

Strain Differentiation Among Indian Clinical Isolates of *M. tuberculosis* by RFLP Analysis

A.Chander¹, R. Yadava¹, S. Ghosh¹, P.C. Rath¹,
P.K. Yadava¹, I. Orme² and R. K. Saxena^{1*}

¹School of Life Sciences, Jawaharlal Nehru University, New Delhi, India

²Microbiology Department, Colorado State University, Fort Collins, CO, USA

Abstract

A large proportion of the Indian population is asymptomatic carrier of *Mycobacterium tuberculosis* (Mtb). The respite from successful combination therapy against Mtb seems to be only short-lived with the emergence of opportunistic infections in the post-AIDS scenario and also of the drug-resistant strains of the bacterium. It has become necessary to be able to identify the bacterial strains in order to arrive at the right course of treatment. We here illustrate the use of direct repeat (DR) probes in strain differentiation of clinical isolates from northern India.

Introduction

Mycobacterium tuberculosis, the causative agent of tuberculosis, infects half of the world's population and causes one of the most important diseases in the world from the standpoint of human morbidity and mortality. The dramatic re-emergence of tuberculosis world-wide, has rendered the present control and preventive methods inadequate. Mycobacterium has also emerged as a highly prevalent opportunistic pathogen in AIDS patients. Moreover emergence of drug-resistant strains of *M. tuberculosis* has posed additional problem in treatment of tuberculosis. A safe and effective vaccine against *M. tuberculosis* is urgently needed, which in turn depends on understanding the mechanisms of protective immunity against tuberculosis. In our ongoing studies on the role of secretory antigens in inducing a protective immunity in tuberculosis patients of Indian origin, it was considered essential to characterize the clinical isolates of *M. tuberculosis*, and to look for genetic diversity amongst them with reference to the standard strain of *M. tuberculosis* H37Ra and Erdman. Fingerprinting by restriction fragment length polymorphism [RFLP] analysis has been a useful tool for studying the strain differentiation of *M. tuberculosis*. Among the various genetic elements that have been found to contribute to DNA polymorphism in *M. tuberculosis*, the insertion element IS 6110 has been studied extensively [5,12,16,23]. Although most of the *M. tuberculosis* strains carry multiple copies of IS 6110, strains with a single copy or no copy of IS 6110 have been reported in India and in many other countries [4,6,22]. Other polymorphic DNA markers are being explored to differentiate such *M. tuberculosis* strains. In addition to IS 6110, four other targets for RFLP analysis have been used viz.; (i) IS 1081, an

insertion element related to the *Staphylococcus aureus* transposable element IS 254 [3,18]; (ii) a polymorphic GC-rich repetitive sequence (PGRS) present on the recombinant plasmid pTBN12 [13,2]; (iii) the major polymorphic tandem repeat (MPTR) sequence [8] and (iv) the 36-bp direct-repeat (DR) sequence [9]. In the present study, we have used the 36-bp DR sequence from *M. bovis* BCG, of which multiple copies are present in the hot-spots for IS 6110 integration in *M. bovis* genome, to overcome the limitations of IS 6110, for differentiating Indian strains of *M. tuberculosis*. We have used this probe to differentiate isolates obtained from pulmonary tuberculosis patients from Northern India. Our results indicate that all seventeen clinical isolates examined by us had distinct RFLP patterns using DR probe. Many strains also showed an expansion of DR sequence in their genomes.

Results and Discussion

Mycobacterial Isolates: Seventeen isolates were obtained from sputum samples of tuberculosis patients at New Delhi tuberculosis center, after initial diagnosis of pulmonary tuberculosis. The isolates were identified as *M. tuberculosis* on the basis of standard biochemical tests [21] as listed in Table 1. Cultures were maintained on Lowenstein-Jensen (L-J) medium. *M. tuberculosis* H37Ra and Erdman strains were used as standards for comparison of RFLP patterns of clinical isolates. For routine propagation, mycobacterial cells were grown in Sauton's medium. One loopful of mycobacterial culture was picked up from L- J slants and inoculated into 100 ml of Sauton's medium and allowed to grow in an incubator shaker (100 rpm) at 37°C. Growth of cells appeared generally in 2-3 weeks (A580 = 0.1 to 0.2). **DR-RFLP:** Genomic DNA samples were prepared as described earlier [1]. The DNA extracted from the isolates was subjected to restriction digestion overnight at 37°C with Alu I (New England Biolabs, USA). The fragments were separated electrophoretically in 1% (w/v) agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) with voltage gradient of 10 V / cm. The DNA fragments were transferred on to Pall Biodyne nylon membranes (Gibco-BRL, NY, USA) and cross-linked by exposing to UV light in a Stratalinker (Stratagene, USA) as recommended by the manufacturers. The membranes were pre - hybridized in the buffer containing 6X SSC (1X SSC consists of 0.15 M NaCl and 0.015 M Sodium citrate, pH 7.0), 5X Denhardt solution (1X Denhardt is 0.02 % Ficoll, 0.02 % polyvinyl pyrrolidone, 0.02 % bovine serum albumin), 0.5 % SDS and 100 mg/ml sonicated denatured Salmon sperm DNA at 40°C for 2 h. The synthetic 36-mer DR sequence (5'GTTTCCGTCCTCCCTCTCGGGGTTTTGGGTCTGACGAC3') of *M. bovis* BCG [8] was end-labeled with γ 32P-ATP (8X10⁶ cpm/pmole), by incubating with T4 polynucleotide kinase (NEB, USA) at 37°C for 1 hr [11]. Labeled probe was added to the pre-hybridized membrane for locating the fragments containing complementary sequence. Hybridization was carried out at 40°C for 16 h in the same buffer as was

Table 1. Properties of clinical isolates used in the present investigation

ISOLATE NO	NO OF BANDS IN THE RFLP	NIACIN TEST	NITRATE TEST	PNB TEST	DRUG RESISTANCE
JNU53	5	+	+	-	NOT KNOWN
JNU7	6	+	+	-	NOT KNOWN
JNU13	5	+	+	-	INH,RIF
JNU14	3	+	+	-	INH,RIF
JNU4	6	+	+	-	NOT KNOWN
H37Ra	5	+	+	-	NOT KNOWN
ERDMAN	4	+	+	-	NOT KNOWN
JNU1	4	+	+	-	NOT KNOWN
JNU2	5	+	+	-	NOT KNOWN
JNU51	7	+	+	-	NOT KNOWN
JNU11	5	+	+	-	NOT KNOWN
JNU52	4	+	+	-	NOT KNOWN
JNU10	7	+	+	-	NOT KNOWN
JNU19*	0	+	-	+	INH,RIF,TBI
JNU18*	1	-	-	+	INH,RIF,TBI,KAN
JNU20	5	+	+	-	INH,RIF
JNU22	4	+	+	-	Sensitive to all
<i>M.bovis</i>	3	+	+	-	NOT KNOWN
JNU31	5	+	+	-	INH,RIF
JNU32	5	+	+	-	INH,RIF

*=Atypical Mycobacteria ;INH=Isoniazid,RIF=Rifampicin,KAN=Kanamycin,TBI=Thiacetazone

used for pre-hybridization. Excess probe was washed with 4XSSC containing 0.2% SDS for 10 min at room temperature and then with 2X SSC at room temperature. After the final wash the membrane was blotted dry on tissue paper and autoradiographed, on Hyper filmTM MP (Amersham, UK).

To investigate systematically, the genetic diversity of *M. tuberculosis* isolates from pulmonary tuberculosis patients, DNA preparations from 17 clinical isolates from northern India were subjected to RFLP analysis by using Alu-1 and the 36-mer long DR sequence probe. RFLP patterns of 17 isolates along with *M. bovis* BCG are shown in Figure 1. These results indicated that all of these isolates were distinguishable based on polymorphism in terms of both the copy number and the size of Alu I fragments containing DR sequence.

It is remarkable that DNA from most of the strains showed 3 to 7 polymorphic bands. Only two strains showed one or no band and these two strains were found to be

belonging to mycobacteria other than tuberculosis (MOTT) group. A high degree of Alu I RFLP was thus observed among all the strains under study and no two strains were found to be identical. Several genetic repetitive DNA markers have been used to study chromosomal DNA polymorphism in *M. tuberculosis*, IS 6110 and PGRS have been found to be most commonly used RFLP markers amongst them. IS6110 was however found to be of limited value for differentiating strains of Indian origin [4]. IS 1081 element was also found to be less informative for strain differentiation because most of the five to seven copies of IS 1081 are present at identical positions in the *M. tuberculosis* genome [18]. The DR sequence has been found to be highly polymorphic, generating distinct signatures of *M. tuberculosis* strains [4,6,22].

Recently, a number of RFLP studies have evaluated the utility of DR and IS 6110 sequences. A molecular fingerprinting study of clinical isolates of *M. tuberculosis* on a Caribbean Island with IS 6110 and DR probes

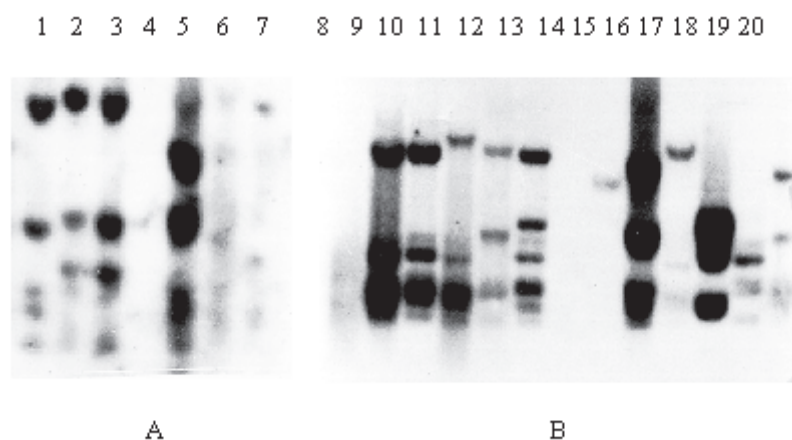


Figure 1. RFLP patterns for various clinical isolates and reference strains generated by hybridization of *AluI* fragments of genomic DNA with DR probes. Panel A and B represent two independently probed gels. Lanes 1-5 correspond to JNU53, 7, 13, and 4. Lanes 6 and 7 represent reference strains H37Ra and Erdmam while lanes 8-20 represent JNU strain numbers 1, 2, 51, 11, 52, 10, 19, 18, 20, 22, 31, and 32 respectively. For further description of these strains please see Table 1.

produced similar patterns of bands [15]. In an earlier study, *M. tuberculosis* strains from the People's Republic of China and Mongolia formed a remarkably homogeneous family of strains, evolved from recent clonal expansion based on IS 6110 and other genetic markers (DR and PGRS) [20]. In another study done in French Polynesia [17], strains exhibiting similar IS 6110 RFLP types also exhibited identical DR RFLP patterns, suggesting linkage between IS 6110 and DR sequences. However, 16 strains originating from six different countries differed in their PGRS and DR restriction fragment patterns, although a majority of these strains contain a single copy of IS 6110 on a 1.5 Kb Pvu II restriction fragment [19]. It is interesting that the DR-containing Alu I fragment patterns among the Indian strains appeared to be less polymorphic compared with those among the strains from other countries. This would suggest that the Indian isolates belong to an evolutionarily related group of strains which is distinct from strains isolated on other continents [19]. A very important factor common to all countries in Southeast Asia is BCG vaccination, which has been used for last two to six decades. Isolates of *M. tuberculosis* from Ethiopia displayed greater DNA polymorphism than those from Tunisia [10], although the incidence of tuberculosis in Ethiopia is about five times that in Tunisia. Since BCG vaccination is not a common practice in Ethiopia, it has been suggested that BCG vaccination in Tunisia may have favoured the selection of *M. tuberculosis* strains that resist BCG-induced immunity [10]. In this light, it will be interesting to characterize isolates from asymptomatic carriers to verify if the Mycobacteria surviving in these individuals are compromised in terms of their virulence and whether their RFLP patterns are convergent. The asymptomatic carrier state has mainly been attributed to the immunological competence of these individuals. Scarce information is available about the heterogeneity within clinical isolates of *M. tuberculosis* on Indian sub-continent. The present study suggests for high genetic heterogeneity of Indian clinical isolates of *M. tuberculosis*, which may at least partially be a consequence of BCG vaccination. Das et al. [4] and Sahadevan et al. [14] at the Tuberculosis Research Center in Madras, using *M. tuberculosis* isolates from South-India, have reported 30 different patterns from 96 individual isolates of *M. tuberculosis* by DR RFLP analysis. The present study indicates that the RFLP patterns of all the 17 isolates from North-India, were distinguishable from each other. These may represent interesting geographical variations within *M. tuberculosis* strains prevalent in India.

Acknowledgements

This work was supported by an Indo-US Vaccine Action Program grant.

References

- Bose, M., A. Chander, and R.H. Das. 1993. A rapid and gentle method for the isolation of genomic DNA from mycobacteria. *Nucl. Acids. Res.* 21: 2529-2530.
- Chaves, F., Z. Yang, H. El Hajj, M. Alonso, W.J. Burman, K. D. Eisenach, F. Drona, J. H. Bates, and M.D. Cave. 1996. Usefulness of the secondary probe pTBN 12 in DNA fingerprinting of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 34: 1118-1123
- Collins, D.M., and D.M. Stephans. 1991. Identification of insertion sequence, IS 1081, in *Mycobacterium bovis*. *FEMS Lett.* 83: 11-16.
- Das, S., C.N. Paramasivam, D.B. Lowrie, R. Prabhakar, and P.R. Narayanan. 1995. IS 6110 RFLP typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, South India. *Tubercl. Lung. Dis.* 76: 550-554.
- Eisenach, K.D., J.T. Crawford and J. H. Bates. 1988. Repetitive DNA sequences as probes for *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 26: 40-45.
- Fomukong, S.G., T.H. Tang., S.Al-Maamary., W.A. Ibrahim., S.Ramayah., M.Yates., Z.F. Zainuddin and J.W.Dale. 1994. Insertion sequence typing of *Mycobacterium tuberculosis*; Characterisation of a wide spread sub type with a single copy of IS 6110. *Tubercl. Lung. Dis.* 75: 399-408.
- Groenen, P.M.A., A.E. Bunschoten., D.van Soolingen and J.D.A. van Embden. 1993. Nature of DNA polymorphism in the direct-repeat cluster of *Mycobacterium tuberculosis*: application for strain differentiation by a novel typing method. *Mol. Microbiol.* 10: 1057-1065.
- Hermans, P.W.M., D.van Soolingen and J.D.A. van Embden. 1992. Characterisation of a major polymorphic tandem repeat in *Mycobacterium tuberculosis* and its potential use in the epidemiology of *Mycobacterium kansasii* and *Mycobacterium gordonae*. *J. Bacteriol.* 174: 4157-4165.
- Hermans, P.W.M., D. van Soolingen., E.M. Bik., P.E.W. de Haas., J.W.Dale and J.D.A.van Embden.1991. The insertion element IS 987 from *Mycobacterium bovis* BCG is located in a hot-spot integration region for insertion elements in *Mycobacterium tuberculosis* complex strains. *Infect. Immun.* 59: 2695-2705.
- Hermans, P.W.M., F. Messadi., H. Quebexabher., D. van Soolingen., P.E.W. Haas., H. Heersma., H.de Neeling., A. Ayub., F. Portaels., D. Frommel., M. Zribi and J.D.A. van Embden. 1995. Usefulness of DNA typing for global tuberculosis epidemiology. *J. Infect. Dis.* 171: 1504-1513.
- Maniatis, T., E.F. Fritsch and J.Sambrook. 1989. *Molecular cloning; A Laboratory Manual.* Cold Spring Harbour Laboratory, Cold Spring Harbour, NY.
- McAdam, R.A., P.W.M. Hermans, D. van Soolingen, Z.F. Zainuddin, D. Catty, J.D.A. van Embden and J.W. Dale. 1990. Characterisation of *Mycobacterium tuberculosis* insertion sequence belonging to the IS 3 Family. *Mol. Microbiol.* 4: 1607-1613.
- Ross, C., K. Raios., K.Jackson and B.Dwyer. 1992. Molecular cloning of a highly repeated element from *Mycobacterium tuberculosis* and its use as an epidemiological tool. *J. Clin. Microbiol.* 30: 942-946.
- Sahadevan, R., S.Narayanan., C.N. Paramasivam., R.Prabhakar and P.R. Narayanan. 1995. Restriction fragment length polymorphism typing of clinical isolates of *Mycobacterium tuberculosis* from patients with pulmonary tuberculosis in Madras, India, by use of Direct- Repeat probe. *J.Clin.Microbiol.* 33: 3037-3039.
- Sola, C., L. Horgen., K.S. Goh and N. Rastogi. 1997. Molecular fingerprinting of *Mycobacterium tuberculosis* on a Caribbean Island with IS 6110 and Drs probes. *J. Clin. Microbiol.* 35: 843-846.
- Thierry, D., A. Brisson-Noel, V.Vincent-Levy-Frebault, S.Nguyen, J.Guesdon and B.Gicquel. 1990. Characterisation of a *Mycobacterium tuberculosis* insertion sequence, IS 6110, and its application in diagnosis. *J. Clin. Microbiol.* 28: 2668-2673.
- Torrea, G., G. Levee., P. Grimont., C. Martin., S.Chanteau and B. Gicquel. 1995. Chromosomal DNA fingerprinting analysis using the Insertion Sequence IS 6110 and the repetitive element DR as strain-specific markers for epidemiological study of tuberculosis in French Polynesia. *J. Clin. Microbiol.* 33: 1899-1904
- van Soolingen, D., P.W.M. Hermans, P.E.W. de Haas and J.D.A. van Embden. 1992. Insertion element associated restriction fragment length polymorphism in *Mycobacterium tuberculosis* complex species; a reliable tool for recognising *Mycobacterium bovis* BCG. *J. Clin. Microbiol.* 30: 1772-1777.
- van Soolingen, D., P.E.W. de Haas., P.W.M.Hermans., P.M.A. Groenen and J.D.A. van Embden. 1993. Comparison of various repetitive DNA elements as genetic markers for strain differentiation and epidemiology of *Mycobacterium tuberculosis*. *J. Clin. Microbiol.* 31: 1987-1995.
- van Soolingen, D., L. Qian., P.E.W. de Haas., J.T. Douglas., H. Traore., F. Portaels., H.G. Qing., D. Enkhsaikan., P. Nymadawa and J.D.A. van Embden. 1995. Predominance of a single genotype of *Mycobacterium tuberculosis* in countries of East Asia. *J. Clin. Microbiol.* 33: 3234-3238.
- Vestal, A.L. 1975. Procedures for the isolation and identification of *Mycobacteria*. US Department of Health, Education and Welfare, Centers for Disease Control, Atlanta, GA.
- Yuen, K.W.Z., B.C. Ross., K.M. Jackson and B.Dwyer. 1993. Characterisation of *Mycobacterium tuberculosis* strains from Vietnamese patients by Southern blot hybridization. *J. Clin. Microbiol.* 31: 1615-1618.
- Zainuddin, Z.F and J.W. Dale. 1989. Polymorphic repetitive DNA sequences in *Mycobacterium tuberculosis* detected with a gene probe from a *Mycobacterium fortuitum* plasmid. *J. Gen. Microbiol.* 135: 2347-2355.

