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Random amplified polymorphic DNA (RAPD) analysis of *Mycobacterium bovis* strain in India

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ABSTRACT

The usefulness of random amplification of polymorphic DNA (RAPD) analysis for typing Indian strains of *Mycobacterium bovis* was investigated. *M. bovis* AN_5, *M. bovis* BCG and 20 field strains of *M. bovis* were subjected to RAPD-PCR using 7 random decamer primers. All 7 primers were differentiative and produced specific RAPD profiles. The polymorphic amplicons served as RAPD markers for *M. bovis*. The dendrograms, obtained by different primers, showed the discriminatory ability of the primers. RAPD analysis provided a rapid and easy means of identifying polymorphism in *M. bovis*, and it was a valuable alternative epidemiological tool. In addition, the results of the present study showed heterogeneity in the *M. bovis* strains in the population studied.

Key words: India, *Mycobacterium bovis*, Random primer, RAPD, Typing

Mycobacterium bovis is the primary pathogen of tuberculosis in cattle and other domestic animals. Culture of micro-organism is the definitive method of diagnosis in cattle, however, culture takes 4 to 8 weeks and as such culture is of little value for the epidemiological purposes (Thoen and Bloom 1995). Investigations of the epidemiology of tuberculosis have been hampered due to the lack of strain specific markers that can be used to differentiate *M. bovis* strains. *M. bovis* strains have uniform characteristics and cannot be meaningfully distinguished from each other by conventional methods using biochemical properties, serotyping (Morris and Ivangi 1985), phage typing (Redmond *et al.* 1979), protein profiling (Borrow 1986). RAPD analysis with arbitrary oligonucleotide primers that produces a set of amplified DNA products is a simple and promising molecular method for determination of genetic heterogeneity based on DNA sequence diversity (Williams *et al.* 1990, Caetano-Anolles 1991). The technique is rapid and the RAPD markers are easier to generate and identify the nucleotide polymorphism. This technique has been used to differentiate and to type mycobacterial species and other bacterial strains like *E. coli* (Chansiripornchai *et al.* 2001), *P. multocida* (Dziva *et al.* 2001) and *Staphylococcus aureus* (Hermans *et al.* 2001). The present study reports RAPD analysis of *M. bovis* strains to know heterogeneity among these strains.

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MATERIALS AND METHODS

Details about *M. bovis* strains are given in the Table 1. *M. bovis* strains used included 2 standard strains *M. bovis* AN, *M. bovis* BCG and 20 strains from cattle, buffalo, and deer. All mycobacterial strains were typed by conventional morphological (Ziehl-Neelsen staining) and biochemical tests (Verma and Srivastava 2001) and maintained on Lowenstein-Jensen (LJ) medium at the Mycobacteria Laboratory, Indian Veterinary Research Institute, Izatnagar, India. Genomic DNA was extracted as per van Soolingen *et al.* (1999). Amplification of mycobacterial DNA using random decamer primers (Table 2) was performed in 25 µl. The reaction mixture contained 1 unit of Taq DNA polymerase, 1.5 mM $MgCl_2$, 200 µM of each dNTP, 30 pmol primers, and 50 ng of template DNA. Amplification was carried out in a thermal cycler. Cycling condition consisted of an initial denaturation step for 5 min at 94°C, followed by 45 cycles of 94°C for 1 min denaturatuon step, an annealing step for 1 min at 36°C, and an extension step for 1 min at 72°C and a final extension at 72°C for 5 min. The products obtained from RAPD-PCR were analyzed on 1.5% agarose gel stained with ethidium bromide. Subsequently, the gel was visualized and was photographed using a gel documentation and analysis system. The banding patterns obtained by RAPD were noted from photograph. A data matrix composed by the numerals 1 and 0 was built on the basis of presence [1] or absence [0] of any DNA band appearing in replicates of each isolate. Only distinct and prominent bands were scored and used in

Table 1. Mycobacterial strains, source and RAPD profile

Isolate No.	Species	Source	RAPD profiles/patterns						
			Primer OPN–02	Primer OPN–05	Primer OPN–06	Primer OPN–10	Primer OPN–20	Primer BG–65	Primer BG–66
227/95	*M. bovis*	Deer lung	A1	B1	C1	D1	E1	F1	G1
30/88	*M. bovis*	Bovine lymphnode	A1	B2	C2	D2	E1	F1	G1
83/91	*M. bovis*	Buffalo lung	A2	B1	C3	D3	E2	F2	G2
4/86	*M. bovis*	Bovine lung	A1	B1	C2	D4	E3	F1	G1
1/87	*M. bovis*	Bovine lung	A1	B2	C4	D5	E1	F1	G1
24/88	*M. bovis*	Bovine lymphnode	A1	B2	C2	D4	E3	F1	G1
3/87	*M. bovis*	Bovine lung	A1	B2	C2	D4	E1	F1	G1
324/96	*M. bovis*	Bovine lung	A1	B1	C2	D6	E1	F1	G2
39/89	*M. bovis*	Bovine lymphnode	A1	B1	C2	D7	E1	F1	G1
93/91	*M. bovis*	Buffalo lung	A1	B1	C2	D4	E1	F3	G1
90/91	*M. bovis*	Buffalo lung	A3	B2	C2	D1	E1	F1	G1
259/95	*M. bovis*	Bovine lung	A3	B3	C5	D8	E2	F2	G1
3/86	*M. bovis*	Bovine lymphnode	A4	B4	C3	D9	E2	F2	G1
2/86	*M. bovis*	Bovine lymphnode	A1	B1	C6	D2	E1	F1	G2
417/99	*M. bovis*	Bovine lung	A1	B1	C7	D2	E1	F1	G1
356/96	*M. bovis*	Deer lung	A1	B1	C6	D10	E1	F1	G1
391/98	*M. bovis*	Bovine lung	A1	B1	C2	D4	E1	F1	G1
85/91	*M. bovis*	Buffalo lung	A1	B1	C2	D10	E1	F1	G1
57/90	*M. bovis*	Bovine lymphnode	A1	B1	C2	D4	E1	F1	G1
89/91	*M. bovis*	Buffalo lung	A5	B1	C2	D11	E4	F4	G1
AN	*M. bovis*	Standard strain	A6	B5	C8	D12	E5	F3	G3
BCG	*M. bovis*	Standard strain	A7	B1	C2	D6	E6	F1	G1

assessing RAPD patterns. The molecular size of bands was calculated using software. The size of bands, which were differing by ± 5% on the different gel, was considered as same bands. The genetic diversity of strains was analyzed by RAP Distance version 1.04 software that works on the principle of UPGMA clustering methods.

RESULTS AND DISCUSSION

The RAPD-PCR showed different size amplicons in *M. bovis* strains. In this study, several fragments were amplified in each sample, and most of these fragments were common among different strains. However, there were some fragments unique to certain *M. bovis* strains. All 22 *M. bovis* strains showed high polymorphism upon RAPD analysis (Fig.1). The number of RAPD patterns generated by each of the primer is shown in Table 1. All the 7 primers showed discriminatory patterns. The primer OPN–10 and the primer BG–65 generated maximum and minimum number of amplimers respectively (Table 2). Among the 7 primers,

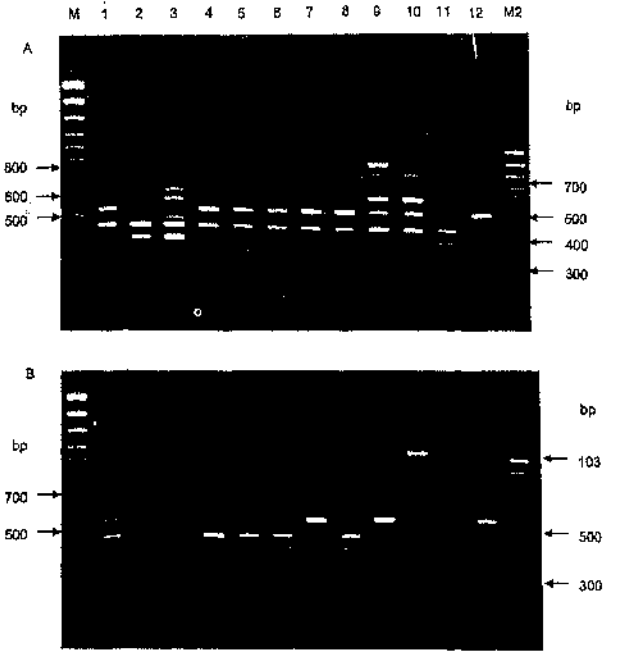

Fig. 1. RAPD profiles of *Mycobacterium bovis* isolates with primer OPN–1. Lane M–100 bp DNA marker ladder; 1-*M.bovis* (227/95); 2-*M.bovis* (30/88); 3-*M.bovis* (83/91); 4-*M.bovis*(4/86); 5-*M.bovis*(1/87); 6-*M.bovis* (24/88); 7-*M.bovis* (3/87); 8-*M.bovis* (324/96); 9-*M.bovis* (39/89); 10-*M.bovis* (93/91); 11-*M.bovis* (AN5); 12-*M.bovis*(BCG); Lane M2–50 bp DNA marker ladder. Lane M–100 bp DNA marker ladder; 1-*M.bovis* (90/91) 2-*M.bovis* (259/95) 3-*M.bovis* (3/86); 4-*M.bovis* (2/86); 5-*M.bovis* (417/99); 6-*M.bovis* (356/96); 7-*M.bovis* (391/98); 8-*M.bovis* (85/91); 9-*M.bovis* (57/90); 10-*M.bovis* (89/91); 11-*M.bovis* (AN5); 12-*M.bovis* (BCG); lane M2–50 bp DNA marker ladder.

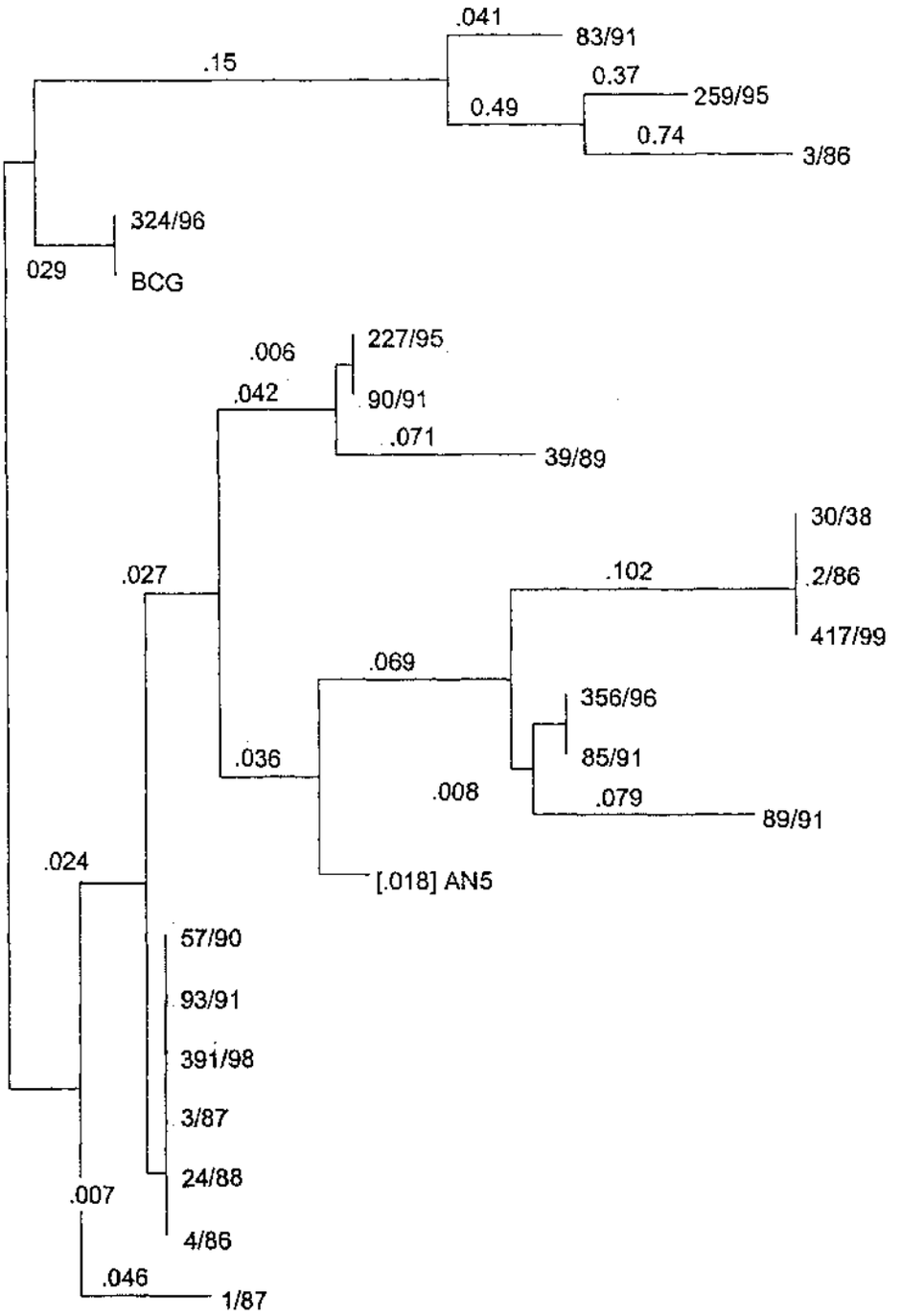

Fig. 2. Dendrogram: Showing genetic relatedness among *M. bovis* isolates with primer OPN–10.

OPN–10 showed a maximum of 12 RAPD patterns while BG–66 showed only 3 patterns (Tables 1, 2).

Upon dendrogram analysis with primer OPN–10, 6 strains, viz. 57/90, 93/91, 391/98, 3/87, 24/88 and 4/86 fell on the same level and farmed largest cluster. *M. bovis* strain AN5 and 1/87 remained separate while BCG fell with clinical isolate 324/96. Rest 12 strains formed 4 small clusters with 3 strains in each cluster (Fig. 2). *M. bovis* strains 391/98 and 57/90 have given similar RAPD patterns with all the 7 primers showing almost identical genetic relatedness with each other, conversely strain AN5 have given unique pattern with all the primers except identical band sharing with strain 89/91 by primer BG–65 (Table 1).

Detailed epidemiological studies of *M. bovis* have been

Table 2. Oligonucleotide primers sequences, GC contents, No. of amplified fragments with their size ranges and patterns of *M. tuberculosis* isolates with 7 different primers

Primer	Sequence	G+C content (%)	No. of bands generated	Band size (approx. range) (bp)	No. of RAPD patterns
OPN–02	ACCAGGGGCA	70	16	1630–325	7
OPN–05	ACTGAACGCC	60	14	1585–140	5
OPN–06	GAGACGCACA	60	11	1570–375	8
OPN–10	ACAACTGGGG	60	10	1165–235	12
OPN–20	GGTGCTCCGT	70	9	1760–280	6
BG–65	CTCGAGCGGC	80	8	1345–245	4
BG–66	CGACGCTGCG	80	10	1070–185	3

hampered by difficulties in differential characterization of causative strains. The ability to distinguish strains of *M. bovis* would be useful for investigating the source of infection, the relatedness of strains recovered from different animals, and the identities of multiple strains recovered from the animals from similar localities. Infections caused by *M. bovis* are known to be transmitted from human to human (Blazquez *et al.* 1997), animal to human (Cosivi *et al.* 1998), and animal to animal (Perumaalla *et al.* 1999). In a tuberculosis infection, it is often important to establish source of infection and determine whether the disease is due to a new strain or relapse of a single strain that is disseminating in a particular population. In India, the status of *M. bovis* infection in animals as well as in humans is poorly understood.

In the present study, RAPD profoundly showed similarity and dissimilarity among *M. bovis* strains (Figs 1, 2). All primers amplified scorable fragments in each strain analyzed. The common or monomorphic bands among the different strains probably represent the highly conserved regions in the genome. Clusters of *M. bovis* consisting of the largest number strains showed a possible close genetic relationship though these were isolated at different occasions from different organs of different animals. Therefore, the outcome of our results is consolidated in a comprehensive manner to draw a phylogenetic relationship, which conforms, to findings of earlier workers (Linton *et al.* 1994, Singh *et al.* 2002, Verma *et al.* 2002). It was interesting to note that, in a previous study of RFLP using IS 6110 and IS 1081 of these *M. bovis* strains showed no polymorphism (Singh *et al.* 2004) which suggested that RFLP could not discriminate within *M.bovis* strains. However, in this RAPD analysis, we found discrimination within *M.bovis* strains. Standardization of PCR mixtures and conditions are very important for reproducibility of RAPD-PCR results. We found that it was necessary to perform RAPD-PCR in duplicate for true analysis of results. Our results indicated that RAPD-PCR yielded good typing ability and reproducibility under precise conditions of assay. RAPD analysis may help to establish the molecular relatedness of *M. bovis* strains, their host range and zoonotic importance in an agrarian country like India, where there is a close association between livestock and human beings.

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