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Original Research Article

Analysis of the molecular diversity of *Mycobacterium tuberculosis* isolates from patients attending central referral hospital, East Sikkim based on the IS6110 element

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ABSTRACT

Background: Using the marker IS6110, which is rated the gold standard for molecular research of *Mycobacterium tuberculosis* complex (MTB), this study seeks to determine the type of circulating strain of *M tuberculosis* complex (MTB). MTB genotype was determined from clinical samples of patients exhibiting pulmonary and extrapulmonary tuberculosis at the Central Referral Hospital, SMIMS, Sikkim.
Methods: The PCR using IS6110 and 38kDa markers were used for identifying the isolates of *M tuberculosis* complex in LJ slants and restriction fragment length polymorphism methodology for identifying number of copies of IS6110 had been used to classify 100 *M tuberculosis* isolates (RFLP-PCR).
Results: IS6110 comprised a number of bands ranging between 0 to 2, with the majority of isolates having a single copy, followed by two copies, and a few isolates bearing no copy.
Conclusions: This study showed a low copy of IS6110 the most predominant circulating strain in the community of Sikkim with high frequency of 85% of single copy of IS6110.

Keywords: Pulmonary tuberculosis, Extrapulmonary tuberculosis, IS6110, 38kDa, PCR, RFLP

INTRODUCTION

Mycobacterium tuberculosis (MTB) remains one of the deadly infectious diseases that are responsible for millions of people worldwide with 85% of cases of MTB and 98% of deaths occurring in developing countries.[1]The classification of strain or subtyping has become important epidemiologically for tracing outbreaks of infection, detecting the cross-transmission of nosocomial pathogens knowing the source of infection recognizing the particularly virulent strains of organisms and monitoring vaccination programs.[1] Advancement in the field of molecular technique is considered to be a sensitive method for detecting and studying the genetic diversity of viruses and bacteria in pathological samples.[1,2] Typing of strain by any DNA fingerprinting system is expected to help to trace the movement of individual strain within the country and even across the country borders for gaining a proper understanding of the disease.[3]

This study has adapted the molecular techniques for the identification of Mycobacteria from the solid culture media by making use of the gene coding for 35kDa and insertion sequence 6110(IS6110) repetitive sequence from *M tuberculosiss.*[4] Insertion sequence IS6110 is a 1,355 base pair long sequence that was exclusively detected in *M. tuberculosis* complex. The number of copies of IS6110 present in the genome is species and strain-specific. The strain typing of MTB using IS6110

RFLP isolates has been studied in different parts of India.[3-9] One of the studies was conducted with the samples collected from different parts of India with the outcome of low copy number, intermediated copy number and high copy of not more than 19 copies of IS6110.[4] The attempt of the present study was more of finding out the type of strain based on the number of copies of IS6110 circulating within the community.

METHODS

Study location

The present study was conducted from December 2016 to May 2020 in the TB laboratory of the department of microbiology of Sikkim Manipal Institute of Medical Sciences (SMIMS), Sikkim.

Bacterial isolates

A total of 100 isolates were obtained from total 592 clinical samples of which 308 were from pulmonary tuberculosis and 284 samples were from extrapulmonary tuberculosis, the samples collected from pulmonary samples were four gastric aspirate and 304 sputum samples and samples collected from extrapulmonary tuberculosis were Ascitic fluid:40, Pleural fluid:95, pus:40, Lymph node:30, CSF:29, ET secretion:11, cold abscess:6, tissue biopsy:11, pericardial fluid:9 and 13 other fluids grown on Lowenstein-Jensen (LJ) agar slants during the study period following RNTCP guidelines. Positive cultures were confirmed for *M. tuberculosis* complex by growth and colony morphologic characteristic, Ziehl Neelsen stain, and with the PCR kit using primer IS6110 and 38kDa.Isolates were then advanced for strain typing using the RFLP technique with Insertion sequence IS6110.

Isolation, identification, and strain typing

The samples (pulmonary and extrapulmonary) were isolated on LJ culture medium. Positive cultures were confirmed for M tuberculosis complex (MTB) by growth and colony morphologic characteristic, Ziehl Neelsen stain, and then finally confirmed with the PCR test using two primers IS6110 and 38kDa specific for MTB. After confirmation of MTB with PCR test in LJ slants, the isolates were then advanced for strain typing using the RFLP technique using Insertion sequence IS6110.

Inclusion and exclusion criteria

The samples were collected from all clinically suspected pulmonary and extra-pulmonary tuberculosis patients attending the central referral hospital of SMIMS till 100 isolates were included. The samples with culture negative of clinically suspected cases were excluded for further strain typing.

Decontamination of clinical samples and extraction of DNA

The clinical samples were decontaminated using modified Petroff's method.[10] The DNA from isolates was extracted using HELINI™ pure fast bacterial genome DNA mini spin prep kit. Isolates were inactivated by boiling at 80ºc for 20 minutes then extracted using a DNA extraction kit (HELINI). The extracted DNA was further divided into aliquots for confirmation by PCR test and restriction digestion.[11] Briefly, the isolates from the LJ slants was suspended in 500ul of distilled water by vortexing in a sterile 2 ml centrifuge tubes.180ul of digestion buffer and 20ul of lysozyme was added followed by gentle vortex for 10 seconds, briefly centrifuged and incubated at 37℃ for 15 mins. Then 200ul of binding buffer and 20ul of proteinase K was added and mixed well. Brief centrifugation was done and incubated at 56℃ for 15 mins followed by the addition of 200 ul of absolute ethanol (100%) and mixed well by inverting several times. The entire sample was pipetted into the Pure fast® spin column. Centrifuged at 8000 rpm for 1 minute. The flow-through was discarded and placed the column back into the same collection tube. 500 ul of wash buffer-1 was added to the Pure fast® spin column, centrifuged at 10000 rpm for 1 min and the flow-through discarded. Twice 500 ul of wash buffer-2 was added to the Pure fast® spin column, centrifuged at 10000 rpm for 1min and flow-through discarded. The Pure fast® spin column was inserted into a fresh 1.5 ml microcentrifuge tube. A pre-warmed 100 elution buffer was added to the centre of the Pure fast® spin column membrane. Incubated for 2 min at room temperature and centrifuged at 13000rpm for 1 minutes purified DNA was stored at -20°C before use.

PCR amplification and identification

The isolates were identified by PCR test using HELINI *Mycobacterium tuberculosis* (MTB) PCR kit (Dual target-IS6110 and 38 kDa). One part of the aliquot was used for the PCR test. As per the manufacturer's instruction, all reagents were thawed completely, mixed, and centrifuged. Briefly, the primer used was IS6110 &38kDa with a product size of 575bp and 400bp respectively. The amplification protocol for thermal cycler was initial denaturation of 95°C for 30 mins followed by annealing at 60°C for 30 sec and final extension at 72°C for 5 minutes for 35 cycles. The PCR product was then loaded in 2% agarose gel containing ethidium bromide. The gel was run at 100 volts then visualized under Gel doc. The result was evaluated in light of the performance of appropriate positive and negative controls and DNA ladder of 100 base pairs (bp) to avoid cross-contamination and false-positive reaction.

IS6110 RFLP-PCR

Digestion of chromosomal DNA for RFLP: After identification by PCR (IS6110 & 38 kDa), as described

by van Embden et al,[12] the other part of the aliquots were digested using Pvu II restriction enzyme. Briefly, the genomic DNA (5ug) from the isolates after extraction was digested with restriction Enzyme Pvu II in a final volume of 50ul as recommended by the manufacturer (Thermo Scientific). PCR for the digested product: After digestion with Pvu II the digested product was amplified using specific and exclusive IS6110 primer for in vitro detection of MTB complex. The amplification procedure was done as per the manufacturer's instruction (HELINI Mycobacterium tuberculosis) PCR Kit. Separation of DNA fragments by Gel electrophoresis: After PCR amplification with IS6110 specific primer the PCR product was size-fractionated on 2% agarose gel along with the DNA ladder of 100 bp at 100 volts and the band was visualized and recorded under UV light in Gel documentation system.

Statistical analysis

The F-test in excel was used to analyse the significant effect of different ethnic group on IS6110 strains. The rest of the study outcome was calculated in percentage.

RESULTS

Identification by PCR

The study includes 100 LJ cultures isolated both from pulmonary and extrapulmonary samples. Of a total of 100 isolates 80(80%) isolates were from pulmonary tuberculosis and 20 (20%) from extrapulmonary tuberculosis. There were 55 isolates (55%) belonging to males and 45 (45%) belonging to female patients. Of the total of 80 pulmonary isolates, 45 (56.25%) were males and 35 (43.75%) were females whereas from a total of 20 extrapulmonary isolates 10 (50%) belonged to males &10(50%) belonged to females. The PCR test was conducted using 38 kDa marker and IS6110 marker for identification of the *M tuberculosis* complex. Of the total of 100 isolates the PCR test with 38 kDa marker gave 100% positive results whereas PCR with IS6110 gave 95% positive results with 78 (97.5%) positive results from isolates of pulmonary tuberculosis and 17 (85%) positive results from isolates of extrapulmonary tuberculosis. Of the 5 (5%) negative results 4 (4%) isolates belonged to female patients and 1 (1%) belonged to a male patient. Overall results are summarized in (Table 1).

IS6110-RFLP (restriction fragment length polymorphism)

The range of IS6110 copies among isolates varied from 0 to 2 (Figure 1). Based on the copy number of IS6110 the isolates of MTB complex have been classified into different strains. As depicted in table 2 the IS6110 was lacking in 2 (2.5%) of the isolates from Pulmonary tuberculosis and 03 (15%) from extrapulmonary tuberculosis. Most of the isolates had a single copy of IS6110 with 68(85%) for pulmonary and 17 (85%) for extrapulmonary tuberculosis and 10 (12.5%) of the isolates from pulmonary tuberculosis had two copies of the IS6110 element whereas no isolates from extrapulmonary tuberculosis showed more than one copy of IS6110 element.

Table 1: Positivity of ZN smear and PCR test in pulmonary and extra pulmonary isolates.

Nature of clinical samples	Total samples In LJ medium	ZN Positive N (%)	ZN Negative N (%)	PCR(38 kDa) N (%)	PCR IS6110 gene N (%)
Pulmonary (Sputum)	80	68 (85)	12 (15)	80 (100)	78 (97.5)
Extra pulmonary	20	01 (5)	19 (100)	20 (100)	17 (85)

Pus:10, Cold Abscess:1, Lymphnode:09

The bands for IS6110 after amplification of restriction digested product by PuvII enzyme of the isolates of *Mycobacterium tuberculosis* complex are depicted in (Figure 1).

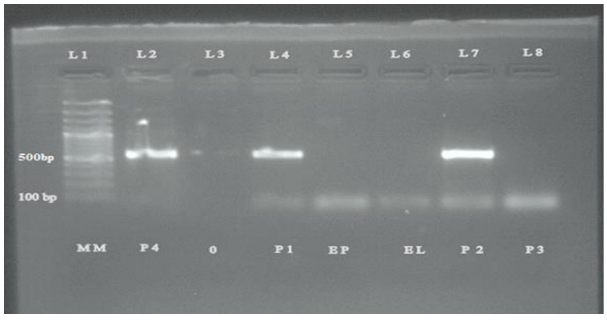

Figure 1: Overview of the gel electrophoresis.

The Lane 1 is a molecular weight marker of 100 base pair, Lane 2 & 8 shows a single band of IS6110 from the isolates of pulmonary tuberculosis, Lane 4 &7 shows two bands from the isolates of pulmonary tuberculosis, Lane 5 & 6 shows single band from the isolates of extra pulmonary tuberculosis and Lane 3 shows null band for IS6110 element. As per the gender-wise distribution of IS6110 strains. Of the total 100 isolates 55, were males and 45 were females. In males (N=55) the single copy with 100 bp was 25 (45.45%) and in females (N=45) it was 21(46.66%), single copy with 500 bp was 23 (42%) in males and 16 (35.55%) in females, strains with two copies of IS6110 was 6 (10.91%) in males and 04 (8.89%) in females and strains lacking IS6110 was 1 (1.82%) in males and 4 (8.89%) in females. Other various factors have been included in the study with IS6110 strains as given in (Figures 2-6).

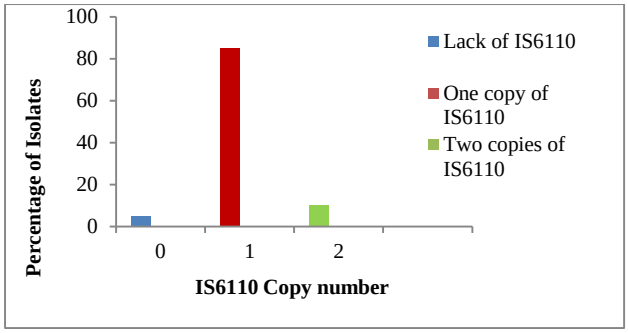

Figure 2: Distribution of IS 6110 copy number in *M. tuberculosis* isolates. Data depicted as percentage of isolates showing a particular copy number.

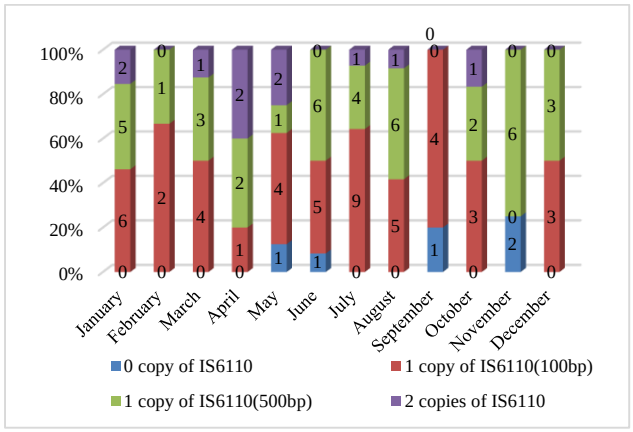

Figure 3: Distribution of different IS6110 strain in different months (pulmonary & extra pulmonary).

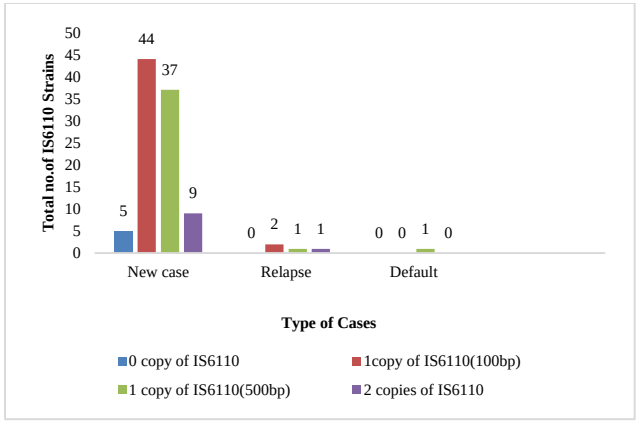

Figure 4: Distribution of IS6110 strains in different cases (pulmonary & extra pulmonary).

DISCUSSION

Polymerase chain reaction (PCR) has already been proven to be useful for early detection of *M tuberculosis* directly from clinical samples.[13]In this study, the PCR test was done for the identification of *M tuberculosis* complex from the isolates grown in LJ culture replacing conventional biochemical test. There are several specific gene targets used for the detection of *M. tuberculosis.* In the present study, the two gene target had been selected for the identification of the *M. tuberculosis* complex from the isolates grown in LJ medium culture.

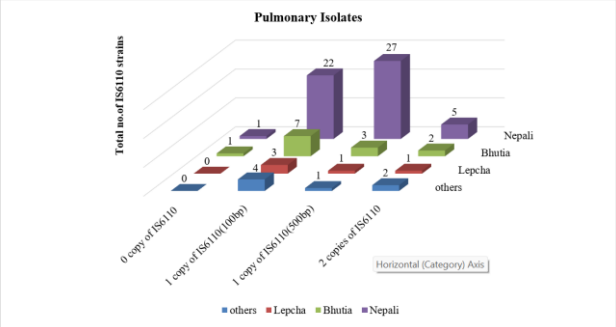

Figure 5: Distribution of IS6110 strains in different ethnic group from pulmonary isolates.

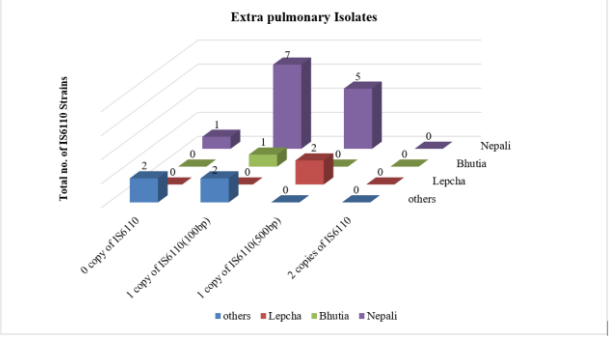

Figure 6: Distribution of IS6110 strains in different ethnic group in extra pulmonary isolates.

Table 2: The distribution of IS6110 copies shown for both pulmonary & extra pulmonary tuberculosis.

Variables	Number of IS6110 copies	Number of strains	% of isolates
Pulmonary (N=80)	0	02	2.5
	1	68	85
	2	10	12.5
Extra pulmonary (N=20)	0	03	15
	1	17	85
	2	00	00

One of the gene targets used for PCR was IS6110 useful for the identification of MTB complex as well as for confirmation of presence or absence of IS6110 sequence. Since the absence of IS6110 sequence had already been reported in previous studies in Asian strain.[14] Therefore, IS6110 was complemented with another target 38 kDa gene-specific for *M. tuberculosis* complex to increase the sensitivity of PCR result. After thorough validation, the PCR kit targeting 38 kDa protein was launched in the market in August 2009 in collaboration with the Department of Atomic Energy's commercial department, BRIT (board of radiation and isotope technology).[15] A

study by Savita Kulkarni et al have shown these two targets (38 kDa and IS6110) to be useful in avoiding false negativity. Of the total 100 isolates of MTB. The PCR with 38 kDa marker gave 100(100%) positive results whereas PCR using IS6110 marker gave 95% positive results. The percentage of positivity for the amplification of 38kDa (100%) was higher than that of IS6110 (95%). This may be due to the absence of IS6110 copy in the genome.[4] The PCR test targeting the IS6110 sequence may not be very useful for identification of MTB complex from the isolates with lacking IS6110 element indicating the need for alternative gene targets for diagnosis and epidemiological point of view. The objective of this study was to analyse the type of circulating strain of *M. tuberculosis* complex with the use of IS6110 element which is thoroughly studied and used for strain type and molecular epidemiology due to its variation in insertion site and the number of its repeated copy. Though many have raised a question of using this IS6110 marker in India due to its presence of either in low copy or its complete absence from the genome of MTB the study from North India have proved the utility of IS6110-RFLP a promising genotyping method with good discriminatory power to differentiate strains in MTB isolates in India.[16] This insertion element IS6110 is present in multiple copies and can range from 0-25.[12] Among the 100 isolates that were tested by IS6110 RFLP in this study, with a male gender being higher than the female in the ratio of 11:9 where globally more men are seen to be diagnosed with TB than women with a ratio of approximately 60:40 between men and women due to various reasons.[17]In the present study, the type of strain found with IS6110 PCR-RFLP ranged from 0 to 2. Similar low copies of IS6110 has also been seen in regions like Delhi, South India, the Indian Ocean region, Madagascar, Southern Asiatic regions and other countries like Germany & Poland.[9,14,18-21] Strains with a single copy of IS6110 were frequent in Indian ocean region. The study in the Indian ocean regions has mentioned that the high IS6110 single copy strains could be due to the presence of Asian migrants there. In the present study, the isolates were classified into different strains based on the range of copies from 0-2. From the total 100 isolates, total isolates from pulmonary tuberculosis were 80 (80%) and total isolates from extrapulmonary tuberculosis were 20 (20%). Overall the IS6110 RFLP analysis provided three types of strains with 5 strains (5%) having null IS6110 element, 85 strains (85%) with one copy and 10 strains (12.5%) with 2 copies of IS6110 (Figure 2). Unlike the other studies with a low copy of IS6110, the unique characteristics of this study with a low copy of IS6110 are their identical banding pattern which are either of a single copy of 100 bp and a single copy of 500 bp and 2 copies either of 500 bp or 100 bp comparatively to the Delhi type where the low copy was found to be of high molecular weight DNA fragments bearing IS6110. The most common pattern seen worldwide is the 1.45kb IS6110 band.[9] The study in Southeastern Asian countries showed a high proportion of single copy of IS6110 (21%±5) ($p<0.005$) but their fragment differed: fragments in Vietnamese isolates were either of 1.3 kb, 1.5 kb and 4.5 kb; among Malaysian isolates, the fragment were of 1.5 kb and 4.5 kb the fragments in Thai isolates were of 1.5 kb and 4.5 kb.Although isolates of China and Mongolia had more copies of IS6110.However, in many of their isolates, the multiple fragments were found to be of less than 1.4 kb by IS6110 RFLP analysis.[19] Of the total 100 isolates, 55 were males and 45 were females. Out of 5 strains, zero IS6110 was higher in females compared to males (80% vs. 20%).Of total 85 strains with a single copy of IS6110was found more in male than in female(56.47 vs. 43.52%). From total of 10 strains having two copies of IS6110 was also seen more in males (60% vs. 40%) than in females. In pulmonary isolates (N=80) out of a total of 68 (85%) single copy (both 100 bp & 500 bp) the strains with a single copy of IS6110 (32.35%) was found to be the highest between age 20 to 29. Out of the total 10 strains having two copies of IS6110 was found maximum (40%) between age 10 to 20 and of the total 2 strains lacking IS6110 were recovered equally between age 10 -19 (50%) and 20-29 (50%). This data depicts that the strains with single copy and strains lacking IS6110 were more predominant in the younger generation. The strains with 2 copies were seen maximum between 10-20 but had variation in distribution among different ages. Whereas the distribution of strains differed in extrapulmonary isolates. In extrapulmonary isolates (N=20), of the total 17 (85%) strains with a single copy was found highest between age 30-39 (41.17%).The strains lacking IS6110 was found between age 20-29 (66.66%)and 50-59 (33.33%) whereas no strain was found in extrapulmonary isolates having two copies of IS6110. The seasonality of TB incidence has been widely reported in different parts of the world. In one of study, it was assumed that the risk of transmission of *M. tuberculosis* appeared to be greatest during winter month due to various factors however; other studies have reported conflicting incidence peaks of TB in spring, summer and winter.[22-27] Likewise, in this study, the maximum number of isolates had been recovered from the samples collected in July compared to other months. In one of the studies in Japan, the highest peak month for notified TB cases was July in the female group and June-July in the male group.[28] With this concept of the season as one of the factors for TB incidence peaks, the observations were made to determine the seasonal influences on strain distribution. In this study, no such specific seasonal influences were observed on the distribution pattern of strains (Figure 3). This could probably be useful for future studies in combination with other genotyping. Examining the distribution of various strains in various sorts of cases (Figure 4). The greatest number of isolates was found in new patients. From relapse instances, two strains had a single copy of the 100 bp fragment, one strain had a single copy of the 500 bp fragment, and one strain had two copies of IS6110.The default instance yielded only one isolate, a strain with a single copy of the 500 bp fragment IS6110. As a result, the predominant strain recovered from relapse cases and default cases was a single copy of IS6110, which was

similar to a study from South India in which isolates from patients with relapse cases had a single copy of IS6110, but the molecular weight of DNA fragments differed from this study.[18]

Sikkim is situated in the Himalayan Mountains; the state is flanked by Nepal toward the west, China toward the north and Bhutan toward the east. Sikkim is inhabited by different ethnic groups. The ethnic group of Sikkim is comprised of Bhutia, Lepcha, Nepali & Indian other.[29] Analyzing the distribution of strains in different ethnic groups in Pulmonary isolates (Figure 5), the strains with the null copy (N=2) was seen in Nepali (50%) & Bhutia (50%) with equal distribution. The strains with a single copy (N=68) in descending orders were Nepali (72.05%) followed by Bhutia (14.70%), the Indian others (7.35%) and then Lepcha (5.88%). The strains with 2 copies (N=10) of IS6110 were maximum in Nepali (50%) followed by Bhutia (20%) and in Indian others (20%) and least in Lepcha (10%). In extrapulmonary isolates, the strains distribution in descending orders were as follows: The strain with a null copy (N=3) of IS6110 was maximum in Indian others (66.66%) followed by Nepali (33.33%) and no strains recovered from Lepcha and Bhutia. The strains with single copy (N=17) was maximum in Nepali (70.58%) followed by the Indian other (11.76%) & Lepcha (11.76%) and least in Bhutia (5.88%). There were no strains with 2 copies of IS6110 recovered from extrapulmonary cases (Figure 6). Statistically using the F distribution with $\propto = 0.05$ there was no significant effect of ethnic group on IS6110 strains for both pulmonary and extrapulmonary cases ($F_{3,10}$=2.462, p>0.05 & $F_{3,3}$ =1.957, p>0.05 respectively). One of the most recent investigations used advanced molecular techniques such as MIRU-VNTR and spoligotype. In Sikkim, the Beijing spoligotype was discovered to be the most prevalent spoligotype.[30] The results of this study, which show a high degree of a low copy of the IS6110 strain, as well as the results of a recent study with dominant Beijing spoligotype, point to the high frequency of tuberculosis transmission in Sikkim, given that the Beijing strain has been known to cause epidemic outbreaks in several countries.[31] As a result, the study using IS6110 RFLP in combination with MIRU-VNTR and the spoligotype could be one of the best options for understanding *M. tuberculosis* transmission dynamics in today's environment. Furthermore, the current IS6110 strain study's findings may be valuable in supplying data to the global surveillance program.

Limitations

Limitations of current study were; an attempt was also made to understand the frequency of strain distribution in different regions, but no noteworthy results were found. This could be related to the fact that the hospital-based study only included a small number of samples, as well as the presence of a low copy number of IS6110.

CONCLUSION

As per the findings of this study, a low copy of IS6110 is the most predominant circulating strain in the community of Sikkim, with such a significant proportion of a single copy of IS6110.

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Ethical approval: The study was approved by the Institutional Ethics Committee

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