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ORIGINAL ARTICLE

Evaluating the sensitivity of three primers using PCR-restriction fragment length polymorphism analysis for rapid identification of *Mycobacterium simiae* isolated from pulmonary tuberculosis patients

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ABSTRACT

Background: Nowadays the molecular methods widely use for rapid identification of *Mycobacterium* other than *tuberculosis* (MOTT). The *Mycobacterium simiae* isolates are cause of majority of human pulmonary diseases compared with other atypical mycobacteria. As sensitivity of primers and digestion patterns for diversified fragments is different, this survey evaluated the three various fragments using the PCR- restriction fragment length polymorphism analysis (PRA) for rapid diagnostic of *M. simiae* isolates.

Patients and methods: Strains that were identified as *M. simiae* (17 isolates) by phenotypic (photochromogen and positive niacin) methods were selected for this study. The fragments of the 16S-23S rRNA gene spacer and *hsp65* gene were amplified by PCR. Subsequently the amplicons were digested with three restriction enzyme namely *Ava*II, *Hph*I and *Hpa*II for a 644bp region of *hsp65* DNAs, *Bst*EII and *Hae*III endonucleases for 439bp region of *hsp65* gene (TB11 and TB12 fragment) and *Hae*III digestion for 225bp region of 16S-23S rRNA gene spacer.

Results: Of 962 culture positive specimens, 17(1.7%) were identified as *M. simiae* species; majority of them were multidrug-resistance (12; 70.5%). The overall detection rate by Tb11, Tb12 and SP primers were 82.3% whereas *hsp65* primer was 100% ($p>0.005$). We also found out that the *Hpa*II and *Hph*I enzymes were more specific to distinguish *M. simiae* species than other restriction enzyme used in this study.

Conclusion: The high discriminative power of *hsp65* pattern particularly *Hpa*II digestion, provide an exact and cost-effective method for rapid identification of *M. simiae* strains among registered pulmonary cases.

Keywords: *Mycobacterium simiae, PRA method, Rapid identification.*
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INTRODUCTION

Recently, there have been increasing evidences that species of mycobacteria other than tubercle bacilli (MOTT), which are also called environmental mycobacteria (EM) or non-tuberculosis mycobacterium (NTM), are implicated in a variety of human disease (1). *Mycobacterium simiae* complex is comprised of several phylogenetically related species, including *M.*

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simiae, *M. triplex*, *M. genaveuse*, *M. heidelbergens* and *M. lentiflavum* (2). The most common species associated with human pathology has been *M. simiae* which was first isolated from rhesus macques in 1965 (2,3), although disease in human was described several years later on (4-6). Recently *M. simiae* has been recognized as an agent of human pulmonary disease in patients with AIDS, as well as in immunocompetent patients (7). This species is a slow-growing photochromogen, appearing rust-colored after exposure to light, and is the only non-tuberculosis mycobacterium (NTM) that, like *M. tuberculosis*, is niacin positive (2). *M. simiae* strains are resistant to commonly used disinfectants and the appearance of multidrug-resistant strains have raised the question about the prevalence of *M. simiae* strains among registered pulmonary tuberculosis (PTB) cases. Therefore, there was an intensified need for the increased use of rapid methods for detection of such cases.

One of the molecular methods for rapid identification of *M. simiae* isolates is PCR-restriction fragment length polymorphism analysis (PRA) (1,8-11). PCR assays using genus group specific amplification followed by restriction enzyme analysis, have been described for the analysis of gene regions like *hsp65* (1,8,10, 12) and rRNA gene spacer (9,13,14-16) which have been found to be useful for identification of different mycobacteria (7).

With respect to the high prevalence of *M. simiae* in respiratory specimens, we aimed to determine which of the previously advised methods are more sensitive for rapid detection and identification of *M. simiae* isolates.

PATIENTS and METHODS

This study was performed in the National Research Institute of Tuberculosis and Lung Diseases (NRITLD, Tehran, Iran), which acts as the reference unit for National Tuberculosis Program. In this centre, diagnosis and treatment of disease caused by non-tuberculosis mycobacteria fulfils with regard to the American Thoracic Society (ATS) criteria (17).

Bacterial strains: Primary isolation and culturing of mycobacteria isolated form sputum specimen were followed in accordance to prior studies (18). The isolates were identified as *M. simiae* using biochemical tests, including production of niacin, catalase activity, nitrate reduction, pigment production and growth rate (7,18), then were confirmed with PCR-RFLP methods (8-10). Drug susceptibility testing against isoniazid (INH), rifampicin (RF), streptomycin (SM), ethambutol (ETB) and pyrazinamide (PZA) were performed by the proportional method on Lowen Stein-Jensen media at a concentration of 0.2, 40, 4.0 and 2.0 µg/ml, respectively (19). DNA extraction was performed by phenol-chloroform method.

TB PCR-RFLP typing: Five microliters of lysate was added to each reaction tube. The composition of the PCR mixture (50µl) was 50mM KCl, 10mM Tris- HCl (pH 8.3), 1.5mM $MgCl_2$, 200µM (each) deoxynucleoside triphosphate, 0.5µM (each) primer, and 1.25U of *Taq* polymerase (Roche). The reaction was subjected to 45 cycles of amplification (1min at 94°C, 1min at 60°C, 1min at 72°C); followed by 10 min of extension at 72°C. Primers Tb11 (5´-ACCAACGATGGTGTGTCCAT) and Tb12 (5´-CTTGTCGAACCGCATACCCT) amplified a 439bp fragment between positions 398 and 836 of the published gene sequence (20). PCR products were digested separately with restriction enzyme *Hae*III and *Bst*EII according to the recommendations of the manufacturers and electrophoresed on %2 agarose gel.

*16S-23S rRNA gene spacer PCR-RFLP typing:*Amplification of a part of the 16S-23S spacer was performed with primers Sp1 (5'-ACC TCC TTT CTA AGG AGC ACC-3') and Sp2 (5'-GAT GCT CGC AAC CAC TAT CCA-3'). The amplification was done with a 50-µl reaction mixture containing 10mM Tris-HCl (pH 8.3),

50mM KCl, 1.5mM $MgCl_2$, 200µM (each) deoxynucleoside triphosphate (dATP, dGTP, dCTP, and dUTP), 75ng of each primer, 1U of *Thermus aquaticus* DNA polymerase (Roche), and 5µl of DNA. The thermal profile involved initial denaturation for 5 min at 96°C and 38 cycles with the following steps: 1-min denaturation at 94°C, annealing at 59°C, and extension at 72°C (9). *Hae*III enzyme digestion of the amplification product was performed essentially as described previously (9).

Hsp65 PCR-RFLP typing: A set of primers [forward primer HSPF3 (5'-ATC GCC AAG GAG ATC GAG CT-3'), reverse primer HSPR4 (5'-AAG GTG CCG CGG ATC TTG TT-3')] were used to amplify 644 bp *hsp65* DNAs from mycobacteria strains and sputa. Template DNA (50ng) and 20pmol of each primer were added to a PCR mixture tube containing 1U of *Taq* DNA polymerase, 250µM of each deoxynucleoside triphosphate, 50mM Tris-HCl (pH 8.3), 40mM KCl, and 1.5mM $MgCl_2$. The reaction mixture was subjected to 30 amplification cycles (60 s at 95°C, 45 s at 62°C, and 90 s at 72°C) followed by a 5-min extension at 72°C (8). After confirming the successful amplification of the 644bp PCR products, they were subjected to restriction enzyme digestion by 3 enzymes, *Ava*II, *Hph*I and *Hpa*II according to the described method in the protocols (8). Following digestion, mixture was electrophoresed on %2 agarose gel.

RESULTS

Of 962 isolates obtained from PTB cases, 17(1.7%) were identified as *M. simiae* using the biochemical and phenotypic tests. All of *M. simiae* specimens were confirmed with PCR-RFLP methods. *M. simiae* was isolated from both Iranian and Afghan TB patients. Interestingly, 12 (70.5%) were MDR strains.

Detection by Tb11, Tb12 primers and RFLP patterns: The size of PCR products (340bp) was the same in both Tb11 and Tb12 primers, however, their digested patterns were different when restriction enzyme (*Hae*III and *Bst*EII) were applied. *Bst*EII patterns displayed two fragments of 245 and 220bp, in contrast, the *Hae*III displayed 200 and 135bp. Our results showed that 13 (76.4%) *M. simiae* produced standard pattern by *Bst*EII enzyme, whereas by *Hae*III only 11(64.7%) isolates had the standard patterns. Therefore, the sensitivity of *Bst*EII was higher than *Hae*III.

Detection by rRNA gene spacer primers and RFLP patterns: The size of PCR products that obtained by 16S-23S rRNA gene spacer primer was different in various *M. simiae* species (range from 200-300bp); the standard *M. simiae* (ATCC) had 250bp. Of 17 isolates, 14 (82.3%) produced three fragments of 85, 80 and 42bp after digestion by *Hae*III restriction enzyme. Therefore, sensitivity of this endonuclease was estimated 82.3%.

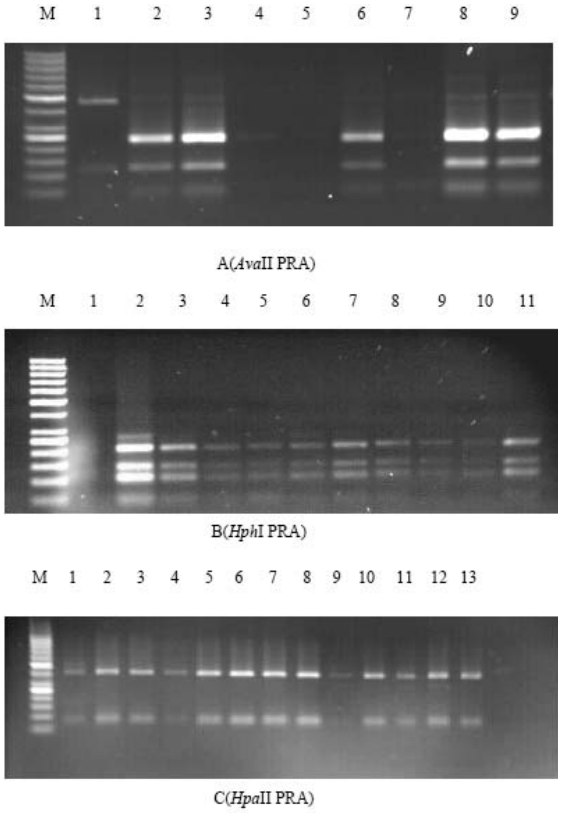

Figure 1. The identification of *Mycobacterium simiae* isolates by hsp65 PRA. The amplified hsp65 DNAs (644bp) of *M. simiae* strains were digested with AvaII (A), HphI (B) and HpaII (C) and electrophoresed on 2% agarose gel. M: marker DNA (50bp ladder).

Detection by hsp65 primers and RFLP patterns: The *hsp65* primer produced a 644bp fragment of the gene encoding 65-kDa heat-shock protein. PCR products were digested with three different restriction endonucleases (*Ava*II, *Hph*I and *Hpa*II). In *M. simiae*, the 644bp fragment produced three fragments of 234 and 119bp by *Ava*II digestion. In our setting, 13(76.4%) samples had the standard pattern, in contrary; *Hph*I enzyme displayed three fragments of 206, 141 and 103bp. Results showed that 15(88.2%) isolates had standard pattern. In contrast, the sensitivity of *Hpa*II-PRA for slow growing species of *M.simiae* relative to *Ava*II and *Hph*I was 100%, representing 80 and 399bp fragments (figure 1).

DISCUSSION

Hsp65 pattern provides a rapid method for identification of *M. simiae* which is the major agent of human pulmonary disease in comparison to other NTM. *M. simiae* is the only non-tuberculosis mycobacterium (NTM) that, like *M.tuberculosis*, is niacin positive (2). Hence, it is not easy to differentiate *M. simiae* from *M. tuberculosis* based on traditional tests like niacin production and molecular methods must be used. *M. simiae* has been recognized as an agent of human pulmonary disease in patients with AIDS (21) or associated with human T lymphothropic virus type 1(HTLV1) (6). Recent studies revealed that *M. simiae* is the most common pathogenic NTM in respiratory specimens (22).

In the present study, of 962 isolates obtained from PTB cases, 5% were identified as MOTT by phenotypic tests; 35% of these NTM cases were *M. simiae.* In fact, *M. simiae* is a common isolate from clinical specimens in Iran, that majority of them (70%) are multidrug-resistance.

Since *M. simiae* is transmitted by inhalation of aerosols or by inoculation (22) and with respect to its resistance patterns (21), rapid identification of *M. simiae* is of utmost importance. The detection and timely treatment of pulmonary infection with MDR can prevent disease transmission, reduce the risk of drug resistance and avoid further lung damage (23).

Several molecular methods were suggested for rapid identification of *M. simiae*, of which PRA method has advantages due to its ease and rapidity (8). The sensitivity of PRA method targeting various fragments is different, therefore, the purposes of this study was to compare three various fragment-PRAs for identification of *M. simiae* strains.

Telenti et al suggested a reliable and rapid procedure for routine identification of mycobacteria by amplification of TB11, TB12 fragment and digestion pattern (10). Although, in their study TB PRA pattern differentiated mycobacterial isolates successfully, the number of *M. simiae* isolates was very low (1 isolate) and could therefore not determine sensitivity of PRA-TB method for identification of this strain. In our study, 17 *M. simiae* were isolated by biochemical tests, of which 76% were differentiated using this method.

Roth et al suggested a novel diagnostic algorithm for identification of mycobacteria using genus-specific amplification of the 16S-23S rRNA gene spacer and restriction endonucleases. In their study, of 678 acid-fast isolates 15 strains were identified as *M. simiae* (9).

The 16S-23S rRNA gene spacer PRA in comparison to phenotypic tests was selected as superior method for rapid identification of mycobacteria by several previous studies (13,14), but sensitivity of this method was not collated with other molecular techniques for rapid identification of *M. simiae.* In our setting, of 17 *M. simiae* 14(82.3%) strains were correctly identified by SP PRA pattern using the *Hae*III enzyme.

PRA schemes targeting *hsp65* have been most widely used, since this molecule is conserved in all mycobacteria and related strains, and because it shows sufficient sequence variation to allow

mycobacteria differentiation at the species or strain level (8,12). The *hsp65* PRA allowed differentiation between subspecies of many of mycobacteria strains (1,8,12). Hong Kim et al demonstrated new PRA method targeting the 644bp *hsp65* gene that has better resolution than the previous PRA method. The PRA-*hsp65* pattern, when compared with other methods, showed short running time of electrophoresise, fragments with apart sizes and lower time for obtaining results. Recently, studies have showed that the 644bp *hsp65* gene PRA is easier, more rapid and cost-effective method than 16S rRNA gene for differentiation of mycobacteria (24). Chimara et al exploited hsp65 algorithm for identification of 333 isolates prosperously, however, there was no *M. simiae* strain among them (12). In this study, the 644bp fragment of *hsp65* gene was amplified from culture positive specimens. Also amplification of this fragment directly from 2 and 3 plus sputum samples was performed successfully. This approach was followed using three restriction enzymes. The high discriminative power of *Hpa*II pattern propounded it as a superior enzyme for *hsp65* profile. Our results revealed that *Ava*II digestion had lower specificity (76%) when compared with other patterns. Enzyme digestion of *hsp65* profile has proven similar effectiveness in other studies (24). Up to now, investigators have not particularly evaluated sensitivity of different methods for rapid identification of *M. simiae* species, however, we suggest *hsp65* PRA specially *Hpa*II pattern for differentiation of *M. simiae*.

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