INTERNATIONAL JOURNAL OF AGRICULTURE & BIOLOGY
ISSN Print: 1560–8530; ISSN Online: 1814–9596
12–1347/2013/15–3–607–611
http://www.fspublishers.org

Full Length Article

The Presence and Copy Number of Insertion Sequence IS*6110* in *Mycobacterium tuberculosis* Isolates from Pakistan

Rubina Tabassum Siddiqui[1]*, Muhammad Waheed Akhtar[2] and Javed Anver Qureshi[1]
[1]*Health Biotechnology Division, National Institute for Biotechnology and Genetic Engineering (NIBGE), P.O. Box #577, Jhang Road, Faisalabad, Pakistan*
[2]*School of Biological Sciences, University of the Punjab, Lahore, Pakistan*
*For correspondence: tabassum.rubina@gmail.com

Abstract

The insertion element IS*6110* has been used extensively for the detection and strain differentiation of *M. tuberculosis.* The presence and copy number of IS*6110* varies geographically. We have investigated the presence and copy number of this insertion sequence in *M. tuberculosis* isolates collected throughout Pakistan. All 92 confirmed cultures of *M. tuberculosis* from Pakistan were found to contain this insertion sequence when checked by PCR using primers based on IS*6110* sequence. The copy number determined by the standard IS*6110* restriction fragment length polymorphism varied from 2–18. Among 46 cultures used for copy number estimation 32 strains (69.5%) contained more than 10 copies of IS*6110*. The percentage of isolates having up to 5 copies of IS*6110* was 6.25, while 21.7% isolates had copy number between 5 and 11. Our results confirmed the presence of IS*6110* in all isolates of *M. tuberculosis* and showed that IS*6110* is a useful marker for the detection and DNA fingerprinting of *M. tuberculosis* strains originating from Pakistan. © 2013 Friends Science Publishers

Keywords: Tuberculosis; IS*6110* copy number; RFLP; *Mycobacterium tuberculosis*

Introduction

Tuberculosis is an infectious disease caused by *M. tuberculosis*. The *M. tuberculosis*-complex includes *M. tuberculosis, M. bovis, M. bovis*-BCG, *M. microti* and *M. africanum*. It is a facultative intracellular parasite, generally invading macrophages. It usually attacks the lungs, but can attack almost any part of the body. Despite the availability of effective short-course chemotherapy and the Bacille Calmette Guerin (BCG) vaccine, TB causes more deaths than any other infectious disease. It is resurfacing as one of the worst public health problem globally. The main reasons for re-emergence of this disease include poverty, crowding, homelessness, substance abuse, the widespread emergence of drug resistant strains and the AIDS epidemic. TB occurs in every country of the world, but the highest incidence is found in Asia and Africa, while in recent years it has re-emerged as a major public health issue in Russia and other Eastern European countries. Tuberculosis remains a high burden disease with estimated 7.8 million new cases in 2011 and around 1.4 million deaths (WHO, 2012). This makes TB the commonest cause of death among women in the developing world and worldwide it ranks 7th in the list of causes of loss of healthy life (WHO, 2012). Pakistan with a total population of 180 million globally ranks 6th by estimated number of cases of tuberculosis. Pakistan carries 44% burden of this deadly disease in World Health Organizations Eastern Mediterranean region comprising 23 countries. The delay in diagnosis, non-compliance and drug resistance are the major reasons for this increasing burden of the disease in Pakistan. Molecular methods for the diagnosis and epidemiology of tuberculosis to identify disease transmission pattern may help in reducing this burden (Schürch and van Soolingen, 2012).

Among the various genetic elements that have been found to contribute to DNA polymorphism in *M. tuberculosis*, the insertion element IS*6110* has been studied most intensively (Lönnroth and Raviglione, 2008). This 1,355-bp insertion sequence element is related to the IS*3* family of ISs and has been found exclusively in all isolates of the *M. tuberculosis* complex group of mycobacteria (Thierry *et al*., 1990). Because of the highly variable number of copies and the variable sites of integration in the chromosome, epidemiologically unrelated strains display an extremely high degree of polymorphism of IS*6110*-containing restriction fragments (van Soolingen *et al*., 1993). This polymorphism is presumably due to the ability of IS*6110* to transpose within the genome without much target sequence specificity. Despite the high degree of IS*6110*-associated restriction fragment length polymorphism (RFLP) among *M. tuberculosis* strains, no IS*6110* transposition has been observed during prolonged *in vitro* culturing or *in vivo* growth, suggesting that the frequency of transposition is relatively low (Cave *et al*., 1991). Among several molecular methods used for analyzing the presence and diversity of *M. tuberculosis*

To cite this paper: Siddiqui, R.T., M.W. Akhtar and J.A. Qureshi, 2013. The presence and copy number of insertion sequence IS*6110* in *Mycobacterium tuberculosis* isolates from Pakistan. *Int. J. Agric. Biol*., 15: 607–611

isolates, the use of insertion sequence IS*6110* is the most widely used method. The ability to distinguish between strains is based on the variability both in the number and in the chromosomal positions of IS*6110* (Thorne *et al*., 2011). It has been shown that some strains of *M. tuberculosis* from Asia either do not contain this insertion sequence or contain only one or two copies of the IS*6110* insertion element, making the use of this sequence unreliable for the detection and typing of *M. tuberculosis* for such isolates (Das *et al*., 1993).

The aim of this study was to establish the presence and the copy number of insertion IS*6110* in *M. tuberculosis* isolates from Pakistan.

Materials and Methods

M. tuberculosis Isolates

M. tuberculosis cultures were kindly provided by TB Clinic, Italian Cooperation for Development, Peshawar, Khyber Pakhtunkhwa, Ojha Institute of Chest Diseases, Karachi, Sindh and Sardar Bahadur Khan Institute for Chest Diseases, Quetta (Table 1). *M. tuberculosis* isolates from Punjab were obtained from clinical specimens collected from various hospitals and cultured on LJ medium (Lowenstein, 1931). Any bacterial growth was checked by ZN staining (Gerhardt *et al*., 1981).

DNA Isolation from *M. tuberculosis* Cultures

DNA was isolated from *M. tuberculosis* strains following the procedure described by (Amaro *et al.*, 2008). Briefly, a loop full of mycobacterial culture on LJ slope was taken in 400 µL TE, heat inactivated at 80°C for 20 min and cooled to room temperature. The bacterial suspension was incubated with lysozyme at 37°C for 1 h, with SDS/proteinase K at 65°C for 20 min and with pre-warmed CTAB/NaCl at 65°C for 10 min. The DNA was then purified and precipitated using chloroform–isoamylalcohol–isopropanol extraction.

PCR for the Detection of *M. tuberculosis*

All *M. tuberculosis* cultures were tested for the presence of IS*6110* using primers given in Table 2. More than one set of primers was used to rule out false positives due to carry-over contamination. A positive control which was DNA isolated from a standard strain H37Rv was included in all PCR reactions, while PCR reaction without *M. tuberculosis* DNA or DNA isolated from *E. coli* served as negative controls. The PCR reaction mixture consisted of 50 mM KCl, 10 mM Tris-HCl (pH 8.7), 2.5 mM $MgCl_2$, 0.2 mM (each) dNTPs, 25 pmole of each primer and 1 unit of Taq polymerase. PCR products were resolved on 1.5% agarose. The amplification of IS*6110* was confirmed by dot-blot hybridization using a cloned PCR product as probe.

Table 1: Copy number of IS*6110* in *M. tuberculosis* isolates from Pakistan determined by RFLP analysis

Strain	Origin	Number of IS*6110*
P60	Khyber Pakhtunkhwa	10
P62	Khyber Pakhtunkhwa	10
P64	Khyber Pakhtunkhwa	12
P65	Khyber Pakhtunkhwa	7
P66	Khyber Pakhtunkhwa	12
P68	Khyber Pakhtunkhwa	14
P69	Khyber Pakhtunkhwa	13
P72	Khyber Pakhtunkhwa	13
P74	Khyber Pakhtunkhwa	14
P76	Khyber Pakhtunkhwa	15
P77	Khyber Pakhtunkhwa	10
P78	Khyber Pakhtunkhwa	8
P79	Khyber Pakhtunkhwa	10
P80	Khyber Pakhtunkhwa	15
P81	Khyber Pakhtunkhwa	13
P82	Khyber Pakhtunkhwa	12
P83	Khyber Pakhtunkhwa	12
P85	Khyber Pakhtunkhwa	12
P86	Khyber Pakhtunkhwa	8
P87	Khyber Pakhtunkhwa	11
P88	Khyber Pakhtunkhwa	12
P89	Khyber Pakhtunkhwa	11
P90	Khyber Pakhtunkhwa	11
P91	Khyber Pakhtunkhwa	14
P93	Khyber Pakhtunkhwa	15
P94	Khyber Pakhtunkhwa	11
P95	Khyber Pakhtunkhwa	10
P96	Khyber Pakhtunkhwa	14
Q7	Balochistan	5
Q20	Balochistan	9
18	Punjab	14
19	Punjab	14
23	Punjab	12
28	Punjab	14
R414	Punjab	11
R413	Punjab	11
R383	Punjab	15
F56	Punjab	8
1)1-9	Sindh	14
3)1-29	Sindh	18
5)1-46	Sindh	7
13)10-4	Sindh	2
11)10-12	Sindh	13
9)2-4	Sindh	12
K3	Sindh	13
K4	Sindh	2

RFLP Analysis of *M. tuberculosis* Isolates

Genomic DNA from *M. tuberculosis* isolates and from standard strain Mt14323 was digested with *Pvu*II (van Soolingen *et al*., 1994). After agarose gel electrophoresis, the digested DNA was transferred to Hybond N+ nylon membrane. Radiolabelled (α^{32}P dCTP) probe was then hybridized and the signal was detected on x-ray film. The copy number of IS*6110* was determined by counting the number of bands appearing on the x-ray film.

Results

Presence of IS*6110* in *M. tuberculosis* Isolates

To determine the presence of IS*6110* in *M. tuberculosis*

Table 2: Primer sets used for PCR amplification of different regions of IS*6110* with their sequences and references

Primer	Sequence	Annealing temperature	Size of PCR product	Reference
Pt3	5'-GAACGGCTGATGACCAAACT -3	65°C	188bp	McAdam *et al*., 1990
Pt6	'5'-ACGTAGGCGAACCCTGCCCA -3'			
Pt8	5' -GTGCGGATGGTCGCAGAGAT -3'	65°C	541bp	McAdam *et al*., 1990
Pt9	5'-CTCGATGCCCTCACGGTTCA- 3'			
Pt10	5'-AGCACGATTCGGAGTGGGCA-3'(probe)			
INS1	5'-CGTGAGGGCATCGAGGTGGC-3'	68°C	245bp	Hermans *et al*., 1990
INS2	5' -GCGTAGGCGTCGGTGACAAA- 3'			
TB294	5'-GGACAACGCCGAATTGCGAAGGGC- 3'	64°C	578	Wilson *et al*., 1993
TB850	5' -TAGGCGTCGGTGACAAAGGCCACG- 3'			
TB505	5'-ACGACCACATCAACC- 3' (nested PCR primers)	50°C	181	
TB670	5'-AGTTTGGTCATCAGCC- 3' (nested PCR primers)			

from Pakistan, primers based on IS*6110* (Table 2) were used in PCR and amplified product was detected by agarose gel electrophoresis (Fig. 1). More than one set of PCR primers based on IS*6110* were used to rule out possible false positives. All isolates of *M. tuberculosis* were positive for IS*6110* with more than one set of IS*6110* based primers. The results showed that all 92 isolates tested for the presence of the sequence were found to contain the IS*6110*, suggesting that this sequence is a reliable source for the detection and discrimination of *M. tuberculosis* isolates from Pakistan (Table 3).

Standardization of RFLP Methodology using Mt14323 as Standard

The standard RFLP methodology was used for determination of copy number of IS*6110* in *M. tuberculosis* isolates from Pakistan (van Soolingen *et al*., 1994). In each gel a lane containing DNA from Mt14323, which when digested with *Pvu*II and probed with IS*6110* region probe (amplification product using INS1/INS2 primers), gives 10 equally spaced bands was included as standard. The digestion of *M. tuberculosis* DNA with *Pvu*II was monitored through agarose gel electrophoresis.

RFLP Analysis Revealed High Copy Number of IS*6110* in *M. tuberculosis* Isolates

After initial establishment, RFLP analysis was used to assess the copy number of IS*6110* in *M. tuberculosis* isolates from Pakistan. The results of RFLP analysis are given in Table 3. Some representative RFLP patterns are given in Fig. 2 and 3. The copy number of IS*6110* was found to vary from 2–18. However, the majority of strains (32 out of 46) were found to contain 10–12 copies of IS*6110*. Several bands in RFLP pattern were common among different isolates and represent hot-spots for integration of IS*6110*. The data showed that isolates from Pakistan contain high copy number of this sequence and thus, it can be used reliably for the detection and strain typing of *M. tuberculosis* isolates from Pakistan. The samples taken from Sindh showed highest level of diversity and reflect ethnic and cultural diversity that exist in urban Sindh. The results presented here represent diversity

Table 3: PCR based detection of IS*6110* in *M. tuberculosis* isolates from Pakistan

Province	No. of isolates	Isolates having IS*6110*
Khyber Pakhtunkhwa	50	50
Punjab	22	22
Balochistan	5	5
Sindh	15	15
Total	92	92

All isolates were tested for the presence of IS*6110* with more than one primer set

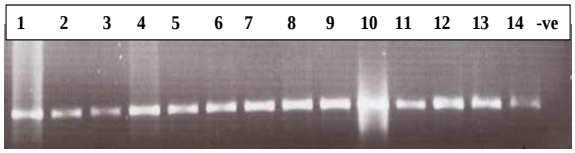

Fig. 1: The presence of IS*6110* in *M. tuberculosis* cultures from Pakistan determined by PCR. The figure shows the specific amplification of IS*6110* using Pt8/Pt9 primer pair when DNA isolated from cultures was used as template

and copy number of IS*6110* found in both Pakistan and Afghanistan since strains originating from Khyber Pakhtunkhwa also included those obtained from Afghan refugees living in Pakistan. Some *M. tuberculosis* strains were repeated in different gels to confirm results. The results of such isolates were the same in different gels, validating copy number analysis.

Discussion

The insertion sequence IS*6110* is one of the most common DNA target sequence used for diagnosis and fingerprinting of *M. tuberculosis.* However, it is essential to document the presence and copy number of the sequence to establish its usefulness in isolates originating from Pakistan. This is a pioneer work where we documented the presence and copy number of IS*6110* among isolates collected from all four provinces of Pakistan. The copy number of IS*6110* varies in different geographical regions and it was important to establish the presence and copy number in *M. tuberculosis* isolates from Pakistan. The study has established that all *M. tuberculosis* isolates from Pakistan contain 2–18 copies of

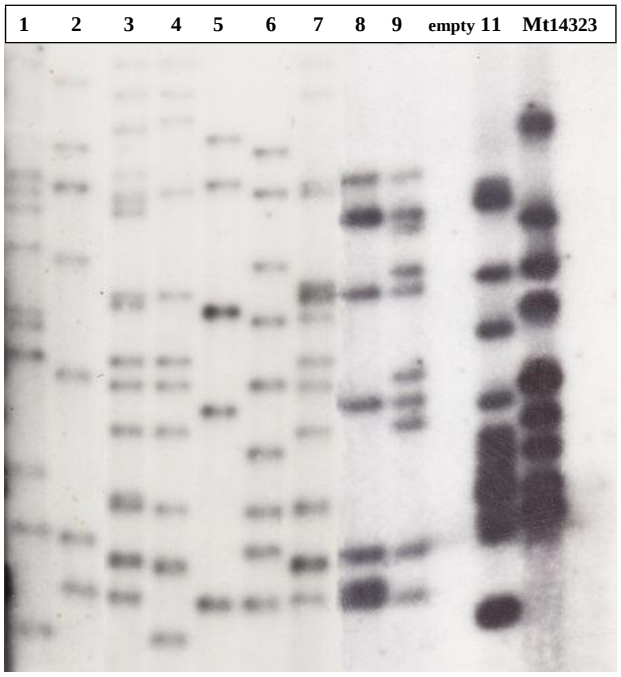

Fig. 2: IS*6110* based RFLP analysis of *M. tuberculosis* strains originating from Pakistan. Mt14323 was used as standard strain. The figure shows RFLP pattern of *M. tuberculosis* isolates originating from Khyber Pakhtunkhwa, Pakistan. The copy number of IS*6110* in isolates shown here, varied from 5 to 15

IS*6110* and thus is a useful target for both diagnosis and finger printing. The study was important in view of the findings that certain strains of *M. tuberculosis* from India contain either one or no copy of IS*6110* thus making usefulness of the IS*6110* for clinical isolates questionable (Das *et al*., 1993). Recent studies from India show that the majority of *M. tuberculosis* strains contain high copy number of IS*6110* (Chauhan *et al*., 2007; Mathuria *et al*., 2008).

Results presented in this study unequivocally showed the presence and high copy number of IS*6110* in cultures originating from Pakistan. A study using RFLP analysis of *M. tuberculosis* isolates from Iran showed that the copy number of isolates ranged from 5 to 18 and the average number per strain was 11 (Doroudchi *et al*., 2000). The strains used in this study included *M. tuberculosis* obtained from patients originating from Afghanistan and suggest that the copy number of IS*6110* found in Pakistan is similar to those found in Iran and Afghanistan and northern India rather than those found in southern India (Siddiqi *et al*., 2002). The conclusion is also consistent with historic contacts of people from Iran and Afghanistan with population in Pakistan. Differences in the prevalence of strains within the country have been reported in a study from Tunisia, suggesting persistent micro-epidemics (Hermans *et al*., 1995). Interestingly, RFLP analysis of

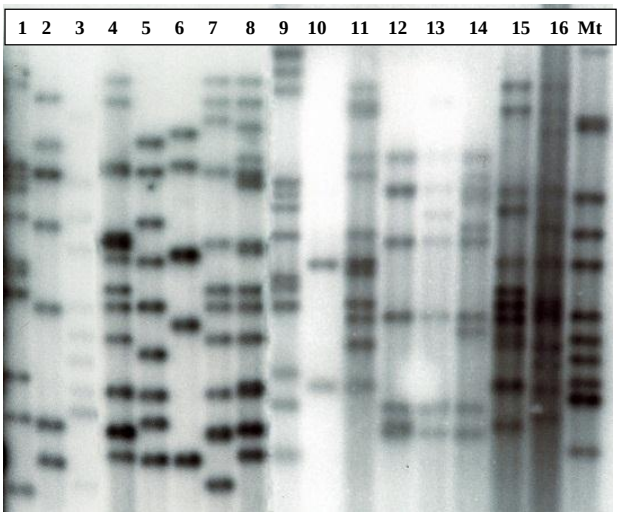

Fig. 3: IS*6110* based RFLP analysis of *M. tuberculosis* strains originating from Pakistan. The *M. tuberculosis* isolates present in the blot originated from Sindh, Khyber PakhtunKhwa, Balochistan and Punjab. Lane 10 represents isolate originating from Sindh with only two copies of IS*6110*, the lowest copy number found in isolates studied here. Mt stands for Mt14323

M. tuberculosis strains originating from northern India revealed high copy number and highly diverse RFLP patterns (Varma-Basil *et al*., 2011) as we found in our study. PCR analysis also confirmed the presence of IS*6110* in all isolates. The copy number of IS*6110* was found to vary from 3–20. The percentage of isolates having up to five copies of IS*6110* was found to be 6.5%, while 21.7% isolates have a copy number between 5 and 10. Strains with more than 10 copies were 32 out of 46 (69.5%). Our data further confirmed and extended a limited study carried out on *M. tuberculosis* strains originating from north of Pakistan (Sechi *et al*., 1996) where copy number of IS*6110* was reported from 4 to 13, confirming our data. However, the study had limited scope, as samples originating from a particular ethnic group were included. Our results provide a comprehensive evaluation of the copy number in strains originating from diverse regions of the country.

Acknowledgements

The authors are thankful to Dr. Ehsan and Dr. Abdul Rehman, Gulab Devi Hospital, Lahore; Mr. Aftab Bhatti, PMRC Centre for Tuberculosis and Chest Diseases, Lahore; Dr. Aitazaz A. Jan, Sardar Bahadur Khan Institute for Chest Diseases, Quetta; Dr. Sabira Tehseen, Ojha Institute for Tuberculosis and Chest Diseases, Karachi and Italian Cooperation Development (ICD), Peshawar for providing *M. tuberculosis* cultures or clinical specimens used in the present study. We are also thankful to Mr. Abdul Ghaffar and Mr. Shan Elahi for technical assistance.

References

Amaro A., E. Duarte, A. Amado, H. Ferronha and A. Botelho, 2008. Comparison of three DNA extraction methods for *Mycobacterium bovis*, *Mycobacterium tuberculosis* and *Mycobacterium avium* subsp. *avium*. *Lett. Appl. Microbiol*., 47: 8–11

Cave, M.D., K.D. Eisenach, P.F. McDermott, J.H. Bates and J.T. Crawford, 1991. *IS6110*: Conservation of sequence in the *Mycobacterium tuberculosis* complex and its utilization in DNA fingerprinting. *Mol. Cell Probes*, 5: 73–80

Chauhan, D.S., V.D. Sharma, D. Parashar, A. Chauhan, D. Singh, H.B. Singh, R. Das, B.M. Aggarwal, B. Malhotra, A. Jain, M. Sharma, V.K. Kataria, J.K. Aggarwal, M. Hanif, A. Shahani and V.M. Katoch, 2007. Molecular typing of *Mycobacterium tuberculosis* isolates from different parts of India based on IS*6110* element polymorphism using RFLP analysis. *Ind. J. Med. Res*., 125: 577–581

Das, S., C.N. Paramasivan, B. Lowrie, R. Prabhakar and P.R. Narayanan, 1993. IS*6110* restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, South India. *Tuber. Lung Dis*., 76: 550–554

Doroudchi, M., K. Kremer, E.A. Basiri, M.R. Kadivar, D. van Soolingen and A.A. Ghaderi, 2000. IS*6110*-RFLP and spoligotyping of *Mycobacterium tuberculosis* isolates in Iran. *Scand. J. Infect. Dis*., 3: 663–668

Gerhardt, P., R.G.E. Murray, R.N. Costilow, E.W. Nester, W.A. Wood, N.R. Krieg and G.B. Phillips, 1981. *ZN Staining.* Manual of methods for general microbiology. ASM Press, Washington, DC, USA

Hermans, P.W., A.R. Schuitema, D. van Soolingen, C.P. Verstynen, E.M. Bik, J.E. Thole, A.H. Kolk and J.D.A. van Embden, 1990. Specific detection of *Mycobacterium tuberculosis* complex strains by polymerase chain reaction. *J. Clin. Microbiol.*, 28: 2051–2058

Hermans, P.W., F. Messadi, H. Guebrexabher, D. van Soolingen, P.E. de Haas, H. Heersma, H. de Neeling, A. Ayoub, F. Portaels, D. Frommel, M. Ziribi, J.D.A. van Embden, 1995. Analysis of the population structure of *Mycobacterium tuberculosis* in Ethiopia, Tunisia, and The Netherlands: usefulness of DNA typing for global tuberculosis epidemiology. *J. Infect. Dis*., 171: 1504–1513

Lönnroth, K. and M. Raviglione, 2008. Global epidemiology of tuberculosis: prospects for control. *Semin. Respir. Crit. Care Med*., 29: 481–491

Lowenstein, 1931. Die Zachtung der Tuberkelba zillen aus dem stramenden Blute. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig.*, 120: 127

Mathuria, J.P., P. Sharma, P. Prakash, J.K. Samaria, V.M. Katoch and S. Anupurba, 2008. Role of spoligotyping and *IS6110*-RFLP in assessing genetic diversity of *Mycobacterium tuberculosis* in India. *Infect. Genet. Evol.*, 8: 346–351

McAdam, R.A., P.W. Hermans, D. van Soolingen, Z.F. Zainuddin, D. Catty, J.D. van Embden and J.W. Dale, 1990. Characterization of a *Mycobacterium tuberculosis* insertion sequence belonging to the IS3 family. *Mol. Microbiol*., 4: 1607–1613

Schürch A.C. and D. van Soolingen, 2012. DNA fingerprinting of *Mycobacterium tuberculosis*: from phage typing to whole-genome sequencing. *Infect. Genet. Evol*., 12: 602–609

Sechi, L.A., S. Zanetti, B. Delogu Montinaro, A. Sanna and G. Fadda, 1996. Molecular epidemiology of *Mycobacterium tuberculosis* strains isolated from different regions of Italy and Pakistan. *J. Clin. Microbiol*., 34: 1825–1828

Siddiqi, N., M. Shamim, S. Hussain, R.K. Choudhary, N. Ahmed, Prachee, S. Banerjee, G.R. Savithri, M. Alam, N. Pathak, A. Amin, M. Hanief, V.M. Katoch, S.K. Sharma and S.E. Hasnain, 2002. Molecular characterization of multidrug-resistant isolates of *Mycobacterium tuberculosis* from patients in North India. *Antimicrob. Agents Chemother*., 6: 443–450

Thierry, D., A. Brisson-Noel, V. Vincent-Levy-Frebault, S. Nguyen, J.L. Guesdon and B. Gicquel, 1990. Characterization of a *Mycobacterium tuberculosis* insertion sequence, *IS6110*, and its application in diagnosis. *J. Clin. Microbiol.*, 28: 2668–2673

Thorne, N., S. Borrell, J. Evans, J. Magee, D.G.D. Viedma, C. Bishop, J. Gonzalez-Martin, S. Gharbia and C. Arnold, 2011. IS*6110*-based global phylogeny of *Mycobacterium tuberculosis*. *Infect. Genet. Evol*., 11: 132–138

van Soolingen, D., P.E.W. Haas, P. Hermans, P. Groenen and J.D.A. van Embden, 1993. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. *J. Clin. Microbiol*., 31: 1987–1995

van Soolingen D., P.E.W. de Haas, P.W.M. Hermans and J.D.A. van Embden, 1994. DNA fingerprinting of *Mycobacterium tuberculosis*. *Methods Enzymol*., 235: 196–205

Varma-Basil, M., S. Kumar, J. Arora, A. Angrup, T. Zozio, J.N. Banavaliker, U.B Singh, N. Rastogi and M. Bose, 2011. Comparison of spoligotyping, mycobacterial interspersed repetitive units typing and *IS6110*-RFLP in a study of genotypic diversity of *Mycobacterium tuberculosis* in Delhi, North India. *Mem. Inst. Oswaldo Cruz*., 106: 524–525

Wilson, S.M., R. McNerney, R., P.M. Nye, P.M., P.D. Godfrey-Faussett, P.D., N.G. Stoker and A. Voller, 1993. Progress toward a simplified polymerase chain reaction and its application to diagnosis of tuberculosis. *J Clin. Microbiol.*, 31: 776–782

World Health Organization (WHO), 2012. *Global Tuberculosis Report 2012*. Geneva, Switzerland

(Received 12 December 2012; Accepted 11 February 2013)