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Amplification of Hsp 65 Gene and Usage of Restriction Endonuclease for Identification of Non tuberculous Rapid Grower Mycobacterium

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Summary
The rapid grower mycobacteria have emerged as significant group of human pathogen amongst the Runyon group IV organisms that are capable of causing infection in both the healthy and immunocompromised hosts. In this study, we analyzed and identified 121 rapidly growing mycobacterium isolated from clinical samples by polymerase chain reaction – restriction enzyme analysis (PRA) at a national reference laboratory. The results were analyzed and compared with standard biochemical test.

In this study, 8 different types of rapid grower mycobacteria were identified by analyzing the fragment generated through restriction enzymes. More than 50% of the isolates were from the pulmonary samples sputum. In pulmonary as well as extrapulmonary samples the most common isolate was M. chelonae (57/121). All strains of M. chelonae were having the same band fragments size. The others species identified in this study were M.fortuitum (42), M. abscessus (11), M. immunogen (06), M. peregrinum (02), M. smegmatis (01), M. wolinskyi (01), M.goodii(01). The study showed that in pulmonary as well as extrapulmonary sample M. chelonae was the most common isolate.

PCR- REA is a rapid accurate system with concordance of 119/121 (98.34%) when compared to standard biochemical tests for identification of clinically important species of rapidly growing mycobacterium.

Keywords: *Rapid grower mycobacterium;REA; PCR.*

Introduction

India is an agriculture based economy, where majority of rural people are exposed to soil, water, plants and livestock constantly. These are potential sources of nontuberculous mycobacterium infection. The importance of several mycobacterial species other than the mycobacterium tuberculosis complex has increased in the recent past. It is now well recognized that NTMs too cause pulmonary infection, lymph node infection, brain, bone, kidney, genital tract and skin /soft tissue infections in human beings. The guidelines for diagnosis, treatment and doses have been laid down by American Thoracic Society (ATS) and Infectious Disease Society of America (IDSA). The antibiotic susceptibility pattern and drug treatment is different from mycobacterial tubercular infections[1,2]. Therefore, there is a growing need to identify mycobacteria to the species level especially in tertiary care TB & chest referral centres. Analysis of PCR products has been the recent focus of considerable interest for separation of mycobacteria. Practice of molecular methods, polymerase chain reaction –restriction enzyme analysis (PRA) test has been considered as promising method for species identification of mycobacterium. Use of restriction enzyme Bst II and Hae III and analysis of the enzymatically digested amplified 65-kDa heat shock protein-encoding gene (hsp65) has been successfully applied by several researchers for the identification of mycobacterial species [3,4,5]. This method is simple, rapid, sensitive, time and labour saving than traditional biochemical methods as has been described previously also [6,7]. Previous studies have discussed the PRA test for NTM but only small number of species of rapidly growing mycobacteria (RGM) were included [8,9].

The aim of the present study was to evaluate the PRA method and to extend it to other mycobacterial species with particular emphasis on the differentiation of rapidly growing nonchromogenic mycobacteria that are often found in tap water and laboratory environment and are of significant clinical importance because of their multiple drug resistance nature.

Materials & Methods

The study was conducted during October 2013 to September 2014. The twice NTM positive samples were collected from the liquid culture section.Themucolytic N-acetyl L cysteine, sodium hydroxide(NALC- NaOH) treated samples were inoculated in BBL 7ml MGIT media and placed in MGIT-960 system. Whereinthe reading procedure is automatic and reading isrecorded on hourly basis by the system. The positive culture tubes were indicted by the system when the growth unit reading of the system comes out to be more than 100. Positive tubes were taken out from the system, checked for acid fast bacilli by Ziehl Neelsen smear examination and further identification was done by immunochromatographic assay and PNB test.The two samples from the same patient when positive for NTM were further subjected for species identification. The identified NTM were divided into two groups on the basis of growth rate on solid Lowenstein Jensen media and examining the media daily for one week. The mycobacterium which grew in less than one week time were grouped as rapid grower and those which grew in more than one week time were grouped in slow grower mycobacterium. Species amongst the rapid grower mycobacterium was further identified by molecular method; polymerase chain reaction –restriction enzyme analysis (PRA) of a segment of the genes encoding 439-bp portion ofthe mycobacterial 65-kDa heat shock protein.In order to avoid contamination, different steps like sample processing, reagent preparation, amplification and detection were done in separate room.

The DNA was extracted by heat lysis method and it was amplified using primers primer TB 11 and TB 12 (Forward (TB11): 5’ACC AAC GAT GGT GTG TCC AT 3’ and Reverse (TB12): 5’ CTT GTC GAA CCG CAT ACC CT 3’) along with 1.25 unit Taq DNApolymerase (5). The amplified PCR product was digested with BST II and Hae

III restriction enzymes. The digested product was electrophoresed with 3% agarose containing 1µg/ml of ethidium bromide in a mini- sub cell electrophoresis system (Bio –Rad Richmond California) at 100 volt for 1.5 to 2 hours. Fragment band size was estimated using 100 base pair ladder (Sigma). Restriction fragment smaller than 50 bp was ignored in the results. The species were identified by matching the band patterns as described by telnati et al and PRASITE Web site (http://app.chuv.ch/prasite/index.html).chuv.The results were analyzed and compared with standard biochemical test.

Results

In this study, total 121 rapidly growing mycobacterium were analyzed (table 1). They were isolated from different clinical samples amongst which commonest was sputum (72) followed by lymph node aspirate (18), pleural fluid (11), skin (5), tracheal aspirate (3), ascitic fluid (3), CVP tip (4) and pus (5) (table -2). DNA from the isolates was amplified using primers specific for hsp65 gene (Figure 1). All the species showed distinctive band patterns for Bst II and Hae III enzymes except *M.goodii* and *M.wolinskyi*. The minimum band size with Bst digestion was of 80bp and maximum band size was of 325 bp. Similarly, in Hae digestion the minimum band size was of 55 bp and maximum band size was of 210 bp. By using 100 base pair ladder the base pair were calculated by its proximity to neighboring base pair size. Altogether 12 different band patterns were seen following the Bst enzyme digestion and 14 different sizes of bands were seen with Hae restriction enzyme (Figure 2). With the help of 26 different band patterns the rapid growers were divided into 8 species (table 3). The most common species in restriction enzyme analysis was *M.chelonae* (57) (table 2). All the chelonae strains showed same restriction pattern of 325,140 and 125bp with Bst enzyme and only one band size of 210bp with Hae restriction (table 1). The growth characteristic, growth rate and other biochemical reactions were not confirmed as *M. abscess* as the colonies were small pinpoint and visible growth was observed on 9th day of incubation. But in the restriction enzyme analysis the band pattern clearly indicated the growth as *M. abscess* showing band 235,210 bp with Bst digestion and 145,70, 60, 55 bp after Hae digestion. Similarly, *M.goodii* was not confirmed in biochemical identification was identified as *M.goodii* in restriction enzyme analysis. The study showed that *Mycobacterium chelonae* species were identified as the most common isolates in pulmonary as well as extrapulmonary samples.

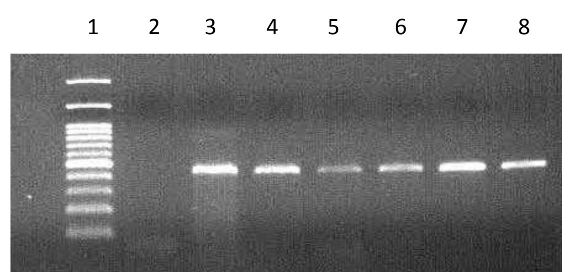
Discussion

In this study, the highly conserved hsp 65 gene is targeted for amplificationas it has a high discriminating power to differentiate Mycobacterium species as described earlier by Buchanan et al[10]. The restriction analysis of a 439-bp fragment within this gene after BstEII and HaeIII digestions is highly effective for differentiating mycobacteriaat the species level.The results of amplification of hsp 65 gene followed by restriction enzyme analysis of rapid grower mycobacterium helped in discriminating 8 different types of rapid grower mycobacterium which were well correlated with biochemical identification results similar to Ringuet H et al [11]. Differentiation between *M.chelonae* and *M.abscessus* by biochemical method is a difficult task, hence PRA test was used for rapid identification. Steingrubeet al has also discussed that Bst II, Hae III along with other enzymes AciI and CfoI gave the best separation of rapidly growing mycobacteria[12]. In their study it showed that sixty percent of all RGM taxa studied were differentiated by HaeIII digests alone. All species studied by PRA test were readily discriminated from each other. *M. fortuitum* and *M. smegmatis*, the two species with the highest degree of similarity were also identified by this test.

Table 1 : Laboratory phenotypic features of the clinically important species of rapidly growing mycobacterium

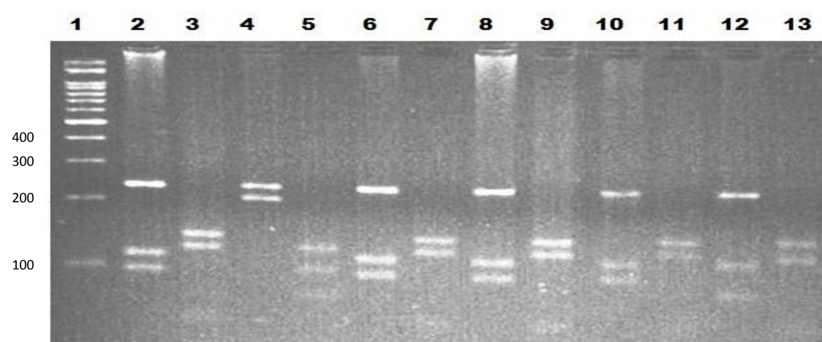
S. No	Mycobacterial species	Pigment	Arylsul-3days	Mac with no CV	Nitrate	Iron uptake	5% NaCl	Growth on		
								Sod citrate	Mannitol	Inositol
1	*M.chelona*	-	+	+	-	-	-	-	-	-
2	*M.fortuitum*	-	+	+	+	+	+	-	-	
3	*M.peregrinum*	-	+	+	+	+	+	-	+	-
4	*M.abscessus*	-	+	+	-	-	+	-	-	-
5	*M.immunogenicum*	-	+	-	-	-	-	-	-	-
6	*M.smegmatis*	+	-	+	+	+	+	+	+	+
7	*M.wolenskii*	-	-	+	+	+	+	+	+	+
8	*M.goodii*	-	-	+	+	+	+	+	+	+

MAC; MacConkey, CV; Crystal Violet



Lane 1 molecular marker; lane 2; negative control; lane 3 to 8 amplified product of Hsp65 gene of 439 bp

Restriction enzyme analysis



Lane 1 ; DNA marker
Lane2,3; Bst: 80, 125,245, Hae; 135, 155 M.fortuitum
Lane 4,5; Bst: 220, 245, Hae 60,160 M.chelonae
Lane 6,7 ; Bst: 80,125, Hae; 135,150 M. fortuitum variant
Lane 8,9; Bst: 110, 125, 245 Hae; 120,140 M.gordonae
Lane 10,11; Bst: 85,125 Hae; 135,150 M.fortuitum variant
Lane 12,13; Bst: 85,245 Hae; 135,150 M.abscessus

Table 2: Species Identification and Isolation Sources of 121 Rapidly Growing Mycobacteria

Sl. No	Species	Sources							
		Sputum	Lymph node	Pleural fluid	skin	Tracheal aspirate	Ascetic fluid	CVP tip	Pus
1	*M.chelonae*(57)	35	11	06	01	01	01	01	01
2	*M. fortuitum*(42)	28	04	03	02	02	01	01	01
3	*M.abscessus*(11)	05	02	02	01	-	-	-	01
4	*M.immunogenum* (06)	03	01	-	-	-	01	-	01
5	*M. peregrinum* (02)	01	-	-	01	-	-	-	-
6	M. smegmatis (01)	-	-	-	-	-	-	01	-
7	*M. wolinskyi* (01)	-	-	-	-	-	-	01	-
8	*M. goodii* (01)	-	-	-	-	-	-	-	01

CVP; central venous pressure

In our study, 8 different types of RGM were identified through the same way as discussed by Telenti et al [5] and Plikaytis et al [13] for identification of RGM species.The study indicates that application of PCR-based methodology is useful at program level in order to differentiate the NTM in short span of time. After the introduction of liquid culture system at national level for diagnosis of tuberculosis,rapidly growing mycobacterium are common isolates in clinical samples in many laboratories. Without proper identification, these may inadvertently be designated as 'MDRTB'. PRA test is particularly useful in identifying mycobacteria when biochemical identification methods not able to differentiate the closely related species. The restriction enzyme digestion patterns obtained in the present study provides species separations similar to those described by Telenti et al [5], with the exception of few band size measurements. In addition, the usual recommendations to prevent PCR-linked contamination may be useful to avoid false identification. We used separate manipulation rooms for reagent preparation, specimen preparation,amplification and detection. Even specimen centrifugations were performed in a separate aerosol-free area (distinct from the above three areas).

The study conclude that restriction enzyme analysis for identification of rapid grower mycobacterium is a simple, sensitive, rapid, labour-saving and can be performed in routine. M.chelonae & M.fortuitum constitute 87% of total rapid growers and M.chelonae was the most common isolate in pulmonary as well as extrapulmonary specimen. Such a system should not be difficult to implement in reference laboratories, which would then be enabled to provide species identification of clinical isolates of RGM within a short span of 1 or 2 working days. The focus on the members of the rapidly growing mycobacteria will help in tackling the emerging important mycobacterial species in cases of sporadic infection or outbreaks.The lacunae of the test observed in the study was analyzing and reporting the result with 5-10 bp band difference is difficult.

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