J. Vet. Sci. (2006), **7**(2), 181–187

JOURNAL OF
Veterinary
Science

Random amplified polymorphic DNA (RAPD) analysis of *Mycobacterium tuberculosis* strains in India

J. P. N. Singh[1], Rishendra Verma[1,*], P. Chaudhuri[2]

[1]*Division of Bacteriology and Mycology, Indian Veterinary Research Institute, Izatnagar-243122, U.P., India*
[2]*Division Veterinary Biotechnology, Indian Veterinary Research Institute, Izatnagar-243122, U.P., India*

The usefulness of random amplification of polymorphic DNA (RAPD) analysis for typing Indian strains of *M. tuberculosis* was investigated. *M. tuberculosis* H37Rv, *M. tuberculosis* DT and 42 clinical isolates of *M. tuberculosis* were subjected to RAPD-PCR using 7 random decamer primers. All 7 primers were found to be differentiated and produced specific RAPD profiles. The polymorphic amplicons served as RAPD markers for *M. tuberculosis*. 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. tuberculosis* isolates, and it was found to be a valuable alternative epidemiological tool. In addition, the results of the present study showed heterogeneity in the *M. tuberculosis* strains in the population studied.

Key words: *Mycobacterium tuberculosis*, RAPD, typing

Introduction

The *Mycobacterium tuberculosis* complex group includes: *M. tuberculosis*, *M. bovis*, *M. africanum*, *M. microti* [19] and a newly described species *M. canetti* [24]. *Mycobacterium tuberculosis* is the primary causative agent of human tuberculosis, but may also infect animals in contact with infected humans [15]. Tuberculosis has re-emerged as one of the leading causes of death worldwide, causing nearly three million deaths annually [2]. In India alone, half a million people die of TB every year, i.e. more than 1000 people every day, and one patient every minute [29]. Both *M. tuberculosis* and *M. bovis* have been isolated from humans and animals in India [26]. However, the origin and transmission of infection between animals and humans have not been investigated. Therefore, in view of the global prevalence of tuberculosis, there is an urgent need to develop techniques that not only identify and characterize tubercular bacilli, but also facilitate epidemiological studies to trace the source of infection thereby facilitating formulation of effective control strategies.

Rarely does the antibiotic susceptibility patterns including: serotyping [10], biotyping and bacteriophage typing [11,18] allow for strain differentiation. DNA based techniques are now available for molecular characterization of *M. tuberculosis.* Restriction fragment length polymorphisms (RFLP) using probes for insertion sequences IS986, IS1081 and IS6110 that have been extensively used to differentiate strains of *M.tuberculosis* [12,21]. However, the relatively complex nature of the standard methods, as well as the lack of utility of some of the probes (such as IS6110) for some of the Indian strains indicates the need for alternate rapid procedures. Random amplification of polymorphic DNA (RAPD) is a multiplex PCR-based molecular system [27,28]. This method uses short oligonucleotide primers of an arbitrary sequence, and low-stringency PCR, to amplify discrete DNA fragments that can be used as molecular markers. RAPD analysis is rapid, inexpensive, easy to perform and can be used for determination of genetic heterogeneity based on DNA sequence diversity [3,4,27,28].

This method, which requires no previous genetic knowledge of the target organism, relies on the presence of low-stringency priming sites, for a single arbitrary primer on both strands of the DNA molecule, close enough to permit PCR amplification. This DNA fingerprinting has been successfully used to type *M. tuberculosis* [8,13,17,22] and other bacteria including: *E. coli* [5], *P. multocida* [7] and *Staphylococcus aureus* [9]. The present study reports on the use of RAPD analysis of *M. tuberculosis* strains to identify the heterogeneity in these strains.

Materials and Methods

Mycobacterial strains

Details of the *M. tuberculosis* strains used in the present study are given in the Tables 1. *M. tuberculosis* strains used included 40 strain isolated from human patients with

*Corresponding author
Tel: +91-581-2301757; Fax: +91-581-2301757
E-mail: rishendra_verma@yahoo.com

Table 1. The sources, origin and RAPD profiles of *Mycobacterium tuberculosis* strains

Sample No.	Isolate No.	Source	RAPD profiles						
			Primer OPN-01	Primer OPN- 02	Primer OPN-05	Primer OPN-09	Primer OPN-20	Primer BG-65	Primer BG-66
1	162/93	Human sputum	A1	B1	C1	D1	E1	F1	G1
2	186/96	Human sputum	A2	B1	C1	D1	E1	F1	G1
3	12/87	Human sputum	A2	B1	C1	D1	E1	F1	G1
4	321/96	Human sputum	A2	B1	C1	D1	E1	F1	G1
5	380/98	Human sputum	A3	B2	C1	D1	E1	F1	G1
6	191/94	Human sputum	A4	B1	C1	D1	E1	F1	G1
7	199/94	Human sputum	A1	B1	C1	D1	E1	F1	G1
8	197/94	Human sputum	A1	B1	C1	D1	E1	F1	G1
9	193/94	Human sputum	A1	B1	C1	D1	E1	F1	G1
10	425/2	Human sputum	A1	B1	C1	D1	E1	F1	G1
11	H37Rv	Reference strain	A3	B2	C1	D1	E1	F1	G1
12	MT-DT	Reference strain	A5	B2	C2	D1	E1	F1	G1
13	11/S	Human sputum	A6	B2	C3	D2	E2	F2	G1
14	3/S	Human sputum	A7	B2	C3	D3	E1	F1	G1
15	6/S	Human sputum	A8	B2	C1	D1	E1	F3	G1
16	5/S	Human sputum	A9	B2	C2	D3	E1	F1	G1
17	9/S	Human sputum	A9	B2	C2	D3	E1	F1	G1
18	SpS10	Human sputum	A4	B2	C1	D2	E3	F4	G2
19	SpS11	Human sputum	A4	B2	C1	D1	E4	F1	G2
20	SpS16	Human sputum	A4	B2	C1	D1	E1	F4	G1
21	SpS22	Human sputum	A4	B3	C1	D1	E1	F4	G1
22	SpS8	Human sputum	A4	B2	C1	D1	E1	F4	G1
23	SpS19	Human sputum	A10	B4	C4	D4	E5	F5	G3
24	SpS17	Human sputum	A1	B2	C1	D1	E1	F1	G1
25	SpS14	Human sputum	A1	B2	C1	D1	E1	F4	G1
26	SpS13	Human sputum	A2	B5	C5	D5	E6	F4	G4
27	SpS4	Human sputum	A3	B5	C5	D5	E6	F4	G2
28	SpS6	Human sputum	A3	B2	C1	D1	E1	F1	G1
29	45/90	Human sputum	A4	B1	C3	D1	E1	F1	G1
30	2/S	Human sputum	A4	B1	C3	D1	E1	F1	G1
31	4/S	Human sputum	A2	B2	C1	D1	E1	F1	G1
32	8/S	Human sputum	A4	B1	C3	D1	E1	F1	G1
33	7/S	Human sputum	A4	B1	C1	D1	E1	F1	G1
34	10/S	Human sputum	A3	B2	C1	D1	E1	F1	G1
35	12/S	Human sputum	A4	B1	C1	D1	E1	F1	G1
36	13/S	Human sputum	A4	B1	C3	D1	E1	F1	G1
37	14/S	Human sputum	A4	B2	C1	D1	E1	F1	G1
38	15/S	Human sputum	A4	B2	C1	D1	E1	F1	G1
39	1/86	Bovine LN	A4	B2	C1	D1	E1	F1	G1
40	203/94	Human sputum	A4	B2	C1	D1	E1	F1	G1
41	125/92	Swine lung	A1	B2	C1	D1	E1	F1	G1
42	320/96	Human sputum	A1	B2	C1	D1	E1	F1	G1
43	439/1	Human sputum	A1	B2	C3	D1	E1	F1	G1
44	128/92	Human sputum	A4	B2	C1	D1	E1	F1	G1

pulmonary tuberculosis from the Medical Hospital, IVRI, Izatnagar (U.P.), India and from the District Tuberculosis Hospital, Bareilly, India, 2 reference strains (H37Rv and MT-DT) and 1 strain each from bovine and swine samples.

Table 2. Analysis of RAPD patterns of *M. tuberculosis* isolates with 7 different primers

Sample No.	Primer	Sequence	G+C content (%)	Band size Approx. range (bp)	No. of RAPD patterns
1	OPN-01	CTCACGTTGG	60	1795 (275)	10
2	OPN-02	ACCAGGGGCA	70	1925 (400)	5
3	OPN-05	ACTGAACGCC	60	1940 (330)	5
4	OPN-09	TGCCGGCTTG	70	1895 (400)	5
5	OPN-20	GGTGCTCCGT	70	1605 (275)	6
6	BG-65	CTCGAGCGGC	80	1865 (265)	5
7	BG-66	CGACGCTGCG	80	1375 (280)	4

All of the mycobacterium strains were typed by conventional morphological (Ziehl-Neelsen staining) and biochemical tests [26] and maintained on Lowenstein-Jensen medium at the Mycobacteria Laboratory, Indian Veterinary Research Institute, Izatnagar, India.

RAPD analysis

A number of primers were used for RAPD analysis of mycobacterium isolates. Details of the primers used in the present study are given in the Table 2. All of the primers from the OPN-series were obtained from Operon Technologies (USA); the primers for the BG-series were synthesized by M/s Bangalore Genei Pvt. Ltd. India. Genomic DNA was extracted as per the method of van Soolingen *et al.* [23]. Amplification of mycobacterium DNA, using random primers, was performed in a total volume of 25 µl. The reaction mixture contained 1 unit of Taq DNA polymerase (Bioloine GmBH, Germany), 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 (Eppendorf, Germany). The cycling conditions consisted of an initial denaturation step for 5 min at 94°C, followed by 45 cycles of 94°C for 1 min denaturation 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 a 1.5% agarose gel stained with ethidium bromide. Subsequently, the gel was visualized and photographed using a gel documentation and analysis system (AlfaImager, Germany). The banding patterns obtained by RAPD were noted on a photograph. A data matrix composed of the numerals 1 and 0 was built on the basis of presence (1) or absence (0) of a DNA band appearing in replicates for each isolate. Only distinct and prominent bands were scored and used in assessing RAPD patterns. The molecular size of bands was calculated using software provided by AlfaImager (Germany). The size of the bands, that differed by ±5% on different gels, was considered to be the same bands. The genetic diversity of isolates was analyzed by RAPDistance version 1.04 software that operates on the basis of UPGMA clustering.

Results

RAPD-PCR revealed the presence of amplicons of a variety of sizes in *M. tuberculosis* strains. In this study, several fragments were amplified in each sample, and most of these fragments were observed to be common to different strains. However, there were some fragments unique to certain strains. All 44 isolates of *M. tuberculosis* showed a high degree of polymorphism with RAPD analysis (Fig. 1A, B, C). The number of RAPD patterns generated by each primer is shown in Table 1. All 7 primers revealed discriminating patterns. The primer OPN-01 and the primer BG-66 generated the maximum and minimum number of amplimers, respectively. Among the seven primers, OPN-01 showed maximum discrimination for the ability to type mycobacterium isolates, and produced 10 RAPD patterns (Table 2).

Upon dendrogram analysis, with primer OPN-02, four clusters were formed, the largest cluster consisted of 17 strains, the second largest cluster contained 10 strains and the remaining two smaller clusters contained 5 strains in each; genetic relatedness was closest among strains within clusters (Fig. 2). Five strains were identified in different clusters along with *M. tuberculosis* strain DT, while the *M. tuberculosis* strain H37Rv was found in one of the smaller clusters. Two strains, 5/S and 9/S, were in the same cluster but remained separated from the rest of the strains and clusters (Fig. 2). Strain SpS19 demonstrated a unique pattern with all of the primers used (Table 1). Bovine strain 1/86 and swine strain 125/92 were identified within the largest and second largest clusters respectively (Fig. 2).

Discussion

Detailed epidemiological studies of *M. tuberculosis* have been hampered by difficulties in differential characterization of causative strains. The ability to distinguish strains of *M. tuberculosis* would be useful for investigating the source of outbreaks of infection, the relatedness of strains recovered from different patient, and the identities of multiple strains

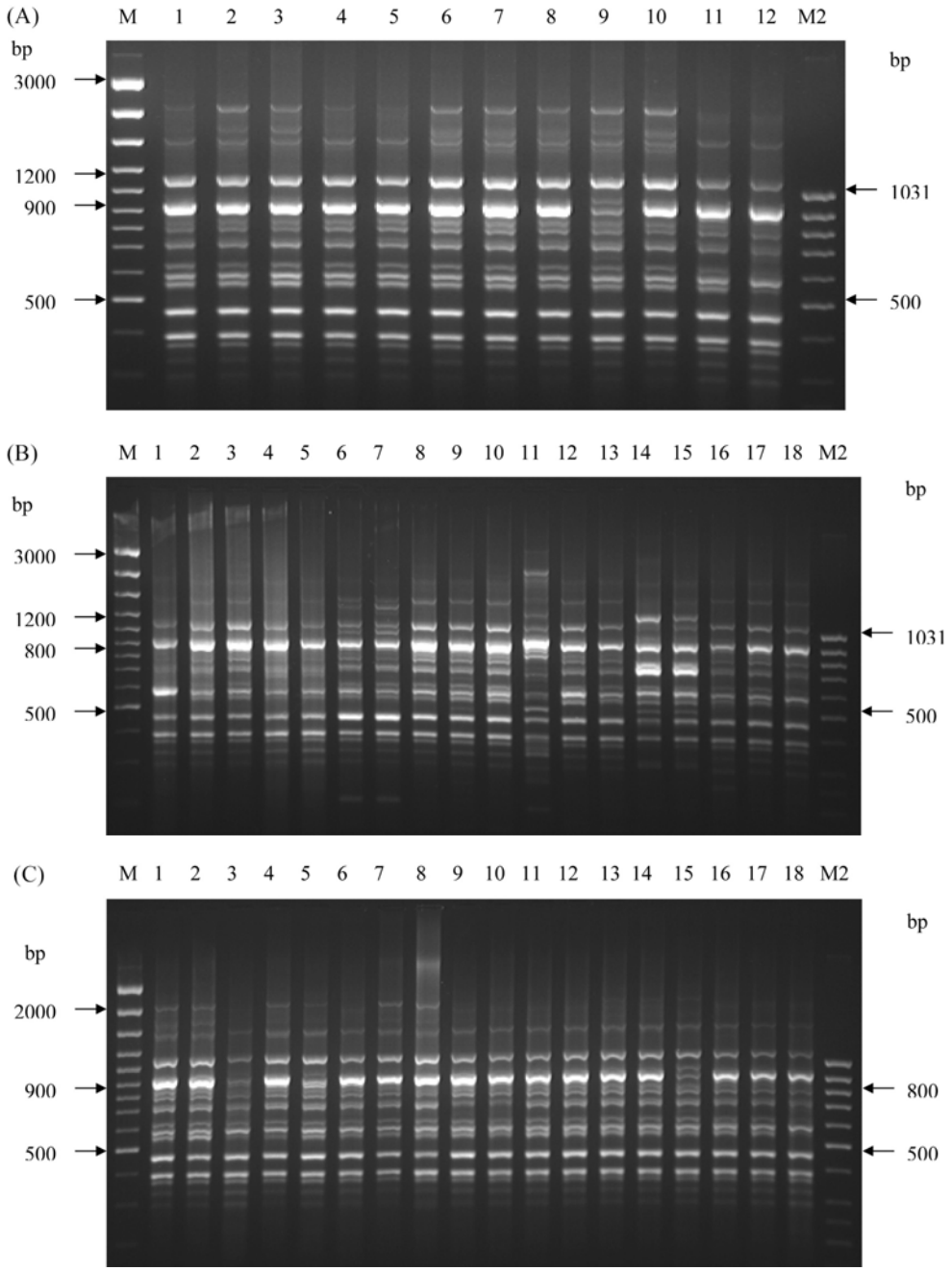

Fig. 1. RAPD profiles of *Mycobacterium tuberculosis* strains with primer OPN-02. (A) Lane M-100 bp DNA marker ladder; 1-(162/93); 2-(186/96); 3-(12/87); 4-(321/96); 5-(380/98); 6-(191/94); 7-(199/94); 8-(197/94); 9-(193/94); 10-(425/2); 11-H37Rv; 12-DT; Lane M2-50 bp DNA marker ladder. (B) Lane M-100 bp DNA marker ladder; 1-(11/S); 2-(3/S); 3-(6/S); 4-(5/S); 5-(9/S); 6-(SpS10); 7-(SpS11); 8-(SpS16); 9-(SpS22); 10-(SpS8); 11-(SpS19); 12-(SpS17); 13-(SpS14); 14-(SpS13); 15-(SpS4); 16-(SpS6); 17- H37Rv; 18-DT; Lane M2-50 bp DNA marker ladder. (C) Lane M-100 bp DNA marker ladder; 1-(45/90); 2-(2/S); 3-(4/S); 4-(8/S); 5-(7/S); 6-(10/S); 7-(12/S); 8-(13/S); 9-(14/S); 10-(15/S); 11- (1/86); 12-(203/94); 13-(125/92); 14-(320/96); 15-(439/1); 16-(128/92); 17- H37Rv; 18-DT; Lane M2-50 bp DNA marker ladder.

recovered from the patients from similar localities. Infections caused by mycobacterium are known to be transmitted from human to human [1], animal to human [6], and animal to animal. [16] In an outbreak investigation of tuberculosis, it is often important to know whether the disease is due to a new strain or relapse of a known strain. This information has a special bearing on our understanding of the emergence of multi-drug resistant disease. In India, the status of *M. tuberculosis* infection in animals is poorly understood.

In the present study, RAPD showed both similarities and

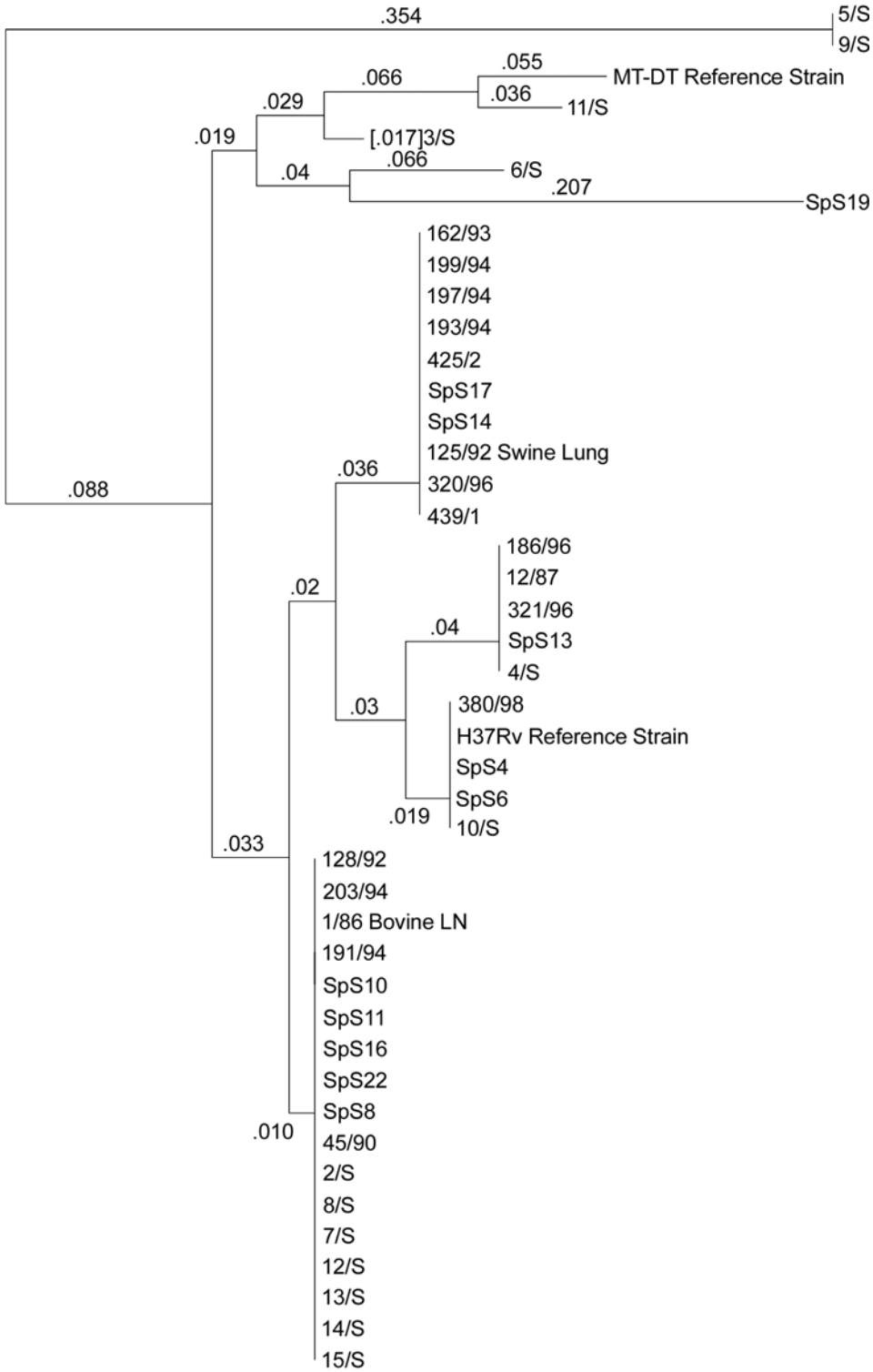

Fig. 2. Dendrogram showing genetic relatedness among *Mycobacterium tuberculosis* strains with primer OPN-02.

differences among *M. tuberculosis* strains. All primers amplified scorable fragments in each strain analyzed. The common or monomorphic bands among the different strains likely represent highly conserved regions in the genome. Clusters of *M. tuberculosis,* consisting of the largest number strains, showed a possible close genetic relationship; these were isolated at different occasions from human sputum and from two animals, one from a bovine lymph node and the

other from a swine lung. Therefore, the outcome of our analysis is consolidated, in a comprehensive manner, to draw a phylogenetic relationship, which is consistent with prior reports [13,20,25].

It was interesting to note that a previous study of RFLP using IS 6110 and IS 1081, of these *M. tuberculosis* strains (13 overlapping *M. tuberculosis* stains, including H37Rv), showed no polymorphism; [21] this suggested that this RFLP could not differentiate these *M. tuberculosis* strains. However, in this study using the RAPD analysis, we found differentiation among the *M. tuberculosis* 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 to obtain valid results. Our findings show that RAPD-PCR yields reliable and reproducible results under precise assay conditions.

Isolation of *M. tuberculosis* from animals is not common. *M. tuberculosis* strains from a bovine lymph node and a swine lung were similar to the *M. tuberculosis* strain from human sputum; this suggests a possible transmission of infection from humans to animals. RAPD analysis may help to establish the molecular relatedness of *M. tuberculosis* strains, their distribution and zoonotic importance in an agrarian country like India, where there is a close association between livestock and human beings.

Acknowledgments

The authors are grateful to Director, Indian Veterinary Research Institute, Izatnagar, India, for providing necessary facilities.

References

1. **Blazquez J, Espinosa de Los Monteros LE, Samper S, Martin C, Guerrero A, Cobo J, van Embden J, Baquero F, Gomez-Mampaso E**. Genetic characterization of multidrug-resistant *Mycobacterium bovis* strains from a hospital outbreak involving human immunodeficiency virus-positive patients. J Clin Microbiol 1997, **35**, 1390-1393.
2. **Bloom BR, Murray CJ.** Tuberculosis: Commentary on a re-emergent killer. Science 1992, **257**, 1055-1064.
3. **Bowditch BM, Albright DG, Williams JG, Braun MJ.** Use of randomly amplified polymorphic DNA markers in comparative genome studies. Methods Enzymol 1993, **224**, 294-309.
4. **Caetano-Anolles G.** Amplifying DNA with arbitrary oligonucleotide primers. PCR Methods Appl 1991, **3**, 85-94.
5. **Chansiripornchai N, Ramasoota P, Sasipreeyajan J, Svenson S B**. Differentiation of avian pathogenic *E. coli* (APEC) strains by random amplified polymorphic DNA (RAPD) analysis. Vet Microbiol 2001, **80**, 75-83.
6. **Cosivi O, Grange JM, Daborn CJ, Raviglione MC, Fujikura T, Cousins D, Robinson RA, Huchzermeyer HF, de Kantor I, Meslin FX**. Zoonotic tuberculosis due to *Mycobacterium bovis* in developing countries. Emerg Infect Dis 1998, **4**, 59-70.
7. **Dziva F, Christensen H, Olsen JE, Mohan K.** Random amplification of polymorphic DNA and phenotypic typing of Zimbabwean isolates of *Pasteurella multocida*. Vet Microbiol 2001, **82**, 361-372.
8. **Harn HJ, Shen KL, Ho LI, Yu KW, Liu GC, Yueh KC, Lee JH.** Evidence of transmission of *Mycobacterium tuberculosis* by random amplified polymorphic DNA (RAPD) fingerprinting in Taipei City, Taiwan. J Clin Pathol 1997, **50,** 505–508.
9. **Hermans K, De Herdt P, Baele M, Devriese LA, Haesebrouck F**. Sequence analysis or a RAPD band differentiating high and low virulence *Staphylococcus aureus* strains from rabbits. Vet Microbiol 2001, **82,** 61-67.
10. **Jones WD Jr, Kubica GP**. Fluorescent antibody technique with mycobacteria. III. Investigation of five serologically homogenous groups of mycobacteria. Zentralbl Bakteriol Orig 1968, **207**, 58-62.
11. **Jones WD Jr.** Geographic distribution of phage types among cultures of *Mycobacterium tuberculosis.* Am Rev Respir Dis 1990, **142**, 1000-1003.
12. **Katoch VM, Singh D, Chauhan DS, Sharma VD, Singh HB, Das R, Srivastava K.** Newer DNA fingerprinting techniques for tuberculosis-relevance in control. In: Mahajan RC, Therwath A (eds.). Multi-drug resistance in emerging and re-emerging diseases. pp. 87-96, Indian Science Academy & Narosa Publishing House, New Delhi, 2000.
13. **Linton CJ, Jalal H, Leeming JP, Millar MR.** Rapid discrimination of *Mycobacterium tuberculosis* strains by random amplified polymorphic DNA analysis. J Clin Microbiol 1994, **32,** 2169-2174.
14. **Menard C, Brousseau R, Mouton C.** Application of polymerase chain reaction with arbitrary primer (AP-PCR) to strain identification of *Porphyromonas (Bacteroides) gingivalis.* FEMS Microbiol Lett 1992, **74**, 163-168.
15. **Michalak K, Austin C, Diesel S, Bacon JM, Zimmerman P, Maslows JN.** *Mycobacterium tuberculosis* infection as a zoonotic disease; transmission between humans and elephants. Emerg Infect Dis 1998, **4**, 283-287.
16. **Perumaalla VS, Adams LG, Payeur J, Baca D, Ficht TA.** Molecular fingerprinting confirms extensive cow-to-cow intra-herd transmission of a single *Mycobacterium bovis* strain. Vet Microbiol 1999, **70**, 269-276.
17. **Richner SM, Meiring J, Kirby R.** A study of the genetic diversity of *Mycobacterium tuberculosis* isolated from patients in the eastern province of South Africa using random amplified polymorphic DNA profiling. Electrophoresis 1997, **18,** 1570–1576.
18. **Snider DE Jr, Jones WD, Good RC.** The usefulness of phage typing *Mycobacterium tuberculosis* isolates. Am Rev Respir Dis 1984, **130**, 1095-1099.
19. **Rynyon EH, Karlson AG, Kubica GP, Wayne LG.** Mycobacterium. In: Lennette EH, Balows A, Hausler Jr WJ, Truant JP (eds.). Manual of Clinical Microbiology. 3rd ed. pp. 150-179, American Society for Microbiology, Washington DC, 1980.
20. **Singh HB, Chauhan DS, Singh D, Das R, Srivastava K,**

Yadav VS, Kumar A, Katoch VM, Sharma VD. Rapid discrimination of Indian isolates of *M. tuberculosis* by random amplified polymorphic DNA (RAPD) analysis - A preliminary report. Indian J Med Microbiol 2002, **20**, 69-71.

21. **Singh SK, Verma R, Shah DH**. Molecular fingerprinting of clinical isolates of *Mycobacterium bovis* and *Mycobacterium tuberculosis* from India by restriction fragment length polymorphism (RFSP). J Vet Sci 2004, **5**, 331-335.
22. **Tazi L, El Baghdadi J, Lesjean S, Locht C, Supply P, Tibayrenc M, Banuls AL.** Genetic Diversity and Population Structure of *Mycobacterium tuberculosis* in Casablanca, a Moroccan City with high incidence of tuberculosis. J Clin Microbiol 2004, **42**, 461-466.
23. **van Soolingen D, de Haas PEW, Kremer K.** Restriction Fragment Length Polymorphism (RFLP) typing of mycobacteria. National Institute of Public Health and Enivronment, Bilthoven, Netherlands, 1999.
24. **van Soolingen D, Hoogenboezem T, de Haas PE, Hermans PW, Koedam MA, Teppema KS, Brennan PJ, Besra GS, Portaels F, Top J, Schouls LM, van Embden JD.** A novel pathogenic taxon of the *Mycobacterium tuberculosis* complex, *canetti*: Characterization of an exceptional isolate from Africa. Int J Syst Bacteriol 1997, **47**, 1236-1245.
25. **Verma R, Singh HB, Sharma VD, Katoch VM.** Molecular characterization of Indian *Mycobacterium bovis* isolates by Random Amplified Polymorphic DNA (RAPD) Analysis – A Preliminary Report. Indian J Vet Res 2002, **11**, 39-41.
26. **Verma R, Srivastava SK**. Mycobacterium isolated from man and animals; twelve year record. Indian J Anim Sci 2001, **71**, 129-132.
27. **Welsh J, McClelland M.** Fingerprinting genomes using PCR with arbitrary primers. Nucleic Acids Res 1990, **18**, 7213-7218.
28. **Williams JG, Kubelik AR, Livak KJ, Rafalski JA, Tingey SV.** DNA polymorphism amplified by arbitrary primers are useful as genetic markers. Nucleic Acids Res 1990, **18**, 6531-6535.
29. **World health organization (WHO)**. Global tuberculosis control. WHO report 2001. Geneva: WHO, 2001.