

Restriction Fragment Length Polymorphism (RFLP) typing of *Mycobacterium tuberculosis* isolates from Nepal

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Abstract

Introduction: Many studies to characterize *Mycobacterium tuberculosis* by RFLP have shown that predominance of Beijing genotype in China and many neighboring countries. Nepal is just bordering in southern part of China. We have tried to differentiate *Mycobacterium tuberculosis* isolates of Nepal by RFLP in first time in Nepal.

Methods: A standard protocol was followed for isolation, Identification and RFLP typing of 60 strains of *Mycobacterium tuberculosis* of Nepal.

Results: Of the 60 isolates interpretable result was found in 31 isolates. The study detected that the Beijing family 12 (38.7%), was predominant type followed by single band 12 (25.5%). Indigenous type, not reported from other part of the world was also detected in 7 strains (22.6%).

Conclusion: Beijing type is the predominant RFLP type of *Mycobacterium tuberculosis* in Nepal and some other endogenous type also prevails which needs further study.

Key words: *Mycobacterium tuberculosis*, RFLP, Nepal

Introduction

Tuberculosis is a global health problem and Tuberculosis now ranks together with HIV as leading cause of death worldwide ¹. Globally, there were an estimated 9.27 million incident cases of tuberculosis in 2007 of which most of the estimated cases were in Asia (55%), Africa (31%) with small proportions of cases in Eastern Mediterranean Region (6%), the European Region (5%) and the region of Americas (3%) of the total cases². Nepal is situated in between China and India. China, India and other neighboring countries of Nepal Bangladesh, Pakistan are among the high burden countries³. Nepal had 28 million populations in 2014 and Nepal reported all together 35277 new and relapse cases of tuberculosis of which 15947 were pulmonary bacteriologically confirmed ⁴.

To control the tuberculosis disease, it is necessary to know the existing rate of active transmission of the disease and

also differentiating whether the disease is due to recent or reactivation of previous infection or is exogenous re-infection and whether the epidemic characteristics are similar in population; likewise transmission dynamics of *Mycobacterium tuberculosis* in the population or group specific level; differentiating transmission to specific group versus outbreak sporadically. Molecular epidemiology made possible to address some of these problems⁵. Restriction fragment length polymorphism (RFLP), is one of the method of molecular typing and RFLP analysis is a DNA fingerprinting technique which has been used to distinguish strains of many species of bacteria. RFLP method makes use of repetitive sequence of DNA, IS6110 which is found almost in all strains of *Mycobacterium tuberculosis*. Differences in the number and location of IS6110 within strains of chromosomal DNA are revealed by RFLP analysis which yields strain specific patterns. There appears to be a sufficient diversity of patterns to ensure that epidemiologically

unrelated pattern has different RFLP patterns whereas related strains have identical patterns⁶.

We have performed RFLP typing of *Mycobacterium tuberculosis* strains to compare the variability of strains of *Mycobacterium tuberculosis* within the country and to compare it with other region and to know the epidemiological aspects of *Mycobacterium tuberculosis* strains of Nepal.

Methods

This study was prospective and was conducted from 2005-2009. Out of 420 isolate from Tribhuvan University Teaching Hospital and National TB Centre, Bhakatur 60 isolates were randomly selected for RFLP typing. Those culture positive isolates in Ogawa medium were identified as *Mycobacterium tuberculosis* by standard biochemical tests, including niacin accumulation test, nitrate reduction test, and heat-labile catalase test⁷.

RFLP typing of *Mycobacterium tuberculosis*:

Genomic DNA was extracted in Central Department of Microbiology, Faculty of Science, Tribhuvan University, Kathmandu, Nepal and RFLP typing was done in Department of Microbiology, Faculty of Science, Mahidol University, Bangkok, Thailand.

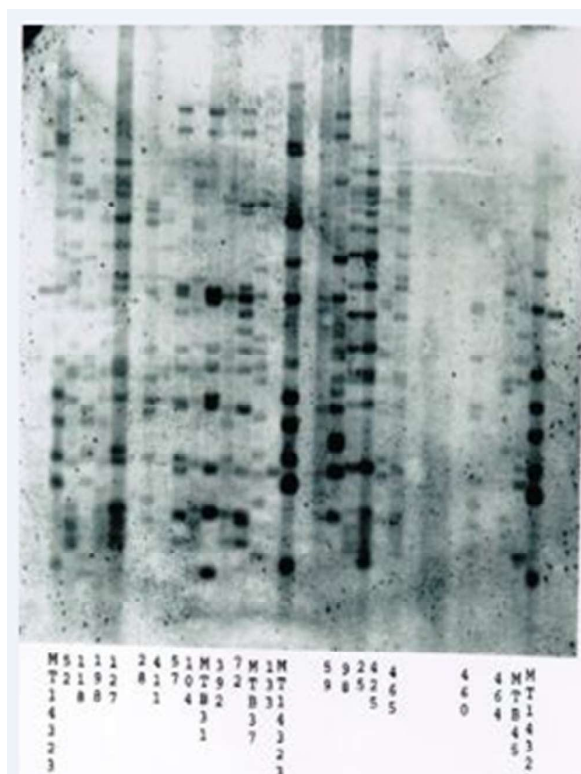
Genomic DNA was extracted from *Mycobacterium tuberculosis* isolates by following the previously standardized methodology. Briefly, two to three loopful of *Mycobacterium tuberculosis* culture mixed with TE buffer in presence of Proteinase- K, Lysozyme and SDS. Genomic DNA was extracted by Chloroform/ Isoamyl alcohol genomic isolation protocol⁸.

The genomic DNA extracted from the clinical isolates of *Mycobacterium tuberculosis* were digested with restriction enzyme puvII and the DNA fragments (IS6110) were separated by electrophoresis and hybridized using pDC73 probe and detected as per kit manufacturers instruction (Boreinger Mannheim, Germany). Briefly, the double stranded DNA extracted as above was digested using restriction enzyme puvII. A total of 30µl of the mixture was prepared taking distilled water, DNA sample, puv buffer and puvII enzyme and incubated overnight at 37°C, allowing the enzyme to digest the DNA fragment. Thus obtained DNA fragments were separated by electrophoresis. The separated DNA fragments were transferred to capillary membrane. Hybridization was done by using pDC73

probe. After hybridization the membrane was washed and detection was done^{8,9}.

Results

Of the 60 isolates of *Mycobacterium tuberculosis* subjected for RFLP typing, only 31 isolates of *Mycobacterium tuberculosis* gave interpretable RFLP result. Out of 31 isolates, 12 (38.7%) were Beijing type, 8 (25.8%) single banded, 4 (12.9%) Heterogeneous (H) and 7 (22.6%) isolates gave result of heterogeneous (h) type; which were not matching to any previously typed strains and were labeled as Heterogeneous (h) Nepal type (Table I, Picture II).



Photograph I : RFLP pattern of *M.tuberculosis* isolates of Nepal

Standard *M. tuberculosis* strain: MT 14323

Beijing family(BJ): 52,198, 127 ,98,25,465

Single banded(SB): 133

Heterogeneous Nepal type (h):57, MTB31,72,59,425, 460

Heterogeneous(H): 118,28, MTB37, 460

Not typable: 411,104,392,464, MTB45

Table 1 RFLP typing of *Mycobacterium tuberculosis* isolated from Nepal (N=31)

S. No	RFLP type	No	%
1	Beijing family (BJ)	12	38.7
2	Single banded (SB)	8	25.8
3	Heterogeneous (H)	4	12.9
4	Heterogeneous(h), Nepal type	7	22.6
	Total	31	100

Discussion

The objective of this study was molecular characterization of *Mycobacterium tuberculosis* isolated from Nepal and for this purpose we used IS6110 to determine the genetic diversity of *Mycobacterium tuberculosis* isolates. RFLP typing was interpreted according to IS6110 band patterns ⁸, The Beijing family which is shown (picture 1) contained 15 to 20 copies of IS6110, the single banded has only one copy of IS6110; whereas Nonthaburi group, which was only reported in Thailand has hybridization patterns similar to each other but different from the member of the Beijing family contained 11-15 copies of IS6110 and shared at least 78% of the IS6110 hybridized bands with another isolates in the group. Heterogeneous group (H) has less than 6 hybridized bands, Heterogeneous (h) containing 8-12 copies of IS6110 and first time reported from isolates of *Mycobacterium tuberculosis* of Nepal.

The RFLP typing showed that of 31 stains of *Mycobacterium tuberculosis*, 12 (38.7%) were Beijing family. The current study revealed that Beijing family was most common RFLP type of the characterized isolates from Nepal. "Beijing family" has been described because greater part of these strains were initially derived from province of Beijing and vast majority of them were genetically closely related; these isolates shared the majority of their IS6110 DNA containing restriction fragments, and also the DNA polymorphism associated with other repetitive DNA elements, like the polymorphic GC- rich sequence and the direct repeat, was very limited. The strains of Beijing family were also predominantly found in neighboring countries such as Mongolia, South Korea and Thailand. While

low prevalence of Beijing type was observed in other countries. It was also described that the strain of Beijing family recently spread out from the single ancestor who has selective advantage ⁹. The predominance of Beijing type was observed in this study and many countries of Asia. In China, 86% of 49 investigated isolates were Beijing type, 50% of the 20 isolates in Mongolia, 43% of the 14 isolates of South Korea were Beijing type. On the other hand, 5% of the 63 isolates of India were Beijing type, similarly, 4% of the 28 isolates in Iran, 20% of the 20 isolates in Moscow, Russia, 3% of the 2594 isolates from Netherlands, 0% of the 21 isolates of Greenland, 0% of the 76 isolates in Spain, and 15% of 46 isolates of South Africa were Beijing type ⁹.

This study also showed that 8 isolates (25.8%) were single banded and 7 (22.6%) was heterogeneous (h), Nepal type. Single banded type were also found in other studies, however Heterogeneous (h) was not detected in other part of the world until now. The RFLP pattern from the Indian Ocean Region revealed that of the 475 strains, 474 had IS6110, the copy ranging from 1 to 26 in number. Of which strains with only one IS6110 were frequent (16.8%) and also 131 had less than five IS6110 copies ¹⁰. which is comparable to present study. RFLP analysis of *Mycobacterium tuberculosis* from countries of Western Pacific Region showed that of mixed pattern. The study demonstrated that China, Hong Kong, Philippines and Korean isolates had eight or more copies Is6110 (95%; +/- 5%), whereas Thai, Malaysian and Vietnamese had fewer IS6110 ((proportion with eight or more copies, 60% +/- 4%) ¹¹. In contrast, The study in India showed that 40% of the patients showed a single copy of IS6110 indicating predominance of single copy of IS6110¹² (Das et al,1995).

Conclusion

The current study has demonstrated that Nepal has diverse type of *Mycobacterium tuberculosis* strains; the leading strain is Beijing type and also has some indigenous type.

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Conflict of interest- None declared

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