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Genetic diversity of *Mycobacterium tuberculosis* isolates obtained from three distinct population groups in the Central Province, Sri Lanka

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PEER REVIEW

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Comments

The authors have performed genotyping of 150 *M. tuberculosis* strains isolated from three different population of Sri Lanka. Both spoligotyping and VNTR_MIRU typing techniques have been used for genotyping.
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ABSTRACT

Objective: To characterize the *Mycobacterium tuberculosis* (*M. tuberculosis*) isolates by spoligotyping and mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing to understand how *M. tuberculosis* strains transmit among the study population.

Methods: Spoligotyping and MIRU-VNTR were used to genotype *M. tuberculosis* isolates obtained from three distinct population groups in Sri Lanka. General population suspected of having tuberculosis attending the Chest Clinic, Kandy (n = 78), patients having tuberculosis in Bogambara prison, Kandy (n = 22) and estate workers having tuberculosis in the Central Province, Sri Lanka (n = 50), from January 2012 to April 2014 were included in the study.

Results: Among 150 isolates, a total of 19 distinct families were observed including 6 major spoligotyping-based families; East-African-Indian (39.33%), Haarlem (20%), Beijing (8.6%), Central European family T (6.5%), European family X (5.2%) and Central and Middle Eastern Asian (0.6%). Beijing strains were only identified among the general population. MANU strains were significant (36.36%) among the prisoners who had clustered with the MANU strains of the general population indicating contact cases and a possible transmission index within a particular geographical area. Haarlem 3 (34%) was the predominant strain among the estate workers. There was a close epidemiological relationship between the prisoners and the estate workers in the population.

Conclusions: The first insight of 15 loci MIRU-VNTR typing in conjunction with spoligotyping in a population in Sri Lanka demonstrated the feasibility and the applicability of these techniques to differentiate strains, their heterogeneity and the predominance of several worldwide distributed spoligotypes.

KEYWORDS

MIRU-VNTR, *Mycobacterium tuberculosis*, Spoligotyping, Sri Lanka, Tuberculosis

1. Introduction

Tuberculosis is considered a global health problem and the leading cause of morbidity and mortality especially in developing countries[1]. Although it is considered that Sri Lanka is a low prevalent country in the South-East Asia region[2], around 9 000 new tuberculosis cases are reported every year[3]. The prevalence rate of tuberculosis in Sri Lanka in 2012 was 109/100 000 population

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and tuberculosis mortality rate was 5.9/100 000 population[4]. Strain typing using molecular methods along with conventional approaches has become powerful tools in the epidemiological surveillance and control of disease transmission[5]. Spoligotyping enables differentiation of strains of *Mycobacterium tuberculosis* (*M. tuberculosis*) present in clinical specimens without the need for culture[6]. Typing using mycobacterial interspersed repetitive units-variable number tandem repeats (MIRU-VNTR) have proven that it is a reproducible typing method that is reliable in the discrimination of strains comparable to that of IS6110 typing[7,8]. Epidemiological data about the tuberculosis genotypes prevalent in Sri Lanka are scarce. Hence, tracking the transmission patterns of tuberculosis by epidemiological investigation is of utmost importance. Therefore, this study was performed to characterize the *M. tuberculosis* isolates prevalent in selected population groups in Sri Lanka by spoligotyping and MIRU-VNTR genotyping techniques.

2. Materials and methods

2.1. Study population

A total of 150 acid fast bacilli positive sputum samples from first visit patients were collected over a period from January 2012 to April 2014 from the three groups studied. Group I were general population suspected of having tuberculosis attending the Central Chest Clinic, Kandy, Sri Lanka (n = 78), Group II were prisoners suspected of having tuberculosis in Bogambara prison, Kandy (n = 22) and Group III were estate workers suspected of having tuberculosis (n = 50). Ethical clearance was obtained from the Ethical Review Committee, Faculty of Medicine, University of Peradeniya, Sri Lanka.

2.2. Collection of data and specimens

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

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

Decontamination of sputum was done using the modified Petroff's method using 4% NaOH[9]. Lowenstein-Jensen medium were inoculated with the suspension and all slopes were observed for occurrence of growth for 8 weeks. Genomic DNA was extracted from culture positive isolates (n = 150), $H_{37}Rv$ and *Mycobacterium bovis* standard strains, using standard CTAB/NaCl method[10].

2.4. Spoligotyping

Spoligotyping was performed as previously described[6] and as per the spoligotyping kit supplier's instructions (Occimum Biosolutions, Hyderabad, India). The obtained spoligotyping hybridization patterns were compared with the patterns of the strains previously reported in the SPOTCLUST[11] and SITVIT databases[12].

2.5. MIRU-VNTR typing

HotstarTaq DNA polymerase kit (QIAGEN, Hilden, Germany) was used in the preparation of PCR premixes for 15 loci MIRU-VNTR[13]. Briefly, DNA amplification was carried out in rotor- gene Q thermal cycler (Qiagen, Germany). The PCR fragments were analyzed using 2% agarose gels and the sizes of the amplicons were assessed manually by using the Genetool Software (Syngene, UK). The MIRU copy number per locus was calculated by using the conventions described[13]. $H_{37}Rv$ was used to verify the results for a particular locus by comparing with the allele number assigned for the locus used. MIRU-VNTR data were analyzed using the web application MIRU-VNTR*plus*[14].

3. Results

3.1. Spoligotyping using SPOTCLUST and SITVIT databases

Spoligotyping of 150 isolates generated 95 distinct spoligo patterns (Figure 1). Of the 95 spoligo patterns, 18 (83 isolates) were exact and matched with the existing spoligo patterns in the SpolDB4 database[15]. According to the study, majority of circulating *M. tuberculosis* strains belonged to a limited number of families. A total of 19 distinct families were observed including 6 major families; East-African-Indian (EAI-59; 39.33%), Haarlem (H-30; 20%), Beijing (13; 8.6%), Central European family T (10; 6.5%), European Family X (8; 5.2%) and Central and Middle Eastern Asian (Delhi/CAS-1; 0.6%). Additionally, MANU (19; 12.6%), EAI3-IND (4; 2.6%), EAI6-BGD1 (2; 1.3%), *M. tuberculosis* S/ Québec (1; 0.6%), Family36 (2; 1.3%) and Family35 (1; 0.6%) sub-lineages were also identified. Of the 13 isolates belonging to U (unknown/undesignated) lineages, six 'U likely H' (SIT124), five 'U likely H3' (SIT124) and two 'U likely EAI' (SIT523) were identified. The predominant group corresponded to EAI which was also the predominant strain among the general population studied (Table 1). Beijing strain was only identified among the general population where shared type (ST) 1 of Beijing lineage being the most prevalent ST among the Beijing isolates in this study (12; 8%). Haarlem (20%) was the predominant strain among the estate workers. Results obtained from SITVIT were found to be similar to SPOTCLUST database. Family33 from SPOTCLUST was identified as EAI from SITVIT database. Nonclustered spoligotypes in the SITVITWEB were designated as "orphan" strains. When all three groups (Group I, Group II and Group III) were compared, three types of common lineages were identified (Table 2); EAI, MANU and Haarlem3. Haarlem1 and EAI6_BGD1 strains were common

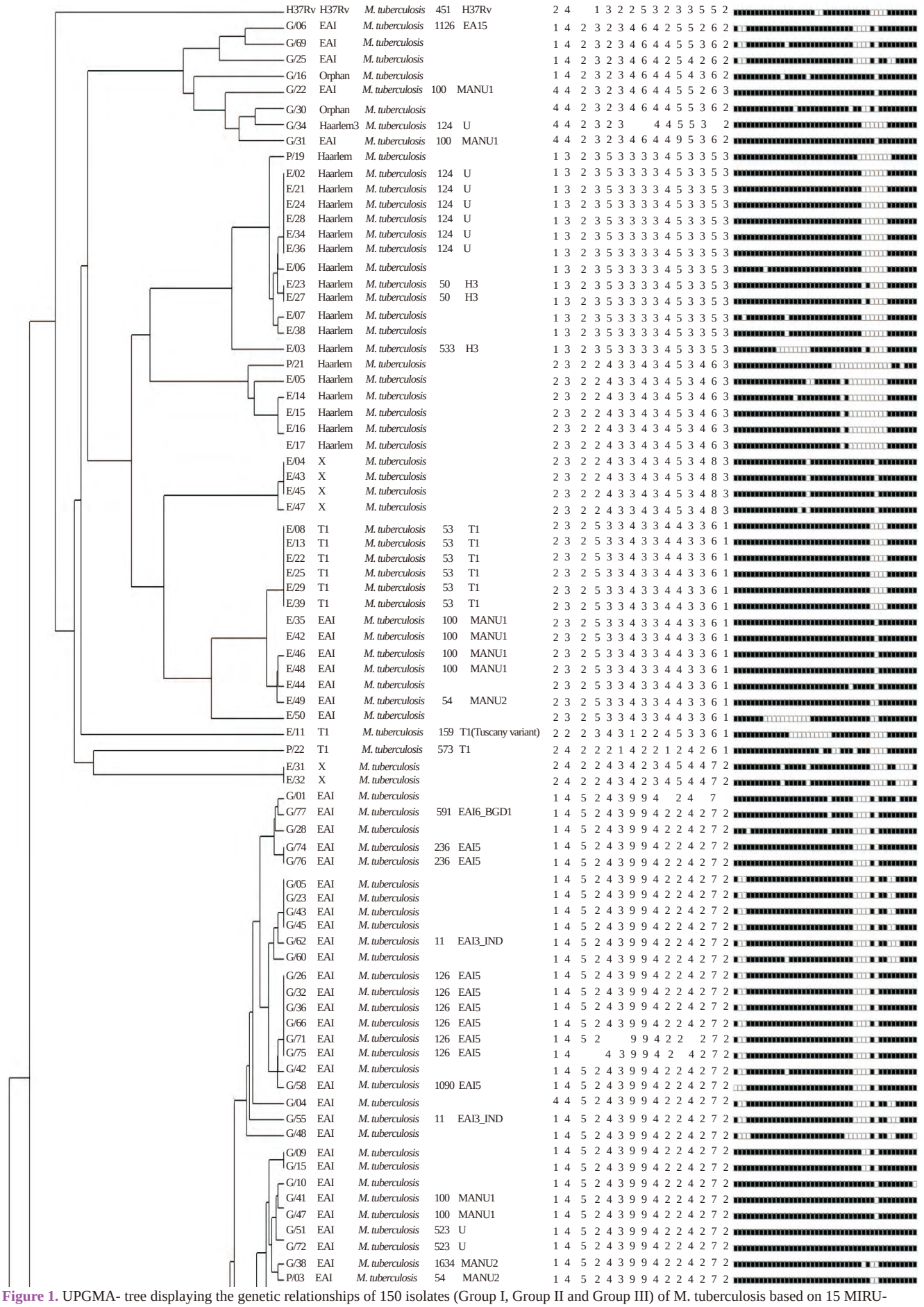

Figure 1. UPGMA- tree displaying the genetic relationships of 150 isolates (Group I, Group II and Group III) of M. tuberculosis based on 15 MIRU-VNTR loci and spoligotyping. The linkage distance scale is indicated at the bottom.

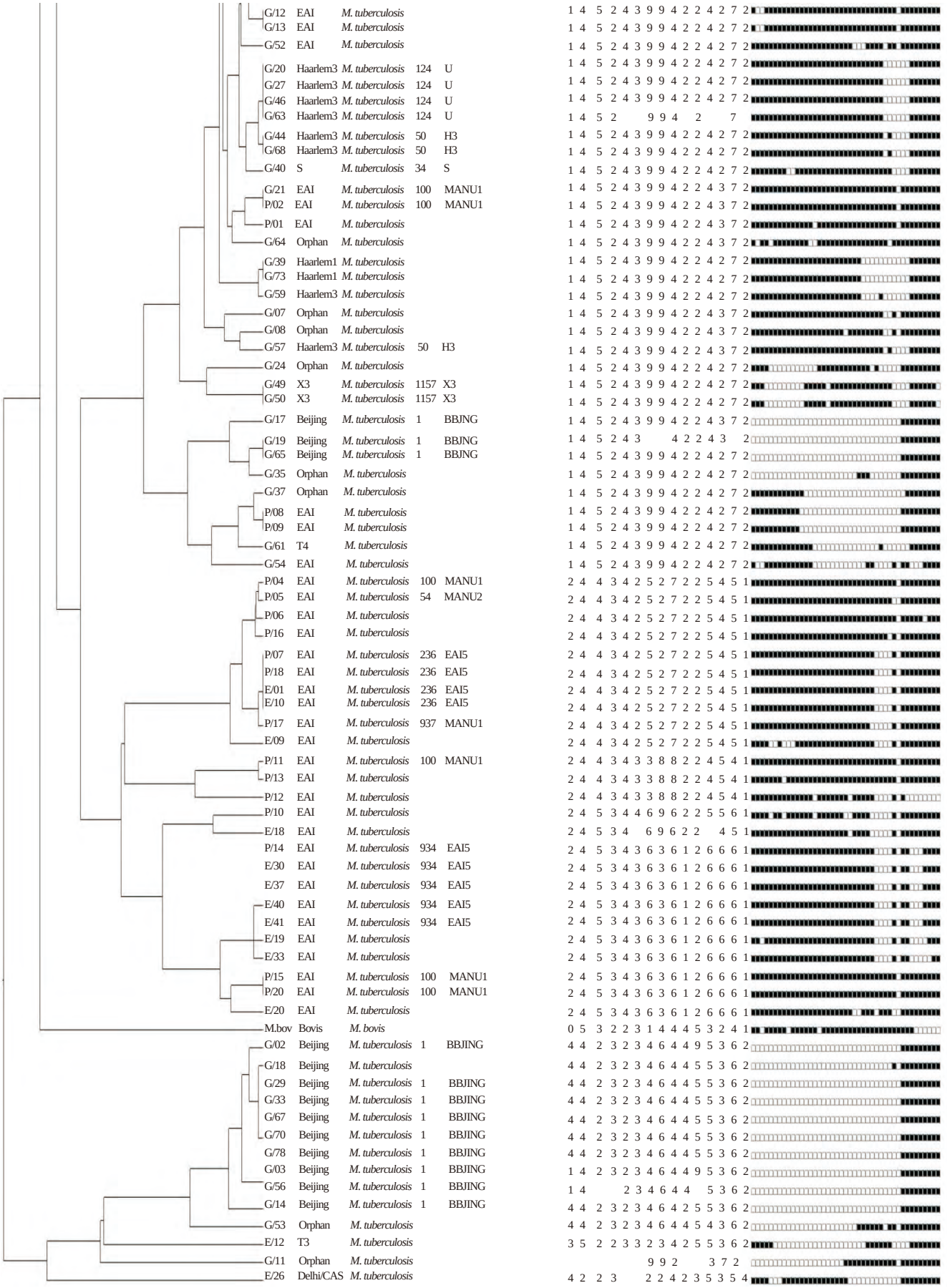

Figure 1, Continued. UPGMA- tree displaying the genetic relationships of 150 isolates (Group I, Group II and Group III) of M. tuberculosis based on 15 MIRU-VNTR loci and spoligotyping. The linkage distance scale is indicated at the bottom.

among Group I and Group II categories. T1 lineage was common among Group II and Group III (Table 2).

Table 1

Distribution of strains of *M. tuberculosis* isolates in the study population.

SpolDB4 lineage or sub-lineage	Genotype/ SpolDB4 SIT	No. of isolates with identical spoligotype pattern	Total no. of strains in lineage [*n* (%)]	Lineage
$H_{37}Rv$ (standard)	451	1	1	
Beijing	1	12	13 (8.7)	East-Asian
	Orphan	1		
EAI5/ Family33	126	7	34 (22.7)	EAI
	2674	2		
	1090	1		
	236	6		
	934	5		
	138	2		
	Orphan	11		
EAI/ Family33		25	25 (16.7)	EAI
EAI6-BGD1	591	2	2 (1.3)	EAI
EAI3-IND	11	2	4 (2.6)	EAI
	Orphan	2		
Haarlem 3	124	17	27 (18.00)	Euro-American
	533	3		
	50	7		
Haarlem 1	1952	2	3 (2.00)	Euro-American
	47	1		
Family33-MANU1	100	14	15 (10.00)	Indo-Oceanic
	937	1		
Family33-MANU2	1634	1	4 (2.7)	Indo-Oceanic
	54	3		
Family35	Orphan	1	1 (0.7)	Indo-Oceanic
Family36	Orphan	2	2 (1.3)	Indo-Oceanic
Delhi-CAS1	Orphan	1	1 (0.7)	EAI
X3	1157	2	2 (1.3)	Euro-American
X2	2180	2	2 (1.3)	Euro-American
X1/ Family33	Orphan	4	4 (2.6)	Euro-American
T1	573	1	8 (5.3)	Euro-American
	53	6		
	Orphan	1		
T3	Orphan	1	1 (0.7)	Euro-American
T4	159	1	1 (0.7)	Euro-American
M. tuberculosis S/Quebec	34	1	1 (0.7)	EAI

3.2. MIRU-VNTR analysis

A total of 77 distinct clusters were observed among the 150 isolates where the largest cluster comprised of 38 isolates of EAI family including family 33 and MANU sub-lineages. MANU1 and MANU2 strains were significant (36.36%) among the prisoners who had clustered with the MANU strains of the general population. Haarlem3 (34%) was the predominant strain among the estate workers. There was a close epidemiological relationship between the prisoners and the estate workers in the population studied. There were significant 26 clusters in which 2 clusters of EAI5 clustered with the isolates of prison and estate workers and another cluster of MANU1 had 2 isolates of general population and prison clustered together (Figure 1). Other MANU1 strains of the three groups clustered separately in three distinct clusters. Six strains of 'U likely H' from estate workers and four strains of U likely H3 of general population grouped in two separate clusters though their SIT numbers were same. Overall, the Haarlem family strains of Group I and Group III was clustered separately. Considering isolates of estate workers, three strains of X family and six strains of T1 family were grouped in two separate clusters. X3 strains of general population was grouped in a cluster. The EAI strains of all groups had similarities with at least one of the three groups, when considering the spoligo banding pattern and 15 MIRU-VNTR loci set. Beijing strains (*n* = 13) identified only in general population (Group I) were scattered in to three clusters due to the differences in the alleles identified (Figure 1). The orphan strains of G/07 and G/08 shared the same branch with a Haarlem3 strain of Group I. This means, these orphan strains share certain phylogenetic similarity with the strains of Haarlem family.

3.3. Genotyped strain families in association with the demographic characteristics of patients

According to demographic data, the patients were from 11 districts

Table 2

Comparison of lineages/ sub-lineages identified in the total study population (Group I, II and III) using MIRU-VNTR and spoligotyping.

Lineages/sub-lineages identified in Group I		Lineages/sub-lineages identified in Group II		Lineages/sub-lineages identified in Group III		Total No. of identified lineages in the study population	
Lineage	No. of isolates	Lineage	No. of isolates	Lineage	No. of isolates	Total No. of strains	Total No. (%) of strains
EAI5	21	EAI5/Family33	7	EAI5	6	34	22.7
EAI/Family33	15	EAI/Family33&35	3	EAI/Family33	7	25	16.7
Beijing	13	-	-	-	-	13	8.7
MANU1/Family33	5	MANU1/Family33	6	MANU1/Family33	4	15	10.0
Haarlem3	9	Haarlem3	1	Haarlem3	17	27	18.0
EAI3_IND	4	-	-	-	-	4	2.6
Haarlem1	2	Haarlem1	1	-	-	3	2.0
Family36	2	-	-	-	-	2	1.3
X3	2	-	-	-	-	2	1.3
MANU2/Family33	1	MANU2/Family33	2	MANU2/Family33	1	4	2.7
EAI6_BGD1	1	EAI6_BGD1	1	-	-	2	1.3
T4	1	-	-	-	-	1	0.7
Family35	1	-	-	-	-	1	0.7
S	1	-	-	-	-	1	0.7
-	-	T1	1	T1	7	8	5.3
-	-	-	-	CAS	1	1	0.7
-	-	-	-	X2	2	2	1.3
-	-	-	-	X1/Family33	4	4	2.6
-	-	-	-	T3	1	1	0.7
Total	78	Total	22	Total	50	Total: 150	Total: 100%

of Sri Lanka. Of 150 patients, 79.3% were males and the majority (30.66%) was in the age group between 46-60 years. Beijing, EAI, Haarlem and MANU strains were the prevalent strains in Kandy district. EAI lineage and its sub lineages were identified in Kandy, Ampara, Badulla, Trincomalee, Polonnaruwa, Gampaha, Kegalle, Galle and Nuwara Eliya districts among which Kandy district had the highest prevalence rate of 82.5%.

4. Discussion

In this study, a detailed picture of tuberculosis transmission pattern among three different population groups in Sri Lanka was attained by combining spoligotyping and MIRU-VNTR genotyping. Analysis of results revealed that the majority of circulating *M. tuberculosis* strains in Kandy (a district of the Central Province of Sri Lanka with a population: 109 343) belonged to nineteen distinct families including six major spoligotyping-based families; Beijing, EAI, Haarlem, CAS, European family X and Central European family T had been previously identified in Kandy[16]. The most predominant group was EAI, which was previously described as a major worldwide epidemic strain belonging to the predominant ancestral genotypic lineage in India[17]. Delhi/CAS family was identified among the estate workers from Nuwara Eliya district. According to Goh *et al.*, CAS clade is evolutionarily younger and more prevalent worldwide[18]. Sarkar *et al.* have reported that Delhi/CAS is largely confined to Indian subcontinent and to some regions in East Africa that have experienced a significant Indian migration[19]. Therefore, it is hypothesized that ''modern'' *M. tuberculosis* lineages are better human pathogens with enhanced virulence which facilitates their near-worldwide penetration[18]. EAI3_IND (ST11) and EAI6_BGD1 (ST591) sub-clades were identified among the EAI clade. The higher percentage of ST11 (EAI3_IND) in South India suggested the probability of the point of entry of this strain and its transmission to the rest of the country[20]. According to the studies reported by Joseph *et al.* and Macías *et al.*, Beijing, Haarlem, CAS and *M. tuberculosis* H_{37}Rv are ''modern'' *M. tuberculosis* families which are prevalent worldwide, whereas the EAI clade represents an ''ancestral'' *M. tuberculosis* lineage which contributes to the global disease burden substantially[17,21]. According to analysis, a substantial part of today's Sri Lankan paternal gene pool was contributed by EAI lineages. It is suggested that in the past, ST26 belonging to CAS clade was introduced from Central Asia to the North India and with human migration, EAI ancestral strains spread back from Asia to Africa through India. It is then noted that this evolution gave rise to the CAS lineage, and possibly to all ''modern'' tuberculosis lineages[22]. The second most common family was Family33 and a recently described clade MANU, an Indian origin belongs to the same family[16,23]. The ancestral isolates of ST126 (EAI5) and ST100 type (MANU) were found to be much more concentrated in Mumbai and Southern Andhra Pradesh, India[24]. These results were not surprising because Sri Lanka is a popular tourist destination located in the Northern Indian Ocean off the southern coast of the Indian subcontinent and because tuberculosis affects the elderly, many of whom were first or second generation immigrants from India being the closest neighbors. In our study, seven clusters each with at least three or more isolates were from the general population. This clustering implied a common strain among them, *i.e*, EAI strain variants. These patients having tuberculosis were epidemiologically linked as they were all identified to be from the Kandy district indicating a possible transmission of the EAI strain among the general population who were having tuberculosis. The age group of 46 and older was significantly more often infected by EAI clade which was similar to a study done in India[25]. Therefore, EAI isolates were found more often in older patients probably reflect that EAI were endemic isolates and have been circulating in the country for long. A close relationship between prison isolates and those from the general population was observed, which was similar to a previous study done in Colombo, Sri Lanka[26]. Of the 22 strains isolated from the prisoners, there were clear similarities between several isolates. When the results of this study was compared to the previous publication of spoligotyping patterns from Sri Lanka[16], similarity was observed in all of the clades except for three clades namely EAI3-IND, EAI6-BGD1 (first identified in Bangladesh by Rahim *et al.*)[27] and *M. tuberculosis* S strains. Among the study population, only 13 (8.6%) strains were identified that belonged to Beijing lineage which is known to be dominant in East and Southeast Asia[28]. Previous studies have shown that more recently evolved sub-lineages of Beijing strain were prevalent (n = 13; 92.85%) in Sri Lanka, mainly in Colombo and Galle districts[29]. In the neighboring countries in Asia, rates of infection with the Beijing family strains were higher than those in the more distant countries, indicating the spread of Beijing family strains from the Beijing area to other regions[5]. In Sri Lanka where tuberculosis is endemic, it is critical to identify predominant strain types in order to study transmission patterns within the country and to understand the epidemiology of the disease. This is all the more important as Sri Lanka is a host to a very large number of immigrant populations and migrant workers from neighboring tuberculosis endemic countries (India, China and Bangladesh), and the movement of these populations would influence strain distribution in the entire region. Geographically, India provides us with the closest comparison. The *M. tuberculosis* strains in Sri Lanka (EAI5, EAI3-IND, EAI6-BGD1, MANU, Delhi/CAS, Family33, Family35, Haarlem, Tuscany-T, X) identified from this study were similar to spoligotypes identified from India[24,25]. The identification of a dominant spoligotype common to India and Bangladesh (EAI6-BGD1) illustrates an important trend in the *M. tuberculosis* infection pattern in the South Asian region.

Some strains of EAI family (G/06, G/69 and G/25) had a slightly different banding pattern with that of the other EAI strains in the same population group, but grouped together with their similarities. Haarlem strains (P/19, E/06, E/07, E/38) had different banding pattern with that of the other Haarlem family strains. X family strain of E/47 did not have the spacer 16 which was different to that of other X strains in the same cluster of estate workers. In some families, however, small changes in the banding pattern have been noted, compatible with strain evolution[30].

An optimal 15-locus VNTR has been proposed as a new worldwide standard method for discriminating tuberculosis genotypes and for the improved epidemiological studies[13].

MIRUs 10, 26, 40, 31, 16, Mtub04, ETRA, ETRC, QUB4156, Mtub21 and QUB26 are known to be highly discriminatory and Mtub30 and 39 are moderately discriminatory for routine epidemiological studies[31]. In this study, the 10 isolates that were differentiated by 15 MIRU-VNTR (Isolate No. G/02, G/18, G/29, G/33, G/67, G/70, G/78, G/03, G/56 and G/14) showed some similarities, which then suggested that these isolates were epidemiologically related as they were all from the Beijing spoligotype family, all with the same shared type ST1. Isolates G/17, G/19 and G/65 did not show any relatedness even though they were all from the Beijing family with the same shared type ST1. Six isolates of T1 family strains of estate workers (E/08, E/13, E/22, E/25, E/29, E/39) were epidemiologically related with the same shared type ST53, whereas the other T1 family strains exhibit different STs. Estate worker with a T1 strain (E/11) had ST159 and a prisoner with a T1 strain had ST573 and appeared to be geographically partitioned from other T1 strains. This suggested that some strains had no epidemiological relatedness. T family (modern strain of tuberculosis) is an ill-defined strain with over 600 unclassified sequences[32]. It's one of the three most frequent families (LAM, Haarlem and T) in Europe, Africa, America and Middle East, corresponding to around 30% of isolates[32]. Haarlem3 (34%) was the dominant strain among the estate workers from Nuwara Eliya district. This indicated a possible intermixing of the populations from different places in Sri Lanka who were infected with diverse tuberculosis strains. Furthermore, some lineages remain poorly differentiated by either MIRU-VNTR or spoligotyping, making phylogenetic grouping difficult.

The use of 15 loci MIRU-VNTR pattern of *M. tuberculosis* strains in conjunction with spoligotyping in a population in Sri Lanka demonstrated the feasibility and the applicability of these techniques to differentiate strains, their heterogeneity and the predominance of several worldwide distributed spoligotypes. The *M. tuberculosis* strains in the Kandy district had high degree of polymorphism in their DNA fingerprinting patterns. Thus, molecular epidemiological studies are useful in determining ongoing transmission dynamics of tuberculosis to enhance the tuberculosis control in developing countries like Sri Lanka. This is the first study in Sri Lanka in which the MIRU-VNTR pattern of *M. tuberculosis* strains in conjunction with spoligotyping in a population has been examined.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

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Comments

Background

Transmission dynamics of *M. tuberculosis* is studied by genotyping techniques like spoligotyping and VNTR-MIRU. These techniques are relatively new to study the epidemiology of tuberculosis in Sri Lanka. These authors have successfully used these techniques to study genetic diversity of *M. tuberculosis* in this country.

Research frontiers

Sopligotyping and VNTR-MIRU are straight forward tools for the genotyping of *M. tuberculosis*. These authors have successfully used them for genotyping.

Related reports

In Sri Lanka, tuberculosis is one of the endemic diseases. Population structure of *M. tuberculosis* has been reported rarely from Sri Lanka. In this manuscript, authors isolated tuberculosis bacilli from three geographical locations, and performed genotyping using spoligotyping and VNTR-MIRU typing.

Innovations & breakthroughs

Both spoligo and VNTR-MIRU typing techniques are new genotyping tools, which the authors used to genotype *M. tuberculosis* strains of this manuscript. Use of these two techniques is an innovative approach for characterization of the population structure of *M. tuberculosis* in Sri Lanka.

Applications

This paper describes the population structure of *M. tuberculosis* isolated from three different geographical regions of Sri Lanka. This paper will add new knowledge in the field of population structure of *M. tuberculosis* in this country.

Peer review

The authors have performed genotyping of 150 *M. tuberculosis* strains isolated from three different population of Sri Lanka. Both spoligotyping and VNTR-MIRU typing techniques have been used for genotyping.

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