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Use of 1.8kb Gene Amplification Restriction Analysis (PCR-RFLP) in Species Identification of *M. tuberculosis*.

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Abstract : Conventional identification of a clinical isolate of Mycobacteria primarily based on culture characteristics and biochemical tests needs several weeks and may remain inconclusive. This study was undertaken to evaluate a new rapid method to identify the Mycobacterial isolates at species level by gene amplification restriction analysis using primers encoding 16S-23S rRNA internal transcribed spacer (ITS) region and flanking parts of the 16S as well as 23S rRNA gene. Restriction was carried out with restriction enzyme *Hha I*. This assay was applied on 5 reference strains and 50 clinical isolates of mycobacterial and 5 environmental isolates to validate the technique. Distinct gene amplification restriction analysis patterns were obtained by restriction of amplicons with *Hha I* restriction enzyme which could differentiate various mycobacterial species. The procedure dose not involve hybridization steps for the use of radioactivity and can be completed within 1 working day.

Key words : Gene amplification restriction analysis, 16-23S rRNA - spacer region, Restriction enzyme.

Introduction

Mycobacteria are heterogeneous group of organism. The most common mycobacterial infection in the world is caused by *M. tuberculosis*. However the infection caused by non tuberculous mycobacteria (NTM) is becoming important which necessitates accurate and fast identification. Accurate identification of mycobacteria at species level is very important for patient management and their effective treatment as drugs required for treatment of TB and NTM diseases are usually different (Springer *et al*., 1996)

Conventional identification of a clinical isolate of Mycobacteria primarily based on culture characteristics and biochemical tests needs several weeks and may remain inconclusive. Although various gene amplification based methods have been applied for the rapid detection and identification of mycobacterium species but we preferred the PCR-restriction analysis (PRA) techniques because these are simple and cost effective.

This study was undertaken to evaluate a new rapid method to identify the Mycobacterial isolates at species level by gene amplification restriction analysis using primers encoding 16S-23S rRNA internal transcribed spacer (ITS) region and flanking parts of the 16S as well as 23S rRNA gene.

Materials and Methods

Collection of Mycobacterial isolates: All the sputum samples were collected from the pulmonary tuberculosis

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cases at DOTS centers situated at the old city of Jaipur. These samples were collected in leak proof vials and equal amount of cetylpyridinium chloride (1% CPC and 2% NaCl) was added in the sputum. All isolates were identified by AFB staining and culture method. The 5 environmental isolates (Ev-1, Ev-2, Ev-3, Ev-4, Ev-5) from the same area and standard strains of *M. tuberculosis* (H37Rv), *M. avium* (N-17), *M. terrae* (N-23), *M. vaccae* (J-28) and *M. intracellulare* (N-8) were taken from the Mycobacterial Repository Centre, CJIL and OMD Agra.

Identification of isolates: All isolates were identified by AFB staining and culture on Lowenstein Jensen medium. A battery of biochemical tests including pigment production test, tween 80 hydrolysis, nitrate reduction test, catalase test at 68°C were applied for identification of isolates.

Sample preparation: DNAs were isolated by a physiochemical procedure of freeze boiling and treatment with lysozyme and protienase K method.

Amplification of 1.8 kb fragment of isolated DNA by PCR: Amplification of 1.8 kb fragment of isolated DNA was performed by the polymerase chain reaction by using specific primers designed at CJIL & OMD, Agra (DBT project report 1999). Master Mix was prepared by mixing following reagents as shown in Table 1:

Table : 1

Reagents	Stock Concentration	Final Concentration	Volume/ Sample
PCR Buffer	10X	1.5 mM	5 µl × n
d NTPs	2 mM	200 mM	1 µl × n
P_1	0.7227 µg/µl	0.21681 µg/reaction	0.3 µl × n
P_2	0.6039 µg/µl	0.18117 µg/reaction	0.3 µl × n
Taq Polymerase	5U/µl	2.5 U/100 µl	0.5 µl × n

n = No. of samples

Distilled water - Variable to make up volume to 50 µl.

Primer Sequence

P_1 (CJIL NO. 52) - 5'>GAT TGA CGG TAG GTG GAG AAG AAG<3'

P_2 (CJIL NO. 53) - 5'>CAC GGG CCC GCT GCT ACT CG<3'

48 µl volume of the PCR mix was dispensed in each tube (in DNA addition room) and 10-200 ng (2 µl) genomic DNA was added and overlaid with 2 drops of mineral oil. 1-2 drops of mineral oil was also added in the wells of thermal cycler before loading the samples. Mineral oil overlaying was not required in gold plated thermal cycler. Gene amplification was performed in a thermal cycler as per the following temperature profiles as given in Table 2:

Table : 2

Step	Temp.	Time	
Initial Denaturation	94° C	2 min	
Denaturation	94° C	1 min	} 35 cycles
Annealing	59° C	1 min	
Extension	72° C	2 min	
Final Extension	72° C	5 min	

Analysis of amplicons by agarose gel electrophoresis: The steps used are as follows:

(i) 15 µl of amplified product was added to 5 µl of loading dye solution (6X).

(ii) It was mixed and loaded into the wells of the 1.5% agarose gel.

(iii) 500 ng of molecular weight marker was loaded into the first lane of the gel.

(iv) Electrophoresis run was done at 1.2 V/ cm for 1 to 1.5 hours.

(v) The fragment was observed on the UV transilluminator and Gel Doc System. All samples were observed for having bands corresponding to the 1.8 kb gene region.

Restriction of Amplified DNA

(i) Preparation of Reaction Mixture: Amplified DNA of each species was restricted by adding following reagents:

PCR product of each species	10 µl
Restriction buffer	4 µl (10X)
Restriction enzyme (*Hha I*)	0.5 µl (5U)
B.S.A.	0.5 µl
Distilled water	15 µl
Total volume	30 µl

(ii) Enzymatic Reaction: After addition of these reagents in each tube, the tubes were centrifuged for few seconds and incubated at 37ºC for 2 hours.

Resolution of restricted DNA by agarose gel electrophoresis

(i) Preparation of Loading Samples in Agarose Gel: After 2 hours 6 µl of loading dye (6X) added in the samples and heat 68ºC for 5 to 10 min. for stop the enzymatic reaction.

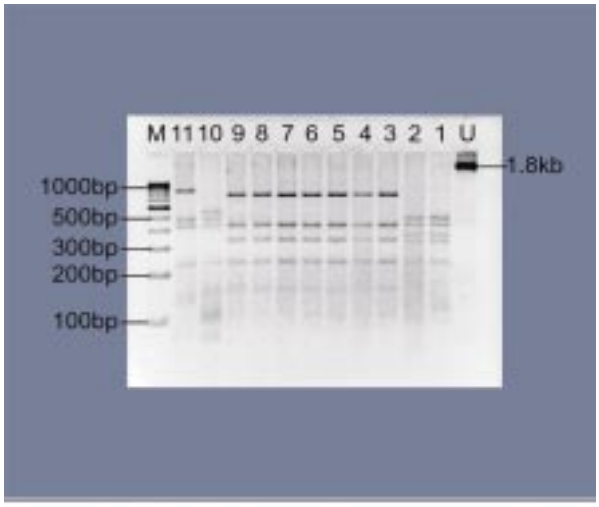
(ii) Loading of Reaction Mixture in a Agarose Gel: Reaction mixture was loaded in a gel (2%) and run for 4 hours at 1.2 V/ cm.

(iii) Analysis of Bands on the Gel Doc System: After 4 hrs. The fragment size of bands obtained were analyzed on Gel documentation system with the help of quantity one software.

Results

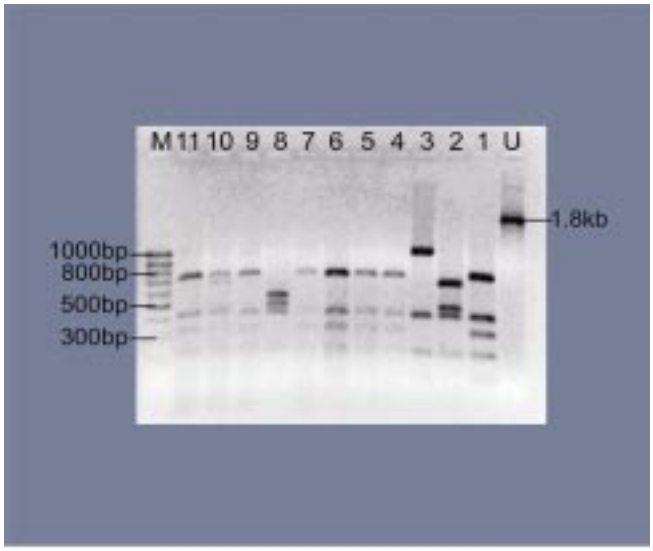
DNAs of all isolates (environmental and clinical) and standard strains were isolated and the 1.8 kb gene was amplified by PCR using mycobacterial specific primers. The amplicon of isolates were restricted by using the restriction enzyme *Hha I* for 1.8 kb gene region. The different band patterns obtained after restriction with *Hha I* enzyme were shown in the Figures 1 and 2 .The results were summarized in the Tables 3 and 4, respectively.

Fig. 1: Gene Amplification Restriction Analysis (PCR-RFLP) of Mycobacterial Isolates Amplicons of 1.8 kb Gene Digested with *Hha I* Enzyme



Lane 0: Uncut Amplicon, Lanes 1 to 2: Environmental isolates, Lanes 3 to 9: *M. tuberculosis* isolates, Lane 10: *M. avium* standard strain, Lane 11: *M. tuberculosis* standard strain, Lane 12: Marker (100bp).

Fig. 2: Gene Amplification Restriction Analysis (PCR-RFLP) of Mycobacterial Isolates Amplicons of 1.8 kb Gene Digested with *Hha I* Enzyme



Lane 0: Uncut Amplicon, Lanes 1 to 3: Environmental isolates, Lanes 4 to 6: *M. tuberculosis* isolates, Lane 7: *M. tuberculosis* standard strain, Lane 8: *M. avium* standard strain, Lanes 9 to 11: *M. tuberculosis* isolates, Lane 12: Marker (100bp ladder).

Table 3: Fragment Size of Restriction Fragments Generated from 1.8 kb Amplicons from Isolates as well as Reference Strains

Lane No.	Sample	Biochemical Identification	Fragment Size (bp)	Molecular Identification
0	Uncut		1800	
1	Ev.-1	Mycobacteria other then tuberculosis	500, 450, 380, 356, 250	Not matched with std. strain of *M. tuberculosis* and *M. avium*
2	Ev.-2	Mycobacteria other then tuberculosis	500, 450, 380, 356, 250	Not matched with std. strain of *M. tuberculosis* and *M. avium*
3	JPK-1	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*
4	JPK-2	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*
5	JPK-3	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*
6	JPK-4	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*
7	JPK-5	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*
8	JPK-6	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*
9	JPK-7	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*
10	N-17	*M. avium* std.	550, 500, 450, 250	
11	H37 Rv	*M. tuberculosis* Std.	810, 450, 365, 250	
12	Marker (100bp)			

Table 4: Fragment Size of Restriction Fragments Generated from 1.8 kb Amplicons from Isolates as well as Reference Strains

Lane No.	Sample	Biochemical Identification	Fragment Size (bp)	Molecular Identification
0	Uncut		1800	
1	Ev.-3	Mycobacteria other then tuberculosis	800, 450, 320, 250	Not matched with std. strain of *M. tuberculosis* and *M. avium*
2	Ev.-4	Mycobacteria other then tuberculosis	700, 500, 450	Not matched with std. strain of *M. tuberculosis* and *M. avium*
3	Ev.-5	Mycobacteria other then tuberculosis	1200, 450	Not matched with std. strain of *M. tuberculosis* and *M. avium*
4	JPK-8	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*
5	JPK-9	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*
6	JPK-10	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*
7	H 37 Rv	*M. tuberculosis* std.	810, 450, 365, 250	
8	N-17	*M. avium* std.	550, 500, 450, 250	
9	JPK-11	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*
10	JPK-12	*M. tuberculosis*	810, 450, 365, 250	Matched with std. strain of *M. tuberculosis*

Observation of 1.8 kb PCR-RFLP

All biochemically identified Mycobacterial isolates, environmental isolates and reference strains were amplified by using specific primers for 1.8 kb gene region and restriction with the *Hha I* enzyme.

- All elicited band patterns were matched with the standard strains of *M. tuberculosis* and the size of bands were 810bp, 450bp, 350bp, 250bp.
- From reference strains of *M. avium* and *M. intracellulare*, bands size obtained were 550bp, 500bp, 450bp, 250bp and 690bp, 500bp, 450bp, 230bp respectively which are distinct from *M. tuberculosis*.
- The five environmental isolates showed the different band pattern. The band size which were obtained from the environmental isolates were

Ev-1: 500 bp, 450bp, 380bp, 365bp, 250bp

Ev-2: 500 bp, 450bp, 380bp, 365bp, 250bp

Ev-3: 800bp, 450bp, 320bp, 250bp

Ev-4: 700bp, 500bp, 450bp

Ev-5: 1200bp, 450bp

Results indicated that the bands sizes from the environmental isolates were different from the standard strain of *M. tuberculosis* and reference strains of *M. avium*, *M. intracellulare*, *M. terrae*, *M. chelonae*, It indicates that these are the different species of mycobacteria.

- All mycobacterial isolates showed the similar band patterns with the standard strains of *M. tuberculosis*; this hereby confirms that these belong to the *M. tuberculosis* species.

Discussion

Of the 121 known Mycobacteria, nearly one third have been observed to be associated with disease in humans (Euzeby, 2006). Non tuberculous mycobecteria have been reported to cause localized or disseminated disease depending on local predisposition and/or degree of immune deficit. In AIDS patients' manifestation may range from localized to disseminated disease (Katoch, 2004; Wayne *et al*., 1992).

Various gene amplification based methods have been applied for the rapid detection and identification (or differentiation) of Mycobacterial species. Among these different methods PCR-restriction analysis (PRA) techniques are preferred because these are simple cost effective. PCR-RFLP analysis of different gene regions such as hsp 65 (Telenti *et al*., 1993), 16S rRNA or its coding rDNA (Vaneechoutee *et al*., 1993), rpo B gene (Kim *et al*., 2001), dna J gene (Takewaki *et al*., 1994), gyr B (Kasai *et al*., 2000), upstream sequences of kat G (Goyal *et al*., 1994) gene etc. have been described. However each technique has its own advantages and disadvantages. In contrast, the 16S-23S rRNA gene region has been considered a suitable target for differentiating species and potentially can be used to distinguish clinically relevant Mycobacterial species (Park *et al*., 2000).

The assay developed can easily distinguish *M. tuberculosis* complex from other non chromogenic mycobacteria like *M. avium* and *M. intracellulare* because the band patterns from *M. tuberculosis* were distinct from *M. avium* and *M. intracellulare*, which are two other clinically relevant non-chromogenic slow growers. It was also observed that different species showed the distnict band patterns, so by using this technique we can identify different species of mycobacteria in one experiment.

This PCR restriction analysis is less time consuming and less labour intensive than traditional methods and should be applied in clinical laboratories. Our assay allowed better and easier differentiation on gels as it targets a larger region than ITS sequences between the 16S-23S rRNA genes alone. The drawback of this system is that it does not differentiate the two important members of *M. tuberculosis* complex (*M. tuberculosis* and *M. bovis*). Use of more restriction enzymes or other assays could be valuable for this purpose.

Conclusion

Restriction patterns with the enzyme used in this study could clearly distinguish *Mycobacterium tuberculosis* complex from other non chromogenic clinically important species *M. avium* and *M. intracellulare*. Results indicated this assay to be a simple, rapid and reproducible method to identify clinically relevant Mycobacteria.

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