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Restriction fragment length polymorphism of *Mycobacterium tuberculosis* strains from various regions of India, using direct repeat probe

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Intraspecies differentiation was studied on 68 *M. tuberculosis* strains obtained from 6 states of India by restriction fragment length polymorphism (RFLP) using a direct repeat probe (DR probe) hybridised with Alu I digest of DNA. Most strains showed polymorphism based patterns that comprised between 2 to 7 bands and were grouped into 26 RFLP types. Of the 11 strains tested from Amritsar, 8 were RFLP type 5; the remaining 3 were of type 11 and were exclusively confined to this region. The strains from other regions were more heterogeneous. We confirm that DR-associated RFLP can be an excellent tool for the differentiation of *M. tuberculosis* strains. Depending on their geographical origin, these strains can be differentiated to a large extent by DR fingerprinting.

Key words DNA fingerprinting - *Mycobacterium tuberculosis* - strain differentiation

Precise epidemiological details of *M. tuberculosis* strains isolated from different clinical settings assume great importance with the upsurge of HIV and unrelenting global increase in the occurrence of multi drug resistant (MDR) tuberculosis. Various phenotypic methods, phage typing, virulence studies, mycobacteria typing *etc.*, were not found to be very useful. Strain specific DNA fingerprint patterns based on the occurrence of repetitive DNA elements in the chromosome of M. *tuberculosis* have provided epidemiologically very useful tools for monitoring the spread of individual strains[1].

Recent studies have shown that *M. tuberculosis* complex bacteria harbour various polymorphic repetitive sequences, which can be exploited for strain differentiation[2]. The most widely used genetic marker for the epidemiology of tuberculosis is the insertion sequence (IS) element IS6110, which is usually present in multiple copies in *M. tuberculosis* [3,5]. IS6110 besides discriminating *M. tuberculosis* strains isolated from diverse geographic regions across the world, was also found to be a unique tool for studying hospital outbreaks with MDR TB, HIV associated transmission and zoonotic transmission of *M. bovis* and *M. tuberculosis* [5,6]. *M. tuberculosis* strains from diverse geographic origins have been compared by RFLP to study the transmission pattern between two geographic regions[7].

However, RFLP typing using insertion element IS6110 showed a high frequency of single copy and a low frequency of strains with no copy of IS6110, which is a major limitation[8]. In contrast to *M. tuberculosis*, isolates of *M. bovis* have often been found to harbour only a single or a few copies of the IS6110 element thus limiting the power to discriminate among different strains[9]. Because of the poor discrimination with

IS6110-associated RFLP in *M. tuberculosis* as well as *M. bovis,* other genetic markers are needed for use in the epidemiology of tuberculosis.

Two other repetitive DNA elements have also been used for strain differentiation of *M. tuberculosis :* The polymorphic GC-rich repeat sequence (PGRS)[10] and the direct repeat (DR) sequence[2,6,7,11]. The nature of the PGRS-associated polymorphism is not understood. In contrast, a recent study[12] on the nature of the polymorphism of the DR region showed that homologous recombination between neighbouring or distant DRs is likely to be involved in the DNA polymorphism of this chromosomal region of bacteria belonging to the *M. tuberculosis* complex. Recently, we undertook a study to compare pretreatment and post-treatment isolates of *M. tuberculosis* to determine the frequency of exogenous reinfection and endogenous reactivation. This study showed that the polymorphism in the *M. tuberculosis* genome of DR-containing fragments allows significantly improved strain differentiation compared with IS 6110 analysis[13].

In this context, we studied the RFLP among *M. tuberculosis* strains from 6 geographic regions of India using the more polymorphic direct repeat (DR) element as probe to decipher unique genotypic variation in the DR region.

Material & Methods

Bacterial strains : A total of 80 strains of *M. tuberculosis* obtained from newly diagnosed pulmonary tuberculosis patients were used for the study. The geographic regions and the number of strains from each region are given in Table I.

Table I. Region-wise distribution of *M. tuberculosis* isolates studied

Geographic region	No. of strains	No. excluded	No. analysed
Amritsar	14	3	11
Andhra Pradesh	9		9
Goa	5	2	3
North Arcot	24	4	20
Pondicherry	14	2	12
Raichur	14	1	13
Total	80	13	68

Bacterial growth and chromosomal DNA isolation. Mycobacterial strains were grown in 10 ml Middlebrooks 7H9 mediurn supplemented with 5 per cent albumin-dextrose complex (Difco Laboratories, Detroit, Mich.) at 37°C in the stationary state. Three week old culture was heated at 80°C for 20 min to kill the cells. After centrifugation the cell pellet was resuspended in 500 µl TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0). Lysozyme was added to a final concentration of 1µg/ml, and the tube was incubated for 1 h at 37°C. To this 70 µ1 of 10 per cent SDS and 6 µl of 10mg/ml proteinase K (Boehringer Mannheim, Germany) were added and the mixture was incubated for 10 min at 65°C. Then 100 µl of 5M sodium chloride and 80 µl of N-cetyl-N,N,N-trimethyl ammonium bromide (CTAB) were added, and the tubes were mixed and incubated for 10 min at 65°C. An equal volume of chloroform/isoamyl alcohol (24:1 v/v) was added and mixed.

After centrifugation for 5 min, the supernatant was transferred to a fresh tube and 0.6 volume of isopropanol was added. The tubes were kept at - 20°C for 30 min to precipitate the DNA. After centrifugation for 15 min, the pellet was washed twice with 70 per cent ethanol, and the air dried pellet was dissolved in 50 µl TE buffer (50 mM Tris-HCl, 1 mM EDTA pH 8.0). Each DNA sample was coded with a random number so that no information regarding its geographic origin was available during RFLP typing.

Restriction enzyme digestion and electrophoresis : Approximately 2 µg of genomic DNA was digested overnight at 37°C with 10µl of Alu I restriction enzyme (New England Biolabs Inc., USA) under the conditions determined by the manufacturer. DNA fragments were separated by electrophoresis in 0.8 per cent horizontal agarose slab gels (10 x 15 cm) containing 0.5 µg/ml ethidium bromide, 1X TBE buffer (0.089M Tris, 0.089M boric acid, 0.002M NaEDTA pH 8.0) at 40 V for 6 h, and the result was recorded photographically. Molecular size standard, lambda DNA digested with Hind III enzyme, was incorporated in the last lane of each gel.

Synthesis and labelling of DR-probe : On the basis of direct repeat (DR) sequence of *M. bovis* BCG[11], 36 base pair oligonucleotide (DR-r) (5'GTTCCGTCCCC-TCTCGGGGTTTTTGGGTCTGACGAC-3') was synthesised using a DNA synthesizer (Applied Biosysterns, USA). These oligonucleotides were gifted by Dr Barry R. Bloom of Albert Einstein College of Medicine, New York, USA. The DR-r was labelled with fluorescein-dUTP and horse radish peroxidase (HRP) using the enhanced chemiluminescence 3'-oligolabelling and detection system (Amersham International plc. Amersham, UK). The detection of HRP labelled probes was carried out by the peroxidase catalysed oxidation of luminol and subsequent enhanced chemiluminescence. The emitted light was detected on X-ray film (Indu Film, Hindustan Photo Films, India).

Southern blotting and hybridisation : The DNA fragments resolved by gel electrophoresis were transferred on to charged nylon membranes (DuPont, NEN research products, Boston, Mass) by vacuum blotting[14,15] (Transvac, TE 80, Hoeffer Scientific Instruments, San Francisco) and were depurinated in 0.25 *M* HCl, and denatured in transfer buffer containing 0.4 *M* NaOH and 0.6M NaCl pH 8.0 and the membrane was rinsed in 2X SSC (1X SSC is 150 mM sodium chloride plus 15mM sodium citrate) and kept damp at - 20°C in a heat sealable plastic bag.

The blots were prehybridised for 30 min in a shaking water bath at 42°C and were hybridised overnight at 42°C in the same hybridisation solution containing the labelled DR-probe. The hybridised blots were washed as per the manufacturers instructions (Enhanced Chemiluminescence 3'-oligolabelling and detection system, Amersham International plc. Amersham, UK). The blots were finally wrapped in cling film (Saran Wrap) and exposed to X-ray film for 5 min and 10 min in a cassette containing an intensifying screen. Autoluminographs were developed and fixed by standard procedure.

Results

Of the 80 isolates used for the RFLP study, 12 isolates were excluded from the analysis due to poor enzyme digestion or hazy bands.

The autoluminograms generated were subjected to visual analysis separately by two investigators, and the band patterns were compared based on their number and molecular size. The isolates were accordingly divided into groups where the members of each group shared the same pattern. A cluster was defined as a group of two or more isolates whose RFLP fingerprints were identical with respect to both number and molecular size of all bands. The isolates that had unique fingerprints were deemed clustered. The different patterns obtained according to the number and molecular size are shown in Fig. 1. Among the 31 patterns 1, 8 and 30 on close scrutiny and repetition belonged to the same group. 21 and 27 were omitted from analysis due to partial digestion.

Thus, RFLP analysis of the 68 isolates from the 6 different geographic regions identified 26 distinct RFLP types based on the number and molecular size of the bands obtained. Among the 26 RFLP types, 15 were unique patterns found in only one patient each. The remaining 11 patterns were found to be shared by two or more isolates. Thus, 53 of the 68 (78%) were in one of the 11 clusters; the clusters ranged from 2 to 17 strains. The RFLP patterns shared by two or more strains are shown in Fig. 2. Seventeen isolates out of 68 (25%) showed an identical RFLP pattern, which is designated as RFLP type-1, and form the most commonly occurring strain. The number of hybridizing bands ranged from 2 to 7, and most of the isolates showing 5 or 6 bands.

RFLP types of the isolates region-wise are presented in Table II. The 11 isolates from Amritsar fall into 2 groups while the isolates from other regions showed more heterogeneity. Of the 11 strains from Amritsar, 8 were of the RFLP type 5 and 3 were of type 11. RFLP type 5, which is common in Amritsar, was not encountered in any other region except for one strain in Andhra Pradesh. The RFLP types 12 to 26, *(i.e.,* 15 types) occurred very infrequently (Table II).

Considerable differences were found in the RFLP

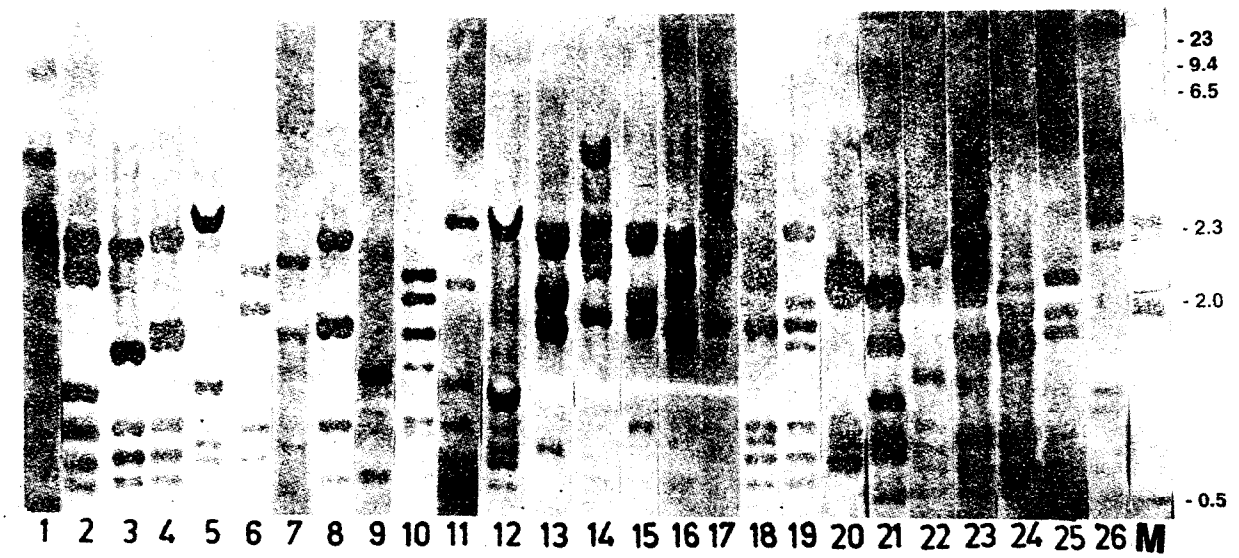

Fig. 1. Lanes 1 to 26 show the total number of patterns obtained from 68 isolates. 'M' indicates molecular weight marker.

Table II. RFLP typing of *M. tuberculosis* strains from six different regions of India

RFLP type	Cluster size no. of strains	Geographic regions					
		Amritsar	Andhra Pradesh	Goa	North Arcot	Pondicherry	Raichur
1	17		I	I	4	4	7
2	2	–	–		1	–	1
3	7	–	–		4	2	1
4	4				3	–	1
5	9	8	1			–	–
6	3		1	–	1	–	1
7	2			–	2	–	–
8	2		I	–	–	1	–
9	2			1	1	–	–
10	2		2	–	–	–	–
11	3	3					
12-26	15		3 (1 each)	1	4 (1 each)	5 (1 each)	2 (1 each)
Total							

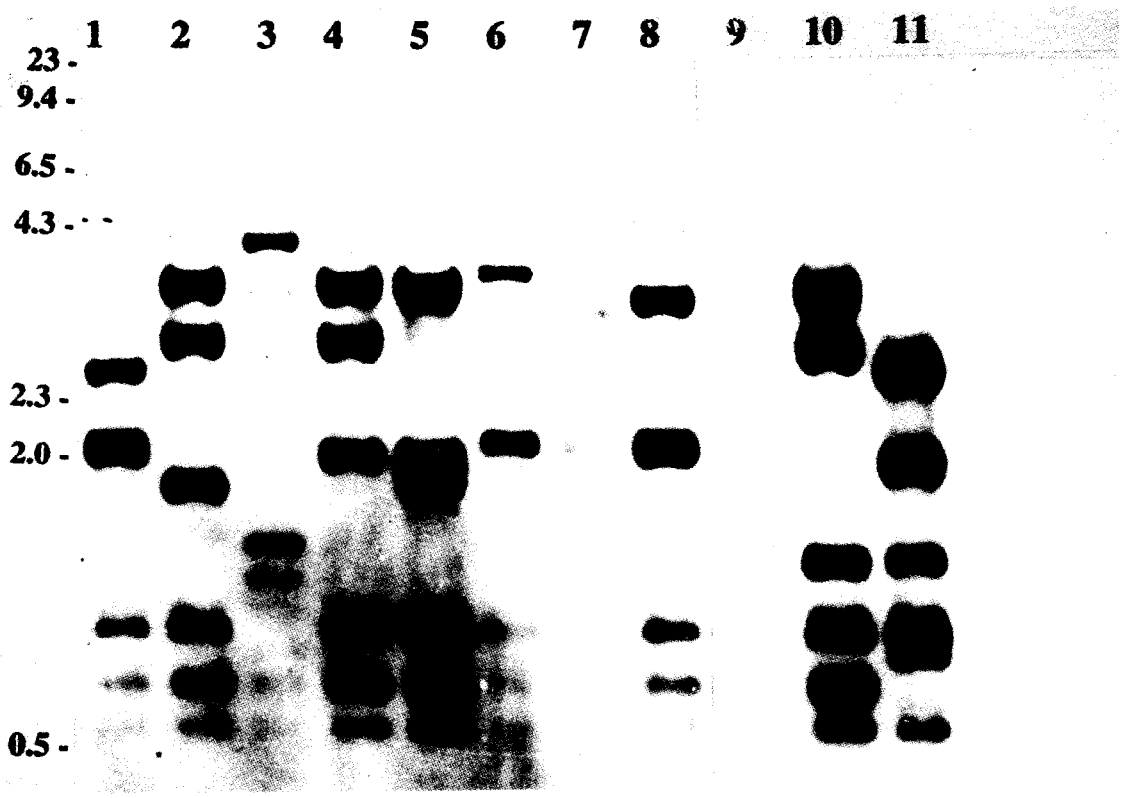

Fig. 2. Lanes 1 to 11 are *M. tuberculosis* DNA digested with Alu I enzyme and probed with DR. Molecular weights are indicated-in kilobases.

patterns of *M. tuberculosis* bacilli from Andhra Pradesh, Goa, North Arcot, Pondicherry, and Raichur. Those from Raichur were most often of type 1 but the other types were almost as frequent. Strains from Pondicherry were predominantly of type 1.

The DNA from these strains were also subjected to DR-probing after Pvu II enzyme digestion. The number of hybridising bands were only 2-3, and the polymorphism obtained with Pvu II digestion was less than that obtained with Alu I digested DNA as shown in Fig. 3.

Discussion

Broad distinctions in the phenotypic characters have been made between isolates of *M. tuberculosis* from south India and other parts of the world. The most prominent of these, the south Indian variant of *M. tuberculosis,* is distinguished by a number of characteristic features, *viz.,* lower virulence in guineapigs, enhanced susceptibility to hydrogen peroxide and thiophene-2-carboxylic acid hydrazide (TCH), natural resistance to thioacetazone and p-aminosalicylic acid, clustering into an intermediate phage type-I and characteristic composition of cell wall lipids[16-19]. Mitchison *et al* [20] showed that *M. tuberculosis* strains from south India had less virulence than the British strains of *M. tuberculosis. M. tuberculosis* strains obtained from the Kashmir valley were resistant to TCH as compared to south Indian strains. Similar intraspecies variations have also been reported in other mycobacterial species like *M. chelonei* [21,22] and *M. kansasii* [23].

The discovery of repetitive DNA elements in *M. tuberculosis* strains and the establishment of DNA fingerprinting techniques for *M. tuberculosis* with suitable genetic markers have made it possible to study the epidemiology of TB at the molecular level and to detect the sources of infection on the basis of clonal differentiation of *M. tuberculosis* isolates. A better understanding of the dissemination of the bacteria in defined areas and population will improve the detection of new

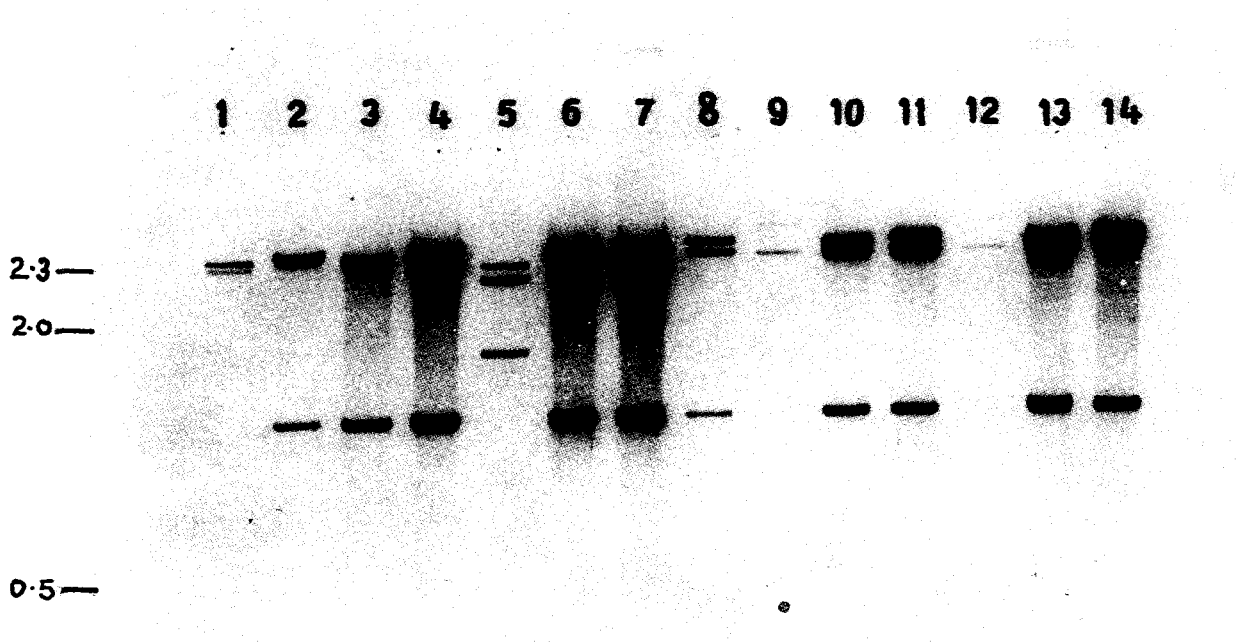

Fig. 3. Lanes 1 to 14 are the RFLP pattern of the DNA from various strains digested with Pvu II enzyme and probed with DR. Molecular weights are indicated in kilobases.

cases and the control of disease transmission. The present study was intended to provide information on the relative frequency of DNA fingerprint patterns based on the genotypic variation in the DR containing region of *M. tuberculosis* isolates from 6 geographic areas.

A greater degree of heterogeneity was observed among the isolates from Andhra Pradesh, Goa, North Arcot, Pondicherry, and Raichur, whereas, the isolates from Amritsar showed a lesser degree of heterogeneity. Although distinct DNA patterns representing exclusively different regions of the country were not observed, generally the degree of polymorphism correlated to an extent to the different regions except for strains studied from Amritsar. This observation is in agreement with previous reports[24] in which it was suggested that geographical separation of a pool of infection may lead to the evolution of distinct bacterial clones. The vast diversity of RFLP patterns in these regions may be due to parallel and divergent evolution. Typing of more number of isolates may give a significant correlation.

Another factor contributing to the degree of DNA polymorphism of *M. tuberculosis* isolates in a population/area is the relative contribution of newly acquired TB versus reactivated infection. Investigations of numerous outbreaks have demonstrated that epidemiologically linked strains of *M. tuberculosis* have identical RFLP patterns, whereas unrelated strains have different patterns [25,26,1] .The substantial diversity of RFLP patterns among the members of the population suggested that the chance occurrence of identical RFLP patterns among unrelated cases would be unusual. It is therefore inferred that cases of tuberculosis caused by strains with unique RFLP fingerprints are primarily due to recently transmitted disease and that cases caused by strains with identical RFLP fingerprints are primarily due to the reactivation of infection. Several findings support this conclusion[27]. The less heterogeneity observed in Amritsar also suggests a clonal dissemination of the particular strain in the area, and thus may have

been epidemiologically linked.

In conclusion, the DR-probe hybridisation studies from 5 of 6 regions (except for strains from Amritsar which had shown a remarkable degree of homogeneity) revealed genotypic heterogeneity among *M. tuberculosis* strains. The polymorphism observed with the DR probe is due to the polymorphism in the intervening sequences resulting from recombination between the DR repeats[12]. Based on the preliminary observations made in this pilot study, additional studies with more number of strains to characterise the RFLP types further will provide information on the relative frequency of DNA fingerprint patterns in geographic areas and populations, and also the extent of spread of related strains and usefulness of DNA fingerprinting in guiding tuberculosis control.

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