Characterization of \textit{Mycobacterium tuberculosis} Strains from Vietnamese Patients by Southern Blot Hybridization

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Despite the decline in the incidence of tuberculosis in highly industrialized nations over the past 50 years, the disease remains a major health problem in the world today. In Australia alone, approximately 600 new cases are still diagnosed annually (2).

As part of efforts to further reduce the incidence of tuberculosis, epidemiological investigations play an important role in the monitoring of control for this disease. The availability of a rapid, simple, and discriminatory typing method for \textit{Mycobacterium tuberculosis} can be of great benefit to such investigations. Studies have shown that restriction fragment length polymorphism analysis of \textit{M. tuberculosis} can detect genotypic variations between strains by using repetitive DNA sequences as probes in Southern hybridization analyses (7, 10, 14).

A DNA fragment containing a repetitive DNA sequence has been cloned in plasmid pTBN12 from \textit{M. tuberculosis} (10). This repeated sequence is highly repetitive in nature, and it has been estimated that at least 30 copies are present in the genome. They are not confined to the \textit{M. tuberculosis} complex but are present in a number of other mycobacterial species (10). By using pTBN12 as a probe, strains from within known clusters of infection demonstrate identical banding patterns whilst nonrelated strains are different.

Two insertion-sequence elements related to the enterobacterial IS3 family, IS6110 and IS986, have also been molecularly cloned from the genome of \textit{M. tuberculosis} (7, 10, 12, 14). Sequence comparisons have shown that IS6110 and IS986 differ from each other by only three nucleotides (7, 10, 14). Although they are probably mobile genetic elements with the potential to translocate within the chromosome, they are stable enough to provide identical restriction fragments for mycobacterial strains from within small clusters of infection (6, 7, 14). Previous studies have reported that all \textit{M. tuberculosis} strains have at least one copy and usually 6 to 15 copies of IS6110 present in their genome (7, 14).

Recently, we have found a group of \textit{M. tuberculosis} strains from two states in Australia with virtually identical pTBN12 restriction fragment patterns which appear to have either one or no copies of IS6110. Most of these strains appeared to be isolated from patients of Vietnamese origin.

In this study, we investigate this finding by analyzing a number of strains from patients of Vietnamese origin in comparison to randomly selected isolates from patients of non-Vietnamese origin.

A total of 79 clinical isolates of \textit{M. tuberculosis} were selected for this study from the following reference laboratories in Australia: Mycobacterium Reference Laboratory, Fairfield Hospital, Victoria; State Health Laboratory, Brisbane, Queensland; Westmead Centre, Westmead, New South Wales; and State Health Laboratory Services, Netherlands, Western Australia. All isolates were identified as \textit{M. tuberculosis} by standard procedures in the various reference laboratories on the basis of positive nitrate reduction, niacin production, pyrazinamidase production, and/or resistance to 5 \textmu g of thiophene-2-carboxylic acid hydrazide per ml. There were no known epidemiological relationships between these patients except for a father and daughter. Of the 79 isolates, 41 were from patients with Vietnamese origin, and as controls, 38 isolates were randomly selected from patients not of Vietnamese origin. These control strains were isolated in Victoria, Queensland, and Western Australia. All \textit{M. tuberculosis} isolates received from interstate were retested in our laboratory and confirmed as \textit{M. tuberculosis}.

Mycobacterial strains were grown and DNA was extracted as previously described (10). The concentration of DNA was estimated by agarose gel electrophoresis stained with ethidium bromide before restriction endonuclease digestion and Southern blot hybridization as described previously (10). RNA and DNA probes used were digoxigenin labelled with a nonradioactive digoxigenin nucleic acid labelling and detection system supplied by Boehringer GmbH (Mannheim, Germany). Chromosomal DNA digested with \textit{AluI} was hybridized with an RNA probe prepared from plasmid pTBN12 (10). DNA digested with \textit{PvuII} was hybridized with a DNA probe prepared from a 123-bp polymerase chain reaction (PCR) product of the IS6110 element (10).

Slot blot analyses were performed as previously described (9). The same amount of DNA for each sample was applied to duplicate nylon filters in the respective slots, baked at 120°C, and hybridized with either a digoxigenin labelled 65-kDa antigen gene probe (13) or an IS6110 probe.

DNA used for PCR was extracted by sonication as described previously (1). Briefly, a colony of \textit{M. tuberculosis} was removed with a bacterial loop and suspended in 200 \textmu l
of distilled water before centrifugation at 12,000 × g for 5 min and resuspension in 50 μl of distilled water. The tube was floated in a water bath containing the sonicator probe (Measuring and Scientific Equipment, Crawley, England) and sonicated for 10 min at 150 W. After sonication, samples were boiled for 10 min and centrifuged for 20 s at 12,000 × g, and the supernatant was collected. DNA samples were amplified in a buffer supplied by the Taq DNA polymerase manufacturer (Promega, Madison, Wis.) in a 20-μl reaction mixture containing approximately 100 ng of DNA, 1 μM primers, 1 U of Taq DNA polymerase, 100 μM dATP, 100 μM dCTP, 100 μM dGTP, and 100 μM dTTP (10). The primers TB1 and TB2 were designed to amplify a 123-bp fragment of IS6110 (4). The reactions were performed in an automated thermal cycler (Hybaid) with a program consisting of an initial denaturation at 95°C for 3 min and then 35 cycles of a two-step PCR of annealing and extension for 1 min at 65°C and denaturation for 1 min at 94°C. The PCR products were then analyzed by electrophoresis through a 10% polyacrylamide gel and stained with a silver staining kit (Bio-Rad Laboratories, Richmond, Calif.) according to the manufacturer’s instructions.

Sequencing of PCR product was performed by the direct incorporation protocol with a fmol DNA sequencing system (Promega) according to the manufacturer’s instructions and with the PCR primer TB1.

The analysis of nine M. tuberculosis isolates from patients of Vietnamese origin by Southern blot hybridization with the probe pTBN12 is shown in Fig. 1. The DNA profiles produced from the patient isolates (lanes 2 to 10) are virtually identical. The only apparent variation was that of a single band difference in one strain marked with an arrow in lane 8. The profile produced by the type strain H37Rv (ATCC 27294) in lane 1 is clearly different from the patient isolates. The similarity between these patient strains suggests an epidemiological relationship.

To verify their relatedness, these same nine isolates were digested with Pvull and hybridized with the IS6110 probe (Fig. 2). Since the 123-bp PCR product of the IS6110 element used in this DNA probe lacks an internal Pvull site, each hybridization band represents one copy of IS6110. The H37Rv strain (lane 1) produced multiple copies of IS6110. In contrast, no bands were visible in four of the Vietnamese isolates (lanes 2 to 5), and the remaining five isolates (lanes 6 to 10) demonstrated one band, implying that these strains have one copy of IS6110. Of the isolates with a single copy of IS6110, four produced a 1.3-kb fragment (lanes 5, 6, 7, and 9) and one produced a 6.0-kb fragment (lane 8). The isolate with the different size fragment is the same strain with a band difference with the pTBN12 probe (Fig. 1, lane 8).

To confirm the Southern blot result, slot blot analysis was performed with DNA probes directed against the mycobacterial common 65-kDa antigen gene and IS6110 (Fig. 3). All mycobacterial strains studied were positive with the 65-kDa antigen probe (Fig. 3A), indicating that sufficient DNA was applied to the membrane to yield a positive result. The four isolates which appeared to lack IS6110 by Southern blot hybridization also failed to hybridize to IS6110 by slot blot hybridization (Fig. 3B). Samples with a single copy and multiple copies were positive with this probe, while species outside the M. tuberculosis complex were all negative.

PCR was performed as a final analysis on the four isolates which lack IS6110 (Fig. 4). The four isolates tested failed to produce a 123-bp fragment (lanes 3 and 4). Two of these isolates produced a 280-bp fragment (lanes 5 and 6) which was sequenced to confirm that it was not related to IS6110 (results not shown).
To determine if this group of nine isolates was representative of a generally low copy number of IS6110 in patients of Vietnamese origin, a further 32 strains were analyzed by Southern hybridization and compared with a group of 38 randomly selected isolates of non-Vietnamese origin. Statistically, M. tuberculosis isolates from Vietnamese patients have an average of 8 ± 7 copies of IS6110. In comparison, isolates from non-Vietnamese patients have an average of 12 ± 4 copies of IS6110. This suggests that the number of copies of IS6110 found in isolates from the Vietnamese population tend to be fewer on average and have a wider variation in IS6110 copy number distribution than isolates from the non-Vietnamese population.

The results of this study demonstrate that IS6110 is not present in the genome of all strains of M. tuberculosis. The four isolates without this element and the five strains which have one copy of IS6110 appear to be genetically related on the basis of their pTBN12 Southern blot profiles (Fig. 1). In contrast, the other 31 Vietnamese strains with multiple copies of IS6110 had variable Southern blot patterns with the pTBN12 probe (results not shown).

It is possible that the similarity of the pTBN12 Southern blot patterns is related to the copy number of IS6110, although an epidemiological relationship between these patients cannot be ruled out. However, it is reasonable to suggest that the movement of IS6110 around the genome of M. tuberculosis contributes to the genetic heterogeneity observed with the pTBN12 Southern blots. Therefore, the lower the copy number of IS6110 in a M. tuberculosis strain, the less genetic diversity there may be over time. Thus, a strain may persist in a particular geographical location such as in Vietnam for a long time without significant alteration to its Southern blot profile because of the low copy number of IS6110. This supposition is supported by finding one strain with a single copy of IS6110 which appears to be in a location different from those of the other single-copy strains (Fig. 2, lane 8). This strain also has a single-band difference by Southern blot with a pTBN12 probe (Fig. 1, lane 8). This result implies that in certain regions of the world, Southern blot hybridization may not be useful as an epidemiological tool.

Published data have reported that IS986, which is virtually identical to IS6110, can remain stable after 2 months of passages in guinea pigs (6) as well as in vitro passaging in liquid medium every week for 6 months (14). Conversely, in our laboratory we have found strains with differences in one or two bands with IS6110 which appear identical with pTBN12 for multiple isolates from the same patient or isolates from a known cluster of infection (3, 8). For this reason, our laboratory employs the probe pTBN12 for primary screening of isolates for epidemiologic relatedness and the IS6110 probe for confirmation and/or subtyping of strains.

Our results also have implications for the application of PCR in laboratory diagnosis. PCR has the potential to provide a more rapid, sensitive, and specific diagnostic assay for M. tuberculosis in clinical specimens (1, 4). This study demonstrates that not all M. tuberculosis strains can be detected with primers directed towards IS6110. This can lead to false-negative results which, according to our data, would occur more frequently in the group of Vietnamese patients reported in this study. Potentially this would present problems in the application of PCR diagnosis and Southern blot hybridization for epidemiology in areas where such strains have a high prevalence.

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REFERENCES


