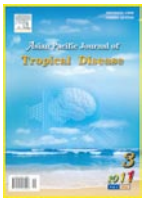
Contents lists available at ScienceDirect

Asian Pacific Journal of Tropical Disease

journal homepage:www.elsevier.com/locate/apjtd



Document heading

Molecular characterization of *Mycobacterium tuberculosis* isolates from Kandy, Sri Lanka

Magana-Arachchi DN[1*], Medagedara D[2], Thevanesam V[3]

[1]*Institute of Fundamental Studies, Hantana Road, Kandy 20000, Sri Lanka*
[2]*Respiratory Unit, Central Chest Clinic, Kandy 20000, Sri Lanka*
[3]*Department of Microbiology, Faculty of Medicine, University of Peradeniya, Peradeniya 20400, Sri Lanka*

ARTICLE INFO

Article history:
Received 20 April 2011
Received in revised form 12 May 2011
Accepted 5 July 2011
Available online 28 September 2011

Keywords:
Epidemiology
IS*6110*
RFLP
Spoligotyping
Tuberculosis
Mycobacterium tuberculosis
Heterogeneity

ABSTRACT

Objective: To determine tuberculosis epidemiology in Kandy, Sri Lanka. **Methods:** IS*6110* RFLP and spoligotyping analyses were performed on 100 *Mycobacterium tuberculosis* (*M. tuberculosis*) clinical isolates from Kandy district, Sri Lanka. RFLP hybridization patterns (n=73) were analysed by the software GeneDirectory. Spoligotypes (n=110) were compared with the international database SPOTCLUST. **Results:** The majority of the circulating *M. tuberculosis* strains in Kandy belong to a single family, but the degree of IS*6110* DNA polymorphism was high. 71 (80%) of the strains displayed distinct RFLP patterns and 63 (71%) were clustered into one main family. Within the family three isolates were grouped into one cluster while the rest isolates were grouped into one. The copy number varied from 1 to 17 while single copy strains were predominant (12) and 15 lacked the IS*6110* element. Spoligotyping revealed a total of 24 families including the 9 major families. Strains were distributed among all the three principle genetic groups PGG1, PGG2, and PGG3. Except for two strains, the rest were not defined in the latest spoligotype database SpolDB4/SITVIT. **Conclusions:** The first study of RFLP and spoligotyping of *M. tuberculosis* strains in Sri Lanka demonstrates the applicability of the genetic marker IS*6110* to differentiate strains and the heterogeneity and predominance of several worldwide-distributed spoligotypes.

1. Introduction

In 2009, there were an estimated 9.4 million incident cases (range, 8.9 million-9.9 million) tuberculosis (TB) globally (equivalent to 137 cases per 100 000 population)[1]. Most of the estimated number of cases in 2009 occurred in Asia (55%)[1]. Sri Lanka is an island in the Indian Ocean, located in Southern Asia, southeast of India, in a strategic location near major Indian Ocean sea lanes. Although India accounts for nearly one-third of the global TB burden, with a population of 19 million Sri Lanka is among the low TB prevalence countries in the region. It is estimated that prevalence of TB is 79 per 100 000 population. The incidence rate of new smear-positive cases is estimated to be 27/100 000 population[2]. The country has achieved the global targets for case detection and treatment success rate. The case detection rate for 2007 was 85.6% and treatment success for the 2006 cohort of new smear-positive cases was 87%[2]. A total of 800 new tuberculosis patients were registered in Kandy (fourth highest population density) for 2009 by the National Programme for Tuberculosis Control and Chest Diseases in Sri Lanka[3]. Of this total, 588 suffered from pulmonary disease, and the balance 212 patients from nonpulmonary disease.

During recent years molecular typing of *Mycobacterium tuberculosis* (*M. tuberculosis*) complex isolates has become a powerful tool used to understand and predict ongoing TB transmission patterns at the regional and national scales[4-8]. The current standardized and most widely used methods are IS*6110* restriction fragment length polymorphism (RFLP) typing[9,10] and, to a growing extent, a combination of interspersed repetitive-unit-variable-number tandem-repeat (MIRU-VNTR)[11-15] typing and spacer oligonucleotide typing (spoligotyping)[16,17]. The last two methods provide equal specificities and sensitivities for the detection of recent transmission chains[17]. Only a few studies have been performed in Sri Lanka applying modern molecular DNA fingerprint techniques that are able to directly trace routes of TB transmission *e.g.*, to analyze

*Corresponding author: Magana-Arachchi DN, Institute of Fundamental Studies, Hantana Road, Kandy 20000, Sri Lanka.
Tel: +94-081-2232002
Fax: +94-081-2232131
E-mail: nayomam@yahoo.com
Foundation Project: Supported by a grant from National Science Foundation Sri Lanka (grant No. RG/2006/HS/07).

the epidemiology of resistant *M. tuberculosis* strains in Sri Lanka[18,19].

There is a lack of information on important issues of TB epidemiology, such as the contribution of recent transmission to the number of annual new cases or the proportion of TB cases attributable to reactivation of latent cases. Hence, improved epidemiological data are urgently needed for better target TB control efforts and forecasting of future epidemiological trends.

Therefore, the present study was focused on characterization of *M. tuberculosis* isolates, obtained from patients in the city of Kandy, by IS*6110* RFLP assay and spoligotyping and the use of RFLP in the study of person-to-person transmission of pulmonary TB among the general population. This is the first study in Sri Lanka in which the RFLP pattern of *M. tuberculosis* strains and the spoligotyping in a population have been examined.

2. Materials and methods

2.1. Study population

A total of 121 mycobacterial isolates collected from first visit patients attending the Central Chest Clinic, Kandy, during February 2007 to 2009 were used for the molecular analysis and were devided into three groups. Group I patients were positive for acid fast bacilli on direct examination of sputum by Ziehl-Neelsen stain and/or culture and/or had radiological findings suggestive of TB (n=125). Group II were recurrent TB patients (n=12). Group III were patients who were negative for acid fast bacilli on direct examination of sputum by Ziehl-Neelsen stain and/or culture and/or had no radiological findings suggestive of TB but had symptoms of other pulmonary diseases (n=25). Ethical clearance was obtained from Faculty of Medicine, University of Peradeniya, Sri Lanka.

2.2. Collection of data and specimens

A questionnaire was administered to the patients of the study population who gave the consent to the study and the sputum samples were collected into autoclavable small wide mouth glass bottles.

2.3. Specimen processing, culture and isolation of genomic DNA from mycobacteria

Decontamination of sputum, isolation of genomic DNA and Southern blotting were performed as described by Warren *et al* previously[20-22].

2.4. Biochemical identification tests

The standard biochemical tests including para-nitrobenzoic acid (PNB) test, thiophen-2-carboxylic acid hydrazide (TCH) test, nitratase test, observation for the growth rate, pigment production and the ability to grow at 27 ℃ were done to differentiate *M. tuberculosis* complex from mycobacteria other than tuberculosis (MOTT).

2.5. IS6110-RFLP and spoligotyping

DNA fingerprinting using IS*6110* as a probe was performed for 120 *M. tuberculosis* strains according to a standardized protocol as described earlier[21]. The software GeneDirectory from SYNGENE was used to compare RFLP hybridization patterns, using the Dice coefficient of similarity and the UPGMA algorithm, with a 1% band position tolerance.

A total of 110 *M. tuberculosis* isolates were subjected to standard spoligotyping as described previously[22]. Spoligopatterns were analyzed using MS Excel data sheets and grouped together for any similarity. The data were further analyzed by comparing with the SPOTCLUST database[23].

3. Results

Of the 125 specimens from the first visit patients inoculated in Lowenstein-Jensen media, 121 grew within eight weeks of incubation. Only one of the 12 specimens from patients with recurrent tuberculosis grew during the eight week incubation period. Of the 25 specimens from control population inoculated in Lowenstein-Jensen media, four grew within five days of incubation.

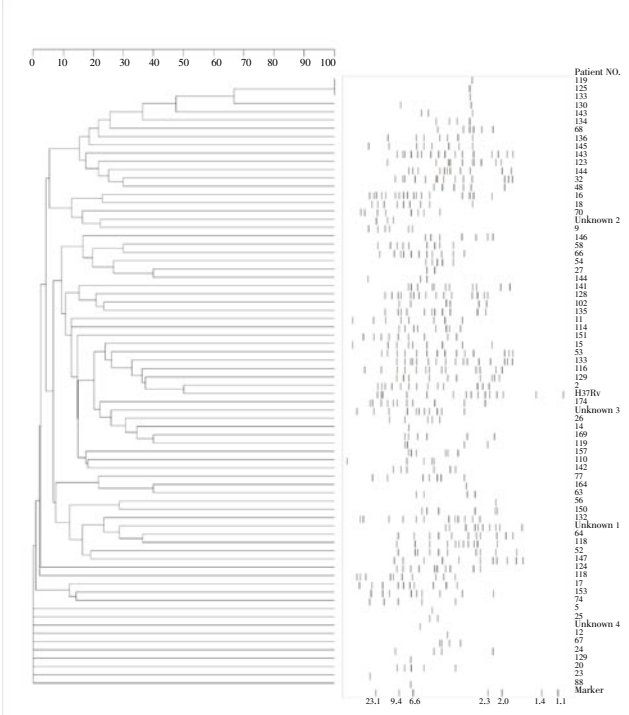


Figure 1. The degree of relatedness among the mycobacterial strains.

3.1. Biochemical identification

Biochemical tests were successfully carried out to differentiate *M. tuberculosis* complex from MOTT in 78 of 121 isolates from the first visit patients. Of these 78 isolates, 76 (97.4%) were identified as either *M. tuberculosis*, or *M.*

tuberculosis complex and 2 (2.6%) as MOTT. The isolate from the recurrent patient and the four isolates from control population were identified as MOTT.

3.2. RFLP analysis

RFLP analysis was successfully carried out to differentiate *M. tuberculosis* complex from MOTT in 100 of 122 isolates from the first visit patients. Figure 1 illustrated the high degree of DNA polymorphism in both banding patterns and number of copies of IS*6110* among strains. None of the isolates had an identical banding pattern except for the three strains with a single copy of IS*6110*.

The number of IS*6110* DNA containing Pvu II fragments in strains varied between 1 and 17 indicating that these strains contain 1 to 17 copies of the IS*6110* element. Figure 2 summarized the number of IS copies found in the strains that were investigated among the study group. Strains containing a single copy of IS*6110* were predominant among the study population (12) and except for three strains, the location of the bands in fingerprints was different and therefore the location of IS*6110* elements in the chromosomal DNA.

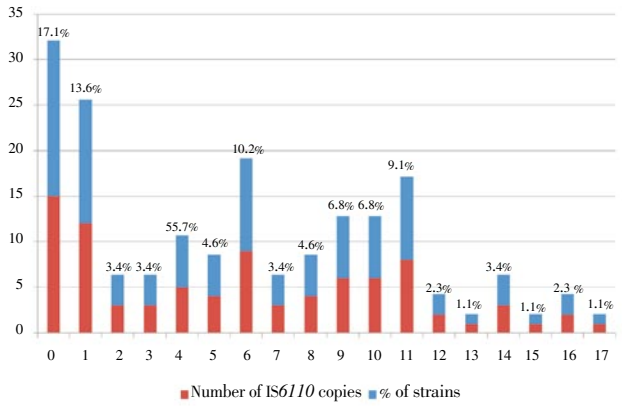


Figure 2. Number of IS*6110* copies found in the strains that were investigated among the study group.

3.3. Computer analysis of DNA fingerprints among the patients

The fingerprints of the 73 strains were subjected to similarity analysis by using the software programme GeneDirectory from SYNGENE. Figure 1 demonstrated the degree of relatedness among the mycobacterial strains. In total 71 distinct IS*6110* patterns were found with strains clustering into one main family (63) and 10 distinct strains. Within the main family three isolates were grouped into one cluster, with closely related isolates while rests of the bacterial strains (60) were grouped into one. Sub clustering pattern of the main family was interesting with total 57 bacterial strains clustering into 3 main groups with 19, 17 and 11 strains, respectively.

Table 1
Spoligotyping-based families that observed in *M. tuberculosis* isolates in patients with tuberculosis in Kandy by SPOTCLUST.

Spoligotyping-based family	Total
Family 33	45
Family 36	13
M. tuberculosis EAI1	7
M. tuberculosis Beijing	7
M. africanum	5
Family 35	5
M. tuberculosis LAM7	5
M. tuberculosis T3	3
M. bovis-BCG	2
M. tuberculosis T1	2
M. microti	2
M. tuberculosis T2	2
M. tuberculosis CAS	1
M. tuberculosis LAM8	1
M. tuberculosis Haarlem3	1
M. tuberculosis Haarlem1	1
M. tuberculosis X3	1
M. tuberculosis H37Rv	1
M. tuberculosis LAM3	1
M. tuberculosis LAM1	1
M. tuberculosis X2	1
M. tuberculosis EAI5	1
M. tuberculosis T4	1
M. tuberculosis Haarlem2	1

3.4. Spoligotyping

A total of 110 *M. tuberculosis* isolates were analyzed by spoligotyping. When spoligo patterns were compared from

Table 2
Major spoligotyping-based families found in the study population.

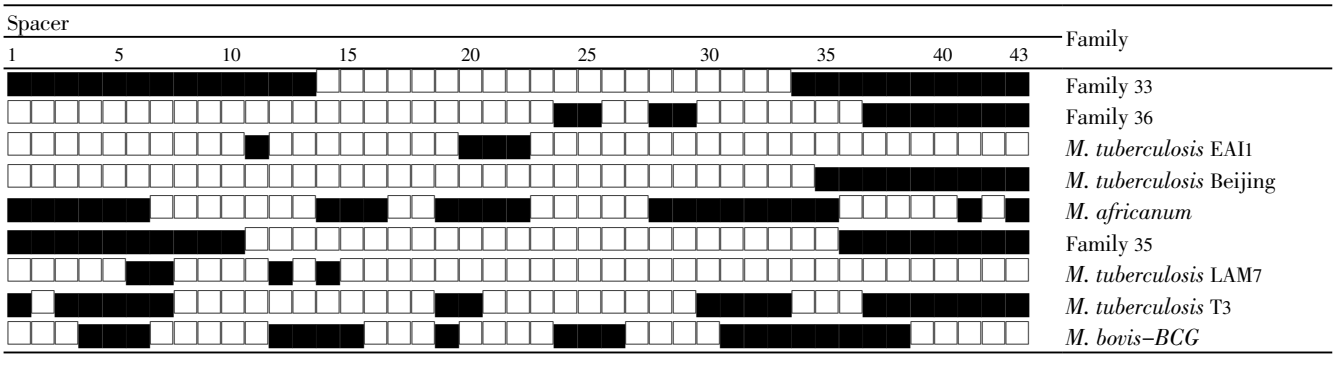


Table 3
Analysis of variables in study population [*n* (%)].

Variables		Category I (*n*=121)	Category II (*n*=25)	*P*-value[a]	*P*-value[*]
Sex	Female	34 (28.1)	13 (52.0)	0.027	-
	Male	87 (71.9)	12 (48.0)	0.027	-
Age	0-15	0 (0.0)	0 (0.0)	-	-
	16-60	112 (92.5)	16 (64.0)	0.004	-
	>61	9 (7.4)	9 (36.0)	0.004	-
Drinkers[#]		67 (55.4)	6 (24.0)	0.074	-
Smokers[#]		69 (57.0)	10 (40.0)	0.729	1.000
Contact history		37 (30.5)	1 (4.0)	0.000	0.005
Past history of TB		0 (0.0)	1 (4.0)	0.307	0.171
Travel abroad		10 (8.3)	0 (0.0)	0.001	0.212
Level of education	No formal education	37 (30.6)	1 (4.0)	0.000	0.005
	Formal education	84 (69.4)	24 (96.0)	0.000	0.005
Being imprisoned		3 (2.5)	0 (0.0)	0.079	1.000

[a]: Two sample proportion test; [#]: Males; [*]: Fisher's exact test.

SPOTCLUST which was based on the SpolDB3 model, 24 distinct families (Table 1) were identified including the nine major spoligotyping-based families (Table 2) *i.e. M. africanum*, *M. bovis*, East African-Indian (EAI), Beijing, Haarlem, Latin American and Mediterranean (LAM), Central and Middle Eastern Asian (CAS), a European family X, and a default family T. Except for two strains 000000000003771 (ST1) and 000000000000031 (ST 585) the tested strains were not defined in the latest spoligotype data bases SpolDB4/SITVIT.

3.5. Analysis of socio-demographic data

Data were analysed for 146 patients. Of these, 121 were positive for acid fast bacilli on direct examination of sputum by Ziehl-Neelsen stain, culture and/or had radiological findings suggestive of TB while rest 25 were from control population. The results of the different parameters analyzed were summarized in Table 3.

In *M. tuberculosis* culture positive category the majority were males (71.9%) with 28.1% females. However, in control population this was different with both females and males were equal in ratio. In Category I, 92% of the population was in the age group of between the ages of 16 and 60, and the less affluent, which nevertheless, form the backbone of the economy of the country. There were significant differences among two categories for contact history of TB, being out of the country (travel-abroad) by the patient, age, sex and level of education.

4. Discussion

In the study presented, a detailed picture of TB, in the district of Kandy, Sri Lanka has been attained by combining IS*6110* RFLP typing, spoligotyping, biochemical analysis and classical epidemiological methods. The data obtained are based on a study period of 2 years in which 160 patients attend the Central Chest Clinic, Kandy, for pulmonary treatment.

Two of the isolates from the first visit patients in this study who were treated as having tuberculosis in the Central Chest Clinic, Kandy were identified as MOTT biochemically. This finding is comparable to the study by Elwitigala *et al* who reported mycobacterial isolates obtained from patients throughout Sri Lanka excluding the districts of Mullaitivu and Kilinochchi, cultured during the period of 2005-2007. In their study, MOTT accounted for 3.27% of the total isolates[24]. The isolate from the recurrent tuberculosis patient was found to be a MOTT strain with rifampin resistance (unpublished data), which explains the reason for the treatment failure in that patient. In RFLP analysis all three strains did not produce any banding pattern with IS*6110* confirming their species variation.

The epidemiological analysis of TB using IS*6110* is based on the observation that the polymorphism of IS*6110* RFLP patterns among unrelated clinical isolates is high, whereas epidemiologically related *M. tuberculosis* strains show identical or similar (one band variation) fingerprints[25]. In this study we observed that the majority of circulating *M. tuberculosis* strains in Kandy belong to a single family, but the degree of IS*6110* DNA polymorphism among strains was high. Interpretation of the clustering of the isolates in the family is complex and the explanation for the high degree of polymorphism in DNA fingerprints can be due to the different origins. Without performing DNA sequencing analysis definite conclusions cannot be made whether the isolates underwent any genetic changes within a given time.

Among the strains tested there were 27 strains that lacked the IS*6110* element. Among these 15 strains were confirmed as *M. tuberculosis* by biochemical testing while three were identified as MOTT with DNA sequencing and biochemical analysis. This has implications for diagnosis of infection when IS*6110* is used as the sequence for DNA amplification. Previous studies showed that *M. tuberculosis* strains carrying one or few IS*6110* copies are often difficult to differentiate by IS*6110* standard RFLP analysis because of a site specific preference for insertion of the IS element. Therefore, to further differentiate the strains other genetic markers such as polymorphic rich GC repetitive sequence (PGRS) and direct repeats (DR) have been used[26,27]. In this

study for DNA fingerprinting restriction enzyme *Pvu* II was used to cleave the chromosomal DNA of the mycobacterial strains. The enzyme cleaves the 1.35-kb IS*6110* element at a single site. In the present study except for three strains, the location of the bands in fingerprints were different and therefore the location of IS*6110* elements in the chromosomal DNA was different. Therefore, *M. tuberculosis* strains carrying one or few IS*6110* copies were differentiated without difficulty. In this study 52% of the isolates had five or less than five copies. This pattern is similar to a previous study in which 68% was rescored from recurrent TB patients[18] and also from other countries in the Asian region, such as India, Malaysia, Oman and Hong Kong[28-36].

We included only culture-positive patients to enhance the possibility of typing actively transmitting strains. Although the exclusion of culture-negative cases could potentially have introduced a bias in the strain composition, we were unable to perform RFLP on all patients due to study constraints. Additionally, we found high strain diversity, with a large number of small clusters, as well as a significant proportion of strains hitherto unreported in the global databases. It has also been noted that the DNA polymorphism could be made use of to identify transmission rates of drug resistance and drug sensitive strains. RFLP typing can be carried out on primary isolates to determine drug resistance. By comparison of these isolates with the existing RFLP patterns of the drug resistance isolates the time taken for determining drug resistance may be much shorter compared with the conventional antibiotic sensitivity testing which takes more than four weeks.

In this study we used the algorithm, SPOTCLUST which incorporates biological information on spoligotype evolution, without attempting to derive the full phylogeny of *M. tuberculosis* complex. Spoligotyping of 110 *M. tuberculosis* isolates revealed a total of 24 families including the nine major families. The most predominant group among the isolates of *M. tuberculosis* corresponded to Family 33. In this family, only spacers 33-34 are absent and recently described clade MANU[37] of Indian origin belongs to this family. When compared to the single publication[19] of spoligotyping patterns from Sri Lanka similarity was observed in only five clades namely Beijing, T1, EAI5, T2 and T3. According to the analysis, bacterial strains were distributed among all three principal genetic groups PGG1, PGG 2 and PGG3. Segregation of *M. tuberculosis* into 'ancestral' versus 'modern' lineages based on PGG indicates that isolates from Kandy have originated from both lineages. In our spoligotyping patterns we found high strain diversity and except for two strains 000000000003771 (ST1) and 000000000000031(ST 585) the tested strains were not defined in the latest spoligotype data bases SpolDB4/SITVIT.

In conclusion, a high degree of polymorphism was observed both in the DNA fingerprinting patterns of *M. tuberculosis* isolates, with a copy number varying from 1-17, while in spoligotyping 24 distinct families were identified including the nine major spoligotyping-based families. However, in the absence of DNA sequencing analysis, conclusions cannot be made whether these isolates were genetically different or they underwent any genetic changes within a given time even though the origin was from a common ancestor. This is the first study in Sri Lanka in which the RFLP pattern of *M. tuberculosis* strains and the spoligotyping in a population have been examined. By using the genetic marker of IS*6110* it was possible to differentiate most of the *M. tuberculosis* isolates. The preliminary inferences from this study plead for a more extensive analysis of the data to study the variability of *M. tuberculosis* strains and their transmission dynamics.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

We thank Ms. Meegahakumbura MGM and Mr. de Silva BAC for technical support. The study was supported by the grant RG/2006/HS/07 from National Science Foundation Sri Lanka.

References

[1] WHO. *Global tuberculosis control*. Geneva: WHO; 2010. [Online] Available from: http://www.who.int/tb/publications/2010/factsheet_tb_2010.pdf. [Accessed on Dec 20th, 2010]

[2] WHO. TB country profile. Geneva: WHO; 2006. [Online] Available from: http://apps.who.int/globalatlas/predefinedReports/TB/PDF_Files/lka.pdf. [Accessed on Aug 8th, 2009]

[3] Sri Lanka. *National programme for tuberculosis control and chest disease. Sri Lanka*; 2009. [Online] Available from: http://203.94.76.60/TBWeb/ Quarterly% 20Reports /2009/Case%20Finding(sum)%20-%202009.pdf. [Accessed on Deb 3rd, 2010]

[4] Alonso M, Borrell S, Martínez Lirola M. A proposal for applying molecular markers as an aid to identifying potential cases of imported tuberculosis in immigrants. *Tuberculosis* 2008; **88**: 641-647.

[5] de Viedma DG, Mokrousov I, Rastogi N. Innovations in the molecular epidemiology of tuberculosis. *Enferm Infecc Microbiol Clin* 2011; **29** (Supl 1):8-13.

[6] Borrell S, Español M, Orcau A, Tudó G, March F, Caylà JA, et al. Factors associated with differences between conventional contact tracing and molecular epidemiology in study of tuberculosis transmission and analysis in the city of Barcelona, *Spain J Clin Microbiol* 2009; **47**: 198-204.

[7] Alonso Rodríguez N, Chaves F, Iñigo J, Bouza E, García de Viedma D, Andrés S, et al. Transmission permeability of tuberculosis involving immigrants, revealed by a multicentre analysis of clusters. *Clin Microbiol Infect* 2009; **15**: 435-42.

[8] Barniol J, Niemann S, Louis VR, Brodhun B, Dreweck C, Richter E, et al. Transmission dynamics of pulmonary tuberculosis between autochthonous and immigrant sub populations. *BMC Infect Dis* 2009; **9**: 197.

[9] van Embden JD, Cave MD, Crawford JT, Dale JM, Eisenach

KD, Gicquel B, et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting: recommendations for a standardized methodology. *J Clin Microbiol* 1993; **31**: 406-409.

[10] McEvoy CRE, Falmer AA, van Pittius NCG, Victor TC, van Helden PD, Warren RM. The role of IS*6110* in the evolution of M*ycobacterium tuberculosis*. *Tuberculosis* 2007; **87**: 393-404.

[11] Scott AN, Menzies D, Tannenbaum TN, Thibert L, Kozak R, Joseph L, et al. Sensitivities and specificities of spoligotyping and mycobacterial interspersed repetitive unit-variable-number tandem repeat typing methods for studying molecular epidemiology of tuberculosis. *J Clin Microbiol* 2005; **43**: 89-94.

[12] Filliol I, Motiwala AS, Cavatore M, Qi W, Hazbón MH, Bobadilla del Valle M, et al. Global phylogeny of *Mycobacterium tuberculosis* based on single nucleotide polymorphism (SNP) analysis: insights into tuberculosis evolution, phylogenetic accuracy of other DNA fingerprinting systems, and recommendations for a minimal standard SNP set. *J Bacteriol* 2006; **188**: 759-772.

[13] Alix-Beguec C, Harmsen D, Weniger T, Supply P, Niemann S. Evaluation and strategy for use of MIRU-VNTRplus, a multifunctional database for online analysis of genotyping data and phylogenetic identification of Mycobacterium tuberculosis complex isolates. *J Clin Microbiol* 2008; **46**: 2692-2699.

[14] Supply P, Allix C, Lesjean S, Cardoso-Oelemann M, Rusch-Gerdes S, Willery E, et al. Proposal for standardization of optimized mycobacterial interspersed repetitive unit-variable-number tandem repeat typing of *Mycobacterium tuberculosis*. *J Clin Microbiol* 2006; **44**: 4498-4510.

[15] Hanekom M, van der Spuy GD, Gey van Pittius NC, McEvoy CRE, Hoek KGP, Ndabambi SL, et al. Discordance between MIRU-VNTR and IS*6110* RFLP genotyping when analyzing *Mycobacterium tuberculosis* Beijing strains in a high incidence setting. *J Clin Microbiol* 2008. doi: 10.1128/JCM.00770-08.

[16] Honisch C, Mosko M, Arnold C, Gharbia SE, Diel R, Niemann N. Replacing reverse line blot hybridization spoligotyping of the *Mycobacterium tuberculosis* complex. *J Clin Microbiol* 2010; **48**(5): 1520-1526.

[17] Oelemann MC, Diel R, Vatin V, Haas W, Rusch-Gerdes S, Locht C, et al. Assessment of an optimized mycobacterial interspersed repetitive-unit-variable-number tandem-repeat typing system combined with spoligotyping for population-based molecular epidemiology studies of tuberculosis. *J Clin Microbiol* 2007; **45**: 691-697.

[18] Magana-Arachchi DN, Perera AJ, Senaratne V, Chandrasekaran NV. Pattern of drug resistance and RFLP analysis on *Mycobacterium tuberculosis* strains isolated from recurrent tuberculosis patients. *Southeast Asian J Trop Med Public Health* 2010; **41**(3): 583-589.

[19] Rajapaksa US, Victor TC, Perera AJ, Warren RM, Seneviranthne SM. Molecular diversity of *Mycobacterium tuberculosis* isolates from patients with pulmonary tuberculosis in Sri Lanka. *Trans R Soc Trop Med Hyg* 2008; **102**(10): 997-1002.

[20] Warren R, de Kock M, Engelke E, Myburgh R, Gey VP, Victor T, et al. Safe *Mycobacterium tuberculosis* DNA extraction method that does not compromise integrity. *J Clin Microbiol* 2006; **44**: 254-256.

[21] van Embden JDA, Cave MD, Crawford JT, Dale J, Eisenach KD, Gicquel B et al. Strain identification of *Mycobacterium tuberculosis* by DNA fingerprinting : recommendation for a standardized methodology. *J Clin Microbiol* 1993; **31**: 406-409.

[22] Kamerbeek J, Schouls L, Kolk A, van Agterveld M, van Soolingen D, Kuijper S, et al. Simultaneous detection and strain differentiation of *Mycobacterium tuberculosis* for diagnosis and epidemiology. *J Clin Microbiol* 1997; **35**: 907-914.

[23] Vitol I, Driscoll J, Kreiswirth B, Kurepina N, Bennett KP. Identifying *Mycobacterium tuberculosis* complex strain families using spoligotypes. *Infect Genet Evol* 2006; **6**: 491-504.

[24] Elwitigala J, Jayawardane KDJHM, Wickramanayake GH. An analysis of drug susceptibility of *Mycobacterium* species isolated at National Tuberculosis Reference Laboratory, Sri Lanka from year 2005 to 2007. *Bull Sri Lanka Coll Microbiol* 2008; **6**: 18-19.

[25] van Soolingen D, Qian L, de Haas PE, Douglas JT, Traore H, Portaels F, et al. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. *J Clin Microbiol* 1995; **33**: 3234-3238.

[26] Flores L, Jarlsberg LG, Kim EY, Osmond D, Grinsdale J, Kawamura M, et al. Comparison of restriction fragment length polymorphism with the polymorphic guanine-cytosine-rich sequence and spoligotyping for differentiation of *Mycobacterium tuberculosis* isolates with five or fewer copies of IS*6110*. *J Clin Microbiol* 2010; **48**: 575-578.

[27] Bauer J, Andersen AB, Kremer K, Miorner H. Usefulness of spoligotyping to discriminate *IS6110* low copy number *Mycobacterium tuberculosis* complex strains cultured in Denmark. *J Clin Microbiol* 1999; **37**: 2602-2606.

[28] van Soolingen D. *Use of DNA fingerprinting in the epidemiology of tuberculosis (thesis)*. Mycobacterial Department, Laboratory for Infectious Diseases and Perinatal Screening (LIS) National Institute of Public Health and the Environment (RIVM), Bilthoven, The Netherlands; 1996.

[29] Wu Q, Zhou P, Qian S, Qin X, Fan Z, Fu Q, et al. Cloning, expression, identification and bioinformatics analysis of Rv3265c gene from *Mycobacterium tuberculosis* in *Escherichia coli*. *Asian Pac J Trop Med* 2011; **4**(5): 266-270.

[30] Olusoji D, Eltayeb O. Prevalence and risk factors associated with drug resistant TB in South West, Nigeria. *Asian Pac J Trop Med* 2011; **4**(2): 148-151.

[31] Tiwari N, Kandpal V, Tewari A, Rao KRM, Tolia VS. Investigation of tuberculosis clusters in Dehradun city of India. *Asian Pac J Trop Med* 2010; **3**(6): 486-490.

[32] Buijtels PCAM, Iseman MD, Parkinson S, de Graaff CS, Verbrugh HA, Petit PLC, et al. Misdiagnosis of tuberculosis and the clinical relevance of non-tuberculous mycobacteria in Zambia. *Asian Pac J Trop Med* 2010; **3**(5): 386-391.

[33] Garberi J, Labrador J, Garberi F, Garberi JE, Peneipil J, Garberi M, et al. Diagnosis of *Mycobacterium tuberculosis* using molecular biology technology. *Asian Pac J Trop Biomed* 2011; **1**(2): 89-93.

[34] Anindita M, Pranita DK, Badole CM, Harinath BC. Prospective study of SEVA TB peroxidase assay for cocktail antigen and antibody in the diagnosis of tuberculosis in suspected patients attending a tertiary care hospital located in rural area. *Asian Pac J Trop Med* 2010; **3**(5): 356-359.

[35] Peter G, Basti SR, Joy AS. Multiple bilateral costo-chondral abscesses due to *Mycobacterium tuberculosis*. *Asian Pac J Trop Med* 2010; **3**(11): 922-924.

[36] Chandrakant Patil, Rashmi Kharat (Patil), Prasad Deshmukh, Sameer Singhal, Blenda D'Souza. Tuberculous retropharyngeal abscess without cervical spine TB. *Asian Pac J Trop Med* 2010; **4**(3): 251-252.

[37] Singh UB, Suresh N, Bhanu NV, Arora J, Pant H, Sinha S. Predominant tuberculosis spoligotypes Delhi, India. *Emerg Infect Dis* 2004; **10**: 1138.