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ORIGINAL ARTICLE

Characterize *Mycobacterium tuberculosis* isolates by Spoligotyping & MIRU-VNTR typing from Gorakhpur District

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ABSTRACT
The present study has been undertaken to assess the comparative efficacy of Spoligotyping and MIRU-VNTR typing in discriminating of Mycobacterium tuberculosis isolate, biotinylated) and DRb (CCGAGAGGGGACGGAAAC) were used as primers to amplify the whole DR region by PCR and for MIRU-VNTR typing 12 MIRU loci (2,4,10,16, from Gorakhpur district. In this present study 21 isolates was selected . Spoligotyping of isolated DNA was done by amplifying DR locus in Mycobacterium tuberculosis, The oligonucleotides DRa (GGTTTTGGGTCTGACGAC20,23,24,26 27,31,39,40). It was conclude that MIRU-VNTR typing analysis is more effective than Spoligotyping in discriminating individual Mycobacterium tuberculosis isolates of Grakhpur District .
Key words: Spoligotyping, MIRU-VNTR, Delhi Strains, Mycobacteium Tuberculosis

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INTRODUCTION

Tuberculosis has been a threatening disease to humanity from ancient time by virtue of high morbidity and mortality associated with it. The etiological agent of the disease belongs to the genus Mycobacterium, which contains human pathogens like *Mycobacterium tuberculosis* and *Mycobacterium leprae* as well as many environmental saprophytes and opportunistic pathogens. The genus mycobacterium causes more suffering and death throughout the world than any other bacterial genus. Tuberculosis is acknowledged as a global problem with 1.7 billion infection and 3 million deaths worldwide per annum. About 10 million new cases appear each year, and in which 90% cases are from developing countries. According to WHO report ,there were 8.8 million new cases of TB reported in 2003,while 9 million new TB cases were reported in 2004 and the other dramatic thing is that, more than 80 % of the TB patient are in economically productive age of 15-49 years resulting in significant economic losses. India has been identified by WHO as, a major hot spot for TB infection[1]. Two of every five Indians are infected with TB bacillus, 10% will develop TB disease during there life time. It has been estimated that about 20% tuberculosis patients are living in India and current rate of infection in India is 1-2% . Almost 1.8 million new cases occur in country, of which almost half are infectious. Patients with infectious pulmonary TB diseases can infect 10-15 persons in a year [1].

More disastrous to the current scenario is the emergence of multiple drug resistant strains of Mycobacterium tuberculosis. Multi drug resistant (MDR-TB) caused several fatal outbreaks worldwide and is an increasing threat of global TB control program [2]. For monitoring transmission of disease, epidemiological and population study DNA fingerprinting technique are shown to be promising. Various methods for genotyping of *Mycobacterium tuberculosis* include RFLP (Restriction fragment length polymorphism), RAPD (Rapid amplified polymorphic DNA), PFGE (Pulse field gel electrophoresis), AFLP (Amplified fragment length polymorphism), Spoligotyping and MIRU-VNTR, represent a portable

approach to global epidemiology of tuberculosis however, all current typing markers suffer from significant draw backs [3].

Among the molecular epidemiology baseds, Spoligotyping (Spacer oligonucleotide typing) method for simultaneous detection and typing of *Mycobacterium tuberculosis* complex bacteria, has been developed [4]. It has emerged as a fast reliable and cost effective alternative to traditional IS 6110-RFLP fingerprinting .This method is very helpful in the study of *Mycobacterium tuberculosis* complex strains , with low IS 6110 copy number (frequently found in some parts of the world) are devoid of these sequence this method is based on polymorphic chain reaction (PCR)amplification of a highly polymorphic locus in *Mycobacterium tuberculosis* genome result can be obtained from a *Mycobacterium tuberculosis* culture within a day . It is useful both for tracking epidemics and to detect new outbreaks. However this method has less power to discriminate among *Mycobacterium tuberculosis* strains than does IS 6110 based genotyping [5]. In Indian settings application of Spoligotyping proved to be very useful. A comparison of RFLP and Spoligotyping with existing isolates suggest the predominance of the Delhi gene group in India subcontinent [6] and similar studies of Mumbai showed distribution of closely related spoligotypes, showing transmission of dominant resistant strains clones [7].

Strain typing by MIRU-VNTR for *Mycobacterium tuberculosis* is based on variable number of tandem repeats of Mycobacterial interspersed repetitive units [8, 9]. Variable number tandem repeat (VNTR) typing is an invaluable tool for genotyping in higher eukaryotes and provides data in a simple and nonambeguous format based on the repetitive number sequences in so called polymorphic micro or mini satellite region. Despite some attempt to develop equivalent approaches for typing bacterial pathogen, any limited application for bacterial molecular epidemiology could be develop up to now. Mycobacterial interspersed repetitive units (MIRUs), VNTR sequences which are scattered throughout the *Mycobacterium tuberculosis* genome. 12 out of 41 MIRU loci present in the *Mycobacterium tuberculosis* H_{37}Rv genome correspond to human mini satellite like VNTR region among non-related isolates of different geographical origin [10]. A PCR based typing method by using these 12 loci provides a resolution comparable to that of IS 6110-RFLP.

The correlation between genetic relationship inferred from MIRU-VNTR and Spoligotyping is highly significant for epidemiological study of tuberculosis . It is important to compare the usefulness of the entire methods in typing isolates for different areas.

MATERIAL AND METHODS

Mycobacterium tuberculosis strains were isolated from patients visited to different PHC and CHC of Gorakhpur district and after confirmation the isolated were coded as GKP-1, GKP-2, GKP-3, GKP-4, GKP-5, GKP-6, GKP-7, GKP-8, GKP-9, GKP-10, GKP-11, GKP-12, GKP-13, GKP-14, GKP-15, GKP-16, GKP-17, GKP-18, GKP-19, GKP-20, GKP-21.

Mycobacterial strains were identified on the basis of growth characteristics (rapid / slow, pigmented / non-pigmented) and biochemical tests. This was done for the species level identification. The biochemical tests used for the identification of mycobacterial isolates [11].

DNA was isolated from cultures using the following standard protocol [12, 13] and all 21 isolates of Mycobacterium tuberculosis were analyzed by spoligotyping [14, 15]. In addition H37Rv and Mycobacterium bovis were included as controls, their amplification was carried out, according to the instruction of manufacturer (Isogen, Bioscience, BV, Maarsen The Netherlands). The hybridization of PCR products of different isolates was detected by chemiluminescence's, detection liquid and exposing using enhanced the membrane for 5 min to Hyper film.

Same 21 isolates were also typed, using the MIRU-VNTR typing method , that were previously typed by spoligotyping method. DNA of these isolates were amplified with specific primers for flanking region of twelve MIRU loci (2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40). The PCR fragments were analyzed by agarose gel electrophoresis with 2% agarose. The size of the amplicons were estimated by comparison with ladder and the MIRU copy number per locus was calculated by using the conventions described by with the help of Gel Documentation system.

RESULTS

With Spoligotyping 14 spoligotype patterns (66.67%) were grouped into 8 shared types (57.1 %) and 6 isolates (42.8%) were orphan (not present in SpolDB4 database). One spoligotype ST-26, (33.34 %) was highly frequent in Gorakhpur district, that represented CAS1 – Delhi family. Six isolates of orphan patterns were further analyzed by "SPOT CLUST D" and represented three families EAI5 (66.67%), CAS (16.67%) & Family-33 (16.67%).

With MIRU-VNTR typing 19 unique MIRU-VNTR profiles were obtained from 21 isolates, that were previously typed by Spoligotyping method. Three isolates (GKP-3,GKP 7 &GKP 18), comprise single cluster (15.79%) and rest of the eighteen isolates had unique patterns. Hunter Gaston Discriminatory index was calculated for each of the 12 loci and it was found to be 0.428, 0.609, 0.800, 0.854, 0.095, 0.838, 0.629, 0.810, 0.267, 0.586, 0.532, 0.595 , for loci 2, 4, 10, 16, 20, 23, 24, 26, 27, 31,39,40 respectively. It showed that MIRU locus 16 was most discriminatory locus that is in accordance with previous study [16] and MIRU locus 20 was found to be least discriminatory.

Table 1.Spoligotyping of Mycobacterium tuberculosis isolates Gorakhpur District:

S. No.	Isolates	Octal Code	Shared Type/Family
1.	$H_{37}Rv$	777767477770371	
2.	M. bovis	676773777776600	ST-820
3.	GKP-1	703777740003771	ST-26/ CAS_1_Delhi
4.	GKP-2	703777740003771	ST-26/ CAS_1_Delhi
5..	GKP-3	703777740003771	ST-26/CAS_1_Delhi
6..	GKP-4	703777740003771	ST-26/ CAS_1_Delhi
7.	GKP-5	703777740003771	ST-26/ CAS_1_Delhi
8.	GKP-6	703777740003771	ST-26/CAS_1_Delhi
9.	GKP-7	703777740003771	ST-26/ CAS_1-Delhi
10.	GKP-8	703677740003771	ST-954/ CAS_1_Delhi
11.	GKP-9	477777777413771	ST-26/EAI5
12.	GKP-10	777777777413700	ST-138/EAI5
13.	GKP-11	777777777760731	ST-52/T_2
14.	GKP-12	777777607760771	ST-42/ LAM9
15.	GKP-13	000000000003771	ST1/ Beijing
16.	GKP-14	477777177413071	ST-1342/ EAI3_IND
17.	GKP-15	777777377413700	ORPHAN/EAI (.96)
18.	GKP-16	777777777757771	ORPHAN/Family33(.99)
19.	GKP-17	703777740003771	ST-1590/ CAS_1_Delhi
20.	GKP-18	703777700001771	ORPHAN/CAS(.99)
21.	GKP-19	703777000000371	ORPHAN/EAI(.99)
22.	GKP-20	777776757413771	ORPHAN/EAI5(.98)
23.	GKP-21	777776757413771	ORPHAN/EAI(.99)

Table 2. Comparative efficacy of MIRU-VNTR and Spoligotyping

Isolates	MIRU-VNTR Typing Results	Cluster	
		Spoligotype	MIRU-VNTR
GKP-1	225525133354	ST-26	MI-1
GKP-2	225525133354	,,	,,
GKP-3	225525133354	,,	,,
GKP-4	125123253263	,,	MI-2
GKP-5	1251221-3363	,,	MI-3
GKP-6	225525144353	,,	MI-4
GKP-7	226323133363	,,	MI-5

DISCUSSION

Complementary to traditional epidemiology, molecular epidemiology based on PCR fingerprinting method such as Spoligotyping and MIRU-VNTR have emerged as a fast, reliable and cost effective alternative to traditional fingerprinting method. Its discriminatory power is close to that of IS110 typing and also useful for differentiating MDR - *Mycobacterium tuberculosis* isolates of Beijing family [16, 17].

Spoligotyping is very practical and reproducible method, which assays the presence or absence of a set of target sequences in a direct repeat locus . It can be performed within a day and used to detect type of *Mycobacterium tuberculosis* strains, present in similar or different clinical specimens such as sputum, tissue or bronchi alveolar lavages and often desirable to distinguish reinfection from reactivation in individual patients. However, Spoligotyping remains unfortunately less discriminatory than IS6110 RFLP

when used alone. In Indian settings application of Spoligotyping proved to be very useful and their Spoligotyping study suggest the predominance of the Delhi gene group in Indian subcontinent and perhaps to specific region in India. Similar studies carried out in Mumbai showed distribution of closely related spoligotypes, showing transmission of dominant resistant clones.

MIRU-VNTR has emerged as a promising approach to identify novel polymorphism as IS6110 typing. It has been found to be allow rapid, reliable, highly throughput genotyping of *Mycobacterium tuberculosis,* molecular epidemiology .However, there is a little information about application of these methods in India [18].

Three isolates clustered by MIRU-VNTR typing, also showed the same genotyping patterns by Spoligotyping. The seven CAS1_ Delhi strains clustered by Spoligotyping were divided into five different MIRU genotypes. Clustered isolates may be involved in the same chain of recent transmission. These epidemiological links is to be established by further investigation.

By applying both of these methods to all 21 isolates, a very close complementarity was found between the result of Spoligotyping and MIRU- VNTR typing methods. The MIRU-VNTR genotyping has good discriminatory power for typing these strains of *Mycobacterium tuberculosis* from Gorakhpur district and locus 16 have been more promising to identify *Mycobacterium tuberculosis* strains of this area. Further studies are necessary for genotyping of large number of *Mycobacterium tuberculosis* isolates from defined localities for knowing its real value in studying molecular epidemiology of TB in this area.

FUTURE PROSPECTS

Molecular epidemiology by using spoligotyping and MITU-VNTR can be applied to large population for better inference to conclude any pattern of infection and strain identification that are dominant to a defined geographical area.

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Conflict of Interest

There is no conflict of interest between the authors.

AUTHOR'S CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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Not applicable

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript.

ETHICS STATEMENT

Not applicable

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