in Figure 1

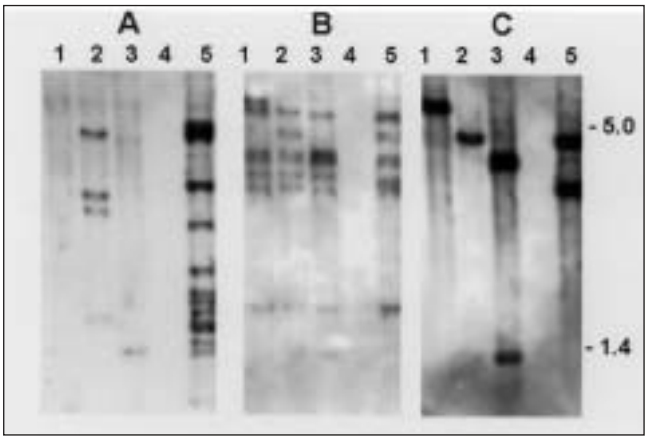

Figure 1) *Restriction fragment length polymorphism analysis of* Mycobacterium tuberculosis *strains. A Southern blot of* PvuII*-digested DNA was probed for IS*6110*, panel A; IS*1081*, panel B; direct repeat, panel C. Lane 1, S384; lanes 2 and 3, clinical isolates; lane 4, blank; lane 5, H37Rv (ATCC 27294). Sizes are indicated in kilobase pairs and were determined from known sizes of IS*6110*-containing fragments from H37Rv. The same area of the blot is shown in each panel*

(panel A). A range of copy numbers of IS6110 was evident with three of the strains of *M tuberculosis* (lanes 2,3,5), including one strain which had only a single copy of the element in a 1.2 kb fragment (lane 3). Except for weak background bands which were present in all sample lanes, no bands were detected for S384 (lane 1), even upon longer exposure (not shown).

Because there may have been insufficient DNA in lane 1 for the detection of specific bands in S384, the blot was rehybridized with a probe to IS1081 which is another insertion element found in the *M tuberculosis* complex (16). IS*1081* is a relatively stable element and produces RFLP patterns with only limited variability (8). Similar, albeit distinguishable, RFLP patterns were observed in the four strains of *M tuberculosis* including S384 (Figure 1, panel B), indicating that there were

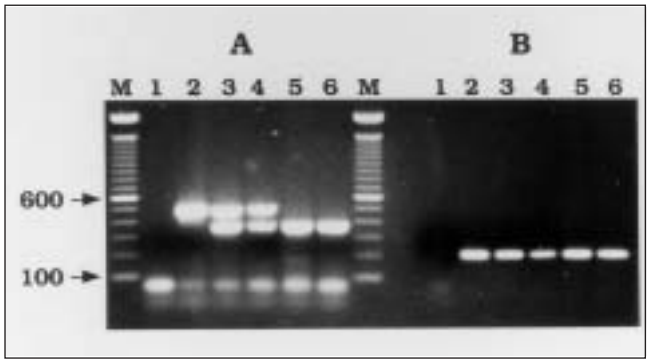

Figure 2) *Polymerase chain reaction amplification from* Mycobacterium tuberculosis *strains. Reaction products were electrophoresed on a 1.5% agarose gel stained with ethidium bromide. Panel A amplification using primers to IS*6110 *and* mpcA*; Panel B amplification using primers to the 16S/23S rRNA gene spacer. Lane 1, water control; lane 2, S384; lanes 3 and 4, clinical isolates of* M tuberculosis*; lane 5,* Mycobacterium bovis *bacillus Calmete-Guérin (BCG) (ATCC 35732); lane 6,* M bovis*; lane M, 100 basepair (bp) ladder (Gibco-BRL). Arrows indicate sizes of molecular weight markers in bp*

adequate and detectable amounts of DNA present for this sample. In addition, the blot reprobed using the oligonucleotide DR-r which hybridizes to the direct repeat (DR), a 36 bp long highly repetitive element (15). All four strains were again detected using the probe to this element (Figure 1, panel C).

To confirm the identification of S384 as a member of the *M tuberculosis* complex, sequence analysis on two regions of the 16S rRNA gene as prescribed by Kirschner and coworkers (17) and on the 16S/23S rRNA gene spacer (18,19) was performed; S384 showed sequence identity in these regions with several well-characterized strains of *M tuberculosis* and *Mycobacterium bovis* (unpublished data).

Next, an attempt was made to detect IS*6110* in S384 using PCR. For this, a multiplex PCR system was chosen in which an additional region of the template was coamplified to control for the presence of PCR inhibitors. In addition to primers designed to amplify a portion of IS*6110*, primers to *mpcA*, a phospholipase gene (20,21), which has been reported to be present in most strains of *M tuberculosis* but not in *M bovis* (22,23), were included. For IS*6110*, primers ISAm and ISB (Table 1) were used which amplify a 373 bp region from the right side of the single *Pvu*II site in the element. For *mpcA*, a region near the 3′ end of the gene known as mtp40 (23,24) was targeted; amplification of this region has been used to distinguish *M tuberculosis* and *M bovis* in PCR assays (22,23). The primers TB1m and TB3 included in the multiplex PCR amplify a 500 bp region of *mpcA* containing mtp40. The two primer sets were designed to have similar annealing temperatures. Separate control reactions were run for each sample using primers to the inernal transcribed spacer (ITS) region between the 16S and 23S rRNA genes; primers ITSms and ITSm2 amplify a 236 bp portion of this spacer region. Products of the PCR reactions were electrophoresed on a 1.5% agarose gel, and the results are shown in Figure 2.

All five samples from the *M tuberculosis* complex produced similar amounts of the ITS control amplicon (Figure 2, panel B), but only four samples produced the 373 bp amplicon from

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Mtb        GAC TTC CAC ATC GAC CCG GGT GAC CAC TTC TCC GGC (180)
           asp phe his ile asp pro gly asp his phe ser gly (60)

M. bovis   ... ... ... ... ... ... ... ... G.. ... ... …
                                           Asp

S384       ... ... ... ... ... ... ... ... ... ..G ... …
                                               Leu
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Figure 3) *Position of missense mutation in* pncA *gene of S384. Partial nucleotide sequence of the* pncA *gene from* Mycobacterium tuberculosis *strain Erdman (Mtb) is shown on the top line with the amino acid sequence beneath it. The point mutations in* Mycobacterium bovis *and S384 are indicated with their corresponding amino acid changes. Sequence of the Erdman and* M bovis *strains and nucleotide and amino acid numbering are those of Scorpio and Zhang (25). '.' indicates nucleotide sequence identical to that of the Erdman strain*

IS6110 (Figure 2, panel A, lanes 3-6). No detectable 373 bp product was generated by S384 (panel A, lane 2), although the template DNA from this sample could be amplified as indicated by the presence of the 500 bp mtp40 product from the same reaction (panel A, lane 2) and also by the control product (panel B, lane 2). Both the IS*6110* and mtp40 amplicons were produced using template DNA from the remaining two samples of *M tuberculosis* (panel A, lanes 3,4). Under the conditions used, copy number of IS*6110* did not have a significant effect on the yield of PCR products; the strain used in lane 3 has 10 copies of IS*6110* while the strain in lane 4 has only a single copy of the element (RFLP analysis not shown), and yet each strain generated similar amounts of the two amplicons. As expected, the *M bovis* isolate and *M bovis* BCG generated only the IS*6110* amplicon in the multiplex PCR (Figure 2A, lanes 5,6). The results of PCR analysis were confirmed using primers specific for a region to the left of the *PvuII* site in IS*6110*. Primers ISI and ISII (Table 1) amplified a 421 bp fragment from control strains of *M tuberculosis* and *M bovis* but not from S384 (data not shown).

Scorpio and Zhang (25) published the results of a study identifying a gene in *M tuberculosis* that encodes an enzyme with pyrazinamidase activity. Certain mutations within this gene, *pncA*, led to a loss of pyrazinamidase activity and conferred resistance to the antimicrobial drug PZA. Point mutations were detected in *pncA* in PZA-resistant strains of *M tuberculosis* and a specific point mutation was shared by strains of *M bovis* and *M bovis* BCG (25), which are naturally resistant to PZA. Because drug-susceptibility testing had indicated that S384 was resistant to PZA at 100 g/mL, it was decided to examine the sequence of the *pncA* gene from this strain.

Comparison with the published sequence of *pncA* showed that S384 had a point mutation at nucleotide position 174. Here, a substitution of a guanine for a cytosine in the third position of a codon resulted in a change from phenylalanine to leucine at amino acid position 58 (Figure 3). Also, whereas *M bovis* strains have an aspartate residue in the preceding amino acid position, S384 has a histidine residue as is found in *M tuberculosis* strains (25). Together with the results of biochemical testing and analysis of *mpcA*, this indicated that S384 was *M tuberculosis* and not *M bovis*.

It is noteworthy that the *M bovis*/S384 mutations occur within a histidine/phenylalanine dipeptide (Figure 3). Examination of the published sequence of *pncA* (25) shows that six of the seven histidine residues in the 186 amino acid polypeptide occur over a 40 amino acid stretch and that of those six residues, four occur within tripeptides containing aspartate and either tyrosine or phenylalanine. The juxtaposition of these amino acids may be important for full enzyme activity.

DISCUSSION

We attempted to detect IS*6110* in *M tuberculosis* strain S384 by RFLP analysis and by PCR, and the results were negative by each approach. To our knowledge, this is the first isolation of a strain of *M tuberculosis* lacking IS*6110* to be reported in Canada. In the multiplex PCR performed to detect IS*6110*, we chose *mpcA* as a second target for amplification, both as a control for the reaction and as a means of distinguishing between *M tuberculosis* and *M bovis*. Although Weil et al (26) reported that two of their strains of *M bovis* produced amplicons using primers to *mpcA*, the sequence of these amplicons was not presented. It is possible that some strains of *M bovis* contain *mpcA* but it is also possible that certain primer sets amplify regions in *M bovis* with weak similarity to *mpcA*. There are a minority of *M tuberculosis* strains that lack *mpcA* (26, 27), though; an absence of the IS*6110* and *mpcA* amplicons in test samples would have indicated a need for an alternative control target in the reaction. For example, del Portillo et al (28) used primers to the 32 kDa alpha antigen (29, 30) to control for the presence of mycobacterial DNA in a multiplex PCR. We attempted a multiplex PCR that included the primer sets for IS*6110*, *mpcA* and the 16S/23S rRNA ITS but due to internal competition between the primers, the ITS product was not always visible and it was, therefore, amplified in a separate reaction.

Susceptibility testing to first-line drugs showed that strain S384 exhibited resistance only to PZA. Although this trait is more usually associated with *M bovis*, other strains of *M tuberculosis* have been isolated which are monoresistant to PZA (25,31). Two reports have indicated that the resistance in *M bovis* and *M bovis* BCG strains is due to a common point mutation that has not been found in either PZA-susceptible or resistant strains of *M tuberculosis* (25,32). S384 did not show this characteristic mutation associated with *M bovis*.

Moreover, the strain was positive for niacin accumulation, resistant to TCH, grew on glycerol-containing medium, and showed the presence of the mpcA gene, all characteristics that support the identification of the strain as *M tuberculosis* (sensu strictu) rather than *M bovis* (22,23,33).

Monoresistance to PZA has not been reported previously among Ontario strains of *M tuberculosis*. In the case of S384, it is not known whether the patient had primary or secondary resistance. While in Canada, she was treated for one week with PZA but then treatment was discontinued when laboratory results revealed that the isolate was already resistant. Unfortunately, the medical history of the patient before her immigration to Canada was not available.

It is important to note that in their study on *pncA*, Scorpio and Zhang (25) raised the concentration of PZA for susceptibility testing from 100 g/mL to 500 g/mL. Mutations were detected in *pncA* only in strains that were resistant to the higher concentration of drug. S384 was not tested for resistance at 500 g/mL, but it is reasonable to expect that the amino acid change from phenylalanine to leucine contributes to some degree of PZA resistance, particularly in view of the proximity of this mutation to that found in *M bovis*.

The presence of a missense mutation within the *pncA* gene may be helpful for identifying the spread of strain S384 because this particular mutation was not among those previously observed (25). However, reports on other isolates of *M tuberculosis* lacking IS*6110* do not indicate whether these strains are PZA-resistant (13,34), and it will first be necessary to determine if any of these also contain this mutation. If the group of strains is closely related, the mutation may be a common feature.

In RFLP analysis of *M tuberculosis*, alternative markers have been used for strains that could not be reliably differentiated using IS*6110* because they had low copy numbers of this element. In these cases, both the DR element and the GC-rich repetitive element have been used for genetic typing (8,12,34). In one study from Madras, India in which 45% of strains had either no copies or only a single copy of IS*6110*, use of the DR element allowed some of these strains to be differentiated (12,35). Yuen and co-workers (13) used a probe to the GC-rich repetitive element to analyze strains from Vietnamese immigrants in Australia and found that these strains had highly similar patterns. The authors speculated, though, that in other strains much of the heterogeneity in the RFLP patterns of the G-C rich element could be due to transposition of IS*6110* so that when the latter element is lacking, the RFLP patterns seen for the G-C rich element are more homogeneous even with unrelated strains. The oligonucleotide $(GTG)_5$ has also been suggested as a possible alternative marker (7).

In 1994, the most recent year for which complete statistics are available, immigrants accounted for 57% of the 2074 cases of tuberculosis in Canada and 65% of these patients were from Asia (36). Because of the much higher incidence of tuberculosis among recent immigrants compared with the general population, it is likely that in the majority of these cases, the disease was contracted outside of Canada, thus increasing the likelihood that additional strains lacking IS*6110* will be isolated. While the most immediate concern is the treatment of these patients, implementing the use of alternative genetic markers will become important for identifying any spread of these strains within local communities and farther afield.

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