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STRAIN IDENTIFICATION AND EVALUATION OF PCR BASED MOLECULAR METHODS FOR SUBTYPING OF *MYCOBACTERIUM TUBERCULOSIS* CLINICAL ISOLATES.

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ABSTRACT

Drug susceptibility profile of 53 *Mycobacterium tuberculosis* clinical isolates was studied by conventional solid and liquid culture methods. Forty three isolates were drug resistant to at least one drug and 10 were susceptible. The strain identification was performed by PCR amplification of *mtp40* gene and strain subtyping was performed by Double Repetitive Element-PCR (DRE-PCR), IS*6110* outward primers-PCR and Random Amplified Polymorphic DNA-PCR (RAPD-PCR). Strains were best discriminated by DRE-PCR (15 patterns), followed by RAPD-PCR (11 patterns), and IS*6110*-PCR (7 patterns). The PCR amplification of *mtp40* gene was evaluated for the rapid differentiation of *M. tuberculosis* and *M. bovis*. The DRE-PCR method showed highest reliability than the other methods and provided a useful alternative tool for subtyping of *M. tuberculosis* clinical isolates and thus offers simple procedure to demonstrate that prevalence of TB in Puducherry region.

KEY WORDS: ***Mycobacterium tuberculosis*, Genotyping, PCR typing, DRE-PCR, RAPD-PCR and IS*6110*-PCR.**

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INTRODUCTION

Strain differentiation of *Mycobacterium tuberculosis* by various genetic markers have simplified the prevalence pattern and epidemiology worldwide[1,2,3]. IS*6110*-RFLP based DNA finger printing of *M. tuberculosis* has created a revolution in studying the rate of infection, causative agents and also epidemiology, thus known to be a gold standard genotyping method[4,5]. This approach provides the highest discrimination and being a reference method as it helps in studying the insertion of these elements even five or more copies[3,5,6]. The major disadvantage of this IS*6110*-RFLP based DNA finger printing is that it is not be useful for long term prospective studies[7,8] and also requires huge quantities of genomic DNA and leads to delayed typing[6]. Thus it requires further typing if the IS*6110* element presents fewer than five[5]. There are evidences in India, as approximately 45% of strains having either one or no copy of this element[9-11]. In Kerala, a study revealed that 19 samples among 80 strains had no copy of this element, whereas 31 samples have only one copy of this element[12]. It is obvious that IS*6110*-RFLP have to be added with some other methods with a rapid, less expensive and less laborious PCR methods for diagnosing and typing *M. tuberculosis*[13]. DRE-PCR amplifies DNA segments located between IS*6110* and polymorphic GC-rich repetitive sequence (PGRS)[14]. RAPD-PCR amplifies DNA fragments using primers directed to specific target using a random selected primer[15]. These above mentioned PCR assays are used to generate distinct DNA band patterns or fingerprints to type *M. tuberculosis* isolates[16]. Species-specific *mtp40* genomic fragment amplification is useful in identifying *M. tuberculosis* as these genomic fragments not seen in other members of *M. tuberculosis* complex[17].Hence, we attempted to analyse the clinical applicability and reproducibility of the use of three different PCR typing methods namely, DRE-PCR, RAPD-PCR and IS*6110*-outward primer PCR and direct identification of *M. tuberculosis* in clinical samples done by the amplification of *mtp40* gene with a panel of fifty three *M. tuberculosis* clinical isolates.

MATERIALS AND METHODS

(i) M. tuberculosis clinical isolates

A panel of fifty three *M. tuberculosis* clinical isolates were analysed in Department of Microbiology, at Intermediate Reference Laboratory, State TB training and Demonstration Centre, Government Hospital for Chest diseases, Puducherry and all the isolates were designated with laboratory code numbers (Strain ID) as listed below (Table 1) and subjected for identification of as *M. tuberculosis* by standard mycobacteriology procedures.All clinical specimens were digested and decontaminated by N-acetyl-l-cysteine-sodium hydroxide (NaOH-NALC) method. Processed specimen was inoculated into 7ml MGIT Middle brook 7H9 broth tube (BD Biosciences, Sparks, MD, USA) and a Lowenstein-Jensen (LJ) slant. The MGIT tubes and LJ slants were incubated until they were found to be positive. All the positive isolates were subjected to conventional tests as p-nitrobenzoicacid (PNB) test for species identification.

(ii) In vitro phenotypic drug susceptibility testing

Phenotypic drug susceptibility test-proportion method (Stand and Economic Variant) to isoniazid (INH), rifampicin (RIF), streptomycin (SM) and ethambutol (ETB) was performed using conventional solid LJ[18,19] and BACTEC MGIT 960 SIRE kit (Becton Dickinson, USA) following the manufacturer's instructions[20]. The reference strain *M. tuberculosis* H37Rv ATCC 27294 was used as a control.

MYCOBACTERIAL GENOMIC DNA ISOLATION

One loopful of culture was homogenized in 100 µL of sterile distilled water and treated with 50 µL of lysozyme (10 mg/mL) at 37 °C for overnight incubation. 70 µL of 14% SDS and 6 µL of Proteinase K (10 mg/mL) was added and incubated at 65 °C for 15 minutes. 10 µL of 5 M NaCl and 80 µL of CTAB/NaCl were added and incubated at 65 °C for 10 minutes. 800 µL of Phenol: Chloroform: Isoamylalcohol (25:24:1) mixture was added and centrifuged at 10,000 rpm for 10 minutes. The supernatant was

transferred to a fresh tube and 600 µL of Isopropanol was added to precipitate the DNA and incubated overnight at -20 °C and centrifuged at 12,000 rpm in 4 °C for 10 minutes. The pellet was washed with 70% ethanol and air-dried and dissolved in 20 µL of 1x TE buffer. DNA samples were stored at -20ºC for further analysis[21].

PCR BASED TYPING

Strain subtyping methods were used for targeting IS*6110 &* PGRS, random DNA fragments and IS*6110* flanking regions in this study namely, DRE-PCR[14], RAPD-PCR[22] and IS*6110* outward directed primers[16] respectively. Direct identification and distinction of *M. tuberculosis* from *M. bovis* infection in clinical samples was performed by amplification of *mtp40* gene[17]. Essentially the typing procedures were slightly modified and standardized with the increase in amount of DNA (5µl) and Taq Polymerase (1U-1.5U). A Master Cycler Gradient PCR 5331H52314 (Eppendorf) was used for all amplification reactions.

AGAROSE GEL ELECTROPHORESIS

Ten-microliter of amplified products were run in 1.5% agarose gels at 100 V for 30 minutes. Gels were photographed with UV transillumination, and band sizes were determined by compared with 100-bp DNA ladder standard (HiMedia, India). DNA fingerprints were compared visually for similarity based on the presence or absence of bands, and variations in band intensity were not taken into constitute strain differences. The number of copies of the bands in gel were calculated and typed separately in each method. Variation in the banding pattern was analyzed and documented visually.

RESULTS

1. Drug Susceptibility Pattern

Based on the results of conventional L.J and BACTEC MGIT 960 SIRE kit, 10 isolates were susceptible, 7 isolates were resistant to Rifampicin (RIF), 5 isolates were resistant to Isoniazid (INH), 3 were resistant to INH and Ethambutol (ETB), 3 were resistant to INH and Streptomycin (SM), 3 were resistant to SM, 3 were resistant to ETB and 19 isolates were MDR-TB [8 were resistant to INH and RIF, 3 were resistant to INH, RIF and ETB, 8 were resistant to four major anti-TB drugs (SIRE)].

2. DRE-PCR banding pattern

The agarose gel electrophoresis patterns generated different DRE-PCR patterns (Table 1) which comprised of seven clusters, as I to VII which encloses from zero to five bands. Based on the banding pattern, clusters were framed and strain genotype was identified. Cluster I comprised of four strains (7.54%) with five bands similarity in banding pattern. Cluster II comprised of seven strains (13.2%) with four bands however, banding positions varied between the strains tested. Cluster III was observed in thirty two strains (60.3%) with three bands made higher proportion which contained of unique amplicon bands termed signature bands. Cluster IV contained of six strains (11.3%) with dissimilar in number and pattern of bands. Cluster V & VI contained of one strain in each from no to one band. Two strains (3.8%) had no bands neither did so after repetition. Positions of the bands varied from 1-kb to below 100-bp regions of amplicons lengths are showed in Fig 1.

Figure 1 DRE-PCR assay examples

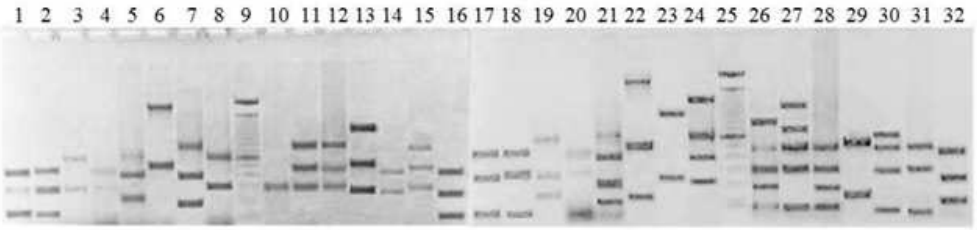

Figure 1

Lane: 1, M. tuberculosis H37Rv ATCC 27294, Lane: 2, HIV (+)ve sample, Lanes: 9 to 25, 100-bp molecular marker, remaining all the lanes shows amplified products of clinical strains (E2051 to E2080).

3. RAPD-PCR banding pattern

On the basis of the DNA fingerprints, different profiles with two to five bands were observed and four clusters were framed by RAPD primers (Fig 2). All the 53 cases were classified as Cluster A-D, based on the number of bands and their pattern similarity (Table 1). 52.8% (28 strains) were cluster A with identical pattern, 22.6% (12 strains) were cluster B which showed pattern identity; 17% (9 strains) were cluster C with dissimilarity in banding pattern. The remaining 7.5% (4 strains) in cluster D were unclassified. Eleven strains demonstrated fewer bands after the second amplification but the position of the stronger bands were identical in all. The most reproducible results were found with primer DKU49, followed by primer pairs of DKU44 plus DKU49; DKU43 plus DKU49, and DKU43 plus DKU44. The use of primer DKU44 resulted in non-identical patterns among five strains.

Figure 2 RAPD-PCR assay examples

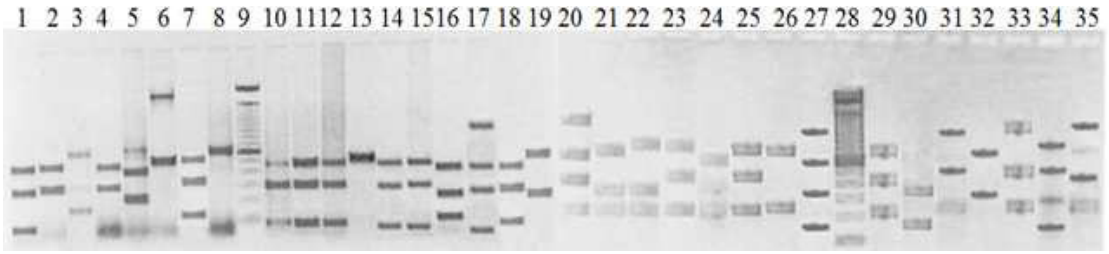

Figure 2

Lane: 1, M. tuberculosis H37Rv ATCC 27294, Lane: 2, HIV (+)ve sample, Lanes: 9 to 28, 100-bp molecular marker, remaining all the lanes shows amplified products of clinical strains (E2051 to E2080).

Table 1

Different banding pattern and cluster analysis of M. tuberculosis clinical isolates

S. No	Strain ID No.	PCR Strain Typing Method			
		DRE-PCR		RAPD-PCR	
		No. of Bands	Cluster	No. of Bands	Cluster
1	H37Rv	3	III	4	B
2	HIV(+)	3	III	3	A
3	E-2051	3	III	3	A
4	E-2052	3	III	4	B
5	E-2053	4	II	3	A
6	E-2055	3	III	3	A
7	E-2056	0	VI	2	C
8	E-2057	2	IV	4	B
9	E-2058	3	III	3	A
10	E-2059	3	III	2	C
11	E-2060	3	III	3	A
12	E-2061	1	V	2	C
13	E-2062	3	III	3	A
14	E-2063	3	III	4	B
15	E-2064	3	III	5	D

16	E-2065	0	VI	3	A
17	E-2066	3	III	3	A
18	E-2067	3	III	3	A
19	E-2068	3	III	3	A
20	E-2069	3	III	4	B
21	E-2070	4	II	2	C
22	E-2071	3	III	2	C
23	E-2072	2	IV	3	A
24	E-2073	4	II	2	C
25	E-2074	5	I	4	B
26	E-2075	5	I	3	A
27	E-2076	4	II	3	A
28	E-2077	2	IV	2	C
29	E-2078	4	II	3	A
30	E-2079	3	III	4	B
31	E-2080	3	III	3	A
32	IRL69	2	IV	4	B
33	IRL41	4	II	3	A
34	IRL42	3	III	2	C
35	IRL934	3	III	3	A
36	IRL380	3	III	3	A
37	IRL387	2	IV	4	B
38	IRL167	5	I	3	A
39	IRL172	3	III	5	D
40	IRL175	3	III	3	A
41	IRL212	3	III	3	A
42	IRL151	3	III	3	A
43	IRL119	3	III	3	A
44	IRL323	4	II	4	B
45	IRL344	3	III	5	D
46	IRL332	0	VI	3	A
47	IRL336	2	IV	3	A
48	IRL168	5	I	4	B
49	IRL41A	3	III	2	C
50	IRL55	3	III	3	A
51	IRL217	3	III	3	A
52	IRL151	3	III	4	B
53	IRL44	3	III	5	D
54	IRL78	3	III	3	A

4. IS6110 outward primer PCR banding pattern

Direct amplification of *M. tuberculosis* DNA with the IS*6110* outward PCR primers generated strain-specific fingerprints composed of a discrete number of DNA bands. A typical agarose gel of fingerprint patterns obtained from hospitalized *M. tuberculosis* clinical isolates is shown in Fig 3. As demonstrated in this gel, the banding pattern from each isolate is distinct and individual isolate fingerprints are readily identified and differentiated by visual comparison. DNA fingerprints of 53 strains examined has revealed three classes of bands: (i) bands shared by all of the *M. tuberculosis* strains examined so far, termed signature bands; (ii) bands shared by many strains, termed common bands; and (iii) bands associated with individual strains, termed strain-characteristic bands.

Figure 3 IS6110 secondary sequences PCR assay examples

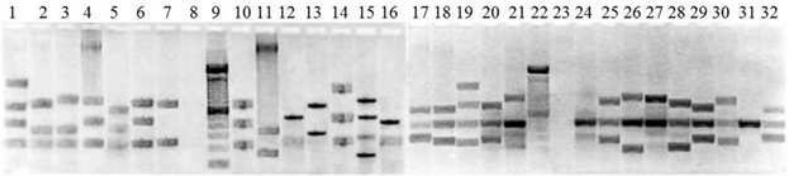

Figure 3

Lane: 1, M. tuberculosis H37Rv ATCC 27294, Lane: 2, HIV (+)ve sample, Lanes: 9 to 22, 100-bp molecular marker, remaining all the lanes shows the amplified products of clinical strains (E2051 to E2080) and the absence of IS6110 element was found in two strains (lanes 8 and 23).

5. PCR amplification of mtp40 gene from clinical samples

A total of fifty three *M. tuberculosis* clinical isolates were tested and amplified 332-bp *mtp40* amplicon (Fig 4); none of the isolates identified as *M. bovis* after PCR amplification. Therefore, the amplification of *mtp40* distinguished *M. tuberculosis* from *M. bovis* strains successfully. Negative controls were used and each set of samples revealed that there was no visible product on agarose gels.

Figure 4 mtp40 PCR assay examples

Figure 4

Lane: 1, 100-bp molecular marker, Lane: 2, Positive Control of M.tuberculosis H37Rv ATCC 27294, Lanes: 3 to 14, shows the amplified products of clinical strains at 332-bp amplicon.

DISCUSSION

There are many molecular methods are available which utilizes PCR based methods to study the epidemiology and evolutionary studies and they also have made rapid tuberculosis identification even in a single reaction[1,13,20,23]. These PCR assays are most sensitive even a loopful of organisms is enough[8]. Intrinsic drug resistance of *M. bovis* to pyrazinamide and its epidemiology have made it to be essential to distinguish them from MTB complex. The amplification product of *mtp40* is helpful in distinguishing *M. tuberculosis* and *M. bovis*. Previous studies[17,24] demonstrated that the *mtp40* gene is not present in all strains of *M. tuberculosis*, but surprisingly in our case we found all the cases of *M. tuberculosis* isolates were *mtp40* positive, thus implies no *M. bovis* strain found in clinical isolates under this study setting.DRE-PCR generated the bands based on the differences in the copy number and the distances between the two different repetitive elements in distinct strains.

32 strains were grouped in cluster III and falling under genotype C according to Varela et al[25] and Friedman et al[14] and suggesting that there is a predominant pattern in drug-resistant strains linked to dissemination in this region (Fig 1). In addition, 3 of the patients infected with cluster III strains exhibited distinct banding pattern that could not establish any epidemiological link. Clinical history of the fifteen strains (cluster III) recovered from patients suggested that they were epidemiologically related and showed the identical banding pattern in the gel. Five of these strains showed the same band profile by DRE-PCR assay (Fig 1, Lanes 11 and 12) and were isolated from patients who lived in same place, which suggests a common origin of infection or transmission between them. Overall the DRE-PCR discriminated with 89% of sensitivity and 100% of specificity.

The degree of polymorphism obtained by RAPD-PCR was almost the same as that obtained by DRE-PCR. 28 strains (52.8%) had unique RAPD fingerprints out of 53 *M. tuberculosis* clinical strains grouped into cluster A (cluster III in DRE-PCR) (Fig 2, Lanes 10-12, 14, 15). These difference RAPD patterns among the members of study population suggests that, the chances of occurrences of identical RAPD fingerprints among unrelated cases would be unlikely[13,15]. Thus it represented the uniqueness of RAPD patterns and also inferred that the cases of TB infection are belonged to similar genotype in this region.IS*6110* outward PCR assay based DNA fingerprints is known to produce analogous patterns similar to standard RFLP method[16]. Clinical isolates of hospitalized TB patients are found to be different fingerprint types and frequently contain multiple isolates of each type. It was also found that at least a pair of isolates with identical fingerprints had identical drug resistance patterns, which was similar as referred by van Embden et al.[15] who designated multiple-drug-resistant type as W were identified to produce distinct, identical PCR fingerprint patterns.

Reproducibility of PCR typing methods

In this study, fifty three different *M. tuberculosis* clinical strains were evaluated in duplicates to acquire the reproducibility of each strain typing procedure and the results were obtained from each typing method. Among the PCR-based methods, the DRE-PCR was highly reproducible, followed by RAPD-PCR. IS*6110* outward-directed primer PCR fingerprints showed weak band pattern and made the analysis difficult, thus the high-intensity bands alone were accounted for the analysis. RAPD fingerprints were initially analysed with computer aided and also by naked eye on the basis of similarity.

CONCLUSION

The DRE-PCR, RAPD-PCR and IS*6110*-PCR assays have been successfully used for rapid differentiation of MDR-TB directly from clinical specimens. Among the drug resistant strains, twenty-three MDR-TB strains showed the same DNA fingerprints (cluster III in DRE-PCR and Cluster A in RAPD-PCR), thus indicating the presence of a clustered transmission of these strains in and around hospital and related facilities. This study also suggested that, *mtp40* amplification of genomic fragment provides a highly sensitive as well as specific technique for the detection and differentiation of *M. tuberculosis* from *M. bovis* strains in clinical samples. In conclusion, DRE-PCR and RAPD-PCR methods produced identical DNA fingerprints among Drug resistant-TB strains demonstrated that, not only that the prevalence of TB infection in Puducherry region is due to transmission rather than reactivation, but that drug resistance also could play a role in tuberculosis dissemination. These direct PCR fingerprint assays are also been a faster and easier molecular epidemiology tool which contributes a major role in control measures against tuberculosis even in poor resource settings.

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