

GENETIC POLYMORPHISMS AMONG MYCOBACTERIUM TUBERCULOSIS ISOLATES FROM PATIENTS WITH PULMONARY TUBERCULOSIS IN NORTHERN INDIA

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Abstract. Restriction fragment length polymorphism (RFLP) based on IS6110 is considered the gold standard for *Mycobacterium tuberculosis* molecular typing. It is useful to discriminate among *M. tuberculosis* strains, investigate outbreaks and distinguish between reactivation and re-infection. We studied polymorphisms among *M. tuberculosis* isolates from northern India using RFLP to determine the presence of a correlation between IS6110 based fingerprints and drug resistance and to look for relapse and transmission among patients and their contacts. RFLP patterns of *Pvu*II digested genomic DNA of 100 *M. tuberculosis* isolates were analyzed using southern blotting with a 245 bp IS6110 probe. Drug sensitivity testing (DST) was conducted for rifampicin (40 g/ml), isoniazid (1 g/ml), ethambutol (2 g/ml) and streptomycin (4 g/ml) using the proportion method. A high degree of polymorphism was seen among the *M. tuberculosis* isolates and the number of IS6110 copies varied from 0 to 14, with a predominance of isolates with 11 bands. Seventy-five isolates had a high number of bands, 9 had an intermediate number, 6 isolates had a low number and 10 isolates had no bands. No correlation between IS6110 band numbers and RFLP banding patterns was found with drug resistance or for any particular geographical area, although clustering was seen amongst MDR-TB cases. No cases of relapses or transmissions were seen.

Keywords: *Mycobacterium tuberculosis*, RFLP, IS6110, MDR-TB, northern India

INTRODUCTION

According to the WHO, nearly 38% of the global tuberculosis (TB) burden is in Southeast Asia where 750,000 deaths

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occur annually due to TB, amounting to 1,500 deaths daily, or one death every minute (WHO, 2004). India contributes to a major part of this burden. There were 64,000 estimated cases of multi-drug resistant TB (MDR-TB) in India in 2010; 2.1% of newly diagnosed cases and 15% of retreatment cases (WHO, 2011). The problem is further compounded by an increase MDR-TB cases among HIV patients (Narain *et al.*, 1992). Molecular

epidemiological studies of *Mycobacterium tuberculosis* strains using DNA fingerprinting have provided valuable information regarding the mechanism of transmission of TB. Restriction fragment length polymorphism (RFLP) of *M. tuberculosis* strains based on the insertion sequence IS6110 is considered the gold standard for molecular typing of this organism. It has been used extensively to discriminate among *M. tuberculosis* strains (Cave *et al*, 1991; Van Soolingen *et al*, 1993), identify transmission chains (Raviglione *et al*, 1995; Barnes *et al*, 1997), investigate TB outbreaks (Kline *et al*, 1995; Valway *et al*, 1998), and distinguish between reactivation and reinfection (Small *et al*, 1993; Das *et al*, 1995; Van Rie *et al*, 1999).

In India, IS6110-RFLP has been used for molecular characterization of *M. tuberculosis* isolates from various regions of the country, either alone or with other typing methods (Mathuria *et al*, 2008; Purwar *et al*, 2011; Varma-Basil *et al*, 2011). A significant proportion (40%) of *M. tuberculosis* isolates from southern India lack this sequence; but a study from Agra in northern India found the majority of isolates had multiple IS6110 copies (Mathuria *et al*, 2008). We investigated IS6110 genetic polymorphism among *M. tuberculosis* isolates from northern India, to determine the correlation between IS6110 based fingerprints and drug resistance profiles among the isolates and to look for transmission and relapse among TB patient contacts. The testing was conducted at the Postgraduate Institute of Medical Education and Research (PGIMER), Chandigarh, India.

MATERIALS AND METHODS

One hundred consecutive isolates of *M. tuberculosis* were collected and sent

to the Mycobacteriology Laboratory, PGIMER, Chandigarh, India from 2001 to 2003. The isolates were obtained from patients from nine Indian states: Uttar Pradesh (25), Punjab (20), Chandigarh (19), Harayana (13), Himachal Pradesh (12), Uttarakhand (8), Bihar (1), Jharkhand (1) and West Bengal (1). Fresh sub-cultures were conducted on Löwenstein-Jensen medium slants (LJ Slants) and incubated at 37°C. When a confluent growth appeared, further identification was carried out using standard biochemical tests: niacin production, nitrate reduction and catalase production (Vestal, 1977).

Written informed consent was obtained from each patient included in the study. The study was approved by the Institute Ethics Committee of PGIMER, Chandigarh, India.

Drug susceptibility testing

Drug susceptibility testing was carried out for rifampicin (40 g/ml), isoniazid (1 g/ml), ethambutol (2 g/ml) and streptomycin (4 g/ml) using the proportion method (Canetti *et al*, 1963; Vestal, 1977).

DNA extraction and polymerase chain reaction

The genomic DNA was extracted from 6-8 week old mycobacterial cultures as described previously (Van Soolingen *et al*, 1991). A 245 bp portion of the IS6110 element located at the 3' end of the *PvuII* restriction site was amplified by PCR. The sequences of the forward and reverse primers used for the PCR were as follows: INS-F (5'CGTGAGGGCATCGAGGTG-GC3'), and INS-R (5'GCGTAGGCGTCG-GTGACAAA3') (Bangalore Genei, India). The 245 bp DNA band was eluted from the agarose gel using a standard protocol with the Qiaquick Gel Extraction Kit (Qiagen, Valencia, CA) and used as the probe.

Table 1
Clustering and drug resistance profiles of *M. tuberculosis* isolates.

Cluster	No. of isolates	Drug resistance profile	IS6110 copy no.
1	3	S – RIF, INH, SM, EMB	1
2	2	S – RIF; R – INH	8
3	6	R – RIF, INH	9
4	2	R – RIF, INH	9
5	2	R – RIF, INH, SM, EMB	11
6	2	R – RIF, INH, SM, EMB	11
7	2	R – RIF, INH	13

EMB, ethambutol; INH, isoniazid; RIF, rifampicin; R, resistant; S, sensitive; SM, streptomycin

Random DNA labeling of probe using digoxigenin

The probe was labeled with non radioactive digoxigenin (DIG) using a random primed DNA labeling technique with a DIG DNA labeling and detection kit (Roche Diagnostics, Mannheim Germany).

Restriction fragment length polymorphism and hybridization

IS6110 RFLP was performed as described previously (Van Embden *et al.*, 1993). Five microliters of the extracted genomic DNA was digested with *Pvu*II restriction enzyme, and RFLP patterns of the digested genomic DNA were analyzed by southern blotting and hybridization with a labeled 245 bp IS6110 probe on 1.5% agarose gel as described previously (Siddiqi *et al.*, 2001). The tests were performed in duplicate for all samples.

The isolates were categorized into 4 groups based on the copy number: 1) those lacking the IS6110 element; 2) those with a low number of copies (1-2); 3) those with an intermediate number of copies (3-5); 4) those with a high number of copies (6-15). Fingerprint patterns were compared using NTSYSpC, Version 2.0 (Applied Biostatistics, New York, NY). A dendrogram was generated based on

the weighted pair group clustering of averages following the manufacturer's instructions (Fig 1). Matching profiles were confirmed visually. A cluster was defined as a group of two or more isolates whose IS6110 fingerprints were identical in number and size for all bands.

RESULTS

Majority of the *M. tuberculosis* isolates (90/100) had an IS6110 element and a substantial degree of polymorphism in terms of copy numbers, ranging from 0 to 14 (Fig 2). Of the 100 isolates, 10% belonged to group 1 (lacking IS6110), 6% to group 2 (low number of copies), 9% to group 3 (intermediate number of copies) and 75% to group 4 (high number of copies). Fig 3 shows the DNA banding patterns for the *M. tuberculosis* isolates.

Different numbers of IS6110 copies were observed by geographic area but the differences were not significant for any specific RFLP pattern. Clustering of isolates was not possible due to a lack of common bands. Seven clusters were found in 19 isolates; 6 clusters had a high number of copies (2 isolates had 8 copies, 8 had 9 copies, 4 had 11 copies, and 2 had 13 copies); one cluster had a single

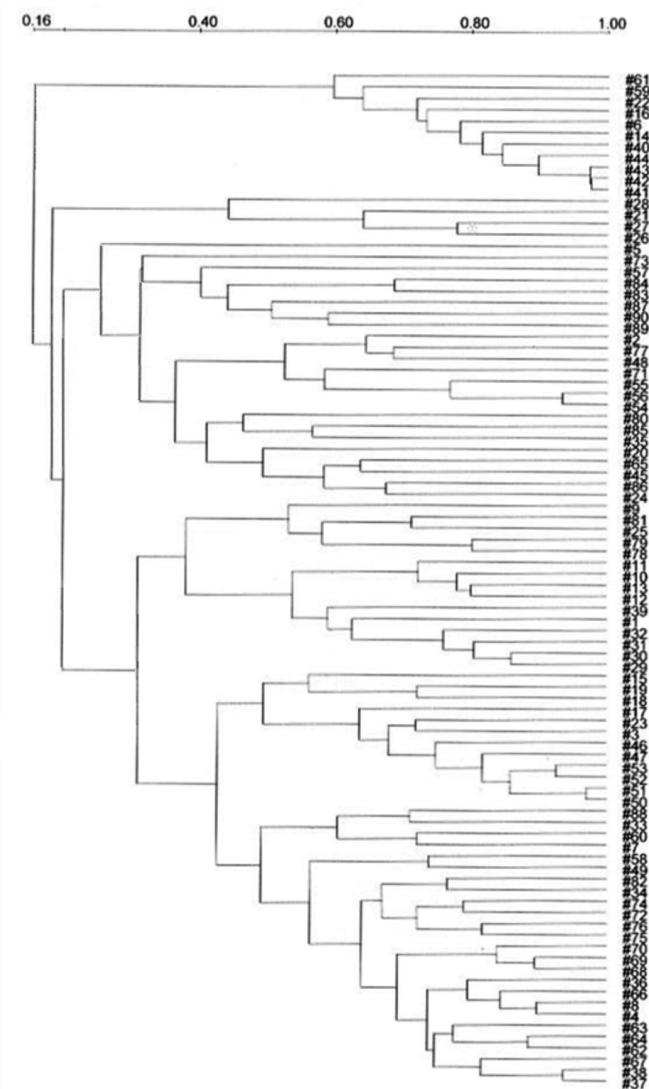


Fig 1—Dendrogram showing relatedness of IS6110 patterns among 90 *M. tuberculosis* isolates.

copy (Table 1). The isolates within each cluster were obtained from patients from different states, and had no geographical correlation.

In this study, 35% of isolates were resistant to rifampicin, 33% were resistant to streptomycin, 25% were resistant to isoniazid and 11% were resistant to ethambutol; multi-drug resistance (MDR) (resistance to both rifampicin and isoniazid) was found in 21% isolates; 5% of isolates

were resistant to all drugs.

In order to observe the relationship between the RFLP pattern and drug resistance, IS6110 fingerprints of isolates resistant to one or more anti-TB drugs were compared with isolates without resistance. There were no noticeable differences in banding patterns and numbers of bands between drug sensitive and drug resistant isolates, suggesting the RFLP pattern has no correlation with drug re-

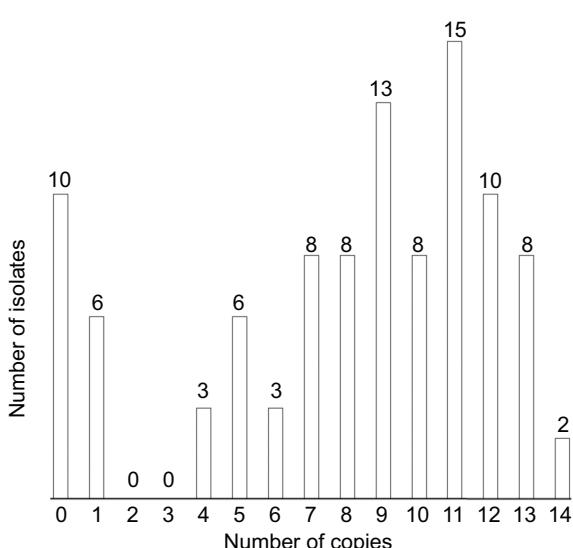


Fig 2-Distribution of IS6110 copy numbers among *M. tuberculosis* isolates.

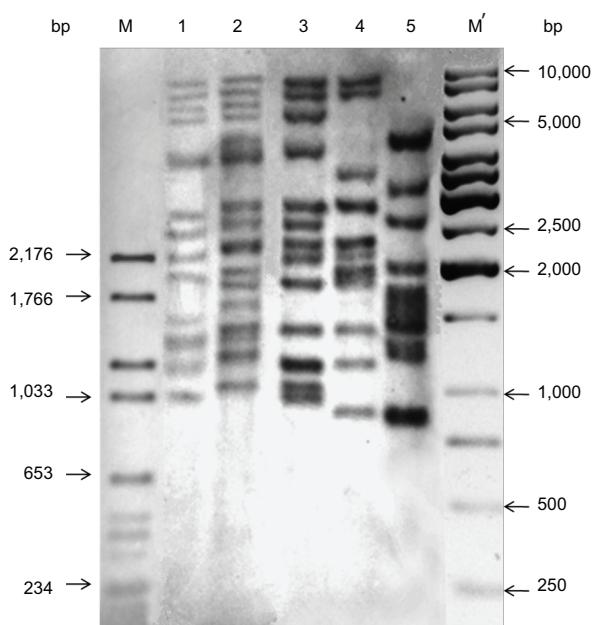


Fig 3-RFLP patterns among *M. tuberculosis* isolates generated by hybridization of *Pvu*II fragments of genomic DNA with a 245 bp IS6110 probe on 1.5% agarose gel. Lane M, DIG labeled molecular weight marker; lanes 1-4, *Pvu*II digested genomic DNA from *M. tuberculosis* clinical isolates; lane 5, *M. tuberculosis* H37Rv DNA digested with *Pvu*II; lane M', 1kb DNA ladder.

sistance. There was a higher degree of clustering seen among MDR-TB patients than drug sensitive patients, although clustering was not seen in the majority of isolates. The 19 isolates forming seven clusters had similar susceptibility patterns within the clusters (Table 1).

While correlating the results with the clinical status of the patients, no cases of relapse or transmission with the same isolate or different isolates were found. Among the family members of one TB patient another *M. tuberculosis* isolate was found but these isolates had different IS6110 fingerprints, suggesting the family member may have contracted the isolate from another source rather than the TB patient in their family.

DISCUSSION

In this study, we identified clustered isolates to ascertain whether there was a correlation between IS6110 fingerprints and drug resistance profiles. The IS6110 fingerprinting revealed a substantial degree of polymorphism. Our hospital is a tertiary care center catering patients from six adjoining states (Chandigarh, Punjab, Himachal Pradesh, Haryana, Uttar Pradesh and Uttarakhand); there are a large number of patients from other areas which could explain the high level of polymorphism seen in our isolates. Siddiqi *et al* (2001) also found a significant degree of polymorphism among *M. tuberculosis* isolates in northern India. In contrast to our study they found a large number of isolates had fewer than 6 bands with predominances of 3 bands and 1 band. They also found

42% of isolates had less than or equal to four copies with the IS6110. In all, there were 60 unique fingerprints in their study (Siddiqi *et al*, 2001). Purwar *et al* (2011) in a study from Kanpur, northern India found 66% of tested isolates had more than 10 IS6110 copies; 17% of isolates had 1-5 copies and another 17% had 6-9 copies. In another study of a small number of patients from Delhi, 77% had multiple copies, similar to our study (Bhanu *et al*, 2002); they also reported that 10% strains had a single copy and one strain had no copies of IS6110. In a similar study from southern India, 41% of *M. tuberculosis* strains had one copy of IS6110, 26% had 2-5 copies and 32% had >6 copies (Narayanan *et al*, 2002).

Ten of our isolates had no copies of IS6110. Although IS6110 has been widely used for PCR diagnosis, a few studies have shown some *M. tuberculosis* isolates may not have any copies of this element. Das *et al* (2005) in a study from southern India found that 40% of clinical strains of *M. tuberculosis* had no copies of IS6110. The large number of strains lacking IS6110 in their study might be partially attributed to the small sample size. Siddiqi *et al* (1998) reported that 5.5% of *M. tuberculosis* isolates from northern India had no copies of IS6110. Thus PCR targeting this sequence may miss such cases in India indicating the need for an alternate gene for use in diagnosis and epidemiology.

No noticeable differences in banding patterns or numbers of bands were seen between drug sensitive and drug resistant isolates in this study suggesting the IS6110-RFLP pattern has no relationship to drug resistance.

This brings to light the fact that genetic movement of the IS6110 element is rare; the phenomenon of drug resistance

is a random selection of mutant isolates. Similar results were found by Siddiqi *et al* (1998) where the number of copies was not related to the resistance pattern of the strain.

Due to a lack of common bands, clustering was not possible in the majority of isolates. Seven clusters were found, of which 6 had high numbers of copies and one cluster had a single copy, which is common in India. The 19 isolates which formed seven clusters had similar susceptibility patterns within the clusters; however, these isolates were obtained from patients from different states and there were no geographical correlations. Higher clustering was seen among MDR-TB strains than drug-sensitive strains which is in agreement with previous studies (Snider *et al*, 1985; Siddiqi *et al*, 1998; Beck-Sague *et al*, 1992; Mistry *et al*, 2002).

The transmission of *M. tuberculosis* has been reported to be 10-58% in developed countries and 20-36% in developing countries (Barnes *et al*, 1996; Warren *et al*, 1996; Braden *et al*, 1997; Haas *et al*, 1999; Garcia-Garcia *et al*, 2000; Godfrey-Faussett *et al*, 2000). In the present study, no cases of relapse or transmission with the same strain were seen.

In conclusion, the present study showed a high degree of polymorphism among *M. tuberculosis* isolates, which implies greater variation among Indian strains, although a larger number of strains is needed to be statistically significant. These findings show that a variety of strains are circulating in the Indian population. Our study confirms the usefulness of IS6110 for molecular characterization of the majority of isolates of *M. tuberculosis* in India. However, using only IS6110 RFLP for *M. tuberculosis* genotyping limits one's ability to analyze

some Indian *M. tuberculosis* strains (Razanamparany *et al*, 2009), and other typing methods, such as spoligotyping, MIRU-VNTR typing, SNP tagging and RD typing need to be carried out in strains which lack IS6110 or have low copy numbers (Varma-Basil *et al*, 2011).

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