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RESEARCH ARTICLE

MOLECULAR EPIDEMIOLOGY OF INDIGENOUS ISOLATES OF *MYCOBACTERIUM TUBERCULOSIS*

***Firoz Naem Khan**

Immunology Laboratory, Centre for Scientific Research and Development (CSRD), People's University, Bhanpur, Bhopal 462037, M.P. (India)

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ABSTRACT

Aim and objectives: To identify *M. tuberculosis* strain prevalent in Bhopal region using microbiological, immunological and molecular techniques. Rapid and accurate detection, identification and susceptibility testing of mycobacteria is important in Indian scenario due to increase in incidence of tuberculosis, resistance to antituberculous drugs and increase in potentially pathogenic species of mycobacterium. It is important to identify false positive reporting from clinical infections. To use Amplified ribosomal DNA restriction analysis (ARDRA) for identification of Mycobacterium species with different restriction enzymes and compare obtained profiles with a library of ARDRA profiles of different species. Microbiological and immunological studies are also important to identify the TB strains.

Methodology: Sputum samples were collected from Peoples Hospital, Bhanpur, Bhopal after consent. The sputum was examined: MICROBIOLOGICALLY: AFB and culture on LJ media, IMMUNOLOGICALLY: Immuno-Dot Blot assay to detect serum Anti-Ag 38 and 65 KDa using secondary conjugate and secondary gold conjugate antibody specific for *M. tuberculosis*. MOLECULARLY: Genomic DNA Isolation, purification, Agarose Gel electrophoresis GENE AMPLIFICATION : Rep-PCR using primer BOX, Gm3f and Gm4r, ARDRA using restriction digestion with *Alu* I, *Msp* I and *Taq* I. Comparative analysis done using NTSYSpc version 2.02i, UPGMA method for phylogenetic relatedness among Mycobacterium species.

Results: 138 suspected TB sputum samples microbiologically were all AFB positive. Culturing showed that 78 samples were of *M. tuberculosis* (MTB) and remaining of other mycobacterium species. Immuno-Dot Blot assay using secondary conjugate and gold conjugate showed 59 samples as MTB affected with elevated level 80-85µg/ml of 38/65 KDa antigen. *M. intracellulare* affected sample showed 73 µg/ml and healthy samples were below 50µg/ml. Genomic DNA was isolated from 117 samples. Rep-PCR showed banding for 124 samples with yield of 193 scorable bands ranging from 700bp - 300bp. 53 samples showed MTB, 39 MTB sub-strains and 34 belonged to other Mycobacterium species. 14 samples showed no banding pattern. In ARDRA the restriction patterns with the enzymes *Alu* I, *Msp* I and *Taq* I for the species presented banding pattern at 500, 300 and 100bp. 16S rRNA sequencing gave 99.8% similarity to MTB. It revealed mutation shifts of 500 and 300bp band replaced by a single fragment of 800bp. 16S rDNA amplification showed merged band of 1500bp for MTB. The AFB positive samples were also identified as non-tuberculous organism with ARDRA. Comparative analysis resulted in the formation of two clusters.

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INTRODUCTION

Tuberculosis (TB) has been present in humans since thousands of years, as evidenced by the bones of ancient Egyptian mummies. Pulmonary TB was known as the "Great White Plague" (causing about one in four deaths) of the 17-18th centuries in Europe, "phthisis" (a Greek term meaning to waste away), "scrofula" (swollen glands of the neck), and "consumption" (progressive wasting of the body) (Daniel *et al*., 2000). From 1700 to 1900, TB was responsible for one billion deaths, more than any other disease (Bloom and Murray, 1992). Prior to the introduction of antibiotics in the 1950s, improved sanitation and living conditions significantly reduced the incidence of TB. The TB is transmitted via the respiratory route as a highly infectious aerosol with varying outcomes of initial *M. tuberculosis* exposure. The outcomes range from immediate organism destruction by the host's immune system to infected individuals developing active primary TB disease within 1–3 years (Flynn and Chan, 2001). However, the majority of individuals infected with *M. tuberculosis* have a non-contagious, clinically-latent infection with an absence of clinical symptoms (Clark-Curtiss and Haydel, 2003). Latently-

****Corresponding author:*** *Firoz Naem Khan,*
Immunology Laboratory, Centre for Scientific Research and Development (CSRD), People's University, Bhanpur, Bhopal 462037, M.P. (India)

infected individuals have a 5– 10% risk of developing reactivation. The discovery of the first antibiotic, streptomycin, to treat TB in 1944 also met with the appearance of the first antibiotic-resistant *M. tuberculosis* isolates (Crofton and Mitchison, 1948). The concomitant HIV in 1980s further complicated the ongoing TB epidemic (Espinal, 2003), and the global TB/HIV coinfection epidemic continues today with TB infecting one-third of the 33.2 million people living with HIV (Raviglione and Smith, 2007). India accounts for one fifth of this global burden of TB, with 1.8 million new cases of active TB each year, more than any other country (Steinbrook, 2007). The Revised National Tuberculosis Control Programme (RNTCP) in India reports that two in five Indians are infected with latent TB (RNTCP report 2009). Molecular epidemiology is the study of distribution and determinants of disease occurrence in human populations using molecular techniques is a blend of molecular biology and epidemiology. Epidemiologic investigations that incorporated DNA fingerprinting of the isolates of *M. tuberculosis* have been used to provide novel information about the spread of tubercle bacilli to analyse the transmission dynamics of TB and to distinguish exogenous reinfection from endogenous reactivation. It is also being used to identify the source of lab contamination, to determine the risk factors for TB transmission in a community, and to track the geographic distribution and spread of clones of *M. tuberculosis* of public health importance (Demay *et al*., 2012; Jagielski *et al*., 2014).

Strains of *M. tuberculosis* resistant to frontline medicines, including isoniazid and rifampicin, (multi- drug-resistant *M. tuberculosis* : MDR-TB) have been responsible for a series of frequently fatal disease outbreaks (Frieden *et al*., 1993). Faced with the slow growth rate of *M. tuberculosis*, it takes weeks to months to identify drug-resistant strains by conventional microbiology. With the prospect of developing rapid DNA based tests for resistance, investigators have focused on identification of the genetic changes responsible for resistance (Telenti *et al*., 1997). Drug resistance in *M. tuberculosis* has been mediated by changes affecting chromosomal genes, with no evidence of involvement of any transmissible genetic elements. Mutations at several different loci can give rise to resistance (De Baere *et al*., 2002), but resistance to rifampicin is almost always mediated by mutation of residues within a short stretch of the gene encoding the β-subunit of RNA polymerase (rpoB) (Telenti *et al*., 1993; Kapur *et al*., 1995). Rifampicin resistance is often associated with resistance to isoniazid, and is considered a useful surrogate marker for MDR-TB (Telenti *et al*., 1997). Investigations in cellular and molecular mechanisms to explain the development of drug-resistant TB strains shows prevalence of strains in the region but other influences like improper or poor health management practices or infrastructure, inadequate therapeutic regimens, antimicrobial misuse, insufficient or unobtainable resources, poor socioeconomic conditions, individual immunocompetence, patient compliance, and complicated personal issues have played roles in the evolution and progression of antibiotic resistance (Dorman and Chaisson, 2007; Sament *et al*., 2013).

Rapid and accurate detection, identification and susceptibility testing of mycobacteria remains important in Indian scenario due to increase in incidence of tuberculosis (Wolinsky, 1992), increased resistance to antituberculous drugs (Espinal *et al*., 2001) and due to an increase in potentially pathogenic species of mycobacterium (Wayne and Sramek, 1992). It is also important to determine the possibility of false positive reporting obtained from the other indigenous mycobacterial and other clinical infections described with species like *M. avium*, *M. bovis* and *M. intracellulare* which have been recognized recently. Current DNA amplification based diagnostic tests are expensive, have limited sensitivity, are usually restricted to the detection of *M. tuberculosis* only and provide no or limited information about susceptibility. Therefore, the need for culture (microbiological approach) has not been circumvented. The Centre for Disease Control (CDC) decided to restrict the use of genotypic tests to confirm smear positive samples, so that they cannot be used to test the large number of specimens processed for mycobacterial detection in an average lab (Doern, 1996). The use of ARDRA for identification of *Mycobacterium* species consists amplification of the 16S rRNA gene (rDNA) and subsequent restriction digestion of the amplicon. The restriction patterns obtained with different restriction enzymes and combination of these patterns into a restriction profile was shown to enable identification of most clinically important mycobacteria by comparison of the obtained profiles with a library of ARDRA profiles obtained for reference strains of different species (Vaneechoutte *et al*., 1993). This PCR-RFLP analysis of the 16S rRNA gene, was published almost simultaneously with the more widely used technique (known as PRA), which is based on the amplification of the *hsp65* gene (Telenti *et al*., 1993; Plikaytis *et al*., 1992; Steingrube *et al*., 1995). The objective of this study was based on identification of *M. tuberculosis* strains prevalent in Bhopal region using microbiological (Acid fast bacilli staining and culture), immunological (Immuno-Dot Blot assay using secondary conjugate and secondary gold conjugated antibody) and molecular techinques (ARDRA, RFLP, Rep-PCR).

Methodology

Collection of sputum samples showing clinical symptom of *M. tuberculosis* was used to create a profile library of the strains prevalent in Bhopal region. The 138 patients sputum samples used in evaluation were collected in routine from Peoples Hospital, Bhanpur, Bhopal, Madhya Pradesh, India with due consent and identified on the basis of microbiological studies.

Microbiological approach

Processing and culturing of the samples

Petroff's decontamination of samples was done by mixing an equal volume of 4% sodium hydroxide. Suspension was incubated at 37ºC for 30 min with frequent gentle mixing and neutralized using 10 ml of 0.5M phosphate buffer (pH 6.8) followed by centrifugation at 12000 rpm for 30 min. The supernatant was discarded and the remaining pellet was suspended in 1ml of phosphate buffer and used for AFB microscopy, culturing and DNA extraction. Ziehl Neelsen staining was done to determine whether the sputum sample contained any bacilli of *M. tuberculosis*. Firstly, smear was

prepared from decontaminated sputum sample and processed according guidelines of Center of Disease and Control Prevention (CDC, USA). A smear of the culture was prepared on glass slide; dried and fixed by gentle heating. Then smear was covered using carbol fuschin and heated from lower side until steam for about 8-10 min. The stain was prevented from boiling or drying. The slide was washed with water and decolorized using Acid Fast decolorizer for 2 min or until film exhibited a faint pink color. Again the slide was washed and counter stained with methylene blue for 30 sec. After counter staining the smear was rewashed and dried. This slide was observed under microscope oil immersion lens (100x) (LABOMED Vision 2000). The prepared LJ media slants (Himedia) were used to culture the Mycobacterium species. For culture of *M. tuberculosis* the concentrated sputum sample was spread on LJ slants and kept for incubation at 37ºC in BOD incubator for 6 weeks and were regularly observed. All the work was carried under Biosafety Level 3 and Biosafety cabinet type IIA (Tortoli *et al*., 1999).

Immunological approach

For immunological identification of Mycobacterium strain, SDS-PAGE was performed for *hsp* 38 and 65KDa antigen and transferred to nitrocellulose using western blotting. Immuno-Dot Blot assay was conducted to detect serum Anti-Ag 38 and 65 KDa using secondary conjugate and secondary gold conjugate specific for *M. tuberculosis*.

Immuno-Dot Blot Assay using secondary conjugate

Nitrocellulose discs containing heat shock protein (Hsp 38 and 65 KDa) were blocked with 3% skim milk in 1x phosphate buffer saline (PBS) and incubated at 37˚C for 1 hr. On incubation, 5µl of serum/sputum (sample) was added and re-incubated at 37˚C for 1 hr. Washing was done with 1X Phosphate buffer saline-Tween 20 (0.05% PBST) and 25µl of Goat Anti-Human IgG-HRP (1:1000) in 1% Bovine serum albumin (BSA) was added and incubated at 37˚C for 1 hr. Again washing was done with 1X PBST and was air dried. After drying 3,3',5,5'-Tetramethylbenzidine (TMB) along with hydrogen peroxide (H_2O_2) was added. The colour developed was taken as positive while no development of colour was negative.

Immuno-Dot Blot Assay using Secondary gold conjugate

Nitrocellulose discs containing Hsp 38 and 65 KDa protein was blocked using 3% skimmed milk in 1X PBS and incubated at 37˚C for 1 hr. Then a suspension of 0.1ml of colloidal gold Goat Anti-Human IgG conjugate and serum/sputum (5:1) was prepared and added to nitrocellulose discs and incubated for 1 hr at room temperature (25˚C) with continuous agitation. Washing with 1X PBST was done to remove unbound particles. The violet colour developed was positive whereas, no colour was taken as negative.

Molecular approach

DNA Isolation: The suspended pellet of sputum was used for DNA isolation of mycobacterium isolates. 500 µl Tris-EDTA (TE) buffer, pH 8.0 was added to the sample and incubated at 80°C for 20 min. Lysozyme was then added to each tube (final concentration, 1 mg/ml), followed by incubation at 37°C for 2 hrs. 10% sodium dodecyl sulfate (final concentration, 1.1%) and proteinase K (final concentration, 0.2 mg/ml) were then added, mixed gently and incubated at 65°C for 20 min. A mixture of N-acetyl-N,N,N-trimethyl ammonium bromide (CTAB; final concentration, 40 mM) and NaCl (final concentration, 0.1 M) was added, followed immediately by the addition of NaCl alone (final concentration, 0.6 M). The tubes were then vortexed until the suspension turned milky and were incubated again at 65°C for 10 min. 750 µl of chloroform-Isoamyl alcohol (24:1 v/v) was thereby added to each tube, and emulsified for 15 min followed by centrifugation at 13,000 rpm for 5 min at room temperature. To the aqueous phase collected equal volume of chilled isopropanol was added and gently mixed. The mixture was incubated for 30 min at -20°C to accentuate precipitation. The precipitated DNA solution was spun at 8000 rpm for 5 min at 4°C to pellet the DNA which was washed with 70% ethanol and air dried. The dried DNA pellet was dissolved in 50 µl of TE (Tris-EDTA) buffer and stored at -20°C until further use. For DNA purification accurate amount of enzyme RNase was added to a final concentration of DNA samples (20µg/ml) and were incubated at 37°C for 20 min. The DNA samples were extracted with equal volume of Chloroform: Isoamyl alcohol (24:1v/v) and emulsified for 15 min followed by centrifugation at 13,000 rpm for 5 min at room temperature. The DNA was precipitated with equal volume of chilled isopropanol, washed with 70% ethanol, air dried and dissolved in 20 µl of TE buffer and stored at -20°C until further use.

Qualitative Analysis of DNA (Agarose Gel electrophoresis)

A submarine horizontal agarose slab gel apparatus (AXYGEN, INDIA) was used. In this technique the open ends of gel tray were sealed with tape. 0.8% of agarose was prepared in 1x TBE (Tris Borate EDTA) buffer and was boiled in microwave oven to dissolve the agarose powder completely. After cooling the molten agarose up to 50°C, Ethidium bromide (EtBr) was added (final concentration of 0.5µg/ml) and mixed properly. Prior to pouring the molten agarose, the comb was fixed over the electrophoresis tray for making wells. The agarose was poured over the gel tray and allowed to solidify at room temperature. The comb was removed after the solidification of agarose and the gel was transferred to an electrophoresis tank containing 1x TBE buffer. The sample DNA mixed with gel loading dye (Bromophenol blue) was then loaded carefully in the wells of casted gel. A DNA ladder (1Kb) was also loaded along with the samples to quantify DNA and electrophoresis was carried out at a constant voltage of 70V. The gel was observed after the dye had run halfway, under ultraviolet light and photographed using the gel documentation system (Gel Doc, Bio Rad).

Amplification of the gene

Rep- PCR: The purified DNA samples were then subjected to Rep-PCR analysis for amplification with test primers BOX (5'GATCGGCAAGGCGACGCTGACG 3' Repetitive sequence), Gm3f (5'AGAGTTTGATCMTGG 3') and Gm4r

(5'TACCTTGTTACGACTT 3') as 16S rDNA sequence. Amplification reactions for PCR analysis were carried out where the final volume of PCR reaction mixture was 25 µl. The contents of master mix prepared for PCR amplification is *Taq* buffer - 1x, dNTPs - 200 µM each dNTP, Primer - 10 pmol (BOX, Gm3f and Gm4r with Tm 78.4 and nmoles 5.5), *Taq* DNA polymerase - 0.65 U, DNA template - 5 ηg and distilled water to make up. The reaction mixture was overlaid with mineral oil to avoid evaporation during PCR. The amplification was programmed in a Mastercycler Gradient (eppendorf) as initial denaturation at 94.7˚C for 2 min, denaturation at 94.7˚C for 1 min, annealing at 62.4˚C for 1 min, extension at 72˚C for 1 min and 35 cycles with final extension at 72˚C for 10 min using the fastest possible transitions between each temperature. The optimum annealing temperature was depending on GC content / length of the primers. The separation of PCR products using agarose gel electrophoresis was carried out with slight changes in the concentration of agarose. The amplified fragments were separated on 1.5% MetaPhor agarose gel / 1x TBE buffer and electrophoresed at a constant voltage of 70 for 2 - 3 hours. Finally, the gel was stained with ethidium bromide (0.5µg / ml), Visualized under ultraviolet chamber and documented in Bio-Rad Gel Doc System. The PCR was conducted as an experiment, with control (distilled water instead of template DNA) to test the purity and viability of reagents.

Amplified Ribosomal DNA Restriction Analysis (ARDRA)

ARDRA for mycobacteria consists of the amplification of the 16S rRNA gene, followed by separate restriction digestion with *Alu* I (Amersham Pharmacia), *Msp* I (Amersham Pharmacia) and *Taq* I (Genei). In aliquots of 10µl of DNA, 10 U of the respective restriction enzymes were added, with 2 µl of the corresponding enzyme buffer (10x□concentrated, final concentration 2x) and each restriction digestion mixture was adjusted to 20 µl with distilled water and incubated during 2 hrs at 37°C. The combination of the three obtained fingerprints is designated an ARDRA profile which can be compared with a library of ARDRA profiles, obtained from well-identified mycobacterial strains (GenBank sequences). In some cases, more discriminatory identification is possible by additional restriction with *Bst*UI. The DNA restriction fragments were electrophoresed in a 2% agarose electrophoresis gel, in the presence of ethidium bromide (50 ng/ml). The gels were photographed on Gel-Doc system, Bio-Rad and the fingerprints were compared visually with the overview gels.

Comparative analysis

A fragment of the gene was sequenced the 16S rDNA sequences obtained in this study indicated difference. All steps of the comparative sequence analysis were performed using the software *NTSYSpc version 2.02i,* Coefficient as Jaccard's and first, pairwise alignment was done using UPGMA method. Finally, a similarity matrix of the aligned sequences was constructed by global alignment homology calculation and a gap penalty of 20 %. The neighbour-joining method was used to construct the dendogram based on this similarity matrix. Bootstrap values were calculated. Theoretical calculation of restriction patterns was done by Restriction Fragment Length polymorphism (RFLP), which makes it possible to obtain restriction patterns using sequences in EMBL format, for every restriction enzyme.

RESULTS

Total 138 suspected patient sputum samples were collected to screen the indigenous strains prevalent in Bhopal region.

Microbiological approach

The AFB staining was performed to overcome the possibility of false positive samples. It was observed that almost all samples showed positive results. Culturing of samples on Lowenstein Jensen (LJ) media showed that 78 out of 138 samples were *M. tuberculosis*, whereas others were relatives of *M. tuberculosis* with different species (Table 1).

Immunological approach

Immuno-Dot Blot Assay using Secondary conjugate and Secondary gold conjugate: Results in both the antibodies (conjugate and gold conjugate) suggests that the analyzed samples showed higher/elevated level of 38/65 KDa antigen in pulmonary and extra-pulmonary TB patient approx 80-85µg/ml. whereas, *M. intracellulare* affected patients sample showed level of 73 µg/ml and for healthy samples level of 38/65 KDa antigen was below 50 µg/ml. Out of the total 138 samples analyzed, 59 samples showed *M tuberculosis* (Fig. 1).

Molecular approach

DNA Isolation : Genomic DNA was isolated from 117 samples and their quality was assessed by electrophoresis on 0.8% agarose gel. The DNA band migrated equivalent to lower than 23.1 kb band of EcoRI / HindIII double digested λ DNA. The DNA samples were diluted for further analysis (Fig. 2).

Amplification of the gene

Rep- PCR: Polymorphic banding patterns amplification was observed in the BOX primer. The amplification products of the 124 samples with the given primer yielded 193 scorable bands, out of which 7 were polymorphic (Fig. 3). The size of amplification products ranged from 700 bp - 300 bp. The highest mol wt of bands (700 bp) was obtained with the *M. tuberculosis* while the lowest mol wt of 300 bp was obtained with *M. tuberculosis* along with *M. intracellulare*. The percentage polymorphism was 16.66. The DNA ladder (GeneRuler Low Range Marker) used as the marker had a molecular range of 1000 to 100bp. While 53 sample showed *M. tuberculosis,* 39 samples showed *M. tuberculosis* sub-strains and 34 samples showed other *Mycobacterium* species. No banding pattern was observed in 14 samples.

ARDRA: The initial study describing the applicability of ARDRA for the identification of mycobacteria (Frieden *et al*., 1993) used universal bacterial primers. This sometimes results in false positive amplification from decontaminated samples of organisms other than mycobacteria. Therefore primer of more specificity for amplification of mycobacteria was used.

Table 1. Sputum examination for *Mycobacterium tuberculosis*

Name of Patients	AFB	Culturing on LJ	DNA from sample	DNA from Culture	PCR	ARDRA		BOX	Mycolic acid (Sputum)	Remark
						Alu 1	Msp I			
L1	+++	+	-	+	+	+	+	+	-	Pulmonary
L2	+++	+	-	+	+	+	+	+	-	Pulmonary
L3	-	-	-	-	-	-	-	-	-	EPT
M. intracellulare ATCC13950	+	+		+	+	-	-	+	-	Standard
L4	+++	+	-	+	+	+	+	+	-	Pulmonary
L5	+++	+	+	+	+	-	-	+	-	Pulmonary
L6	+++	+	-	+	+	-	-	+	-	Pulmonary
M. tuberculosis ATCC25177	+	+	+	+	+	+	+	+	+	Standard
L7	+++	-	-	-	-	-	-	-	+	Pulmonary
L8	+++	-	-	-	-	-	-	-	+	Pulmonary
L9	+++	-	-	-	-	-	-	-	+	Pulmonary
L10	+++	-	-	-	-	-	-	-	+	Pulmonary

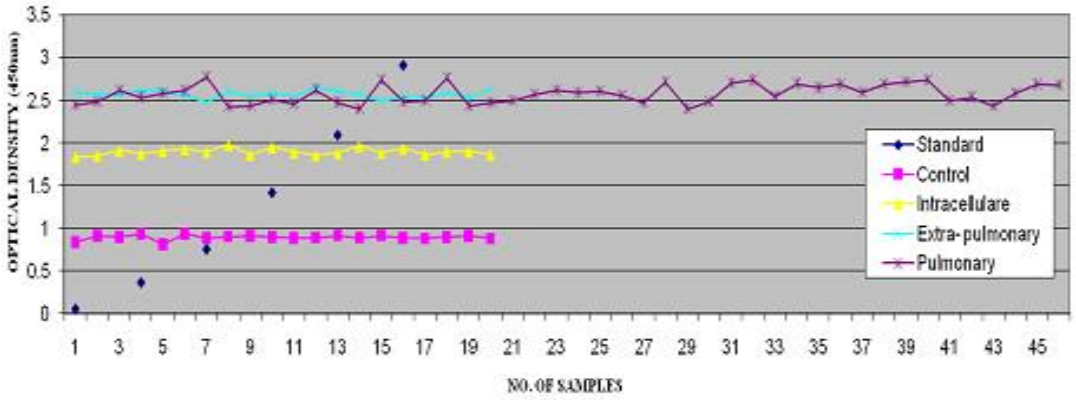

Fig. 1. Immuno-Dot blot assay of sputum samples

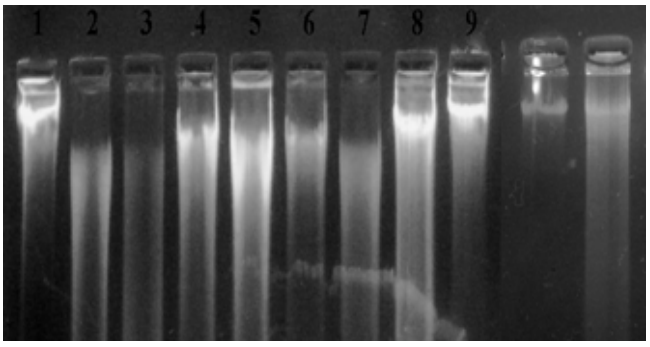

Fig. 2. Genomic DNA isolated from sputum sample: Lane 1-9 Patients Sample; Lane 10: Standard 1 (*M. intracellulare*); Lane 11: Standard 2 (*M. tuberculosis*)

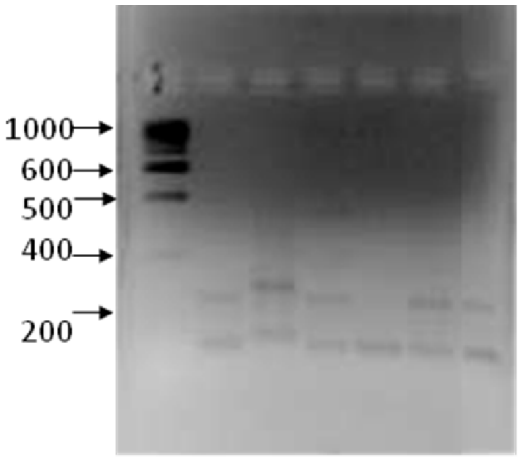

Fig. 3. Rep-PCR employed for determination of phylogenetic relatedness amongst Mycobacterial spp. isolated from TB affected patients. M: 100bp DNA Ladder, Lane 1: *M. tuberculosis*, Lane 2: *M. intracellulare*, Lane 3-6: DNA of TB patients

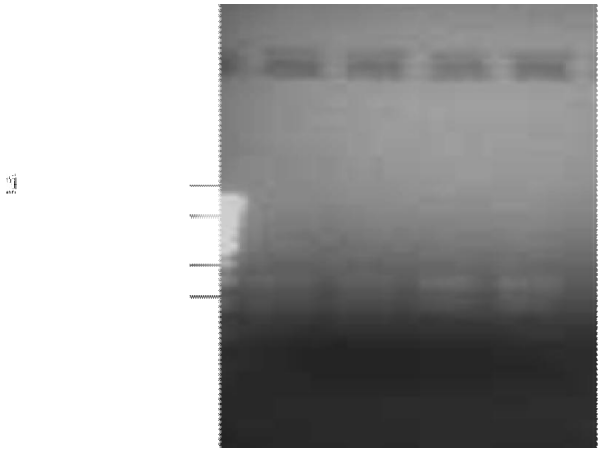

Fig. 4. ARDRA of Sputum samples showing 100 bp DNA Ladder, Lane 1-3: TB sample, Lane 4: *M. tuberculosis* ATCC 25177

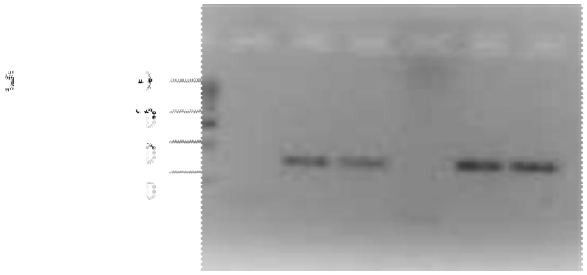

Fig. 5. 16S rDNA amplification showing Lane 1: 1Kb DNA ladder, Lane 2-5: TB sample, Lane 6: *M.tuberculosis* ATCC 25177

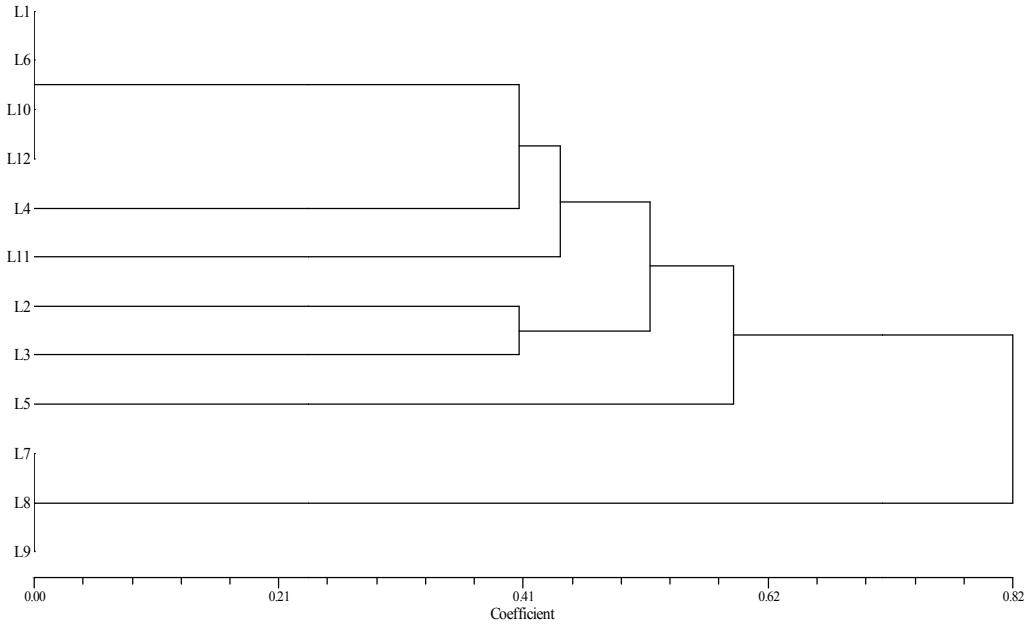

Fig. 6. Dendogram of the relatedness of *M. tuberculosis* samples

The restriction patterns obtained with the enzymes *Alu* I, *Msp*I and *Taq* I for the species presented banding pattern at 500, 300 and 100bp respectively (Fig. 4). The restriction patterns obtained with the same enzymes on published GenBank sequences were similar. The combination of these patterns is designed as ARDRA profiles. For some species the ARDRA pattern obtained with enzyme is already characteristic, e.g. *Hha*I 1 is observed only for species of the *M. tuberculosis* complex. Most mycobacterial species could be readily identified by comparison of the obtained ARDRA profile. However, the species of the *M. tuberculosis* complex cannot be differentiated on the basis of the 16S rDNA sequence, and therefore restriction digestion of this gene is more relevant. Sequencing of the 16S rRNA gave a 99.8 % similarity to the

16S rRNA sequence of the type strain of *M. tuberculosis*. The sequence revealed a mutation shifts because the two fragments of 500 and 300 bp are replaced by a single fragment of 800 bp (Fig. 5). Whereas, 16S rDNA amplification showed merged band of 1500bp for *M. tuberculosis*. In case only the 16S rDNA fragment of 1500 bp is present, the absence of *M. tuberculosis* can be confirmed by ARDRA which also immediately provides with the identification of the *Mycobacterium* species other than tuberculosis. This resulted in an identification of nontuberculous strain, confirming the absence of *M. tuberculosis*. Also in cases the amplifications of the smear positive sample remain negative, this can be interpreted as the absence of *M. tuberculosis*. The AFB positive samples of tuberculosis were also identified as non-tuberculous organism with ARDRA.

Comparative analysis: The cluster were analyzed from the dendogram constructed base on pairwise distance coefficient values the grouping base on the genetic values of unknown samples and the standard with the BOX primer resulted in the formation of 2 clusters (cluster 1 and 2). These clusters further differentiated into 2 sub-clusters each viz. a and b respectively. Average Similarity Index value observed between cluster 1 and cluster 2 was 0.25, indicating genetic diversity amongst species and the dendogram (Fig. 6) ranges from 0.25 to 1.0. The highest and lowest similarity index values observed were 1.0 and 0 respectively.

DISCUSSION

Restriction analysis of the amplified 16S rRNA gene, or amplified rDNA restriction analysis (ARDRA) has been used for several comparable approaches, based on restriction digestion of the amplified rRNA genes and spacer regions have been described (Sament *et al*., 2013; Wolinsky, 1992; Espinal *et al*., 2001; Wayne and Sramek, 1992). This approach is being updated and refined, like increased quality control of gel electrophoresis and pattern interpretation and the use of primers specific for species. Modification became possible because of the improvement of mycobacterial taxonomy and the by PCRRFLP techniques, like ARDRA, to easily adapt to this new information. Indeed, when new species are described, there is no need to develop new probes or primers. Instead, new ARDRA profiles can be easily added to the existing library. Also, ARDRA profiles for newly described species can be predicted by applying computer aided digestion of the available GenBank sequences, given the availability of sequences of sufficient quality (Doern, 1996). ARDRA was found to be a useful tool for identification of mycobacterial isolates in a clinical routine laboratory, because of its speed compared to phenotypic identification, its reliability, practical applicability, flexibility and the possibility to identify most nontuberculous mycobacteria together with and at the same cost as *M. tuberculosis,* at an affordable price. Molecular and cultural identification has a vast difference in interpretation of Mycobacterial strains. The practical applicability of ARDRA is increased due to its time duration (6 hrs) and the average identification time (36 hrs) for obtaining results. Technically, ARDRA is nondemanding, comprising only basic molecular biology techniques like simple DNA extraction, PCR, restriction digestion and submarine agarose gel electrophoresis. If ARDRA is compared to other culture based genotypic identification techniques (Gene Restriction techniques) it is a practical short cut to determine full sequence have been developed (Telenti *et al*., 1993; Steingrube *et al*., 1995; Roth *et al*., 2000; Brunello *et al*., 2001; Wong *et al*., 2001; Malakmadze *et al*., 2005). The study clearly indicates the discriminatory power of these RFLP approaches for identification of mycobacteria which is almost as high as that of sequencing. Restriction digestion of a 439 bp stretch of the *hsp65* gene for identification of mycobacteria was described almost simultaneously with ARDRA and designated PCR-RFLP Analysis (PRA) (Telenti *et al*., 1993). Researchers have published their work using this technique (Steingrube *et al*., 1995; Brunello *et al*., 2001; Wong *et al*., 2001; Negi *et al*., 2007). There had been drawbacks of *hsp65* gene restriction analysis as the smaller sized restriction fragments and the higher intraspecific variability, which makes interpretation more difficult. The small size differences have led to the use of polyacrylamide gel electrophoresis (Brunello *et al*., 2001), which is less practical than agarose gel electrophoresis and the difficulties in interpretation have led to reconsideration of the *hsp65* gene restriction profiles (Wong *et al*., 2001). Comparable remarks can be made for PCR-RFLP analysis of the rRNA spacer region (Demay *et al*., 2012; Jagielski *et al*., 2014; Roth *et al*., 2000; Ashworth *et al*., 2008). The immunological approach for *hsp* 38 and 65 using secondary antibody is supporting along with in interpretation of mycobacterial strains differentiating their level. Several limitations have been addressed in molecular biology based detection in diagnostic bacteriology (Vaneechoutte *et al*., 1993). There are expectations that DNA amplification technologies surpass microscopy, accurately predict culture results and provide an immediate definitive diagnosis were premature and that these claims have to be replaced with a more realistic view of the limitations and of the practical value of molecular diagnostics of tuberculosis (Doern, 1996; Roth *et al*., 1997; Gallina *et al*., 1999). A tremendous effort, both in academic and commercial research, has been put into the applicability of nucleotide amplification techniques for the detection of mycobacteria directly from clinical samples, the CDC approved application of these techniques only for smear positive samples. This implicates that for approx 50% of the culture positive samples with *M. tuberculosis* encountered in this study, DNA technology would not have accelerated detection, since microscopy was negative. Moreover, since direct detection amplification technology is technically demanding or requires specialized equipment and kits, many laboratories carry out these tests only at well-set time intervals (Artiles *et al*., 2001). Finally direct detection without direct antimicrobial susceptibility testing does not obviate the need for culture.

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