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Molecular genotyping to differentiate endogenous reactivation and exogenous reinfection of recurrent tuberculosis

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1. Introduction

Tuberculosis (TB) is one of the leading causes of death worldwide. Currently one third of the world's population is infected by TB. Globally in 2016, 6.6 million people with TB were notified and reported to WHO. Of these, 6.3 million had new or relapse TB. India accounts for more than one quarter of the world's tuberculosis disease and deaths. In 2016, 2.8 million people were infected with TB in India (World Health Organization [1].

In a high burden country like India, there are many challenges faced by clinicians and public health programs in the control of TB even after improvements in TB diagnosis, treatment and prevention. One such problem is recurrent TB. After an effective standard short course of anti-tuberculosis treatment (ATT), some patients develop repeated tuberculosis disease with significant time interval between the first and the second episode of TB called as recurrent TB infection. The rate of recurrent TB varies from 0 to 14% in studies from around the world [2].

The recurrent TB may be due to endogenous reactivation or exogenous reinfection. When the first and the second episode of TB is caused by same strains of *Mycobacterium tuberculosis* it is called relapse or endogenous reactivation and when the first and the second episode of TB is caused by two different strains of *Mycobacterium tuberculosis* it is called exogenous reinfection. DNA finger printing techniques plays an important role to find out whether the recurrent TB infection is due to exogenous reinfection or endogenous reactivation. The genotyping tool uses different genetic markers and their positions in the genome to identify and differentiate the strains of *Mycobacterium tuberculosis* [3].

We have used Spoligotyping and 12 loci mycobacterial interspersed repetitive unit–variable number tandem repeats (MIRU–VNTR) techniques in this study to differentiate relapse and reinfection types of recurrent tuberculosis. Spoligotyping is a simple, rapid and highly reproducible genotyping tool and MIRU–VNTR is a reliable method which is considered as a gold standard genotyping technique [4]. Twelve loci MIRU–VNTR when used along with spoligotyping will increase the discrimination among the strains of *Mycobacterium tuberculosis* than using spoligotyping alone.

The strains of *Mycobacterium tuberculosis* were isolated from patients during each episode of TB at the Department of Clinical Microbiology, Christian Medical College and Hospital, a tertiary care hospital receiving patients with tuberculosis from different parts of India. The first isolate was collected from newly diagnosed patients with tuberculosis and the second isolate was collected when they had any form of recurrent TB.

2. Materials and methods

Out of 500 bacteriologically confirmed patients with TB in this study, 22 patients had 2 episodes of tuberculosis over a period of 2 years from January 2014 and January 2016. The first isolate was collected from these 22 patients prior to TB treatment and the follow up cultures were collected anywhere between 7 to 16 months after the completion of 6 months of standard anti tuberculosis treatment(ATT) for their first episode of TB.

2.1. Culture

The samples were processed as per standard laboratory protocol [5] and were inoculated on Lowenstein–Jensen medium (L–J) and MGIT as per the standard laboratory protocol and manufacturers instruction respectively.

Positive cultures were confirmed as *M.tuberculosis* by performing Ziehl–Neelsen (ZN) staining and *M.tuberculosis complex* by MPT64 detection using an immune chromatographic test by SD BIOLINE TB Ag MPT64 Rapid kit .

Mycobacterium tuberculosis strains which grew in MGIT tubes were subcultured on to LJ slants and the isolates from LJ media were used for genotyping.

2.3. Drug susceptibility testing

Drug susceptibility test [6] of *Mycobacterium tuberculosis* isolates was done using 1% proportion method as per standard laboratory protocol (6). They were tested against first line drugs namely Streptomycin, Isoniazid, Rifampicin, Ethambutol. Doubling dilution of

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Mycobacterium tuberculosis isolates were inoculated on both drug containing and drug free LJ medium and incubated at 37 °C for 6 weeks. Growth of more than 1% on drug containing media as compared to drug free media were taken as drug resistant strains. Strains that were resistant to Isoniazid and Rifampicin were considered as multi drug resistant and they were subjected to second line DST.

2.4. Molecular typing

2.4.1. DNA extraction by CTAB NaCl method

About two loop full of bacteria were heat killed in 500 µl of TE buffer. After cooling, 80 µl of lysozyme (Sigma, USA) solution was added and incubated at 37 °C overnight. Following this 6 µl of proteinase K (Sigma, USA) and 80 µl of 10% sodium dodecyl sulphate (SDS) was added and incubated at 65 °C for 30 min.. After 30 min 100 µl of NaCl and 80 µl of Cetyl Trimethyl Ammonium Bromide–NaCl (CTAB–NaCl) was added and incubated at 65 °C for 30 min. Next 80 µl of chloroform isoamyl alcohol was added and centrifuged at 10,000 rpm for 20 min. The supernatant was transferred to fresh tubes with 500 µl of ice cold isopropanol and incubated at −20 °C overnight. The DNA was precipitated with ethanol and resuspended in 50 µl of 1 × TE buffer.

2.5. Spoligotyping method

Spoligotyping was done at National Institute for Research In Tuberculosis (NIRT), Chennai by the method described by Kamerbeek et al. [7]. The direct repeat region in the genome of MTB complex was amplified using two primers DRa (5-CC AAG AGG GGA CGG AAA C-3) and DRb (5-GGT TTT GGG TCT GAC-3) .Chromosomal DNA of *Mycobacterium tuberculosis* strains H37Rv and *M. bovis* BCG P3 were used as positive controls and molecular grade (MQ) water was used as negative control. The amplified products were hybridized on Biodyne C membrane (Isogene Bioscience) which contained synthetic oligomeric spacer sequence derived from direct repeat region of *M.tb* and *M.bovis* BCG. The presence of spacers was visualized on X-ray films as black squares after incubation with streptavidin–peroxidase and the hybridization signals were detected using enhanced chemiluminescence (ECL) detection system.

2.6. MIRU–VNTR genotyping

Mycobacterial Interspersed Repetitive Unit–Variable–Number Tandem Repeats (MIRU–VNTR) typing has been widely used for genotyping of *M. tuberculosis* [8]. We have used MIRU–VNTR Genotyping Kit (GenoScreen, Lille, France) for the amplification of the 12 MIRU–VNTR loci and the 12 loci used were MIRU 2, 4, 10, 16, 20, 23, 24, 26, 27, 31, 39, 40. These 12 loci were considered as intergenic regions dispersed throughout the genome of *M. tuberculosis*. Chromosomal DNA of *Mycobacterium tuberculosis* strains H37Rv was used as a positive control and milli Q (MQ) molecular grade water was used as negative control.

3. Results

As shown in Fig. 1 we identified 22 patients with recurrent TB and all these patients had pulmonary TB. Out of 22 patients 19 were male and 3 were female. Their mean age was 35 years. Both the initial and the follow up isolates were collected from these 22 patients. Follow up cultures were collected from these patients anywhere between 7 to 16 months after the completion of 6 months of standard ATT treatment for their first episode of TB. In total we had 44 isolates from 22 patients from their 1st and 2nd episodes of their disease manifestation.

We have used two genotyping methods for better discrimination of *M. tuberculosis* isolates. Spoligotyping and 12 loci MIRU- VNTR was done for 44 isolates from these 22 patients. Patients whose 1st and 2nd isolates showed differences in genotype pattern were considered cases of exogenous reinfection, whereas patients those whose 1st and 2nd isolates had the same genotype pattern were considered as cases of endogenous reactivation.

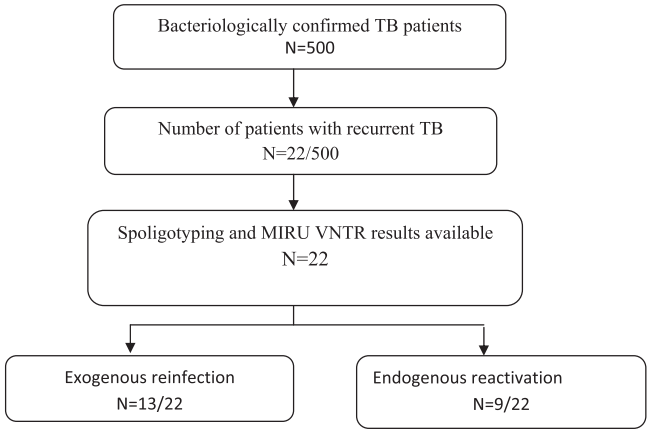

Fig. 1. Details of recurrence among patients with recurrent TB.

Table 1 shows the genotyping results using both Spoligotyping and MIRU–VNTR patterns of all patients. Out of the 22 patients, 13 had exogenous reinfection and 9 patients had endogenous reactivation. Out of 22 patients 14 (57%) had multi drug resistant TB (MDR–TB). Eight patients out of 14 patients with MDR–TB had exogenous reinfection.

Seven different strain lineages were found in this study. They were EAI, Beijing, U, CAS, T, H, and LAM. EAI and Beijing lineages were the predominant lineages in both exogenously and endogenously infected patients.

As discussed in Table 1 there were four sub lineages of EAI. They were EAI3_IND -SIT 11(10), EAI5-SIT138(2) and SIT126(2), EAI1_SOM-SIT 48(1) and EAI6_BGD SIT 292(1).Beijing strains were under two SIT number -SIT 1(9) and SIT 255(1).CAS lineage was found in two sub lineages .Two isolates belonged to CAS1_DELHI sublineage with SIT 26 and CAS with SIT 1345 .There were two strains which belonged to H lineage (H1 SIT 47 and H4 SIT 778).The remaining three isolates were grouped under U, T and LAM lineages (U-SIT 1418, T3-SIT 1655) and LAM9-SIT 42

Out of 22 paired isolates, 21 pairs had correlation between spoligotyping and MIRU–VNTR results but only one pair which belonged to EAI3_IND(SIT 11) with similar spoligotype pattern had 3 loci difference among 12 loci in MIRU–VNTR analysis and hence considered as different strain of *M. tuberculosis*.

4. Discussion

Molecular epidemiological analysis has played a significant role to distinguish recent or reactivated infection where clinical or epidemiological data are absent. In this study, we have compared pretreatment isolates of *M. tuberculosis* with isolates at the time of recurrence with tuberculosis.

In our study recurrent TB was more common among male when compared to female. Out of 22 patients, 19 patients were male and only 3 were female. A study by Corona et al from Mexico have reported the high prevalence of recurrent TB among males was due to poor adherence to anti tuberculosis treatment by men than women [9].

We found recurrent TB among patients with age group between 18 to 65 years and we could not find any significant correlation between recurrent TB and patients occupation. Similarly a study by Gadoev et al in 2017 from Uzbekistan have also reported that recurrent TB was not associated with any specific age group and occupation of patients in their study [10].

Table 1
Genotyping results of paired isolates:.

S.No	Age/Sex	Episodes/Months	Lineages	Spoligotype pattern	SIT No	12 Loci MIRU												Recurrence
						MIRU2 154	MIRU4 580	MIRU40 802	MIRU10 960	MIRU16 1644	MIRU20 2059	MIRU23 2531	MIRU24 2687	MIRU26 2996	MIRU27 3007	MIRU31 3192	MIRU39 4348	
1	22/F	0	EAI3_IND	477,777,777,413,071	11	2	5	3	4	3	2	0	2	2	3	6	4	Endo
		7	EAI3_IND	477,777,777,413,071	11	2	5	3	4	3	2	1	2	2	3	6	4	
2	38/M	0	Orphan	770,377,740,000,140		2	6	4	2	2	4	4	4	2	4	6	1	Exo
		8	Orphan	400,377,740,000,771		2	6	4	5	2	4	4	4	2	4	6	4	
3	37/M	0	EAI3_IND	477,777,777,413,071	11	2	5	3	4	3	0	0	2	2	3	6	4	Exo
		8	EAI3_IND	477,777,777,413,071	11	2	5	2	4	3	2	5	2	2	3	6	4	
4	18/M	0	EAI6_BGD	777,777,757,413,371	292	2	4	2	4	3	2	5	2	2	1	6	2	Exo
		11	Orphan	777,777,774,000,771		2	2	2	4	4	3	2	0	1	4	2	3	
5	40/M	0	BEIJING	000,000,000,003,771	1	2	2	5	3	3	2	5	1	5	3	5	3	Exo
		9	Orphan	777,767,777,513,761		2	5	2	2	4	3	4	5	1	3	3	4	
6	43/M	0	Orphan	777,417,607,560,771		2	5	5	0	4	3	5	3	6	1	2	5	Exo
		8	U	777,777,777,700,771	124	2	2	3	2	3	2	5	1	6	3	3	3	
7	24/M	0	EAI5	777,777,777,413,700	138	2	4	3	4	3	2	7	1	2	3	4	1	Endo
		12	EAI5	777,777,777,413,700	138	2	4	3	4	3	2	7	1	2	3	4	1	
8	30/M	0	CAS1_DELHI	703,777,740,003,771	26	2	2	2	4	4	2	5	1	3	3	5	3	Endo
		9	CAS1_DELHI	703,777,740,003,771	26	5	2	2	4	4	2	5	1	3	3	5	3	
9	45/M	0	BEIJING	000,000,000,003,771	1	2	2	5	3	3	2	5	1	5	3	5	3	Endo
		13	BEIJING	000,000,000,003,771	1	2	2	3	3	3	2	5	1	5	3	5	3	
10	21/M	0	BEIJING	000,000,000,003,771	1	2	2	3	3	2	2	5	7	5	3	5	3	Exo
		12	Orphan	777,777,777,703,771		2	5	5	0	4	3	5	3	6	0	2	5	
11	60/M	0	Orphan	777,777,777,513,760		2	5	5	4	4	3	5	3	6	3	2	5	Exo
		14	Orphan	777,767,777,740,771		2	5	5	3	4	3	5	2	6	3	5	2	
12	40/M	0	EAI3_IND	477,777,777,413,071	11	2	4	3	4	3	2	4	2	2	3	4	3	Endo
		9	EAI3_IND	477,777,777,413,071	11	2	4	4	4	3	2	4	2	2	3	4	3	
13	31/M	0	BEIJING	000,000,000,003,371	265	2	2	5	3	3	2	5	1	5	3	5	3	Exo
		15	Orphan	774,377,777,413,771		2	5	5	1	4	3	5	3	6	1	2	5	
14	24/M	0	T3	777,723,777,760,771	1655	2	1	2	5	1	2	5	1	1	3	2	2	Exo
		9	H1	777,777,774,020,771	47	2	2	3	4	3	2	5	1	5	3	3	2	
15	30/M	0	BEIJING	000,000,000,003,771	1	2	2	3	2	3	2	5	1	5	3	5	4	Exo
		13	LAM9	777,777,607,760,771	42	1	2	5	3	3	2	5	1	4	3	2	2	
16	53/M	0	EAI3_IND	477,777,777,413,071	11	2	5	3	4	3	2	4	2	2	3	4	3	Endo
		16	EAI3_IND	477,777,777,413,071	11	2	5	3	4	3	2	4	2	2	3	4	3	
17	65/M	0	Orphan	777,777,777,753,771		2	3	3	6	5	5	4	4	2	2	4	2	Exo
		10	EAI1_SOM	777,777,777,413,731	48	2	5	3	4	3	2	6	2	2	3	5	2	

(*continued on next page*)

Table 1 (*continued*)

S.No	Age/Sex	Episodes/Months	Lineages	Spoligotype pattern	SIT No	12 Loci MIRU											Recurrence
						MIRU2 154	MIRU4 580	MIRU40 802	MIRU10 960	MIRU16 1644	MIRU20 2059	MIRU23 2531	MIRU24 2687	MIRU26 2996	MIRU27 3007	MIRU31 3192	MIRU39 4348	
18	35/M	0	EAI5	477,777,777,413,771	126	2	2	3	4	3	2	6	1	3	3	4	1	Endo
		14	EAI5	477,777,777,413,771	126	2	2	3	4	3	2	6	1	2	3	4	1	
19	48/M	0	BEIJING	000,000,000,003,771	1	2	3	5	3	3	2	5	1	3	3	5	3	Endo
		13	BEIJING	000,000,000,003,771	1	2	3	5	3	3	2	5	1	3	3	5	3	
20	25/F	0	EAI3_IND	477,777,777,413,071	11	2	5	2	4	3	2	6	2	2	2	4	3	Exo
		14	H1	177,777,764,020,771	1586	2	2	2	4	3	2	2	1	5	3	3	2	
21	20/F	0	EAI3_IND	477,777,777,413,071	11	2	5	3	4	3	2	6	2	2	3	6	5	Exo
		8	CAS	703,777,700,000,371	1345	2	2	3	5	4	2	6	1	7	3	5	1	
22	28/M	0	BEIJING	000,000,000,003,771	1	2	2	3	3	3	2	5	1	3	3	5	4	Endo
		7	BEIJING	000,000,000,003,771	1	2	2	3	3	3	2	5	1	3	3	5	4	

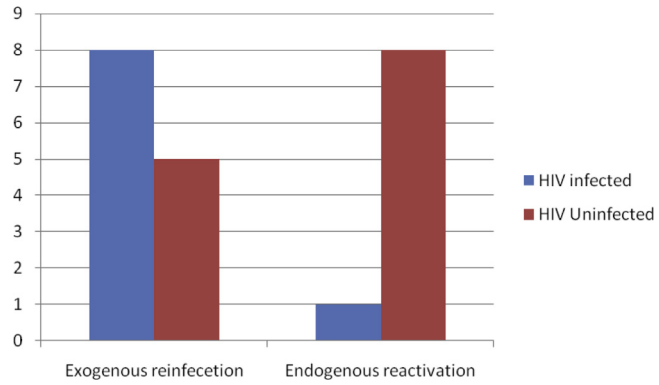

Fig. 2. Recurrent TB among HIV infected and HIV Un infected patients.

Among 22 patients with recurrent TB in this study, 59% had exogenous re infection and 41% had endogenous reactivation. These patients had exogenous reinfection within two years after completion of standard 6 months treatment with ATT. In contrast Vynnycky and Fine [11] from England reported that the exogenous reinfection is rare during the first 2 to 5 years after the first infection. These results suggested that in immunocompetent persons living in an area where tuberculosis is endemic, reinfection and progression to active disease might occur earlier than from patients in non-endemic countries.

We have compared the rate of re infected and relapsed TB among HIV infected and HIV uninfected patients. Out of 22 patients with TB, 9 were HIV positive and the remaining 13 were HIV negative patients. As shown in Fig. 2, 8/9 (88%) HIV positive patients had recurrent TB due to exogenous reinfection. A study by Narayanan et al [12] from India in 2010 have also reported the high prevalence of exogenous re infection among HIV infected patients than HIV uninfected patients. The high rate of re infection among HIV infected patients may be due to the increased disease progression following exposure to TB in high endemic areas. Out of 13 HIV uninfected patients, 8(61%) had recurrent TB due to endogenous reactivation and 5(38%) had exogenous reinfection. Similarly [13] have reported the strong association of endogenous reactivation of TB in HIV uninfected patients.

Many previous studies have reported the contribution of MDR TB to TB recurrences [14].We also found that recurrence of TB was high among patients with MDR TB when compared to patients with drug susceptible TB. Out of 22 patients with recurrent TB, 14(63%) had multi drug resistant TB and 8(36%) patients had drug susceptible TB. Of which only 2 had recurrent TB caused by *M. tuberculosis* isolates which are multi drug resistant and both these patients had recurrence due to exogenous reinfection .The remaining 20 patients had recurrent TB due to drug susceptible *M. tuberculosis* isolates. Similarly a study by Small et al from California have reported exogenous reinfection with MDR strains in patients with recurrent TB and they have also stated that exogenous reinfection with multidrug-resistant *M. tuberculosis* can occur either during therapy for the original infection or after therapy has been completed [15].

In our study *M. tuberculosis* isolates causing exogenous reinfection were found in 7 different lineages. They are EAI, Beijing H, U, T, CAS and LAM .While isolates causing endogenous reactivation were found in only 3 lineage groups like EAI, CAS and Beijing.

We found EAI lineage as the predominant lineage circulating among isolates causing exogenous reinfection as well as endogenous reactivation. Out of 23 isolates causing recurrent TB due to exogenous reinfection, 6 isolates belonged to EAI lineage and in 18 isolates which caused endogenous reactivation, 10 belonged to EAI lineage. Narayanan et al from Chennai in 2010 have reported previously a high prevalence of EAI lineage among isolates causing recurrent TB [16].

Beijing lineage accounted for 10 /44 (22%) of the total number of *M. tuberculosis* isolates and it was the second largest lineage in our

study. Of the 10 Beijing lineage strains 6 were associated with endogenous reactivation. Similarly studies by Sun et al [17] and Lan et al [18] from Singapore and Vietnam respectively have reported the strong association of Beijing genotype with endogenous reactivation or relapse TB when compared to exogenous re infection. Laboratory studies have suggested that Beijing strains are better adapted for intracellular growth [19] and the greater flexibility to adverse conditions, such as by multidrug therapy might be the reasons for its association with relapse or reactivation form of recurrent TB [20].

Conclusion

We have found that molecular genotyping methods like Spoligotyping and MIRU–VNTR have been useful in differentiating *Mycobacterium tuberculosis* strains demonstrating whether a new episode of TB is caused by infection with the same strain as the previous episode or by a different strain. In this study recurrent TB was mainly due to exogenous reinfection than endogenous reactivation. Therefore patients who have been recently treated and cured of the disease should be counseled on possibilities of recurrence and advised on early detection and treatment. More efforts should also be made in educating the public in reducing the transmission of tuberculosis in the community.

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