J. Vet. Sci. (2004), **5**(4), 331–335

JOURNAL OF
Veterinary
Science

Molecular fingerprinting of clinical isolates of *Mycobacterium bovis* and *Mycobacterium tuberculosis* from India by restriction fragment length polymorphism (RFLP)

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Forty mycobacterial strains comprising clinical Indian isolates of *Mycobacterium tuberculosis* (28 field isolates + 1H37 Rv) and *Mycobacterium bovis* (10 field isolates + 1 AN5) were subjected to restriction fragment length polymorphism analysis (RFLP) using IS6110 and IS1081 probes. Most of these strains originated from dairy cattle herd and human patients from Indian Veterinary research Institute (IVRI) campus isolated from the period of 1986 to 2000. Our study showed presence of 8 copies of IS6110 in most of the *M.tuberculosis* (96.6%) strains irrespective of their origin with the exception of one *M.tuberculosis* strain with presence of an extra copy (3.4%). All *M.bovis* strains showed a single copy of IS6110 on the characteristic 1.9kb restriction fragment. RFLP analysis with IS1081 invariably showed the presence of 5 copies in all isolates of *M.bovis* and *M.tuberculosis* at the same chromosomal location. Similarity of IS6110 RFLP fingerprints of *M.tuberculosis* strains from animals and human suggested the possibility of dissemination of single *M.tuberculosis* strain among animals as well as human. It was not possible to discriminate within the isolates of either *M.tuberculosis* or *M.bovis*, when IS1081 was used as target sequence. The IS6110 RFLP is a valuable tool for disclosing transmission chain of *M. tuberculosis* and *M. bovis* among humans as well as animals

Key words: *Mycobacterium bovis*, *Mycobacterium tuberculosis*, Restriction fragment length polymorphism

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Introduction

Mycobacterium tuberculosis complex group comprises of *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* [15] and a newly described species *M. canetti* [21]. *M. tuberculosis* is primarily the causative agent of human tuberculosis, but may also infect animals in contact with infected human [9]. *M. bovis* is pathogenic for many animal species, especially bovidae, cervidae and occasionally carnivores. Human infection with *M. bovis* is well described and historically has been a common cause of tuberculosis (TB) transmitted through contaminated dairy products. It is interesting to note that out of total Asian cattle and buffalo populations, only 6% and less than 1%, respectively, are found in countries where bovine TB is notifiable and a test-and-slaughter policy is used; while 94% of the cattle and more than 99% of the buffalo populations in Asia are either only partly controlled for bovine TB or not controlled at all [4]. Thus, 94% of the human population lives in countries where cattle and buffaloes undergo no control or only limited control for bovine TB. In India alone, half a million people die of TB every year i.e. more than 1000 every day and a patient every minute (WHO, 2001). Both *M. bovis* and *M. tuberculosis* have been isolated from human and animals in India [22]. However, the origin and transmission of infection between human and animals has not been investigated. Therefore, in view of global prevalence of tuberculosis and zoonotic importance of *M. bovis* and *M.tuberculosis*, there is an urgent need to evolve techniques that not only identify and characterize tubercle bacilli but also facilitate epidemiological studies in order to back trace a source of infection thereby facilitating formulation of effective control strategies for both bovine as well as human TB. Rarely do antibiotic susceptibility patterns, serotyping [7], biotyping [14] and bacteriophage typing [6] allow strain differentiation. DNA based technology is now available for molecular characterization

of *M. tuberculosis* and *M. bovis.* Restriction fragment length polymorphism (RFLP) analysis based on IS6110 and IS1081 sequences easily and rapidly discriminates mycobacterial strains for epidemiological purposes [12,17,21]. The present study, was carried out to characterize clinical isolates of *M. bovis* and *M. tuberculosis* isolated from animals and human in India by using IS 6110 and IS 1081 sequence polymorphism based RFLP in order to disclose chain of transmission between human and animals in a restricted geographical location.

Materials and Methods

Mycobacterial strains

Details of clinical isolates of *M. bovis* and *M. tuberculosis* used in this study are shown in Table 1. *M. tuberculosis* strains used in the study included 18 strains isolated from human patients with pulmonary TB from the Medical Hospital, IVRI, Izatnagar (U.P.) India, 8 strains from bovines, 1 strain each from guinea pig and swine. *M. bovis* strains included 9 strains from bovines and 1from deer. These mycobacteria were maintained on Lowenstein-Jensen (LJ) medium with glycerol and with sodium pyruvate (0.5%) at the Mycobacteria Laboratory, Indian Veterinary Research, Institute, Izatnagar, India. The purity of cultures was examined by Ziehl-Neelsen staining and conventional biochemical tests (Verma and Srivastava, 2001).

DNA Techniques

Genomic DNA extraction, digestion of DNA and Southern blotting were performed as described previously [18]. The IS 6110 and IS1081 probes were a 245 bp and 236 bp DNA fragment, respectively amplified by PCR [18]. The probes were labeled with digoxigenin 11-dUTP by the random primed DNA labeling technique using DIG DNA Labeling and Detection Kit as recommended by the manufacturer (Boehringer Mannheim, Germany). The presence of the labeled probe was detected using the alkaline phosphatase conjugated anti-DIG DNA antibodies and NBT/BCIP (4-nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl-phosphate) as per the recommendations of supplier (Boehringer Mannheim). Molecular weights of the probed fragments were calculated by running DIG-labeled electrophoresis weight marker VII (SPP1 DNA, cleaved with *Eco*RI) supplied by Boehringer Mannheim.

Table 1. Mycobacterial strains

Sr. No.	Isolate number	Species	Source
1	3/86	*M. bovis*	Bovine lymph node
2	1/87	*M. bovis*	Bovine lung
3	3/87	*M. bovis*	Bovine lung
4	30/88	*M. bovis*	Bovine lymph node
5	57/90	*M. bovis*	Bovine lung and lymph node
6	89/91	*M. bovis*	Buffalo lung
7	83/91	*M. bovis*	Buffalo lung
8	227/95	*M. bovis*	Deer lung
9	259/95	*M. bovis*	Bovine lung
10	391/98	*M. bovis*	Bovine lung
11	1/86	*M. tuberculosIs*	Bovine lymph node
12	13/87	*M. tuberculosIs*	Human sputum
13	5/87	*M. tuberculosIs*	Bovine lung and lymph node
14	10/87	*M. tuberculosIs*	Bovine lung
15	25/88	*M. tuberculosIs*	Bovine lung
16	29/88	*M. tuberculosIs*	Human sputum
17	34/89	*M. tuberculosIs*	Human sputum
18	36/89	*M. tuberculosIs*	Calf lung
19	37/89	*M. tuberculosIs*	Calf lymph node
20	92/91	*M. tuberculosi*	Calf lymph node
21	91/91	*M. tuberculosIs*	Guinea pig lung and spleen
22	82/91	*M. tuberculosis*	Buffalo lung
23	87/91	*M. tuberculosIs*	Human sputum
24	125/92	*M. tuberculosis*	Swine lung
25	128/92	*M. tuberculosis*	Human sputum
26	162/93	*M. tuberculosis*	Human sputum
27	193/94	*M. tuberculosis*	Human sputum
28	203/94	*M. tuberculosis*	Human sputum
29	197/94	*M. tuberculosis*	Human sputum
30	191/94	*M. tuberculosis*	Human sputum
31	175/94	*M. tuberculosis*	Human sputum
32	198/94	*M. tuberculosis*	Human sputum
33	320/96	*M. tuberculosis*	Human sputum
34	321/96	*M. tuberculosis*	Human sputum
35	373/98	*M. tuberculosis*	Human sputum
36	380/98	*M. tuberculosis*	Human sputum
37	178/99	*M. tuberculosis*	Human sputum
38	425/2000	*M. tuberculosis*	Human sputum
39	AN 5	*M. bovis*	Standard strain
40	H_{37} Rv	*M. tuberculosis*	standard strain

*All the strains used in the study were isolated and characterized at Mycobacteria Laboratory, IVRI, Izatnagar (India) and were derived from animals/human from IVRI campus except the isolate no. 34/89 which was isolated from a human case outside the IVRI campus

Results

In this study, genomic DNA from 40 mycobacterial strains were subjected to digestion with *pvu*II enzyme followed by hybridization with labeled IS6110 and IS1081 probes. The results of RFLP fingerprinting of mycobacterial strains with these probes are shown in Table 2 and 3. Out of 28 field *M. tuberculosis* strains, 27 showed 8 copies of IS6110 (Fig. 1) while 1 strain (34/89) was found to contain 9 copies (Fig. 1, lane 3). The predominant IS6110 fingerprint pattern among *M. tuberculosis* strains was pattern A that consisted of 8 *pvu*II fragments. This pattern was found in 27 of 28 strains

Table 2. Distribution of IS 6110 DNA fingerprint types among *M. tuberculosis* and *M.bovis* strains

Species	No. of strains tested	Fingerprint pattern		
		A	B	C
M. tuberculosis	29	28(96.6%)	1(3.4%)	0
M. bovis	11	0	0	11(100%)

Table 3. Distribution of IS 1081 DNA fingerprint types among *M. tuberculosis* and *M. bovis* strains

Species	No. of strains tested	Fingerprint type D
M. tuberculosis	29	29 (100%)
M. bovis	11	11 (100%)

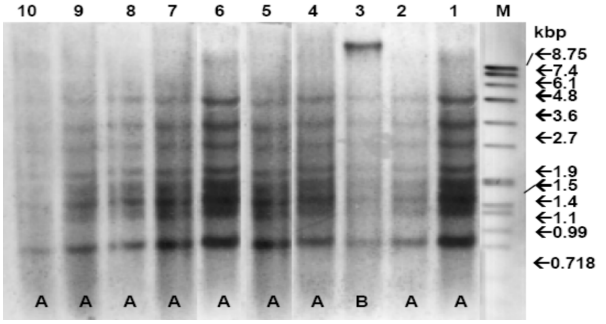

Fig. 1. RFLP analysis of representative *M. tuberculosis* isolates. Southern blots of *Pvu*II-digested chromosomal DNA hybridized with 245 bp DNA of IS 6110 fragment. Lanes: 1 = H37 Rv; 2-10 = Clinical isolates 92/91, 321/96, 34/89,193/94, 10/87, 320/96, 128/92, 203/94 and 25/88 (Table-1). Lane M: Molecular weight of DNA marker in kb pairs (DIG labeled molecular weight marker VII,). The capital letters A & B denote the IS 6110 *Pvu*II fingerprint patterns.

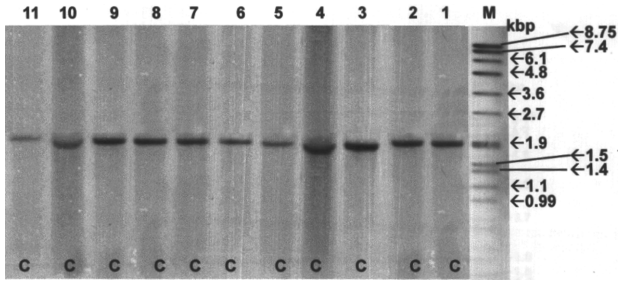

Fig. 2. RFLP analysis of representative *M. bovis* isolates. Southern blots of *Pvu*II-digested chromosomal DNA hybridized with 245 bp DNA of IS 6110 fragment. Lanes: 1 = AN5; 2-11 = Clinical isolates 3/86, 3/87, 83/91, 30/88, 57/90, 227/95, 89/91, 1/87, 259/95 and 391/98 (Table-1). Lane M: Molecular weight of DNA marker in kb pairs (DIG labeled molecular weight marker VII,). The capital letter C denotes the IS 6110 *Pvu*II fingerprint pattern.

tested. The pattern B consisting of 9 *pvu*II fragment was found in only one strain (Table 2). In *M. bovis*, all 10 strains (100%) including reference strain AN_5 showed single copy of IS6110 (Fig. 2). Therefore, the IS6110 fingerprint pattern

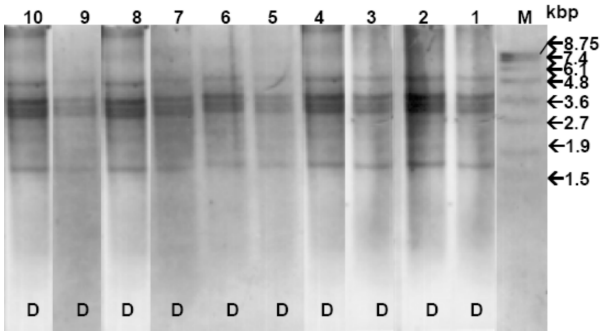

Fig. 3. RFLP analysis of representative *M. tuberculosis* isolates. Southern blots of *Pvu*II-digested chromosomal DNA hybridized with 236 bp DNA of IS 1081 fragment. Lanes: 1 = H37 Rv; 2-10 = Clinical isolates 92/91, 321/96, 34/89,193/94, 10/87, 320/96, 128/92, 203/94 and 25/88 (Table-1). Lane M: Molecular weight of DNA marker in kb pairs (DIG labeled molecular weight marker VII,). The capital letter D denotes the IS 1081 *Pvu*II fingerprint pattern.

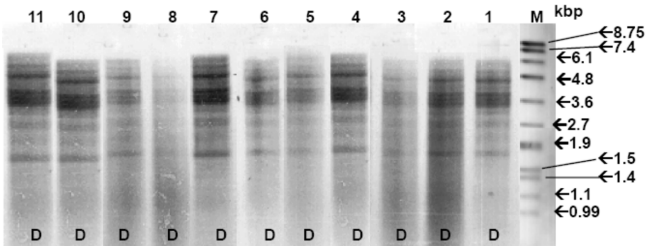

Fig. 4. RFLP analysis of representative *M. bovis* isolates. Southern blots of *Pvu*II-digested chromosomal DNA hybridized with 236 bp DNA of IS 1081 fragment. Lanes: 1 = AN5; 2-11 = Clinical isolates 3/86, 3/87, 83/91, 30/88, 57/90, 227/95, 89/91, 1/87, 259/95 and 391/98 (Table-1). Lane M: Molecular weight of DNA marker in kb pairs (DIG labeled molecular weight marker VII,). The capital letter D denotes the IS 1081 *Pvu*II fingerprint pattern.

among all the *M. bovis* strains was pattern C (Table 2) that consisted of single *pvu*II fragment of 1.9 kb. To increase the accuracy of strain classification, we also used IS1081 fingerprinting for *M. tuberculosis* and *M.bovis.* RFLP with IS1081 probe generated identical IS1081 RFLP types in *M.tuberculosis* and *M. bovis* strains, all of which contained 5 copies of IS1081 on the same chromosomal location (Figs. 3 & 4).

Discussion

Infections caused by *M. tuberculosis* and *M. bovis*, are known to be transmitted from human to human [1], human to animal [9], animal to human [4] and animal to animal [12]. In a tuberculosis outbreak of human or animal, it is often important to establish the 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. Identification and differentiation of strains of *M.*

tuberculosis or *M. bovis* by RFLP provided a better understanding of epidemiology of infection due to these pathogens in developed countries [9,17,8,12]. However, the situation is different in the developing countries like India that harbors more than 30% of the world's cases of human tuberculosis [23] with poorly understood state of *M. bovis* infection in animals as well as human. There are few studies revealing epidemiology of human tuberculosis based on molecular fingerprinting of Indian *M. tuberculosis* strains [5,11,13,16]. However, these studies did not include *M. tuberculosis* strains of animal origin, while the information regarding fingerprinting patterns of *M. bovis* or *M. tuberculosis* of animal origin in India is not available.

In *M. tuberculosis,* copies of IS6110 have been found to vary from 1 to 20 [20]. However, the earlier studies in India, particularly those on *M. tuberculosis* strains from Southern part of the country have been shown to contain either single or no copy of IS6110 [5,13,16]. Interestingly, in the present study we did not find any *M. tuberculosis* strain with single or zero copy of IS 6110, indicating that despite of high frequency of single or zero band isolates reported earlier from India [5,13,16], the discriminatory power of IS6110 based RFLP typing obtained in this study was sufficiently high to use it for clinical and epidemiological purposes. The disparity in the IS 6110 RFLP patterns of *M. tuberculosis* obtained here might be due to the differences in the geographical distribution of *M. tuberculosis* within India since none of the stain used in our study originated from southern part of India. The results of IS6110 RFLP patterns of *M. tuberculosis* obtained in our study therefore indicate that, this approach could be used successfully for discriminating clinical Indian isolates of *M. tuberculosis*.

Our results of RFLP in *M.bovis* differed from earlier reports demonstrating the presence of multiple copies (2 to 13) of IS6110 in isolates from cattle [8], since the RFLP pattern of all the *M. bovis* strains used in the present study was identical with a single copy of IS6110 at unique location of 1.9 kb. Our observations however corroborates with the earlier evidence of presence of single copy IS6110 element at unique chromosomal location of 1.9 kb in *M. bovis* strains [2,19] suggesting its limited discriminatory power.

We found that RFLP fingerprinting with IS1081 probe generated identical fingerprinting patterns among all the strains *M. bovis* as well as *M. tuberculosis* and hence could be of limited use for strain discrimination. The IS1081 fingerprinting could not effectively discriminate *M. tuberculosis* and *M.bovis* strains used in this study. This could be due to the highly stable nature of this insertion sequence that does not allow its easy transposition within genome [19], thereby generating limited polymorphism. As evident from this study and the previous studies which reported either 5 or 6 copies of IS1081, generating limited polymorphism [2,19] we discourage use of IS1081 probe for strain discrimination.

Interestingly, the analysis of geographical distribution of the RFLP patterns revealed that all the 27 strains of *M. tuberculosis* belonging to pattern A originated from the human patients and animals from within the Indian Veterinary Research Institute (IVRI) campus, while one strain belonging to pattern B was isolated from human sputum obtained from out side IVRI campus. 18 out of 28 strains (64.28%) from pattern A were isolated from human patients living or working in IVRI campus. The remaining *M. tuberculosis* strains isolated from bovine [8], guinea pig [1] and swine [1], were also from the animals reared in IVRI Campus. Similarly, all the *M.bovis* strains used in this study were also isolated from IVRI Campus. These animals showed lesions of tuberculosis on autopsy. Since all the strains isolated from the period of 1986 to 2000 originated from limited geographical territory, our findings indicate the possibility of existence of a common focus of infection for animals and human included in this study. The results obtained in this study strongly indicate the possibility of transmission of *M. tuberculosis* between human and bovine herd. We suspect this possibility because infection of animals with *M. tuberculosis* has recently been reported in birds, elephants and other mammals with prolonged contact with humans [9,10]. Further characterization of these clinical isolates using combination of more probes like DR (Direct repeat) and PGRS (Polymorphic GC rich repeat sequence) may be used to generate better discrimination of mycobacterial strains especially *M. bovis* strains in order to analyze the geographical distribution of the RFLP patterns. Further work on large number of *M. tuberculosis* and in particular *M. bovis* strains isolated from different geographical areas of India would be quite useful in disclosing the distribution of various RFLP types and thereby strengthen the understanding of epidemiology of human and bovine TB in India.

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