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Pattern of Circulating *Mycobacterium tuberculosis* Strains in Sri Lanka

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1. Introduction

Historically tuberculosis (TB) has been and today it remains the leading cause of mortality in adults due to an infectious agent, and with the increasing prevalence of TB's resistance to the drugs of choice the problem posed by TB to public health should not be underestimated. The strain classification or sub typing is important epidemiologically for recognizing outbreaks of infection, detecting the cross transmission of nosocomial pathogens, determining the source of infection, recognizing the particularly virulent strains of organisms and in monitoring vaccination programs (Olive and Bean, 1999). Sub typing has been accomplished by a number of different approaches, and if the method to be successful it has to satisfy several criteria. Mainly all the organisms within a species must be type able by the method used and secondly, it must have high differentiation power and the methodologies should be reproducible (Olive and Bean, 1999).

Molecular epidemiology makes use of the genetic diversity within strains of infectious organisms to track the transmission of these organisms in human populations and to evaluate the host and parasite - specific risk factors for disease spread. In the past, efforts to type strains of *M. tuberculosis* in human hosts were hampered by the lack of a strain specific immune response and by an apparent lack of genetic polymorphism in the organism. However advances in the field of TB research paved the way in developing molecular techniques which allowed the identification and tracking of individual strains of *M. tuberculosis*.

1.1 Molecular epidemiology of tuberculosis

1.1.1 Organization and sequence of genome

It is thought that the progenitor of the *M. tuberculosis* complex comprising *M. tuberculosis, M. bovis*, *M. bovis BCG, M .microti* and *M. africanum* arose from a soil bacterium and that the human bacillus may have been derived from the bovine form following the domestication of cattle. The complex lacks inter strain genetic diversity, and nucleotide changes are very rare. The H_{37}Rv strain of *M. tuberculosis* was isolated in 1905, and since then it has found extensive, world - wide application in biomedical research. It has retained full virulence in animal models of tuberculosis and also susceptible to drugs and amenable to genetic manipulation (Cole et al., 1998). The genome comprises 4,411,529 base pairs (bp) with a G + C content of 65.6%, which is relatively constant throughout the genome and the genome is

rich in repetitive DNA, and several regions show higher than average G + C content which corresponds to sequences belonging to a large gene family that includes the polymorphic G + C rich sequences (PGRSs) (Cole et al., 1998).

1.1.2 Insertion sequences and prophages

The genome of H_{37}Rv contains 16 copies of the insertion sequence IS*6110*, and 6 copies of the insertion element IS*1081* (Philipp et al., 1996). Cole et al in 1998 found another 32 different insertion sequences, and of the 13E12 family of repetitive sequences which exhibit some of the characteristics of mobile genetic elements. Most of the insertion sequences belong to the IS*3* and IS*256* families but six of them form a new group (Cole et al., 1998). Most of the insertion sequences in *M. tuberculosis* H_{37}Rv are inserted in intergenic or non-coding regions (close to tRNA genes), with clustering suggesting the existence of insertion hot-spots that prevent genes from being inactivated (Cole et al., 1998). The chromosomal distribution of the insertion sequences is informative and shows selection against insertions in the quadrant encompassing *oriC* and an overrepresentation in the direct repeat region that contains the prototype IS*6110* (Cole et al., 1998). According to Cole et al, two prophages, phiRv1 and phiRv2 (both – 10 kb in length) have been detected in the H_{37}Rv genome sequence and only IS*1532* exhibited significant variability indicating that most of the prophages and insertion sequences are currently stable (Cole et al., 1998).

1.1.3 Insertion sequence IS*6110*

Insertion sequence IS*6110* is a 1361 base pair long sequence that was detected exclusively in members of the TB complex and differences of only a few nucleotides have been detected between the sequenced copies. The sequence is flanked by two 28 base pair repeats and has two open reading frames (ORF) that show homology with genes coding for putative transposases of other elements of the IS3 family, which are typical features of mobile elements (Suffys et al., 1997). Though the transposition of IS*6110* has not been experimentally demonstrated in *M. tuberculosis*, mobility of IS*986* has been observed. The number of copies of IS*6110* present in the genome is species and strain specific (Suffys et al., 1997) and most strains of *M. tuberculosis* carry between 8 to 15 copies in different positions of the genome. Several single copy strains of *M. tuberculosis* had been reported, while other studies found some *M. tuberculosis* isolates, which were devoid of the IS*6110* sequence.

1.2 Genotyping methods

Mycobacterial strain typing by means of molecular methods has become an important instrument for tuberculosis surveillance, control and prevention (van Soolingen, 1998).

1.2.1 Restriction Fragment Length Polymorphism (RFLP)

Among DNA fingerprinting methods which restriction fragment length polymorphism (RFLP) typing is the most common method used has permitted novel investigations of the epidemiology and pathogenesis of tuberculosis. The use of IS*6110*, an insertion sequence which is present in *Mycobacterium tuberculosis*, is generally considered to be the gold standard for tuberculosis molecular epidemiology studies (van Embden et al., 1993), but other molecular typing techniques could be used as adjuncts in selected circumstances

(Cohn and O'Brien, 1998). Das et al., 1995 studied the utility of a standardized IS*6110* / *Pvu* II, RFLP typing method for distinguishing between isolates of *M. tuberculosis*, and assess the potential for distinguishing between relapse versus re infection rates. They concluded that despite the high frequency of single and zero band isolates in the population, the discriminatory power of RFLP typing with IS*6110* is sufficiently high to be useful for clinical and epidemiological studies (Das et al., 1995). Sahadevan et al., 1995 observed that *M. tuberculosis* isolates obtained from patients' sputa on diagnosis and during follow-up after short-course chemotherapy in Madras, had either no copy or only a single copy of IS*6110*. This posed a limitation for DNA fingerprinting with an IS*6110*-based probe to determine the frequency of exogenous re infection versus that of endogenous reactivation. They overcame this limitation by using an alternate probe, the direct-repeat element. Comparison of pre- and post treatment isolates by direct-repeat restriction fragment length polymorphism analysis indicated a high degree of endogenous reactivation among patients who had relapses after the successful completion of chemotherapy (Sahadevan et al., 1995). van Duin et al., investigated an episode of laboratory cross contamination using IS*6110* RFLP typing and it proved to be a useful tool to trace the source of contamination (van Duin et al., 1998).

1.2.2 Spoligotyping

Spoligotyping is a technique based on the polymorphism of the direct repeat (DR) locus present in *M. tuberculosis* DNA. The DR sequences are composed of multiple 36bp copies, interspersed by short non repetitive sequences (Kmerbeek et al., 1997). However, the spacer sequences between any two specific direct repeats are conserved among strains (Kmerbeek et al., 1997). The presence or absence of each non repetitive sequence creates a pattern for each strain when analyzed by spoligotyping. A database of spoligotypes of *M. tuberculosis* has been created (Sola et al., 2001) containing the global distribution and phylogenetic analysis of worldwide spoligotypes and this database is useful for comparing the patterns found in different regions of the world, enabling a better understanding of the dynamics of disease distribution. Simultaneous use of RFLP and spoligotyping methods increases understanding the epidemiological factors that facilitates the spread of tuberculosis inside a country. Studies have revealed that both transmission and reactivation are contributing to the spread of tuberculosis in the world. Another study result highlighted the importance of molecular epidemiology studies of tuberculosis in insufficiently studied regions with a high TB burden, in order to uncover the true extent of genetic diversity of the pathogen.

1.2.3 MIRU

Another genotyping technique which becoming popular is mycobacterial interspersed repeat units (MIRU). MIRU genotyping categorizes the number and size of the repeats in each of 12 independent MIRUs, with the use of a polymerase-chain-reaction (PCR) assay, followed by gel electrophoresis to categorize the number and size of repeats in 12 independent loci, each of which has a unique repeated sequence (Supply et al., 2001). The discriminatory power of MIRU genotyping is almost as great as that of IS*6110*-based genotyping (Supply et al., 2001). Unlike IS*6110*-based genotyping, MIRU analysis can be automated and can thus be used to evaluate large numbers of strains, yielding intrinsically digital results that can be easily catalogued on a computer data base (Supply et al., 2001). A Web site has been set up so that a worldwide data base of MIRU patterns can be created

(Supply et al., 2001). MIRU genotyping is technically simpler than IS*6110*-based genotyping and can be applied directly to *M. tuberculosis* cultures without DNA purification (Barnes and Cave, 2003).

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. 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 the epidemiology of resistant *M. tuberculosis* strains in Sri Lanka.

Therefore, this chapter focus on Molecular Epidemiology of Tuberculosis including the two studies conducted in Sri Lanka on *M. tuberculosis* isolates with IS*6110* RFLP assays and spoligotyping.

2. Genotyping by RFLP & spoligotyping

2.1 Study population

One hundred and seventy sputum smear positive TB patients admitted for re-treatment to Chest Hospital, Welisara, Sri Lanka were enrolled for the first study. There were 24 patients among the chest clinic attendees having a history of imprisonment before being diagnosed as having TB (ex-prisoners). The study population consisted of 131 culture positive re treatment TB patients. Remaining patients were excluded, as their cultures were negative. The second study consisted of 121 mycobacterial isolates collected from first visit patients attending the Central Chest Clinic, Kandy, Sri Lanka who 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.

2.1.1 Specimen processing, culture and isolation of genomic DNA from mycobacteria

Sputum samples were decontaminated using the standard Sodium hydroxide – sodium citrate – N acetyl – L – cysteine method and were inoculated on Lowenstein-Jenson (LJ) medium and Middle brook 7H-10 agar medium to isolate the *M. tuberculosis* strains. The strains of *M. tuberculosis* obtained from these media were used for antibiotic sensitivity testing and RFLP analysis. Isolation of Genomic DNA was performed using standard protocols.

2.1.2 Antibiotic sensitivity testing

In the first study 12 drugs were tested and the criterion for resistance was based on the 1% survival level of the organism in comparison with a control medium without the drug. Resistance was defined as survival of the tubercle bacilli at the following drug concentrations (µg/ml); isoniazid (H), 0.2; rifampin (R), 1.0; streptomycin (S), 2.0; ethambutol (E), 5.0; pyrazinamide (Z), 25.0; *p*-amino salicylic acid (PASER), 2.0; ethionamide (Et), 5.0; cycloserine (Cs), 30.0; kanamycin (Km), 5.0; viomycin (Vm), 5.0; ciprofloxacin (Cx), 2.0 and rifabutin (Rb), 2.0 (Magana Arachchi et al., 2010). For the second study isoniazid and rifampin were tested.

2.2 IS*6110* – RFLP and spoligotyping

In the first study RFLP analysis of the 131 isolates by Southern blotting and DNA hybridization with IS*6110* was performed according to the standard fingerprinting method (van Embden et al., 1993). In the second study DNA fingerprinting using IS*6110* as a probe was performed for 120 *M. tuberculosis* strains according to standardized protocol of van Embden et al., 1993. 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 and the spoligo patterns were analyzed using MS Excel data sheets and grouped together for any similarity. The data was further analyzed by comparing with the SPOTCLUST data base

2.2.1 IS*6110* – RFLP

2.2.1.1 Digestion of chromosomal DNA for RFLP

Genomic DNA (5 μg) per each sample / isolate (obtained from above procedure) was digested with restriction enzyme *Pvu* II in a final volume of 25 μl as recommended by the manufacturer (Pharmacia Biotech).

2.2.1.2 Separation of DNA fragments by electrophoresis

The *Pvu* II digested chromosomal DNA from samples was size fractionated on 1% agarose gels. Along with the samples a DNA marker (λ cleaved *Hind* III/PhiX 174-*Hae* III) and DNA from the reference *M. tuberculosis* strain cleaved with *Pvu* II was included.

2.2.1.3 Southern blotting

Gel was soaked in HCl (0.25 M) for 20 minutes, followed by several volumes of gel soak I solution (1.5 M NaCl, 0.5 M NaOH) for 30 minutes and next in several volumes of gel soak II solution (1 M Tris HCl, pH 8.0, 1.5 M NaCl) for 1 hr at room temperature with constant shaking. The gel was then Southern blotted onto nylon filters.

2.2.1.4 Preparation of DNA probe by PCR

The IS*6110* – specific DNA probe of 245 bp was amplified by PCR using the oligonucleotide primers INS-1 (5'-CGTGAGGGCATCGAGGTGGC-3') and INS- 2 (5'-GCGTAGGCGTCGGT GAC AAA-3') corresponding to bp 631 to 650 and 856 to 875 which are based on the positions of the IS*6110* sequence, respectively.

2.2.1.5 Preparation of the labelled probe for RFLP

The probe was labelled by using a direct nucleic acid labelling and detection kit (ECL, Amersham, RPN 3001) according to the manufacturer's instructions. DNA to be labelled was diluted to a concentration of 10 ng/μl using water. DNA was denatured by heating for 5 min in a boiling water bath. The DNA sample was immediately cooled on ice for 5 min and centrifuged (2 sec, 12000 g). An equivalent volume of DNA labelling reagent was added to the cooled DNA and mixed thoroughly. An equivalent volume of glutaraldehyde was added to the solution, and spun briefly in a micro centrifuge. Next the DNA was incubated for 10 min at 37 ^{0}C. The labelled DNA probe was stored in 50% (v/v) glycerol at –20 ^{0}C until used.

2.2.1.6 Hybridization and detection

The nylon filter was pre hybridized with hybridization buffer (0.125 ml/cm^2) in a sealed plastic bag for 1 hour at 42 ^{0}C. Labelled probe was mixed with the hybridization buffer, and was added to the solution containing the filter. The nylon filter was hybridized overnight at 42 ^{0}C with shaking. Next the hybridized filter was removed from the plastic bag and placed in a clean plastic box and the filter was washed twice (2x10 min) with the pre warmed (55 ^{0}C) primary wash buffer at 55 ^{0}C. Then the filter was placed in a clean plastic box and washed twice with the secondary wash buffer for 5 min at room temperature on a shaking platform**.**

Next the filters were treated with detection reagents as per manufacturers instructions in a dark room and then exposed to Kodak XAR-5 film for overnight at room temperature. The nylon filters were stored under moist conditions at 4 ^{0}C for further use.

2.2.2 Spoligotyping

Spoligotyping was carried out as previously described by Kmerbeek et al., 1997.

2.2.2.1 Preparation of the membrane containing the spacer-oligonucleotides

Standard spacer oligonucleotides (n=43) were diluted to the optimized concentrations in 150 µl 500 mM $NaHCO_3$, pH 8.4. Next Biodyne C membrane was activated by 10 min incubation in 10 ml freshly prepared 16% (w/v) 1-ethyl -3-(3-dimethyl aminopropyl) carbodiimide (EDAC) in demineralized water, in a rolling bottle at room temperature. Membrane was rinsed with water for 2 min, placed on the mini blotter, and filled the slots with diluted oligonucleotides. Next membrane was incubated for 1 min at room temperature and then oligonucleotide solutions were removed by aspiration. Next blot was incubated in 100 mM NaOH for 10 min in a sealed bag to in activate the membrane. Membrane was washed in 250 ml 2x SSPE/ 0.1%SDS for 5 min at 60 ^{0}C and then in 100 ml 20 mM EDTA, pH 8.0 for 15 min at room temperature. Membrane was stored at 4 ^{0}C until used.

2.2.2.2 PCR for DR

PCR was performed with primers Dra (5'-GGTTTTGGGTCTGACGAC-3') (biotinylated 3'end) and Drb (5'-CCGAGAGGGGACGGAAAC-3'). The PCR reaction contained 10 ng of DNA, 1U of *Taq* DNA polymerase, 20 pmol of each primer and 200 µm dNTPs. The cycling parameters were 3 min at 96 ^{0}C, followed by 1 min at 96 ^{0}C, 1 min at 55 ^{0}C and 30 sec at 72 ^{0}C for 30 cycles.

2.2.2.3 Hybridization with PCR product and detection

Membrane was washed in 250 ml 2x SSPE/ 0.1% SDS for 5 min at 60 ^{0}C and was placed in the mini blotter in a way that the slots were perpendicular to the line pattern of the applied oligonucleotides. 20 µl of the PCR product was added into 150 µl 2x SSPE/0.1% SDS and the diluted product was denatured by heating for 10 min at 100 ^{0}C and was immediately cooled on ice. Next residual fluid was removed from the slots and the slots were filled with diluted PCR product and incubated for 1hr at 60 ^{0}C. Next samples were removed and the membrane was washed twice in 250 ml 2x SSPE/ 0.5% SDS for 10 min at 60 ^{0}C. Membrane was placed in a sealed bag and allowed it cool to prevent inactivation of the peroxidase. Membrane was

incubated in 1:4000 diluted streptavidin-peroxidase conjugate: (2.5 µl streptavidin-peroxidase conjugate in 10 ml of 2x SSPE/ 0.5% SDS for 45-60 min in a sealed bag). Next membrane was washed twice in 250 ml 2x SSPE/ 0.5% SDS for 10 min at 42 ^{0}C. Then membrane was rinsed twice in 250 ml 2x SSPE for 5 min at room temperature. For chemiluminescence detection of hybridizing DNA the membrane was incubated for 1 min in 20 ml ECL detection liquid. Membrane was covered with a Saran-wrap and was exposed to an X-ray film overnight at room temperature. Finally, the X-ray film was developed using Kodak developer (1 min) and fixer (3 min).

3. Transmission of tuberculosis

3.1 Pattern of TB transmission in first study

In the first study the persistence of *M. tuberculosis* strains in a population was examined. To estimate the degree of transmission of TB within the general population (Colombo district) and among the prison population, analysis of the RFLP data were carried out in three ways: (I) determination of the degree of clustering of matching DNA types as a measure of transmission within the general population (ii) determination of the clustering and matching DNA types among the prisoners and (iii) degree of clustering and matching types among the prisoners , ex-prisoners and the patients from the general population.

The study showed that the majority of circulating *M. tuberculosis* strains in Sri Lanka belongs to a limited number of families, but the degree of IS*6110* DNA polymorphism among strains was high. Dendogram analysis showed 41 distinct IS*6110* banding patterns (Magana Arachchi et al., 2000). A close relationship between prison isolates and those from the general population was observed in this study (Magana Arachchi et al., 2000). Of the 20 strains isolated from prisoners, none of the strains displayed identical fingerprints (Magana Arachchi et al., 2000). In bacterial isolates of prisoners and ex-prisoners from the general population, there were two strains, which had identical banding patterns, while there were clear similarities between several isolates (Magana Arachchi et al., 2000). Comparative analysis of the study populations, observed five pairs showing identical banding patterns. One pair had a strain from prisoner and the other ex prisoner while another pair had identical banding patterns between an ex-prisoner and a patient from general population (Magana Arachchi et al., 2000). This indicates the spread of TB between prisoners and general population. Analysis of the data showed that ex-prisoners contributed to a substantial population of TB patients in the general population (Magana Arachchi, 2001). Therefore persons entering prison, carry the risk of being exposed to TB and when they leave could potentially carry the TB bacillus (Magana Arachchi, 2001). Reactivation of the latent disease among some will result in many new cases for many years to come. Compounding the tragedy is the fact that the prison is a perfect environment to produce drug resistant strains (MDR TB). Most prisoners in Sri Lanka do not receive follow up medical treatment, as they are lost for further follow up after discharge from prison. Inconsistent drug supplies can lead to strains of TB that are resistant to drugs. Thus inmates when released to their home communities pose a risk to public health as well as to themselves. Therefore continuity of medical care after release should take place in such instances.

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 (van Soolingen et al., 1998). In the first study for DNA fingerprinting restriction enzyme *Pvu* II was used to cleave the chromosomal DNA of the mycobacterial strains (Magana Arachchi, 2001). The enzyme cleaves the 1.35 – kb IS*6110* element at a single site. In the first study the 541 bp DNA probe used for the hybridization corresponds to a piece of the IS*6110* element and the *Pvu* II site is located within that region. By using this DNA probe all of the possible IS*6110* containing restriction fragments were visualised (Magana Arachchi, 2001) and when analysing the fingerprints 2 bands were considered as a single copy. Therefore *M. tuberculosis* strains carrying one or few IS*6110* copies could be differentiated by the DNA probe used (Magana Arachchi, 2001). There were 6 strains among the prisoners who had a single copy of the IS*6110* and among the general population there were 13 strains which had a single IS copy (Magana Arachchi et al., 2010). In this study 68% of the isolates had less than five copies which were similar to that of other countries in the Asian region, such as India, Malaysia, Oman and Hong Kong (Magana Arachchi et al., 2010). According to previous studies, the strains from countries with a high prevalence of TB exhibited less DNA polymorphism than do strains in countries with a low prevalence of infection (van Soolingen et al., 1998). However strains analysed in study I showed an extensive polymorphism in the banding patterns even though the numbers of copies were less.

Number of IS*6110* copies	Number of strains among prisoners, n= 20 (%)	Number of strains among ex-prisoners, n=24 (%)	Number of strains among general population, n=106 (%)
1	6 (30)	6 (25)	13 (10.32)
2	4 (20)	5 (20.83)	11 (8.73)
3	2 (10)	5 (20.83)	24 (19.05)
4	4 (20)	2 (8.33)	22 (17.46)
5	3 (15)	4 (16.67)	20 (15.87)
6	1 (5)	2 (8.33)	10 (7.94)
7	0 (0)	0 (0)	06 (4.76)

Table 1. Comparison of the IS element copies (IS*6110*) found among the different categories from Study I

3.1.1 Pattern of TB transmission in second study

The data included in this section are based on a study conducted over a period of 2 years in which total 121 *M. tuberculosis* isolates were analyzed from the first visit (n=178) and recurrent patients (n=12) who attended 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 mycobacteria other than tuberculosis (MOTT) biochemically. This finding is comparable to a previous study in which mycobacterial isolates obtained from patients throughout Sri Lanka where MOTT accounted for 3.27% of the total isolates. The isolate from the recurrent tuberculosis patient was found to be a MOTT strain with rifampin resistance, which explained the reason

for the treatment failure in that patient (Magana Arachchi, et al unpublished data). In RFLP analysis all three strains did not produce any banding pattern with IS*6110* confirming their species variation.

3.1.1.1 RFLP analysis

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, where as epidemiologically related *M. tuberculosis* strains show identical or similar (one band variation) finger prints (Barnes and Cave, 2003). In this study RFLP analysis was successfully carried out to differentiate *Mycobacterium tuberculosis* complex from mycobacteria other than tuberculosis in 100 of 122 isolates from the first visit patients. A high degree of DNA polymorphism in both banding patterns and number of copies of IS*6110* among strains were observed. 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 (Magana Arachchi et al., 2011). Table 2 summarizes 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 were different and therefore the location of IS*6110* elements in the chromosomal DNA. Therefore *M. tuberculosis* strains carrying one or few IS*6110* copies were differentiated without difficulty (Magana Arachchi et al., 2011). In this study 52% of the isolates had five or less

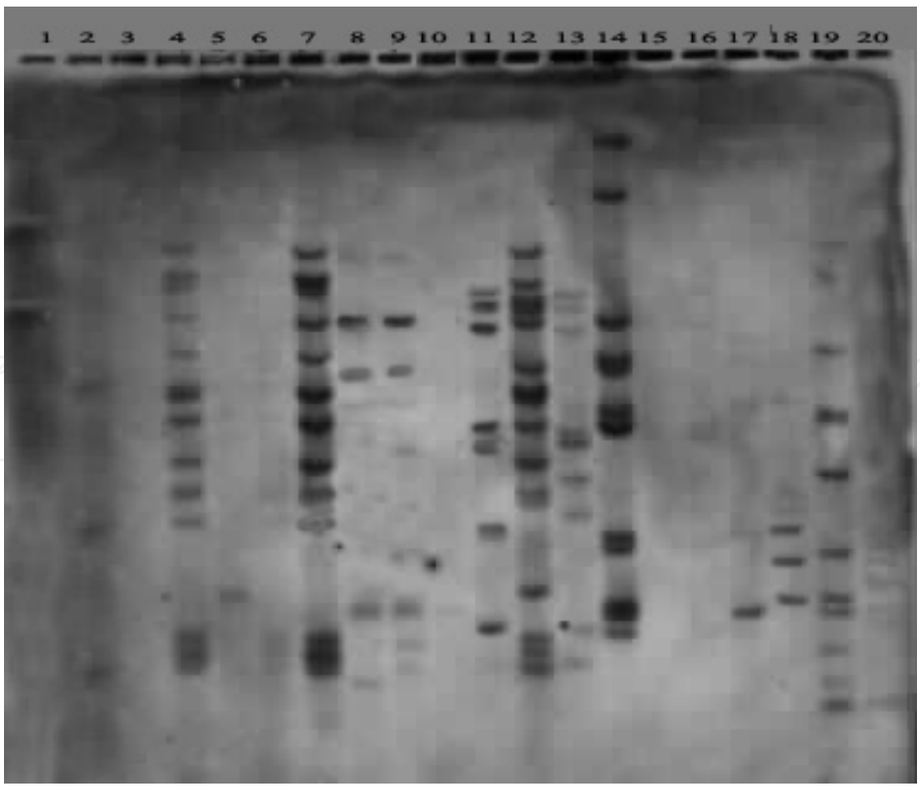


Fig. 1. IS*6110* DNA finger prints of *M. tuberculosis* isolates from study population of Kandy

Number of IS*6110* copies	Number of strains	% of strains
0	15[a]+2 [b]+10[c] = 27	27
1	12	12
2	3	3
3	3	3
4	5	5
5	4	4
6	9	9
7	3	3
8	4	4
9	6	6
10	6	6
11	8	8
12	2	2
13	1	1
14	3	3
15	1	1
16	2	2
17	1	1

[a] = MTC / MTb *Mycobacterium tuberculosis,* [b] = MOTT confirmed, [c] = to be identified

Table 2. IS element copies observed in *M. tuberculosis* isolates from Kandy

than five copies and pattern is similar to the previous study in which 68% was recorded from recurrent TB patients (Magana Arachchi et al., 2011).

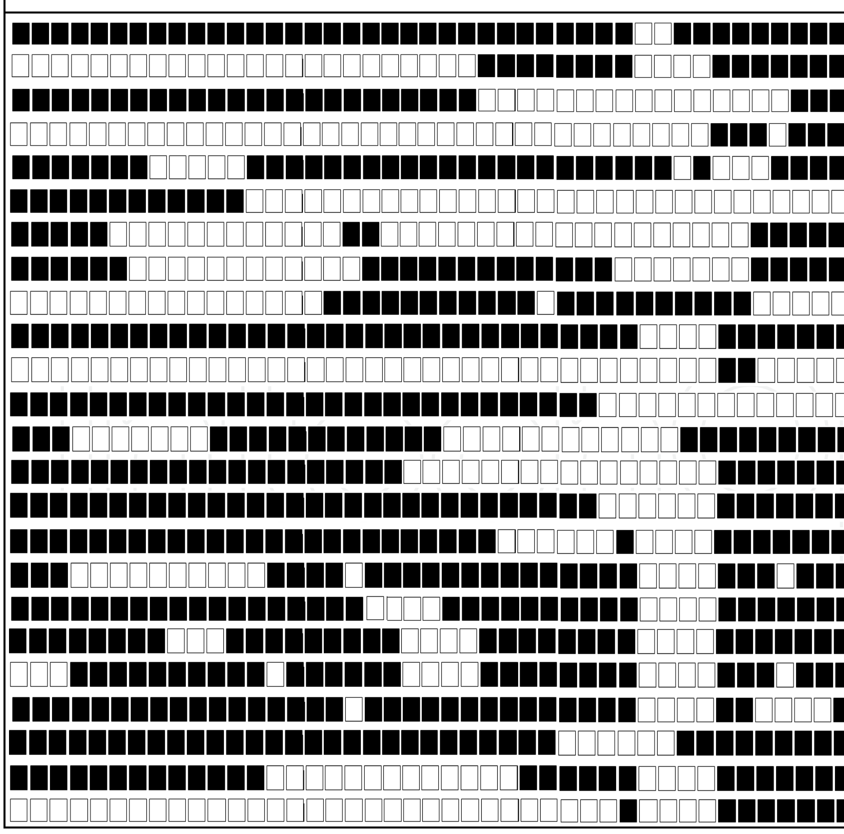
The fingerprints of the 73 strains were subjected to similarity analysis by using the software programme GeneDirectory from SYNGENE. This study showed that the majority of circulating *M. tuberculosis* strains in Kandy belongs to a single family, but the degree of IS*6110* DNA polymorphism among strains was high. 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, 27 and 11 strains respectively (Magana Arachchi et al., 2011). 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 (Magana Arachchi et al., 2011).

3.1.1.2 Spoligotyping

In this study, the used the algorithm SPOTCLUST incorporates biological information on spoligotype evolution, without attempting to derive the full phylogeny of *M. tuberculosis* complex. A total of 110 *M. tuberculosis* isolates were analyzed by spoligotyping. When spoligo patterns were compared from SPOTCLUST which was based on the SpolDB3 model, 24 distinct families were identified including the nine major spoligotyping-based families;

Mycobacterium 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 (Table 3). The most predominant group among the isolates of *M. tuberculosis* corresponded to Family33. In this family, only spacers 33-34 are absent and recently described clade MANU of Indian origin belongs to the same family (Magana Arachchi et al., 2011). When compared to the single publication of spoligotyping patterns from Sri Lanka similarity was observed in only five clades namely Beijing, T1, EAI5, T2 and T3 (Magana Arachchi et al., 2011). According to the analysis, bacterial strains were distributed among all three principal genetic groups PGG1, PGG2 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 the spoligotyping patterns high strain diversity was observed 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 (Magana Arachchi et al., 2011). The cluster analysis on spoligotyping are being carried out and after completing it in due course identifying the risk factors associated with TB transmission as well as the evolution of *M. tuberculosis* in Sri Lanka could be achieved.

Spacer
1 5 10 15 20 25 30 35 40 43



Spacer	Family	T
	Family 33	45
	Family36	13
	*M.tuberculosis*EAI1	07
	M. tuberculosis Beijing	07
	M. africanum	05
	Family 35	05
	*M. tuberculosis*LAM7	05
	M. tuberculosis T3	03
	M. bovis – BCG	02
	M. tuberculosis T1	02
	M. microti	02
	M. tuberculosis T2	02
	M. tuberculosis CAS	01
	M. tuberculosis LAM8	01
	M. tuberculosis Haarlem3	01
	M. tuberculosis Haarlem1	01
	M. tuberculosis X3	01
	M. tuberculosis H37Rv	01
	M. tuberculosis LAM 3	01
	M. tuberculosis LAM 1	01
	M. tuberculosis X2	01
	M. tuberculosis EAI-5	01
	M. tuberculosis T4	01
	M. tuberculosis Haarlem2	01

Table 3. Spoligotyping – based families that observed in *M. tuberculosis* isolates in patients with tuberculosis in Kandy by SPOTCLUST (n=110)

3.2 Evaluation of drug susceptibility

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 to the conventional antibiotic sensitivity testing which takes more than four weeks. Genotyping also permits the evaluation of isolates with different patterns of drug susceptibility. Such an evaluation may helpful in cases in which the original organism developed resistance during or after antituberculosis therapy, the patient was reinfected with a different *M. tuberculosis* strain or cross contamination is suspected (Barnes and Cave, 2003). According to literature higher number of susceptible *M. tuberculosis* strains tends to be in clusters, where as only 22% of the isoniazid (INH) mono resistant strains were found to be clustered. But some studies did not find differences in clustering between susceptible and streptomycin mono-resistant strains (Soolingen et al., 1993). In the first study no difference in clustering was observed among the drug resistance and susceptible isolates, while analysis is being performed for the second study. Most studies have shown that acquisition of drug resistance of *M. tuberculosis in vivo* did not result in any observable IS mediated genetic rearrangements. But in contrast to the findings of others the relative, instability of IS*6110* was found in one of two MDR out break strains, and also four of the nine tested IS*6110* RFLP patterns showed a minor and different alteration. According to them the transposition rate may be strongly related to the *M. tuberculosis* genotype represented. In study I there were 2 pairs of isolates, which had identical banding patterns. However the pattern of drug resistance in the two strains was different and these isolates were collected from patients coming from different districts but from same Western province. Although they come to the same hospital for treatment, the strains were unlikely to be epidemiologically related. The findings of other research showed that non-random association of IS*6110* with *M .tuberculosis* could result in false positive clustering in unselected collections of isolates.

3.3 IS*6110* as a diagnostic tool

Among the strains tested in the first study there were two strains (one strain from general population and the other from DNA amplification studies) that lacked the IS*6110* element. In the second study among the strains tested there were 25 strains that lacked the IS*6110* element. Among these, 15 strains were confirmed as *M. tuberculosis* 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.

3.4 Insights into transmission of TB

Clustered cases of TB are defined as those in which have identical or closely related genotypes with recent transmission while isolates with distinct genotypes generally represent a reactivation or infection acquired in the distant past (Barnes and Cave, 2003). However there are limitations to this concept (Barnes and Cave, 2003). Both studies showed that the majority of circulating *M. tuberculosis* strains in Sri Lanka belongs to a limited number of families, 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. Molecular epidemiologic studies have shown that the dynamics of the transmission of TB vary greatly geographically (Barnes and Cave, 2003). Findings of the two studies indicate the differences observed in two provinces in both banding patterns and number of copies of IS*6110* among strains with Colombo district having 0-7 IS copies while Kandy having 0-17 copies of IS*6110* (Tables 1 and 2).

4. Future use of genotyping

Although the number of copies of IS*6110* can range from 0-25, population based molecular epidemiological studies report that most strains contain 8-18 copies a number sufficient to discrimination between the majority of strains (Burgos and Pym, 2002) and the findings of the two studies clearly emphasize the value of RFLP and spoligotyping in molecular epidemiology. However both studies 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, due to study constraints RFLP and spoligotyping were not performed on all patients. Additionally by performing both RFLP and spoligotyping on culture positives high strain diversity was observed with a large number of small clusters, as well as a significant proportion of strains hitherto unreported in the global databases. But performing spoligotyping alone has advantageous over IS*6110* RFLP typing. As the technique needs only small amounts of DNA the test can be performed on clinical samples directly or on strains of *M. tuberculosis* shortly after their inoculation into liquid cultures (Kmerbeek et al., 1997). Presently the gold standard for molecular epidemiological studies on tuberculosis is changing towards MIRU-VNTR typing because this technique generates easily analyzed numerical results and it is less labour intensive and has a discriminative power comparable to that of IS*6110*-based RFLP (Barnes and Cave, 2003).

Genotyping has been used to study the transmission dynamics of TB in both developed and developing countries. However, only the developed nations are using it to guide tuberculosis control efforts (Barnes and Cave, 2003). This is the first study in Sri Lanka in which both the RFLP pattern of *M. tuberculosis* strains and the spoligotyping in a population has been examined. In this study the feasibility of establishing molecular typing methods in a developing country like Sri Lanka has been demonstrated specially in spoligotyping without using any commercial kits.

5. Conclusions

Typing of *Mycobacterium tuberculosis* isolates is of great potential value for basic and epidemiological studies on tuberculosis. Results obtained from restriction fragment length polymorphism typing and spoligotyping show that the majority of circulating *Mycobacterium tuberculosis* strains in Sri Lanka belong to a limited number of families, but the degree of IS*6110* DNA polymorphism among strains were high. By using the genetic marker of IS*6110* it was possible to differentiate most of the *M. tuberculosis* isolates. The preliminary inferences from these studies plead for a more extensive analysis of the data, to study the variability of *M. tuberculosis* strains and their transmission dynamics. The goal of molecular epidemiology is to quantify the extent of ongoing transmission of infectious

agents and to identify host and strain specific risk factors for disease spread. Molecular methods in epidemiology require the development of both appropriate epidemiologic study design and analytical tools to yield meaningful assessments of disease transmission. Therefore the interpretation of molecular epidemiological studies should largely depend on the study question, the geographical area under study and the typing methods used to prevent the sampling bias in the molecular epidemiology of TB.

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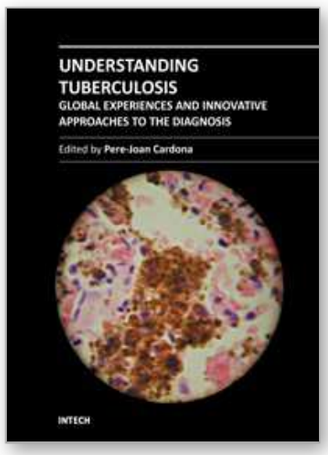

Understanding Tuberculosis - Global Experiences and Innovative Approaches to the Diagnosis

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Mycobacterium tuberculosis is a disease that is transmitted through aerosol. This is the reason why it is estimated that a third of humankind is already infected by Mycobacterium tuberculosis. The vast majority of the infected do not know about their status. Mycobacterium tuberculosis is a silent pathogen, causing no symptomatology at all during the infection. In addition, infected people cannot cause further infections. Unfortunately, an estimated 10 per cent of the infected population has the probability to develop the disease, making it very difficult to eradicate. Once in this stage, the bacilli can be transmitted to other persons and the development of clinical symptoms is very progressive. Therefore the diagnosis, especially the discrimination between infection and disease, is a real challenge. In this book, we present the experience of worldwide specialists on the diagnosis, along with its lights and shadows.

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