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Molecular characterization of *Mycobacterium tuberculosis* isolates from pulmonary tuberculosis patients in Sri Lanka

（スリランカにおいて肺結核患者より分離された結核菌株の分子疫学的解析）

Balapuwaduge Charitha Gayathri Mendis

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ABBREVIATIONS

DR	Direct repeat
IS 6110 RFLP	Insertion sequence 61110 Restriction fragment length polymorphism
LSP	Large sequence polymorphism
MDR-TB	Multidrug resistant tuberculosis
MIRU-VNTR	Mycobacterial interspersed repetitive unit- variable number tandem repeats
MTB	*Mycobacterium tuberculosis*
MTBC	*Mycobacterium tuberculosis* complex
PCR	Polymerase chain reaction
QRDR	Quinolone resistance determining region
RD	Region of differences
SIT	Spoligo international type
SNP	Single nucleotide polymorphism
TB	Tuberculosis
TbD	*Mycobacterium tuberculosis* specific deletion
WHO	World Health Organization

PREFACE

Tuberculosis (TB) is one of the world leading cause of death by infectious disease with estimated 10 million incidences in 2017. It is mainly caused by *Mycobacterium tuberculosis* (MTB). Although TB is curable and preventable disease, due to incomplete understanding of the genetic variations of MTB that contribute to pathogenesis and antibiotic resistance, still we have not succeeded in combating MTB. It is believed that emergence of multidrug resistant TB (MDR-TB), HIV and poor TB control have contributed to the dramatic increase in the TB burden worldwide.

Sri Lanka is a moderate TB prevalence country in South Indian region. The TB incidence and mortality rates in Sri Lanka in 2017 were 64 and 3.2 per 100,000 population (World Health Organization, 2018). Sri Lanka also has a relatively good TB control programme, with a 69% case detection rate and 82.9% treatment success rate (World Health Organization, 2018). However, it is high time to think of more effective strategies to prevent and control TB in Sri Lanka to go in par with World Health Organization (WHO) End TB Strategy and end the TB epidemic by reducing TB deaths and new cases. Specially we have to target the interruption of transmission and prevention of the emergence of drug resistance outbreaks. One of the key factors that we need achieve this goal is national epidemiological data on circulating genotypes of MTB, transmission patterns, gene mutations conferring drug resistance in Sri Lanka.

MTB is one of the members of the *M. tuberculosis* complex (MTBC). It is an acid fast, intracellular, aerobic bacillus with a tough cell wall structure containing high content of mycolic acids, long chain cross linked fatty acids and other cell wall lipids. It is an intracellular pathogen and able to survive by slow growing in an adverse environment such as inside of the macrophage. MTB has a circular chromosome which contains about 4,200,000 base pairs consisiting of 65% GC content.

Genetic characterization of MTB has diversified the human-adapted strains into seven major lineages, which differ in their geographic distribution and association with human sub-populations (Gagneux et al. 2006b). They are lineage 1(Indo-Oceanic), lineage 2 (East-Asian), lineage 3 (East African-Indan), lineage 4 (Euro American), lineage 5 (West African 1), lineage 6 (West African 2) and lineage 7 ('Aethiops vetus'). Though MTB shows a strong phylogeographical population structure, some lineages occur globally while others show a strong geographical restriction. Therefore, understanding the genetic diversity of MTB strains in a given clinical setting is a key factor to inform the introduction of more effective control measures and patient management strategies.

Over the last decades different genotyping tools such as large sequence polymorphism (LSP), spoligotyping, mycobacterial interspersed repetitive unit- variable number tandem repeat (MIRU-VNTR) typing and whole genome sequencing have become beneficial in epidemiological studies by providing a platform to study the genetic diversity, transmission dynamics and phylogenetic analysis of MTB. LSP analysis is a PCR-based method that uses specific primers for the expected Regions of Difference (RD) for each lineage (Gagneux et al. 2006b). By performing LSP analysis MTB isolates can be assigned into lineage 1-6. Spoligotyping is a frequently used PCR-based molecular typing technique which allows the differentiation of MTB strains into different sub lineages. It uses a reverse-hybridization technique to detect variability in the direct repeat (DR) region which consists of multiple copies of a conserved 36-bp sequence separated by multiple unique spacer sequences in the genome of MTB (Kamerbeek et al. 1997). MIRU-VNTR uses the variability in the numbers of repeats present at particular tandem repeat loci in bacterial genomes, and involves PCR amplification of such tandem repeat loci and size calculation to identify the number of repeats at each locus in a given MTB strain (Supply et al. 2006). MIRU-VNTR method has been used along with spoligotyping as the combination of both approaches has more discriminatory power to identify epidemiologically linked strains. With recent advances in next generation sequencing, the analysis of bacterial whole genome sequences has contributed significantly to the understanding of virulence factors and antibiotic resistance of MTB.

However, the exploration of molecular epidemiology of MTB in Sri Lanka is limited to several studies that have been performed using molecular DNA fingerprinting techniques such as IS*6110*-RFLP, spoligotyping, MIRU-VNTR (Rajapaksa et al. 2008; Magana-Arachchi et al. 2010, 2011; Weerasekera et al. 2015, 2019) and whole genome sequencing (Stucki et al. 2016). Therefore, I aimed to perform molecular characterization of MTB isolates from pulmonary tuberculosis patients in Sri Lanka in order to identify the population structure, transmission patterns and lineage 4 specific characteristics among a selected district (Kandy) in Sri Lanka.

Kandy is one of the main cities in Sri Lanka as well as the capital of Kandy District and the Central Province. It had 1,378,803 population and 3rd highest number of TB patients (n=720) in country in 2013. It is a hotspot for foreign and local pilgrims, tourists and traders since ancient time. Historically it is important as the last Sri Lankan monarchy, where mainly native Sri Lankan population resided until Sri Lanka became a dominion of the British Empire in 1815. During Portuguese (1517- 1638) and Dutch (1602- 1796) colonial periods they mainly interact with Kandy for trade, but British lived in Kandy. South Indians who came with the queens of South Indian origin to the Kandyan Kingdom and the Tamil plantation workers who were brought subsequently from South India to Central province by the British in mid-19th century also started living in and around Kandy District. With this background we selected Kandy District as my study site.

The present thesis consists of two chapters. The first chapter contains genotyping of MTB isolates from Kandy by spoligotyping, LSP analysis and MIRU-VNTR typing in order to identify circulating genotypes of MTB and their transmission patterns within Kandy

District, Sri Lanka. As I identified the predominant MTB lineage in Kandy District, Sri Lanka is lineage 4 (Euro-American lineage) and clonal expansion of locally evolved lineage 4/SIT3234 in chapter I, the focus of chapter II was lineage 4. In chapter II for a deeper understanding of the characteristics of lineage 4 specially concerning lineage 4/SIT3234, comparative genomic analysis was performed.

CHAPTER I

Insight into genetic diversity of *Mycobacterium tuberculosis* in Kandy Sri Lanka reveals predominance of the Euro- American lineage

Introduction

Tuberculosis (TB) is one of the ancient diseases known to mankind yet remains a major public health problem in many low- and middle-income countries. It has overtaken HIV/AIDS as the leading cause of death by a single infectious agent, with an estimated 10 million new TB cases with 1.6 million deaths worldwide in 2017. Two thirds of the estimated number of TB cases in 2017 occurred in Asian and African countries: India (27%), China (9%), Indonesia (8%), the Philippines (6%), Pakistan (5%), Nigeria (4%), Bangladesh (4%) and South Africa (3%). While India accounts for more than a quarter of the global TB burden, the neighboring country Sri Lanka (population 21 million) is among the moderate TB prevalence countries in the region. The TB incidence and mortality rates in Sri Lanka in 2017 were 64 and 3.2 per 100,000 population (World Health Organization, 2018).

It is believed that emergence of multidrug resistant TB (MDR-TB), HIV and poor TB control have contributed to the dramatic increase in the TB burden worldwide. In Sri Lanka, the estimated percentage of TB cases with MDR-TB among new TB patients was 0.5% while it was 4.1% among retreatment patients according to the national surveillance conducted in 2018. Sri Lanka also has a relatively good TB control programme, with a 69% case detection rate and 82.9% treatment success rate (World Health Organization, 2018). In addition, the low prevalence (less than 0.1%) of HIV/AIDS in Sri Lanka may also have contributed to it being an intermediate TB burden country.

However due to the changes in the sociocultural environment, an increasing prevalence of diabetes mellitus and use of immunosuppressive therapies, the TB situation in the country could change. Emigration and immigration could also change the current TB situation through the introduction of new *Mycobacterium tuberculosis* (MTB) strains which are more prone to develop drug resistance or more transmissible virulent. Hence monitoring the MTB population will provide important data to monitor and underpin the Sri Lankan TB control programme.

Genetic characterization of MTB has shown that the human-adapted strains are diversified into seven major lineages, which differ in their geographic distribution and association with human sub-populations (Gagneux et al. 2006b). Though MTB shows a strong phylogeographical population structure, some lineages occur globally while others show a strong geographical restriction. For example lineage 2 and 4 are widespread globally, probably due to high virulence, compared to lineage 5 and 6 which are highly restricted to West Africa; distinct lineages therefore appear to have differing propensities to transmit and develop drug resistance (Gagneux 2018). Therefore, understanding the genetic diversity of MTB strains in a given clinical setting is a key factor to inform the introduction of more effective control measures and patient management strategies.

Over the last decades different genotyping tools such as large sequence polymorphism (LSP), spoligotyping and mycobacterial interspersed repetitive unit- variable number tandem repeat (MIRU-VNTR) typing have become beneficial in epidemiological studies by providing a platform to study the genetic diversity, transmission dynamics and phylogenetic analysis of MTB.

LSP analysis is a PCR-based method that uses specific primers for the expected Regions of Difference (RD) for each lineage (Gagneux et al. 2006b). By performing LSP analysis MTB isolates can be assigned into lineage 1-6.

Spoligotyping is one of the most frequently used PCR based molecular typing techniques which allows the differentiation of MTB strains into different sub lineages/ clades. It is a hybridization assay that detects variability in the direct repeat (DR) region of the DNA of MTB (Kamerbeek et al. 1997). The DR region consists of multiple copies of a conserved 36-bp sequence (the DRs) separated by multiple unique spacer sequences. The entire DR locus is amplified by PCR using primers that are complementary to the sequence of the DRs. The PCR products are hybridized to a membrane with 43 spacer oligonucleotides. Each of the spacers produces either a dark band/spot (indicating the presence of the spacer) or no band/spot (indicating the spacer's absence). For each M. tuberculosis isolate, the spoligotyping assay produces a series of bands. The pattern is converted to a 43-digit binary code system that has 1s and 0s (1 means that the band is present and 0 means it is absent). The results can easily be interpreted and compared using SITVIT2 database. The sensitivity of spoligotyping is estimated to be 10 fg of chromosomal DNA.

MIRU-VNTR typing uses the variability in the numbers of repeats present at particular known tandem repeat loci in bacterial genomes. PCR amplification using primers specific for the regions of tandem repeat loci and the determination of the sizes of the amplicons, after electrophoretic migration are the two steps in this method. As the length of the repeat units is known, the sizes of the amplicons reflect the number of repeats in each locus. The final result is a numerical code, corresponding to the number of tandem repeats present in each locus and this serves as a DNA fingerprint of a bacterial isolate (Supply et al. 2006). MIRU-VNTR method has been used along with spoligotyping as the combination of both approaches has more discriminatory power to identify epidemiologically linked strains.

The molecular epidemiology of MTB is poorly explored in Sri Lanka. Although several studies have been performed that applied molecular DNA fingerprinting techniques such as IS*6110*-RFLP, spoligotyping and MIRU-VNTR (Rajapaksa et al. 2008; Magana-Arachchi et al. 2011; Weerasekera et al. 2015, 2019), the results of these studies indicated the requirement for additional molecular epidemiological analysis of circulating genotypes of MTB in Sri Lanka. Therefore, this study was performed to identify circulating genotypes of MTB and their transmission patterns within Kandy district, in the Central Province in Sri Lanka by using spoligotyping, LSP analysis and MIRU-VNTR typing.

Materials and methods

Sample collection

The sputum samples were collected from 100 new pulmonary TB patients (patients with no evidence of past TB) who visited the Central Chest Clinic in Kandy Sri Lanka from December 2012 to October 2013. Only patients above 18 years of age and currently residing in Kandy district were included in this study. The collected sputum samples were processed and cultured on Lowenstein-Jensen medium at the Department of Microbiology in the Faculty of Medicine, University of Peradeniya. Data on patient demographics, risk factors and laboratory investigations were also collected. This study was ethically approved by the Ethical Review Committee, Faculty of Medicine, University of Peradeniya, Sri Lanka

DNA extraction

Suspected MTB colonies grown on Lowenstein-Jensen medium were suspended in 200µl of distilled water and heated for 20 minutes at 95°C. The heat killed bacteria were transported to Hokkaido University Research Center for Zoonosis Control in Japan and stored at -30°C. After several steps of freezing and boiling, the suspensions were centrifuged for 5 min at 10,000 rpm. Finally, the supernatant containing the bacterial DNA was retrieved and used for further molecular analysis.

Sequencing of drug resistance associating genes

Comparative sequence analysis of *rpoB* gene was performed to confirm the bacterial species (Helb et al. 2010; Poudel et al. 2012). Sequencing to detect mutations in genes associated with drug resistance was performed as described previously by Poudel et al. (2012), targeting the rifampicin resistance-determining region (RRDR) in *rpoB*, *katG* coding and *inhA* regulatory regions, and the quinolone resistance-determining region (QRDR) in *gyrA* in order to identify multidrug resistant (MDR) and pre-extensively drug-resistant (pre-XDR) isolates.

The sequences were compared with the wild-type sequences of H37Rv using BioEdit software version 7.0.9 (Hall, 1999). Phenotypic drug susceptibility test results were not available for these isolates.

Spoligotyping

All MTB isolates were analyzed by spoligotyping as previously described (Kamerbeek et al. 1997). The DR region in the mycobacterial genome was amplified by PCR, and the resulting products were hybridized to a set of 43 spacer specific oligonucleotide probes covalently bound to a membrane. Presence or absence of such spacer was determined (with a dark band indicating the presence of a spacer, while no band indicates a spacer's absence) and this pattern is converted to a 43-digit binary code system which was interpreted and compared using the SITVIT2 database (http://www.pasteur-guadeloupe.fr:8081/SITVIT2/) to determine the spoligotype international type (SIT) (Couvin et al. 2019).

Large sequence polymorphism (LSP)

MTB isolates of spoligotype patterns with either no assigned SIT or sub lineage were analyzed by LSP to assign lineages. PCR was performed using specific primers for the expected Regions of Difference, namely lineage 1-RD239, lineage 3- RD750, allowing lineages to be identified based on the size of PCR products as described by Gagneux et al. (2006b) and Tsolaki et al. (2004). Lineage 4 was identified based on the 7-bp deletion in *pks15/1* locus (Marmiesse et al. 2004).

MIRU-VNTR typing

MIRU-VNTR typing was performed by amplifying 24 loci, including 12 MIRU loci (MIRU2, MIRU4, MIRU10, MIRU16, MIRU20, MIRU23, MIRU24, MIRU26, MIRU27, MIRU31, MIRU39, and MIRU40), four exact tandem repeat (ETR) loci (ETR-A, ETR-B, ETR-C, and ETR-F), four Queens University Belfast (QUB) loci (QUB11a, QUB11b, QUB26, and QUB4156), and four VNTR loci (VNTR424, VNTR1955, VNTR2401, and VNTR3690) with modifications as described by Supply et al. (2006) for the selected clusters based on spoligotyping results. The number of tandem repeats for each locus was calculated from the PCR product size by conventional gel electrophoresis. Isolates that didn't show any band or showed multiple bands in more than two loci, suggestive of mixed infection, were excluded from the analysis after confirmation by repeat testing.

Data analysis

Statistical analysis was performed using RStudio (Integrated Development for R. RStudio, Inc., Boston, MA: URL http://www.rstudio.com/). Spoligoforest tree (Fruchterman-Reingold algorithm) was drawn using the spolTools online software (Reyes et al. 2008; Tang et al. 2008) available on http://spoltools.emi.unsw.edu.au/ to identify the evolutionary relationship among spoligotype patterns. A minimum spanning tree (MST) was constructed based on MIRU-VNTR results using BioNumerics software version 6.6 (Applied Maths, Belgium). Clusters were defined as two or more isolates sharing an identical 24-loci MIRU-VNTR pattern and the clustering rate was calculated using the formula: number of clustered isolates/total number of isolates (Glynn et al., 1999).

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Results

MTB isolates

Out of 100 clinical isolates, 89 were confirmed as MTB by *rpoB* gene sequencing. As four isolates showed evidence of mixed infection with MTB in lineage 1 and 4, those were excluded. Finally, 85 isolates were used for molecular analysis. All the TB suspected patients living in the district were supposed to visit the Central Chest Clinic, thus, the 89 samples can be taken as representative of the region and were approximately 1/7 of the expected total TB incidence cases in the Kandy district during the collection period.

Drug resistance conferring gene mutations

Three isolates (3.5%), out of 85 were genotypically resistant to isoniazid. Two isolates had the G944C mutation (i.e. Ser315Thr substitution) in *katG* and one isolate had a mutation T-8A in the *inhA* regulatory region. No mutations were detected in the RRDR in *rpoB* and QRDR in *gyrA.*

Spoligotyping and LSP

Spoligotyping of 85 isolates enabled the detection of 26 distinct spoligotype patterns corresponding to 21 different SITs and 5 new patterns which have not been reported in SITVIT 2 database yet (Table 1). Those new patterns (New Type 1-5) were assigned into lineage 1 and 4 by LSP. The dominant lineage in our study was lineage 4 (n=39, 46.1%), followed by lineage 1 (n=25, 29.6%) and lineage 2 (n=20, 23.6%). We found only one isolate from lineage 3 (1.2%). The ratio of the lineage 4 was significantly higher than other lineages (*p*<0.05, Chi square test or Fisher’s exact test). SIT1 (Beijing, lineage 2) was the most prevalent SIT found (n=19, 22.4%) followed by SIT11 (EAI3_IND, lineage 1; n=16, 18.8%), SIT124 (Undesignated, lineage 4; n=8, 9.4%) and SIT3234 (Undesignated, lineage 4; n=8, 9.4%) (Table 1). Two isolates from Beijing/SIT1 had a *katG* G944C mutation (Ser315Thr) and one isolate from EAI 3_IND/ SIT355 had an *inhA* T-8A mutation.

MIRU-VNTR typing

Based on the spoligotyping results, clusters of: Beijing/SIT 1 (n=17/19; two isolates were excluded when constructing MST due to no bands in several loci); EAI3_IND/SIT 11 (n=16); Undesignated lineage 4/SIT 124 (n=8); and Undesignated lineage 4/SIT 3234 (n=8) were analyzed by 24 loci MIRU-VNTR typing and an MST was constructed (Fig. 2). The clustering rates in SIT 1, SIT 11 and SIT 124 were 41, 56 and 50%, respectively. All 8 isolates in SIT 3234 were in one cluster (clustering rate = 100%) together with 2 isolates of SIT 124. Genetically INH resistant isolates in SIT 1 (n=2) were singletons.

Analysis of patients’ demographics, risk factors and laboratory findings

Complete data on patients’ demographics, risk factors and laboratory findings (smear positivity and time to culture positivity) were available for 55 patients (Table 2). Overall, 42 patients were male and 13 were female (male to female ratio 3.23). The age of the patients ranged from 21-80 years. There was no significant association between variables and the lineage 4 or non-lineage 4 when compared category-wise.

Discussion

MTB, the main causative agent of human TB co-evolved with humans and its diversity has been shaped by human migration out of Africa and distinct human populations (Comas et al. 2013). By adapting to different human populations, lineage 1, 2, 3, 4 evolved and became endemic lineages in the Indian Ocean Region, East Asia, Central Asia and Europe respectively. Brosch et al (2002) found that MTB strains could be divided into “ancestral” and “modern” strains based on the presence or absence of an MTB specific deletion (TbD1) region. Among the four MTB lineages observed in this study, only the lineage 1 (labeled EAI or MANU in the spoligotyping nomenclature) possesses an intact TbD1 locus and is therefore an “ancient” type. Lineage 1 is suggested to have been the first MTB lineage that emerged out of Africa and became the predominant lineage in countries bordering the Indian ocean from Eastern Africa to Melanesia. Later, lineage 3 is thought to have emerged across Southern Asia and dispersed out of the Indian subcontinent (O’Neill et al. 2019). When the distribution of lineages was compared among different geographical areas of India, lineage 1 (EAI/TbD1+) was predominant in southern India while lineage 3 (CAS/TbD1-) was dominant in northern India. This suggests that lineage 1 could be the endemic lineage in Southern Asia, while lineage 3 emerged and spread from the northern to southern area in subsequent periods (Gutierrez et al. 2006; Thomas et al. 2011; Joseph et al. 2013; Varma-Basil et al. 2016; Manson et al. 2017a; Sharma et al. 2017) (Table 3, Table 4). In a previous study in Sri Lanka in which isolates were collected in Colombo, commercial capital on the west coast, lineage 1 was also reported as dominant with 58.2% of isolates belonging to this lineage (Rajapaksa et al. 2008). These findings are similar to those in the nearby region of southern India, suggesting that lineage 1 could be the endemic ‘domestic’ lineage in this location. Furthermore, the prevalence of lineage 3 was found to be less than 1% in Sri Lanka (Table 3, Table 4) suggesting less interaction between Sri Lanka and central or northern India.

In contrast, this study revealed the predominant lineage circulating in the Kandy district was lineage 4, and not lineage 1 as expected. The historical relationship that Sri Lanka has had with European countries may have contributed to this finding. Sri Lanka was colonized by the Portuguese, Dutch and British for hundreds of years (16-17th, 17-18th and 19th-mid 20th century, respectively). I hence hypothesize that the introduction of lineage 4 into Sri Lanka may have happened during the European colonial period. Supporting my hypothesis, population genomic and phylogeographic analysis of MTB lineage 4 have found that dispersal of lineage 4 has been dominated by historical migrations out of Europe (Brynildsrud et al. 2018). This latter study demonstrated an intimate temporal relationship between European colonial expansion into Africa and the Americas and the spread of MTB lineage 4. In Sri Lanka, Portuguese and Dutch settlers mainly colonized the coastal area including Colombo, whereas British settlers scattered over the country and mainly resided in Kandy. Evidence for predominance of lineage 4 in the Kandy district may suggest that it was introduced as a founder MTB population, or alternatively that, as the "modern" lineage 4 (TbD1-) is suggested to have enhanced virulence and an ability to infect distinct human populations with different genetic backgrounds (Stucki et al. 2016), it may have outcompeted the "ancient" lineage 1 (TbD+) that may have been the endemic lineage in Kandy prior to colonization.

I identified 14 distinct spoligotype patterns in the lineage 4 isolates. Half of them were designated as Haarlem, T and X sublineages, which are well known to be prevalent in European countries. Comparison of these spoligopatterns with those circulating in other countries using data present in the SITVIT2 database revealed that SIT50, SIT49 and SIT53 have worldwide distribution including Portugal, Netherlands and the United Kingdom; SIT2 has been mainly distributed in Europe and SIT478 is prevalent in the European region. This again provides circumstantial evidence that Portuguese, Dutch and British settlers introduced lineage 4 to Sri Lanka during the colonial period. SIT50 and SIT53 sublineages seem to be well established in Sri Lanka as they were also reported in previous studies (Rajapaksa et al. 2008; Weerasekera et al. 2015). The other half of the spoligotypes studied, in which the majority of lineage 4 isolates (26/39, 66.7%) were contained, were of a new or undesignated type. An important finding of this study was that 33.1% (27/85) of isolates had new or undesignated spoligotype patterns according to the SITVIT2 database and 96.3% (26/27) of those were identified as lineage 4 by LSP. This finding indicates that lineage 4 has been circulating in Kandy, Sri Lanka for a long time and that micro evolution to adapt to the Sri Lankan host population may have occurred. However, further detailed studies using techniques like such as whole genome sequencing and time-scaled haplotypic density are warranted to confirm the factors that shaped the local population structure of MTB in Sri Lanka.

A spoligoforest tree (Fig.2) revealed the probable parental links between the strains belonging to different sublineages. Most of ancestral lineage 1 (EAI) strains were linked within a parental network with no recent evolutionary connections to the new types. In contrast, the majority of lineage 4 strains were linked within a parental network together with undesignated and new types showing ongoing evolution. SIT124 is a probable descendent of SIT50 (Haarlem, H3) while SIT3234, SIT1952 and new type 1 have evolved from SIT124. MIRU-VNTR analysis using 24 loci showed all isolates (n=8) in SIT3234 were in one cluster together with 2 isolates of SIT124 indicating a clonal expansion of these sublineages in the study group. Out of eight SIT3234 isolates, four patients' demographic data were available and revealed that all the patients lived in different regions and that no direct contact between them was found suggesting this sub-lineage has already widely spread in the area. In the SITVIT2 database, 0.06% of isolates belong to SIT124 with a worldwide distribution that includes India, China, Netherlands and United Kingdom, all of which are known to have deeply rooted historical relations with Sri Lanka. Previous studies also suggested the clonal expansion of this sublineage in Sri Lanka (Rajapaksa et al. 2008; Weerasekara et al. 2015). SIT3234 which was found in China (n=1) and France (n=1) in the SITVIT2 database was also reported in Sri Lanka (Weerasekara et al. 2015). Comparison of 15-loci MIRU-VNTR patterns of SIT124 and 3234 in our study with the SITVIT2 database revealed that identical or similar MIRU-VNTR patterns have not been reported previously. Therefore, clonal expansion of SIT3234 requires attention, monitoring and further characterization as it seems to have evolved in Sri Lanka with local adaptation. It also has a parental link with the Haarlem sublineage which is known to cause drug resistant epidemics (Mardassi et al. 2005; Khanipour et al. 2016; Tarashi et al. 2017). These SIT3234 isolates formed a cluster with Haarlem isolates in a NJ-Tree using 22 MIRU-VNTR loci in MIRU-VNTRplus (https://www.miru-vntrplus.org/MIRU/index.faces, Weniger et al. 2010) (data not shown). Evolutionary ‘modern’ sublineages like Beijing and Haarlem are suggested to be more virulent compared to ‘ancient’ ones such as EAI. Based on this assertion SIT124 and 3234, which showed clonal expansion in this study, could have implications for epidemiology and control of TB in Sri Lanka in the future.

The Beijing sublineage is considered to be one of the predominant MTB sublineages, with a worldwide distribution and particularly dominating in East and South East Asian countries (Tamaru et al. 2012; Merker et al. 2015). The Beijing sublineage is suggested to be more virulent than other sublineages, showing higher pathogenicity and increased mortality in animal studies (Parwati et al. 2010). This lineage also has a higher mutation rate which contributes to its success as a major sublineage responsible for MDR and XDR (Parwati et al. 2010; Merker et al. 2015; San et al. 2018). Ongoing transmission of the Beijing sublineage has previously been detected in Sri Lanka (Rajapaksa et al. 2008; Weerasekera et al. 2015), as well as in our current study. While SIT1 is the most prevalent SIT that found in this study, MIRU-VNTR results (Fig.1) showed highly diverse patterns. The Beijing lineage may have been introduced to Sri Lanka through trading links with South East Asian countries during the period that Sri Lanka was one of the main ports in ancient maritime silk and spice trade routes. Furthermore, continuous migration and emigration between populations in Sri Lanka, China and other South Asian countries that continues up to the present date may be responsible for the higher genetic diversity within this sublineage in Sri Lanka. In addition, there is a hypothesis that Beijing lineage strains may have spread as a result of their increased resistance to BCG induced immunity (Bifani et al. 2002), a suggestion which may also need to be considered for selective transmission of Beijing strains in Sri Lanka as it has a high coverage of BCG vaccination.

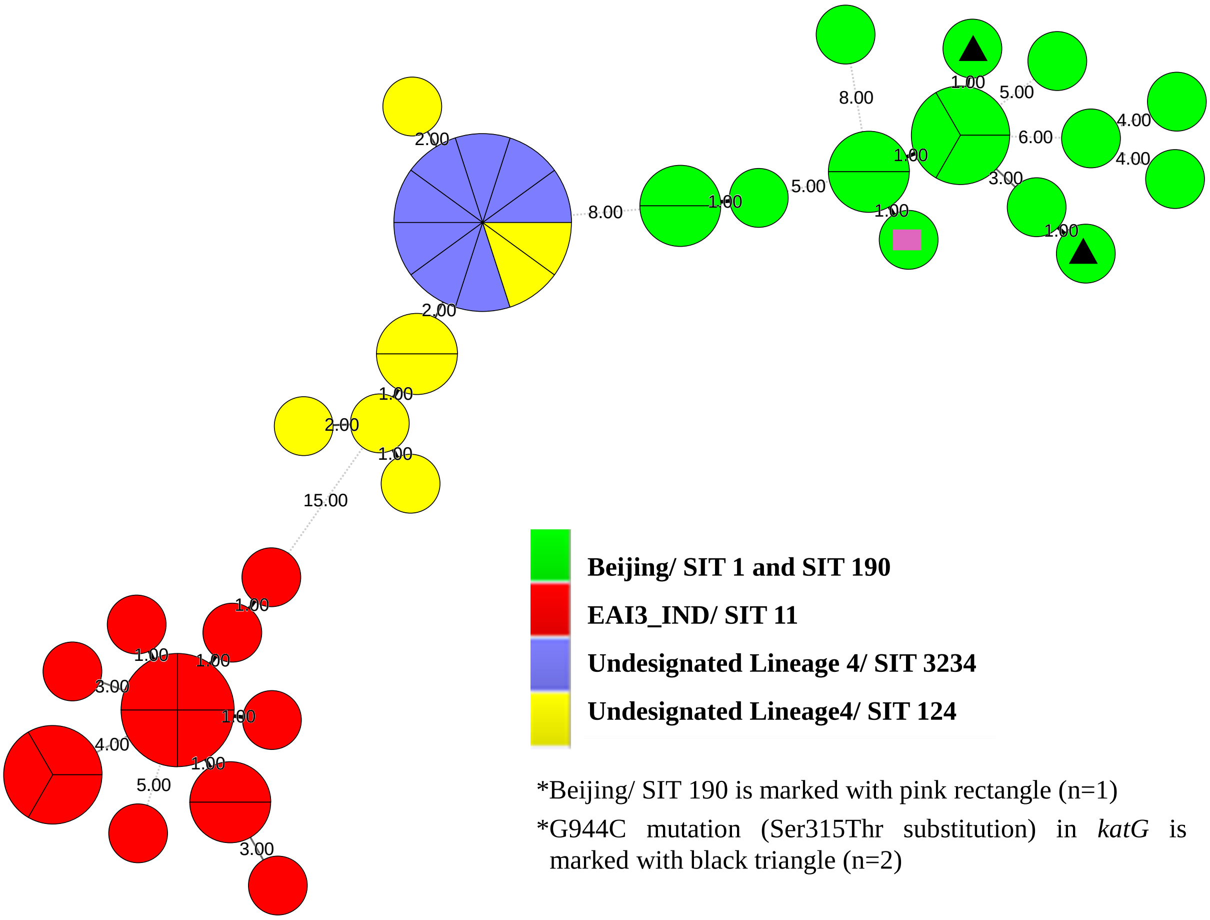
Two isolates from the Beijing/SIT1 clade had a G944C mutation (Ser315Thr) in *katG* suggesting resistant to isoniazid. The *katG* Ser315Thr mutation is a well-known low fitness cost substitution (Gagneux et al. 2006a; Manson et al. 2017b) that supports the maintenance of efficient transmission of drug resistant MTB and is associated with MDR epidemics worldwide (Manson et al. 2017b; Shah et al. 2017; San et al. 2018). The *katG* Ser315Thr mutation is reported to have arisen before mutations that conferred rifampicin resistance across all of the MTB lineages, geographical regions and time periods (Manson et al. 2017b). Monitoring the drug resistant patterns in TB patients in Sri Lanka is highly warranted so as to identify the trends in drug resistance, to inform current control and to prevent future outbreaks. Detection of the harbinger the mutation, katG Ser315Thr, also known as pre-MDR TB mutations, could be advantageous in this aspect.

Considering lineage 1, a high percentage of EAI3_IND/SIT11 was also observed in previous studies in Sri Lanka (Rajapaksa et al. 2008) and South India (Joseph et al. 2013), suggested that South India may represent the probable origin of this sublineage in Sri Lanka due to migratory patterns that stretch back to ancient times. What's more, in a previous study by Rajapaksa et al (2008), the EAI5 sublineage was shown to be prevalent (n=20/98, 20%) in the Western province while present at a much lower prevalence in the Kandy district (n=3/85, 3.5%). These findings suggest that the diversity of the MTB population structure in Sri Lanka. A high proportion of the MANU sublineage was detected in Kandy by Weerasekera et al. (2015), but I was unable to identify any isolate within this sublineage. This discrepancy may occur because the MANU sublineage spoligotype could be constructed by combining more than two spoligotype patterns (Lazzarini et al. 2012; Diab et al. 2016) in situations of mixed infections or a contamination.

In summary, the predominant lineage of MTB in Kandy, Sri Lanka was lineage 4 which may have been introduced by European traders and settlers during the colonial period. As the isolates from lineage 4 were genetically diverse, with most of them were having an undesignated or new spoligotype pattern, I suggest that this lineage has circulated in Sri Lanka for a long time period with microevolution driving the emergence of new descendants which may be adapted to the local Sri Lankan host population. Therefore, the clonal expansion of locally evolved and potentially host-adapted undesignated lineage 4/SIT3234 requires continued monitoring to inform the control of current and future outbreaks. The Beijing/SIT1 clade was the most prevalent SIT found in this study indicating ongoing transmission that reflects the global situation with the Beijing lineage. Though I didn't find MDR TB in my study, two isolates of Beijing /SIT1 from new TB patients had well known pre-MDR *katG* G944C mutation (Ser315Thr) which alarms for monitoring. This study shows that it will be necessary to carry out continuous surveillance of genetic diversity and drug resistant TB to develop a clear picture of prevalence, transmission and evolution of the TB to prevent future epidemics in Sri Lanka.

Summary

Sri Lanka is a country where the molecular epidemiology of *Mycobacterium tuberculosis* (MTB) is poorly explored. Therefore, this study was performed to identify circulating lineages/sub lineages of MTB and their transmission patterns. DNA was extracted from 89 isolates of MTB collected during 2012 and 2013 from new pulmonary tuberculosis patients in Kandy, Sri Lanka and analyzed by spoligotyping, large sequence polymorphism (LSP), mycobacterial interspersed repetitive unit-variable number tandem repeat (MIRU-VNTR) typing and drug resistance-associated gene sequencing.The predominant lineage was lineage 4 (Euro-American, 46.1%), followed by lineage 1 (Indo-Oceanic, 29.6%), lineage 2 (East-Asian, 23.6%) and lineage 3(Central-Asian, 1.2%). Among 26 spoligotype patterns, eight were undesignated or new types and seven of these belonged to lineage 4. Undesignated lineage 4/SIT 124 (n=2/8) and SIT 3234 (n=8/8) clustered together based on 24-locus MIRU-VNTR typing. The dominant sublineage was Beijing/SIT 1 (n=19), with isoniazid resistance *katG* G944C mutation (Ser315Thr) detected in two of them. The population structure of MTB in Kandy, Sri Lanka was different from the South Asian Region. Clonal expansion of locally evolved lineage 4/SIT 3234 and detection of the pre-MDR Beijing isolates from new TB patients is alarming and will require continuous monitoring.



*Beijing/ SIT 190 is marked with pink rectangle (n=1)

*G944C mutation (Ser315Thr substitution) in *katG* is marked with black triangle (n=2)

Figure 1. 24-loci MIRU-VNTR based MST of Beijing/ SIT1, EAI3_IND/ SIT11, Undesignated Lineage 4/ SIT124, Undesignated Lineage 4/ SIT 3234 isolates.

Each node represents a MIRU-VNTR type. The size of the node indicates the number of the isolates in each cluster. The length of the branches represents the distance between patterns while the numbers on the branch denotes the number of loci changes between two patterns. Green: Beijing/ SIT1, Red: EAI3_IND/ SIT11, Yellow: Undesignated Lineage 4/ SIT124, Purple: Undesignated Lineage 4/ SIT 3234

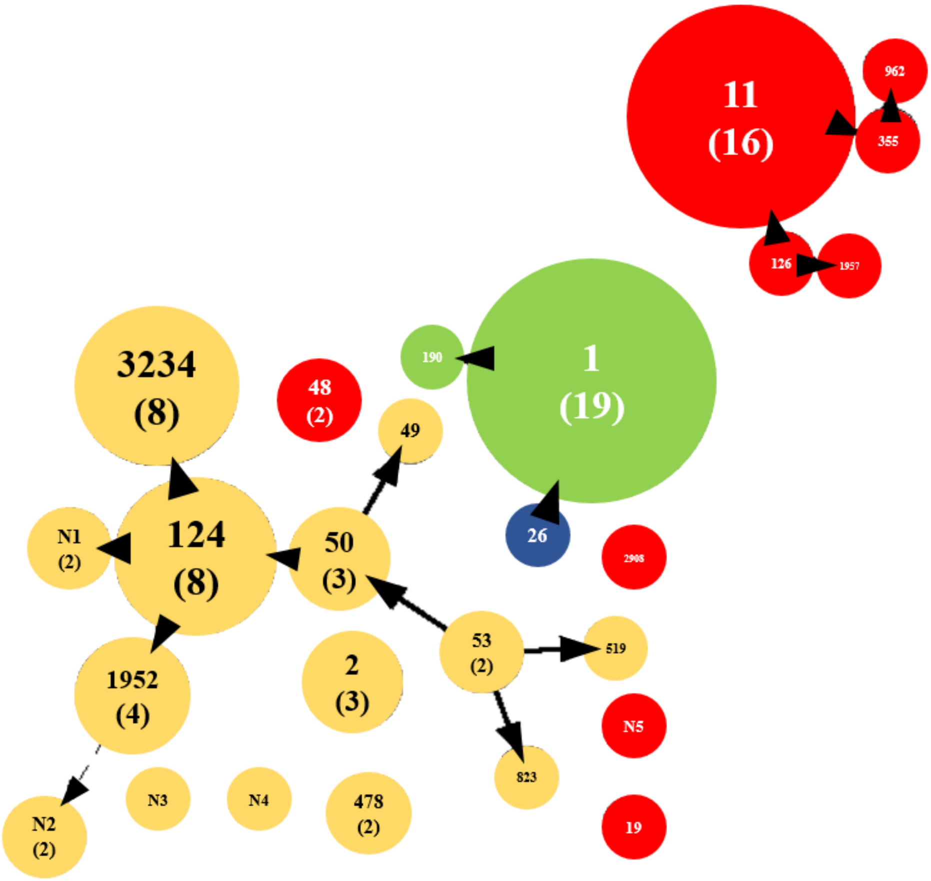


Figure 2. A Spoligoforest tree based on all spoligotypes

Each spoligotype pattern from the study is represented by a node with area size being proportional to the total number of isolates with that specific pattern. Changes (loss of spacers) are represented by directed edges between nodes, with the arrowheads pointing to descendant spoligotypes. The heuristic used selects a single inbound edge with a maximum weight using a Zipf model. Solid black lines link patterns that are very similar, i.e., loss of one spacer only (maximum weigh being 1.0), while dashed lines represent links of weight comprised between 0.5 and 1, and dotted lines a weight less than 0.5. Number inside the circle is SIT number while the number in parenthesis indicates the number of isolates in our study with that SIT.

Table 1

Description of 26 Spoligotype International Type (SITs; n=85 isolates) and corresponding spoligotyping defined sub lineages

Sub Lineage [a]	SIT [b]	Spoligotype Description [c]	Octal Number	No, of isolates	% of isolate
Lineage 1 (Indo- Oceanic Lineage)					
EAI3_IND	11	■□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□■■■■	477777777413071	16	18.8
EAI1-SOM	48	■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■□■■■	777777777413731	2	2.4
EAI3_IND	355	■□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□□■■■	477777777413031	1	1.2
EAI5	126	■□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■■■■■■■■	477777777413771	1	1.2
EAI5	962	□□□■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□■■□□□□■■■	777777777413031	1	1.2
EAI5	1957	■□□■■■■■■■■■■■■■■■■■■■■■■■■□□□□□■□■■■■■■■■■	477777777013771	1	1.2
EAI2_MANILLA	19	■■□■■■■■■■■■■■■■■■■□□■■■■■■■□□□□■□■■■■■■■■■	677777477413771	1	1.2
EAI6-BGD1	2908	■■■■■■■■■■■■■■■■■■■■■■□■■■■■□□□□■□■■■■□■■■■	777777757413671	1	1.2
New type 5		■■■■■■■■■■■■■■■■□■■■■■□□■■■■□□□□■□■■■■□■■■■	777775747413671	1	1.2
Lineage 2 (East - Asian Lineage)					
Beijing	1	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■■■■■	000000000003771	19	22.4
Beijing	190	□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□□■■■■■□■■■	000000000003731	1	1.2
Lineage 3 (East- African- Indian Lineage)					
CAS1-Delhi	26	■■■□□□□■■■■■■■■■■■■■■■□□□□□□□□□□□□■■■■■■■■■	703777740003771	1	1.2
Lineage 4 (Euro-American Lineage)					
Undesignated	124	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■■	777777777700771	8	9.4
Undesignated	3234	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□■■■■■■	777777777600371	8	9.4
Undesignated	1952	■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□■■■■■■■	777777774000771	4	4.7
H2	2	□□□□□□□□□□□□□□□□□□□□□□□□■□□□□□□■□□□□■■■■■■■	000000004020771	3	3.5
H3	50	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■■■■■	777777777720771	3	3.5
T1	53	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■■■■■■■	777777777760771	2	2.4
New type 1		■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■□■■■■	777777777700671	2	2.4
X2	478	■■□□□■■■■■■■■■■■■□■■■■■■■■■■■■■■□□□□■■□□□□■	617776777760601	2	2.4
New type 2	orphan	■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□□□□□□■■■□■■■	777777774000731	2	2.4
H3	49	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□■□□□□■■■□■■■	777777777720731	1	1.2
T1	823	■■■■■■■■□□□□□□□□□□□□□□□□□■■■■■■■□□□□■■■■■■■	776000003760771	1	1.2
T1	519	■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■■□□□□□□■■■■■■	777777777740371	1	1.2
New type 3		■■■■■■■■■■■■□□□□■■■■■■■■■■■■■■■■□□□□■■■□□□□	777703777760700	1	1.2
New type 4		■■■■■■■■■■■■■■■■■■■■■■■■■□□□□■□□□□□□■■■■□■■	777777774100751	1	1.2

[a] Sub lineages were annotated using the SITVITWEB database

[b]SIT (Spoligo International types) were assigned by SITVITWEB database

[c]Closed squares represent positive hybridization (presence of spacer) and open squares represent no hybridization (absence of spacer)

(Undesignated - Spoligotype pattern is available in SITVIT 2 database and has SIT number, but the sub lineage is not assigned; Orphan- A SIT number has not been assigned for the Spoligotype pattern by SITVIT 2 database but has been reported by previous studies; New type- Spoligotype pattern found only in our study)

Table 2

Characteristics of patients (n=55) infected with Lineage 4 and Non-Lineage 4 isolates

	Variants	**Number of Lineage 4 (n=30)**	**Number of non-Lineage 4 (n=25)**	***p*-Value[a]**
Demographics				
Gender	Male	26	16	0.0620
	Female	4	9	
Age	21- 40 years	6	11	0.1654
	41- 60 years	15	10	
	61-80 years	9	4	
Occupation[b]	Office workers/ businessman	8	6	
	Laborers	6	3	
	Estate workers	2	3	
	Drivers	1	2	
	Factory workers	0	2	
	Worked at Elderly home	0	1	
	Worked abroad	0	1	
	Worked in Tourist industry	1	2	
	Housewife	2	5	
	Non respondent	10	0	
Risk factors				
Smoking	Yes	15	12	1.0000
	No	15	13	
Comorbidity	Diabetes mellitus	10	7	0.9268
	Other comorbidities (lung disease, taking chemotherapy, cancer)	4	3	
	None	16	15	
Contact history	Yes	2	1	1.0000
	No	28	24	
Laboratory				

findings				
Direct smear	Positive	19	18	0.5716
	Negative	11	7	
Time for become culture positive	1-2 weeks	8	4	0.3466
	3-4 weeks	16	11	
	5-6 weeks	3	7	
	7-8 weeks	3	3	

[a] A *p*-value of <0.05 was considered significant; determined by Fisher’s exact test.

[b] As number of patients in each variable of “occupation” were low, it was excluded from statistical analysis

Table 3 Summary of *M.tuberculosis* lineages distribution from previous studies in Sri Lanka and India

		Country of isolation %							
		Sri Lanka		India					
		Current study 2019 N=100	Rajapaksa et al. (2008) N= 98	Joseph et al. (2013) N=168	Manson et al. (2017a) N=201	Thomas et al. (2011) N= 101	Sharma et al. (2017) N= 335	Varma-Basil et al. (2016) N= 139	Gutierrez et al. (2006) N=91
Period of sample collection		2012-2013	2005-2006	1998-2005	1999-2005	2000-2005	2005-2007	2010-2011	1996- 2002
Geographical area		Kandy District	Colombo[a]	Kerala[b]	Tamil Nadu[b]	Andhra Pradesh[c]	Ghatampur[d]	Delhi[d]	12 different region
Lineages	**Lineage 1**	29.6	58.2	81.5	70.0	48.5	22.4	23.0	45,0
	Lineage 2	23.6	14.3	2.4	11.0	4.0	3.9	6.5	10.0
	Lineage 3	1.2	0	6.5	16.0	40.6	63.6	53.2	26.0
	Lineage 4	46.1	27.6	9.5	3.0	6.9	10.1	14.4	19.0

[a] Sample collection site was Colombo, however, the residences of patients were unclear [b] Kerala and Tamil Nadu represent Southern India.
[c] Andhra Pradesh represent South Eastern India. [d] Ghatampur and Delhi represent Northern India. [e] U = Undesignated

Table 4 Summary of *M.tuberculosis* sublineages (selected) distributions from previous studies in Sri Lanka and India

		Country of isolation %							
		Sri Lanka		India					
		Current study (2019) N=100	Rajapaksa et al. (2008) N= 98	Joseph et al. (2013) N=168	Manson et al. (2017a) N=201	Thomas et al. (2011) N= 101	Sharma et al. (2017) N= 335	Varma-Basil et al. (2016) N= 139	Gutierrez et al. (2006) N=91
Period of sample collection		2012-2013	2005-2006	1998-2005	1999- 2005	2000-2005	2005- 2007	2010-2011	1996-2002
Geographical area		Kandy District	Colombo[a]	Kerala[b]	Tamil Nadu[b]	Andhra Pradesh[c]	Ghatampur[d]	Delhi[d]	12 different region
Lineages/ sublineages									
Lineage 1	**EAI**	28.4	33.6	64.9	70	20.8	19.1	14.4	32.9
	Manu	0	0	1.2	0	7.9	3.0	1.4	0
Lineage 2	**Beijing**	23.6	14.3	2.4	11	2.0	3.3	6.5	8.5
Lineage 3	**CAS**	1.2	0	4.8	16	26.7	59.1	48.2	19.2
Lineage 4	**Haarlem**	8.2	3.1	1.		2.8	0.6	2.8	0
	T	4.8	5.1	3.6	1.5	1.0	5.07	7.2	4.3
	X	2.4	0	0	0	0	2.68	1.4	0
	LAM	0	1.0	0.6	0	0	0.29	0	0
	U[e]/SIT 124	9.4	6.1	3.0	0	0	0	0	0
	U[e]/SIT 3234	9	0	0	0	0	0	0	0
	U[e]/SIT 1952	4.7	0	1.2	0	0	0.3	0	0

[a] Sample collection site was Colombo, however, the residences of patients were unclear [b] Kerala and Tamil Nadu represent Southern India.

[c] Andhra Pradesh represent South Eastern India. [d] Ghatampur and Delhi represent Northern India. [e] U = Undesignated

CHAPTER II

Genetic signatures of *Mycobacterium tuberculosis* lineage 4 in Kandy, Sri Lanka

Introduction

Tuberculosis (TB) remains a global threat despite of efforts that have been taken towards its control. Although TB is a curable disease, due to incomplete understanding of the genetic variations of *Mycobacterium tuberculosis* (MTB) that contribute to pathogenesis and antibiotic resistance, still we have not succeeded in combating MTB. However, with recent advances in next generation sequencing, the analysis of bacterial whole genome sequences has significantly contributed to better understanding of genetic variability, mycobacterial population dynamics and evolutionary genetics. This knowledge will prime the understanding epidemic potential of strains, differences in virulence, antibiotic susceptibility which will possibly be important for the treatment and control of TB.

There are seven human adapted phylogeograpghic lineages of MTB. Large sequence polymorphisms (LSPs), such as regions of difference (RD) TbD1 (Brosch et al. 2002) and other lineage specific RDs together with additional phylogenetic markers such as single nucleotide polymorphisms (SNPs) (Tsolaki et al. 2004b; Gagneux et al. 2006b; Sreevatsan et al. 1997) allowed the recognition of these main lineages : lineage 1(Indo-Oceanic), lineage 2 (East-Asian), lineage 3 (East African-Indan), lineage 4 (Euro American), lineage 5 (West African 1), lineage 6 (West African 2) and lineage 7 (‘Aethiops vetus’) with evolutionary evidence.

Though the human adapted M. tuberculosis shows a strong phylogeographical population structure, some lineages occurs globally while others show a strong geographical restriction. For example lineage 2 and 4 are most widespread globally probably due to high virulence compared to lineage 5 and 6 which are highly restricted to West Africa (Gagneux 2018).

In first chapter, I identified that the predominant lineage of MTB in Kandy, Sri Lanka was lineage 4 (Euro-American lineage), but not lineage 1 as expected. I hypothesized lineage 4 may have been introduced by European traders and settlers during the colonial period based on the Sri Lankan history. Further I suggest either lineage 4 was introduced as a founder MTB population or the "modern" lineage 4 (TbD1-) may have outcompeted the "ancient" lineage 1 (TbD+) that may have been the endemic lineage in Kandy prior to colonization. As the isolates from lineage 4 were genetically diverse, with most of them were having an undesignated or new spoligotype pattern, I suggested that this lineage has circulated in Sri Lanka for a long time period with microevolution driving the emergence of new descendants which may be adapted to the local Sri Lankan host population. I also revealed the clonal expansion of locally evolved and potentially host-adapted undesignated lineage 4/ SIT 3234 in Kandy, Sri Lanka. Therefore, I proposed continued monitoring of lineage 4 with special attention to SIT 3234 to control and prevent current and future outbreaks

To achieve this goal, we need to have background knowledge on genetic variation in lineage 4 in Sri Lanka and specific genetic variations of SIT 3234 to identify virulent potential and to develop molecular based rapid detection tool for epidemiological studies.

Therefore, the main objectives of this study was to detect the genomic variations in MTB lineage 4 in Kandy, Sri Lanka and to identify the clonality and micro diversity of SIT 3234 isolates.

Materials and Methods

MTB lineage 4 strains used for whole genome sequencing

In chapter I, 39 isolates out of 85 were identified as lineage 4. Based on the DNA concentration, spoligotype pattern and mycobacterial interspersed repetitive unit- variable number tandem repeat (MIRU-VNTR) pattern, 20 isolates were selected for whole genome sequencing (T1/SIT 53: n=2, T1/ SIT 823: n= 1, X2/SIT 478: n= 2, H2/SIT 2: n=1, H3/ SIT 50, H3/ SIT 49, undesignated lineage 4/ SIT 124: n=4, undesignated lineage 4/ SIT 3234: n=4, Undesignated lineage 4/ SIT 1952: n= 1, New type 1: n=2, New type: 3: n= 1) (Table 1). In previous study SIT 3234 showed clonal expansion, clustering 8 isolates with 2 isolates of SIT 124 having similar MIRU-VNTR pattern. Hence one isolate of SIT 124 and 4 isolates of SIT 3234 from that cluster were included in the above mentioned 20 isolates

Whole genome sequencing and analysis

Genomic DNA of the 20 isolates of lineage 4 were sequenced using Illumina MiSeq sequencer, Nextera XT library preparation kits and Miseq reagent kit as instructed by the manufacturer (Illumina, San Diego, CA, USA). Resulting reads were mapped to the MTB H37Rv genome (GeneBank accession number NC_000962.3) using CLC Genomic Workbench version 12. SNPs and INDELS (insertions or deletions) were also detected using CLC genomic workbench version 12 with a minimum depth of 10x. SNPs with low quality evidence were discarded. As for some strains the reference genome coverage was low the large sequence polymorphisms (region of differences) were determined by checking the genome sequences manually. The mapping consensus sequences of isolates were extracted and aligned by MAFFT version 7. (online available at https://mafft.cbrc.jp/alignment/software/_).

Construction of phylogenetic trees

After aligning the whole genomic sequences of strains, phylogenetic tree for 16 isolates and reference strain H37Rv was constructed based on the SNPs. Four isolates with less coverage of reference genome were excluded. The evolutionary history was inferred using the Neighbor-Joining method (Saitou and Nei 1987) with 1000 bootstraps (Felsenstein 1985). The evolutionary distances were computed using the Tamura-Nei method (Tamura and Nei 1993) and are in the units of the number of base substitutions per site. The differences in the composition bias among sequences were considered in evolutionary comparisons (Tamura and Kumar 2002). All positions containing gaps and missing data were eliminated. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

A phylogenetic tree based on Jaccard's similarity coefficient was developed for 20 isolates. Distance was estimated as the Jaccard distance for the presence/absence of the region of deletions. To estimate the pairwise distance between two different strains (eg. X and Y) we used the formula $J(X,Y) = 1-(X\cap Y)/(X\square Y)$. The calculation of distance and construction of phylogenetic were done with RStudio (Integrated Development for R. RStudio, Inc., Boston, MA : URL http://www.rstudio.com/).

Assignment of sublineages

Based on the previously published, sublineage specific regions of differences and SNPs (Coll et al. 2014; Stucki et al. 2016), Sri Lankan strains were assigned in to the sublineages.

SNPs in undesignated lineage 4/ SIT 3234

The SNPs that are common to the SIT 3234 strains were identified using CLC genomic workbench version 12.

Results

Whole genome sequencing

Out of 20 genome sequences, 16 were showed approximately 100% coverage of reference genome. But one sample showed 87% coverage while the another three showed less than 50% coverage. Details of the mapping and annotation are shown in Table 2. All isolates had 65.5% guanine/ cytosine (G/C) content, typical of mycobacteria. The draft genomes had average size of 4,334,630 bp. Per isolate 642-1104 SNPs were determined.

Sublineages of lineage 4

Out of 20 isolates 18 were assigned into sublineages and 2 isolates were excluded because of their less coverage of reference genome. Based on sublineage specific deletion of RDs and SNPs, following sublineages were identified: L4.1.2.1Haarlem, L4.1.1.1 X, L4.3.3 LAM, L4.2.2, L4.4.1 and L4.8. Majority of the isolates (13/18) were identified as L4.1.2.1Haarlem. SIT124, SIT 3234 and new type 1 were classified as L4.1.2.1 Haarlem. However, three isolates which were detected as T1 by spoligotyping were reassigned into L4.8, L4.3.3 LAM and L4.4.2. New type 3 was identified as L4.4.1 while X2/SIT 478 was confirmed its identity as L4.1.1.1 X (Table3).

Phylogenetic distribution of lineage 4 isolates

Phylogenetic tree that was constructed based on SNPs in whole genomes of 16 isolates showed the evolutionary relationship among circulating lineage 4 strains in Kandy, Sri Lanka (Figure 1). This provided evidence that isolates belonging to SIT 124, SIT 3234, New type 1 clustered together with H3/ SIT 49, H3/SIT 50, H2/ SIT 2 into one branch showing their evolutionary relatedness with Haarlem sublineage. This is concordance with sublineage classification (L4.1.2.1) of Stucki et al. (2016). Furthermore, isolates of SIT 3234 clustered into a subclade within that big cluster of Haarlem.

Large sequence polymorphism

Previously reported (Coll et al. 2014; Stucki et al. 2016) sublineage specific RDs: RD 115 for LAM, RD 183 for X2, RD 219 for T1 and RD 182 for Haarlem were found in the isolates in this study (Figure 2). In addition, Haarlem specific deletions: HSD4 and HSD6 reported by Cubillos-Ruiz et al. (2010) were also identified. Furthermore 12 regions of deletions which were not reported previously were also observed and they were named as SL RDs (Table 4). Those deletions showed a different distribution pattern among different sublineages in our study (Figure 2). Isolates in L4.1.2.1 Haarlem sublineage can be further differentiated in to 4 clades based on SL-RDs (Figure 3).

SNPs in SIT 3234

We found 259 SNPs which were common to four isolates belonging to undesignated lineage 4/ SIT 3234. Among them 46 SNPs were in non-coding regions while 213 were in coding regions. We identified 90 synonymous mutations and 123 non-synonymous mutations among 213 SNPs in coding regions.

Discussion

Comparative genomic studies have shown that MTB complex has evolved through irreversible genetic events that occurred in ancient common progenitor strains (Brosch et al, 2002). The major forces that drive MTB genome evolution are mutations, deletions, and transpositions of chromosomal regions, but not the horizontal genetic exchange between MTB strains. In recent past, through analyzing the whole genomes of MTB, number of phylogenetically informative deletions of large genomic sequences and SNPs have been identified. Based on such previously reported RDs and SNPs (Coll et al. 2014; Stucki et al. 2016) we assigned sublineages to lineage 4 isolates found in Kandy, Sri Lanka and they were L4.1.2.1Haarlem, L4.1.2 Haarlem, L4.1.1.1 X, L4.3.3 LAM, L4.2.2, L4.4.1 and L4.8. We were able to classify SIT124, SIT 3234 and new type 1 as L4.1.2.1 Haarlem. With this allocation the total number of isolates belonging to Haarlem sublineage increased to 29 isolates in our original study group in chapter I. It represents 74.4% of lineage 4 MTB isolates and 34.1% of total MTB isolates analyzed in Kandy, Sri Lanka. Therefore, the whole genome analysis revealed the major sublineage of MTB circulating in Kandy, Sri Lanka was Haarlem (34.1%) followed by EAI (28.4%) and Beijing (23.6%).

However, I found incompatibility between spoligotyping based sublineages and RDs, SNPs based sublineages. Three isolates which were identified as T1 by spoligotyping were reassigned into L4.8, L4.3.3 LAM and L4.4.2. in this study. This may have occurred due to the homoplasy in the spaces used in spoligotyping (Comas et al. 2009). Therefore, spoligotyping is an unreliable tool for formal phylogenetic analyses.

Whole genome based phylogenetic tree yielded clearly defined population substructure among locally circulating lineage 4 isolates in Kandy Sri Lanka. It showed SIT 3234 isolates were clustered into a clade in L4.1.2.1 Haarlem branch providing evidence for the clonal expansion of SIT 3234 (Figure 3).

In this study I identified twelve RDs (Table 4) which have not been used for sublineage classifications previously. Specially we noted that three SL- RDs (SL-RD 3,6,9) and RD 182, HSD 4 and HSD 6 were deleted in majority of L4.1.2.1 Haarlem strains (n= 12/14; including SIT 49, SIT 124, SIT 3234, SIT 1952, new type 1). One additional SL- RD (SL-RD 11) was deleted in 3 out of 4 isolates of SIT 3234. The phylogenetic tree based on Jaccard distance (Figure 3) clearly showed the clustering of SIT 124, SIT 3234, SIT 1952, New type 1 together with H3/ SIT 49. This revealed the close evolution relationship among those isolates with H3/ SIT 49. In contrast to the spoligoforest tree (based on spoligotyping) constructed in chapter 1, RD analysis provided evidence that, SIT 124 was a probable descendants of H3/ SIT 49, but not H3/SIT 50.

RD 182 , HSD4 and HSD 6 (Cubillos-Ruiz et al. 2010; Coll et al. 2014; Stucki et al. 2016) are well known Haarlem specific genetic markers which have been detected worldwide. Possibly the combination of SL- RD 3,6,9 could be used as a marker to identify locally circulating Haarlem strains in Sri Lanka. But before making a firm conclusion it is essential to confirm whether the combination of these three SL-RDs are specific to Sri Lankan isolates by performing comparative genomic analysis using Haarlem and non-Haarlem MTB strains from Sri Lanka and other countries.

SL-RD 11 was only deleted in SIT 3234 isolates (3 out of 4 isolates). This may provide evidence that SIT 3234 evolved from SIT 124 and then again went through evolution for local adaptation and loss SL-RD 11 and created two clades of SIT 3234. SL-RD11 is present in SIT 3234/clade I while it has been deleted in SIT3234/clade II. Then SIT 3234/clade II may have clonally expanded in the study group. This hypothesis is supported by the phylogenetic trees (Figure 1 and 3). Furthermore, we need to detect the presence or absence of SL-RD11 in other SIT 3234 isolates in our study to make a firm conclusion. Moreover, SL-RD 11 could be a possible candidate for a specific genetic marker to differentially identify

2 clades of SIT 3234 together with other genotyping methods as we suggested to continue monitoring of SIT 3234 in Sri Lanka to prevent future outbreaks.

Interestingly SIT 3234 isolates with similar spoligotyping and MIRU- VNTR pattern can be further differentiate into two clades based on deletion in RDs. It is also required to perform comparative analysis with SIT 3234 isolates from different countries to identify the uniqueness of tSL-RD11.

As these lineage specific polymorphisms may have important functional consequences for MTB which can affect strategies for disease control. For example, gene *Rv1354c* in HSD4, which codes for the only identified putative diguanylate cyclase in the genome, is associated with the inner membrane and thought to be involved in the turnover of cyclic-di-GMP, a multifunctional second messenger molecule exclusive of the bacterial domain (Mawuenyega et al. 2005). Recently this Rv1354c has been proposed as an ideal target for the design of new drugs (Cui et al. 2009) . However, gene Rv1354c is completely deleted in Haarlem strains. Hence this target is not suitable for drug development. This highlights the implications of strain genetic variation in drug development.

It is important to study the genomic deletions in MTB strains circulating in particular geographical area as those are expected to represent uni directional genetic events that the distribution of the deletions suggests a phylogeny for MTB (Kato- Maeda et al. 2001). As these RDs harbor several important genes and virulence factors and their presence or absence could help to identify lineages of isolates in particular geographical region on an evolutionary time scale (Rao et al. 2005). The other important fact is the virulence properties of strains may have a significant correlation with different RD profiles. Therefore, studying about evolutionary dynamics and virulence mechanisms using animal models is beneficial. Furthermore, the genetic variations including deletion of RDs could have effect on drug and vaccine development as they can have impact on target sites

Genomic variant analysis revealed that four isolates of SIT 3234 has 123 non- synonymous common SNPs in coding regions. These SNPs should be compared with other genomes representing different sub lineages to identify the unique SNPs for SIT 3234. Then virulence genes should be selected and further comprehensive analysis is requisite to confirm the virulence properties and mechanisms.

Summary

The predominant lineage of MTB in Kandy, Sri Lanka was lineage 4 (Euro-American lineage), but not lineage 1 as expected. As the isolates from lineage 4 were genetically diverse, with most of them were having an undesignated or new spoligotype pattern, I suggested that this lineage has circulated in Sri Lanka for a long time period with microevolution driving the emergence of new descendants which may be adapted to the local Sri Lankan host population. I also noted the clonal expansion of locally evolved and potentially host-adapted SIT 3234 in Kandy, Sri Lanka. Therefore, I performed this study to detect the genomic variations in MTB lineage 4 and to identify the clonality and micro diversity of SIT 3234 isolates in Kandy, Sri Lanka. Genomic DNA of the 20 isolates of lineage 4 were sequenced using Illumina MiSeq sequencer. The MTB H37Rv genome (GeneBank accession number NC_000962.3) was used as the reference genome in analysis. Based on sublineage specific deletion of RDs and SNPs, six sublineages: L4.1.2.1Haarlem, L4.1.1.1 X, L4.3.3 LAM, L4.2.2, L4.4.1 and L4.8 were identified. SIT 124, SIT 3234, SIT 1952 and new type 1 were identified as L4.1.2.1 Haarlem and by phylogenetic analysis I revealed SIT 49 was evolutionary closely linked to them. Previously unreported 12 RDs were detected among lineage 4 isolates. Out of them the presence of combination of SL-RD 3,6,9 could be used as a marker to identify locally circulating Haarlem strains in Sri Lanka. The clonal expansion of SIT 3234 was also confirmed by the phylogenetic analysis. Further phylogenetic tree based on Jaccard distance showed SIT 3234 could be separated into 2 clades based on SL-RD11. Deletion of SL-RD 11 in SIT 3234/clade II may have occurred as a local adaptation while evolution before the clonal expansion. SL-RD 11 could be a possible candidate for a specific genetic marker to differentially identify 2 clades of SIT 3234 together with other genotyping methods as I suggested to continue monitoring of SIT 3234 in Sri Lanka to prevent future outbreaks. I found 123 non-synonymous SNPs in coding regions

which were common to SIT 3234. Further analysis is required to identify the virulence properties and mechanisms of SIT 3234.

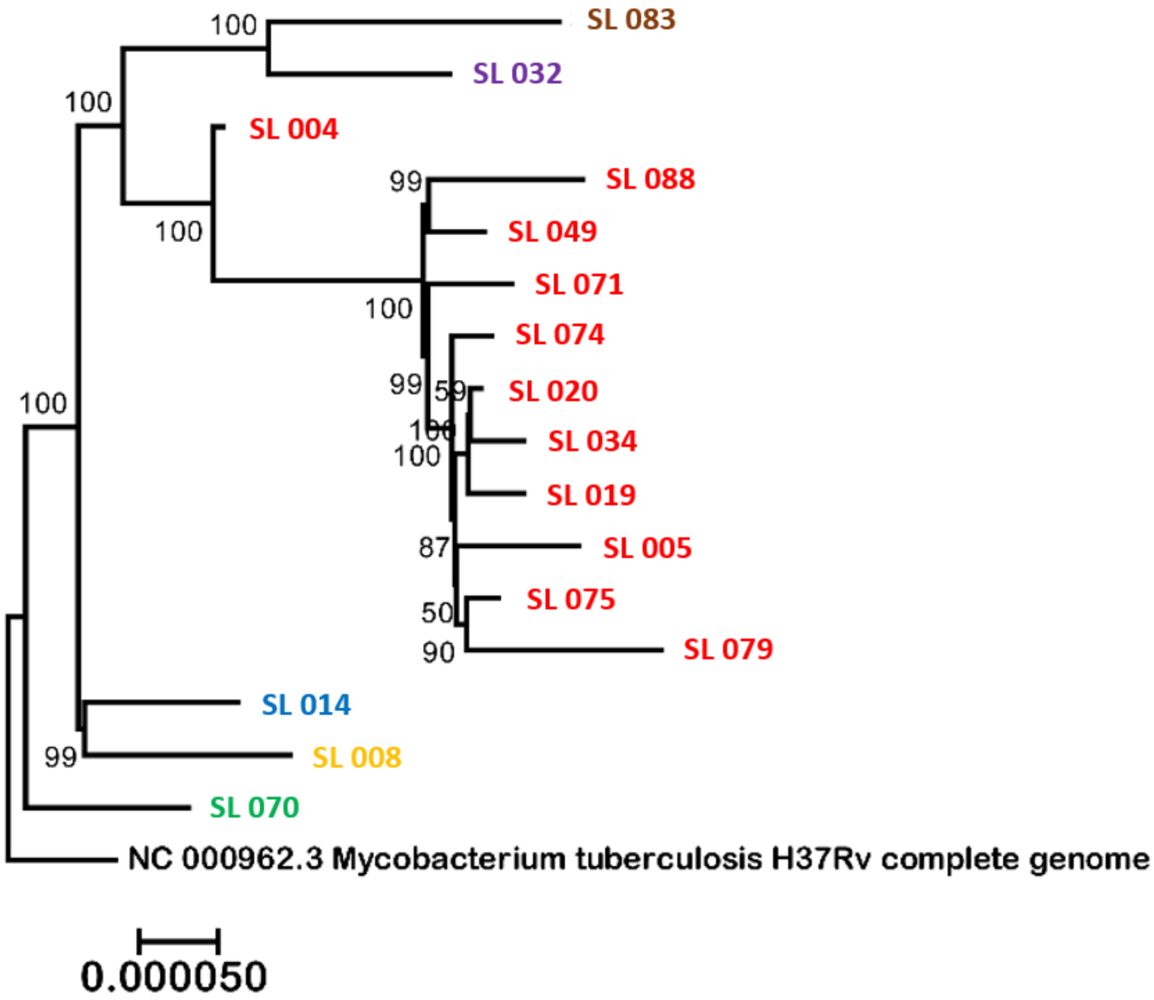


Figure 1. Phylogenetic tree based on SNPs

Phylogenetic tree was constructed based on SNPs using the Nei d the Tamura-Nei method

🟫 L4.2.2
🟪 L4.1.1.1 X
🟥 L4.1.1.1 Haarlem
🟦 L4.4.1
🟨 L4.3.3
🟩 L4.8

Isolate ID	SL-RD 1	SL-RD 2	HSD4	SL-RD 3	SL-RD 4	SL-RD5	HSD6	RD 182	RD 183	SL- RD6	RD 219	SL-RD 7	SL- RD 8	SL-RD 9	SL-RD 10	SL-RD 11	SL-RD 12	RD 115
SL 070										🟦	🟦				🟦			
SL 083				🟦														
SL 008				🟦										🟦				🟦
SL 032									🟦	🟦			🟦	🟦			🟦	
SL 038									🟦	🟦			🟦	🟦			🟦	
SL 071	🟦		🟦				🟦	🟦		🟦		🟦						
SL 049		🟦		🟦			🟦	🟦		🟦								
SL 088			🟦	🟦			🟦	🟦		🟦				🟦				
SL 004			🟦	🟦			🟦	🟦		🟦				🟦				
SL 005			🟦	🟦			🟦	🟦		🟦				🟦				
SL 015			🟦	🟦			🟦	🟦		🟦				🟦				
SL 074			🟦	🟦			🟦	🟦		🟦				🟦				
SL 019			🟦	🟦			🟦	🟦		🟦				🟦		🟦		
SL 020			🟦	🟦			🟦	🟦		🟦				🟦		🟦		
SL 034			🟦	🟦			🟦	🟦		🟦				🟦		🟦		
SL 035			🟦	🟦			🟦	🟦		🟦				🟦				
SL 039			🟦	🟦			🟦	🟦		🟦				🟦				
SL 075			🟦	🟦			🟦	🟦		🟦				🟦				
SL 079			🟦	🟦			🟦	🟦		🟦				🟦				
SL 014					🟦	🟦				🟦				🟦				

Figure 2. Distribution matrix of deleted sequences among 20 isolates of *M. tuberculosis* lineage 4.

Sequences present in H37Rv and absent from the study isolates are shown in blue. Each row represents an isolate and each column is a region of difference. Color codes used for sample IDs are as in Figure 1.

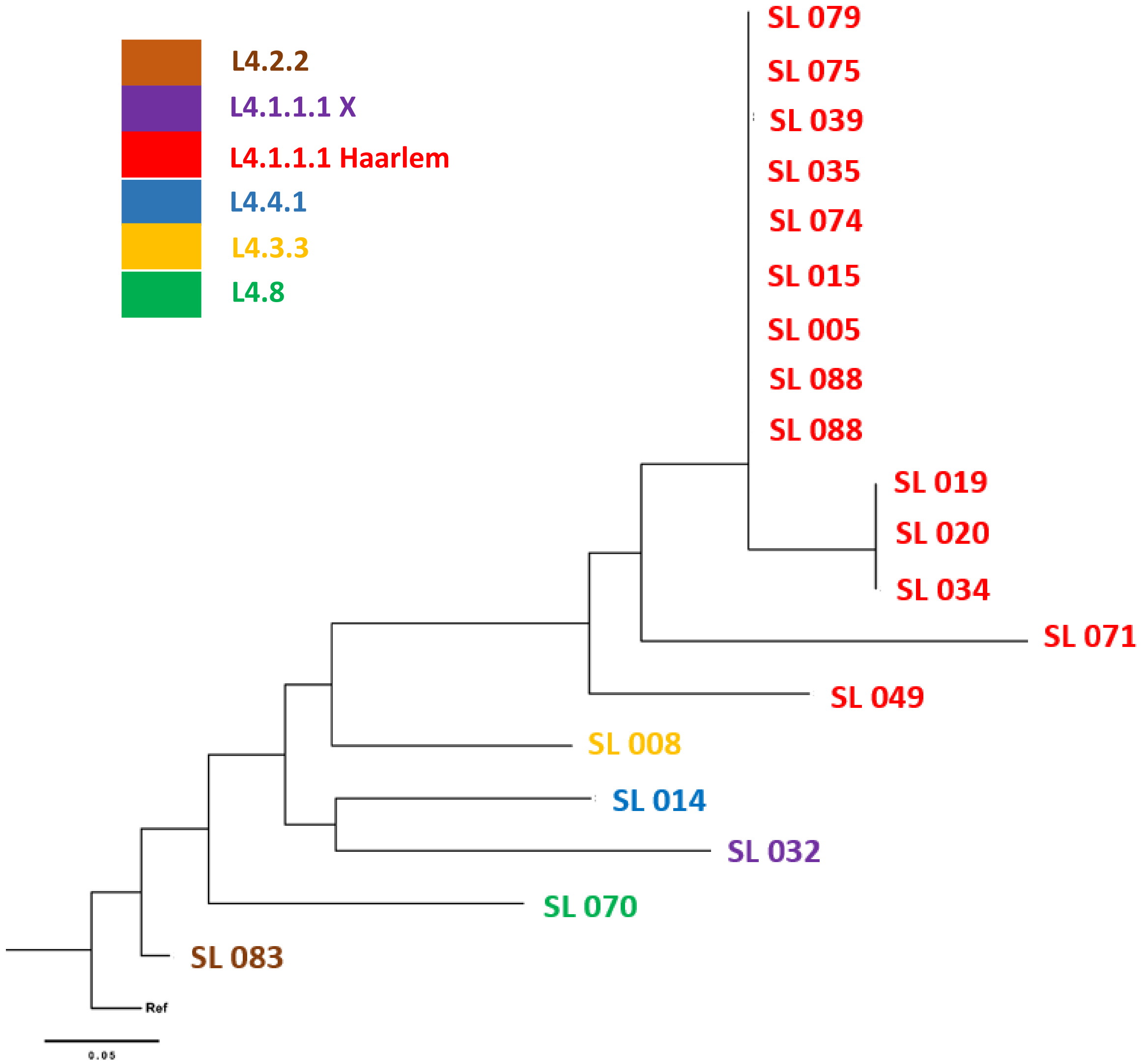


Figure3. The Jaccard phylogenetic tree based on RDs

Distance was estimated as the Jaccard distance for the presence/absence of the RD segments. To estimate the pairwise distance J between two different strains X and Y we used the formula J(X,Y) = 1-(X∩Y)/(X□Y). The distance and phylogenetic were done with R.

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Table 1. ***M. tuberculosis*** **lineage 4 strains analyzed by whole genome sequencing (n=20)**

	Strain ID	Spoligotype pattern (octal number)	SIT	Sublineage based on spoligotyping
1	Sri Lanka 070	777777777760771	53	T1
2	Sri Lanka 083	777777777760771	53	T1
3	Sri Lanka 008	776000003760771	823	T1
4	Sri Lanka 032	617776777760601	478	X2
5	Sri Lanka 038	617776777760601	478	X2
6	Sri Lanka 071	4020771	2	H2
7	Sri Lanka 088	777777777720731	49	H3
8	Sri Lanka 049	777777777720771	50	H3
9	Sri Lanka 004	777777777700771	124	Undesignated
10	Sri Lanka 005	777777777700771	124	Undesignated
11	Sri Lanka 015	777777777700771	124	Undesignated
12	Sri Lanka 074	777777777700771	124	Undesignated
13	Sri Lanka 019	777777777600371	3234	Undesignated
14	Sri Lanka 020	777777777600371	3234	Undesignated
15	Sri Lanka 034	777777777600371	3234	Undesignated
16	Sri Lanka 035	777777777600371	3234	Undesignated
17	Sri Lanka 039	777777774000771	1952	Undesignated
18	Sri Lanka 075	777777777700671		New type 1
19	Sri Lanka 079	777777777700671		New type 1
20	Sri Lanka 014	777703777760700		New type 3

Table 2. Summary of short reads of 20 isolates of *M.tuberculosis* lineage 4

Isolate ID	Total reads - count	Total reads- No of bases	mapped reads	Not mapped reads	Percentage of mapped reads (%)	Mapped reads- Average length	Reference genome coverage (%)
SL 004	21,008,316	5,009,634,748	19,195,409	1,812,907	91.37	241.39	102.97
SL 005	736,380	188,828,866	730,197	6,183	99.16	256.45	100.00
SL 008	1,174,680	286,583,598	1,167,302	7,378	99.37	243.98	101.29
SL 014	1,468,700	344,241,961	1,442,747	25,953	98.23	234.21	101.80
SL 015	348,154	81,463,453	327,030	21,124	93.93	233.42	87.43
SL 019	754,498	193,140,213	686,517	67,981	90.99	255.49	100.19
SL 020	1,105,126	276,239,420	1,097,965	7,161	99.35	249.97	100.96
SL 032	1,111,138	275,826,722	1,105,524	5,614	99.49	248.24	100.94
SL 034	790,814	203,462,503	786,783	4,031	99.49	257.28	100.32
SL 035	175,190	45,135,095	174,357	833	99.52	257.64	46.02
SL 038	1,176,428	214,813,917	230,080	946,348	19.56	158.54	20.42
SL 039	172,124	44,169,182	171,190	934	99.46	256.63	43.82
SL 049	1,084,286	274,873,843	1,077,329	6,957	99.36	253.52	101.08
SL 070	890,028	222,665,057	857,323	35,705	96.00	248.95	100.93
SL 071	1,330,510	325,961,300	1,323,128	7,382	99.45	245.02	101.32
SL 074	853,554	199,438,630	847,713	5,841	99.32	233.72	100.85
SL 075	875,902	217,858,414	867,929	7,973	99.09	248.74	100.62
SL 079	1,752,004	418,454,849	1,742,587	9,417	99.46	238.84	101.49
SL 083	1,282,218	320,069,763	1,189,318	92,900	92.75	249.86	101.07
SL 088	3,148,906	677,359,260	2,601,267	547,639	82.61	215.08	101.93

Table 3 Assignment of sublineages to the 18 isolates of our study according to RDs and SNPs published by Coll et al. (2014) and Stucki et al. (2016)

	Sample ID	Spoligotype pattern (octal number)	SIT	Sublineage based on spoligotyping	Sublineage based on RDs and SNPs
1	Sri Lanka 070	777777777760771	53	T1	L4.8
2	Sri Lanka 083	777777777760771	53	T1	L4.2.2
3	Sri Lanka 008	776000003760771	823	T1	L4.3.3 (LAM)
4	Sri Lanka 032	617776777760601	478	X2	L4.1.1.1 (X)
5	Sri Lanka 071	4020771	2	H2	L4.1.2.1 (Haarlem)
6	Sri Lanka 088	777777777720731	49	H3	L4.1.2.1 (Haarlem)
7	Sri Lanka 049	777777777720771	50	H3	L4.1.2.1 (Haarlem)
8	Sri Lanka 004	777777777700771	124	Undesignated	L4.1.2.1 (Haarlem)
9	Sri Lanka 005	777777777700771	124	Undesignated	L4.1.2.1 (Haarlem)
10	Sri Lanka 015	777777777700771	124	Undesignated	L4.1.2.1 (Haarlem)
11	Sri Lanka 074	777777777700771	124	Undesignated	L4.1.2.1 (Haarlem)
12	Sri Lanka 019	777777777600371	3234	Undesignated	L4.1.2.1 (Haarlem)
13	Sri Lanka 020	777777777600371	3234	Undesignated	L4.1.2.1 (Haarlem)
14	Sri Lanka 034	777777777600371	3234	Undesignated	L4.1.2.1 (Haarlem)
15	Sri Lanka 035	777777777600371	3234	Undesignated	L4.1.2.1 (Haarlem)
16	Sri Lanka 075	777777777700671		New type 1	L4.1.2.1 (Haarlem)
17	Sri Lanka 079	777777777700671		New type 1	L4.1.2.1 (Haarlem)
18	Sri Lanka 014	777703777760700		New type 3	L4.4.1

Table 4 previously unreported region of deletion and affected open reading frames found in isolates in this study

Region of difference	Region in reference genome (H37Rv, NC 000962.3	Length (bp)	Open reading frame (ORF) affected
SL- RD 1	69714- 70599	885	Rv0064
SL- RD 2	474121- 475816	1694	Rv0394c, Rv0395, Rv0396
SL- RD 3	1779168- 1788417	9249	Rv1573- Rv1587c
SL- RD 4	1991454- 1995970	4516	Rv1760, Rv1761c, Rv1762c, wag22
SL- RD 5	2025696- 2028360	2664	PPE26, PE18
SL- RD 6	3378132- 3379259	1127	esxR, esxS
SL- RD 7	3709315- 3710368	1053	moaC3, Rv3324A, moaX
SL- RD 8	3773007- 3774074	1067	Rv3361c, Rv3362c, Rv3363c
SL- RD 9	3842170- 3846705	4535	PPE57, Rv3428c
SL- RD 10	4212851- 4214887	2036	Rv3767c, Rv3768, Rv3769
SL- RD 11	4278145- 4280066	1921	Rv3813c, Rv3814c, Rv3815c
SL- RD 12	4370370- 4373111	2741	Rv388c, eccD2, espG2

CONCLUSION

TB is a major public health problem worldwide with no exception in Sri Lanka. Although Sri Lanka is a moderate TB prevalent country in South Indian region, it is high time to think of more effective strategies to prevent and control TB in Sri Lanka to end the TB epidemic by reducing TB deaths and new cases. One of the key factors that we need achieve this goal is epidemiological data on circulating genotypes of MTB, transmission patterns, gene mutations conferring drug resistance. As the exploration of molecular epidemiology of MTB in Sri Lanka is limited to several studies, I aimed to perform molecular characterization of MTB isolates from pulmonary tuberculosis patients in Kandy district, Sri Lanka.

In first chapter , I identified the predominant lineage in Kandy district, Sri Lanka was lineage 4. The population structure of MTB in Kandy was different from the South Asian Region. Even when I compared our results with limited studies done in Sri Lanka I revealed that the genetic diversity of MTB is highly diverse within the country. I detected the clonal expansion of locally evolved lineage 4/SIT3234 and pre-MDR Beijing isolates from new TB patients which is alarming for continuous monitoring to prevent future out breaks.

As I identified MTB lineage 4 plays a major role in TB burden in Kandy district, in second chapter , I performed whole genome sequencing selecting 20 isolates of lineage 4 to get deep understanding on genetic diversity. Based on sublineage specific deletion of RDs and SNPs, six sublineages were identified and the majority were L4.1.2.1Haarlem. Previously unreported 12 RDs were detected among lineage 4 isolates. Out of them combination of SL-RD 3,6,9 could be used as a marker to identify locally circulating Haarlem strains in Sri Lanka. The clonal expansion of SIT 3234 which was notice in chapter I, was confirmed by the phylogenetic analysis and identified two clades of SIT 3234 based on SL-RD11. Deletion of SL-RD 11 in SIT 3234/clade II may have occurred as a local adaptation while evolution before the clonal expansion. SL-RD 11 could be a possible candidate for a specific genetic marker to differentially identify 2 clades of SIT 3234 together with other genotyping methods. I found 123 non-synonymous SNPs in coding regions which were common to SIT 3234. Further analysis is required to identify the virulence properties and mechanisms of SIT 3234.

When I combined the results of spoligotyping and whole genome sequencing the most common sublineage in Kandy was Haarlem (34.1%) followed by EAI (28.4%) and Beijing (23.6%). Haarlem sublineage is well known to cause out breaks and drug resistant TB mainly in European countries.

I believe this study underlines the need for continuous surveillance of genetic diversity and drug resistant MTB so as to develop a clear picture of prevalence, transmission and evolution of MTB that can underpin the current TB control programme and prevent future epidemics in Sri Lanka.

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