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A new method for species identification and differentiation of *Mycobacterium chelonae* complex based on amplified *hsp65* restriction analysis (AHSPRA)

Suresh B. Selvaraju, Izhar U.H. Khan, Jagjit S. Yadav*

Molecular Toxicology Division, Department of Environmental Health, University of Cincinnati Medical Center, Cincinnati, OH 45267-0056, USA

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Abstract

Members of the *Mycobacterium chelonae* complex (MCC), namely *M. chelonae*, *Mycobacterium abscessus* and *Mycobacterium immunogenum*, have been implicated in nosocomial infections and occupational respiratory illnesses like hypersensitivity pneumonitis (HP) associated with contaminated metalworking fluid (MWF) exposures. Close relationship among these member species makes their differentiation cumbersome using the existing methods. Here we report a simple and rapid method for unambiguous identification and differentiation of the three-member species of the MCC group with PCR-restriction analysis targeting a 667-bp segment of a variable region of the 65-kDa-heat shock protein (*hsp65*) gene. This assay, described as Amplified *hsp65* Restriction Analysis (AHSPRA), can discriminate all the three individual species using a one-step restriction digestion using either *Bbv*I or *Eco*0109I. The enzyme *Nar*I can differentiate *M. immunogenum* from the other two MCC species (*M. chelonae* and *M. abscessus*). The developed method was validated using several non-MCC reference species of other rapidly growing mycobacteria (RGM) and MCC field isolates from MWF samples. Direct cell-lysis was used instead of the conventional DNA template preparation, which improved the rapidity, simplicity and adaptability of the developed method. The results suggest that the developed method can unambiguously differentiate species of the *M. chelonae* complex from other RGM species and from one another.

Keywords: *Mycobacterium immunogenum*; *M. chelonae*; *M. abscessus*; Metalworking fluid; AHSPRA; *hsp65* gene

1. Introduction

Nontuberculous mycobacteria (NTM) are environmentally associated organisms and have emerged as one of the major causatives of opportunistic respiratory infections and illnesses. Among different nontuberculous mycobacteria, the *Mycobacterium chelonae* complex (MCC) is an important group associated with contaminated hospital washing equipment and metalworking fluid environment and consists of three closely related member species, *M. chelonae*, *Mycobacterium abscessus* and the newly described *Mycobacterium immunogenum* [1]. Specifically, the MCC group has been implicated in pseudo-outbreaks involving contaminated bronchoscopes [1] and in metalworking fluid-associated hypersensitivity pneumonitis (HP) [2–4]. Potential clinical significance of individual MCC species and the need for their appropriate specific treatment or control regimen [5] demands the development of a species differentiating method that provides accurate and unambiguous results in a more timely fashion and is simple and adaptable in reference or diagnostic laboratories.

Differentiation of the MCC species with conventional biochemical methods such as sodium chloride tolerance and utilization of citrate [6] requires long time and is labor intensive. Mycolic acid profiling based on high-performance liquid chromatography (HPLC) also has limited resolving potential with this group [7]. Some of the newer molecular techniques used for differentiating the mycobacterial species are either not applicable to MCC or have their own limitations. For instance, the Accuprobe assay, which is

* Corresponding author. Tel.: +1 513 558 4806; fax: +1 513 558 4397.
E-mail address: jagjit.yadav@uc.edu (J.S. Yadav).

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based on 16S rDNA, is not applicable to MCC species. The 16S rDNA sequencing method requires technical infrastructure and time and is relatively expensive [8]. More importantly, the 16S rDNA sequencing based assay has low resolution power due to fewer number of base pair differences among the three-member species [4]. Restriction enzyme analysis of *Mycobacterium* specific PCR products has shown promise in yielding species-specific DNA patterns. In particular, PCR-restriction analysis (PRA) based on 441-bp region of the *hsp65* gene [9] has been used to identify several species of *Mycobacterium* [10–13]. However, this classical PRA method fails to differentiate some nontuberculous mycobacterial species [14] including MCC species [4]. For instance, the classical PRA method led to poor or no amplicon signal in *M. immunogenum* [15], as also demonstrated in Section 3 of this study for field isolates of this species. Further, intraspecies sequence polymorphism for the recommended restriction enzymes used in this method was reported earlier in *M. chelonae* [4,15]. In view of these limitations, a new method designated as Amplified *hsp65* Restriction Analysis (AHSPRA), was developed based on expanded variable region (667-bp) of the *hsp65* gene, newly designed consensus primers and a newly selected set of restriction enzymes. The developed AHSPRA method is simple and rapid, as it requires a single-step restriction analysis as compared to a two-step digestion in the existing method [4] and a rapid direct cell-lysis step for template preparation.

2. Materials and methods

2.1. Mycobacterial strains and culture conditions

The *M. chelonae* complex (MCC) species, used in this study were *M. immunogenum* ATCC 700506, *M. chelonae* ATCC 35752^T and *M. abscessus* ATCC 19977^T. Other non-pigmenting rapidly growing mycobacteria (RGM), *M. fortuitum* ATCC 6841^T, *M. mageritense* ATCC 700351^T, *M. mucogenicum* ATCC 49650^T, *M. peregrinum* ATCC 14467^T, *M. senegalense* ATCC 35796^T, *M. smegmatis* ATCC 19420^T, *M. vaccae* ATCC 15483^T, *M. wolinskyi* ATCC 700010^T and pigmenting RGM species *M. phlei* ATCC 11758^T were used for restriction pattern comparison and validation. In addition to these ATCC mycobacterial reference strains, 13 MCC isolates from metalworking fluids (M-JY1–M-JY4 and M-JY6—M-JY14) and one non-MCC isolate (M-JY5) were included.

The mycobacterial species and isolates were subcultured on agar plates using Middlebrook (MB) 7H10 agar or in liquid cultures using 7H9 broth (Difco, Detroit, MI), both supplemented with Oleic acid–Albumin–Dextrose–Catalase (OADC, BD Biosciences, Sparks, MD) enrichment. The liquid cultures were grown with continuous shaking (150 rpm) at 37 °C until a 120 Klett reading was obtained, as measured using Klett Photoelectric Colorimeter (Klett, New York).

2.2. Direct cell-lysis based hsp65 PCR

PCR was performed using our previously developed single-tube-direct lysis method for template preparation [15]. A single colony was taken directly in the amplification tube (0.2 ml) containing 5 μl of lysis solution and suspended by gentle mixing. The contents were subjected to lysis using a brief thermal regime (98 °C for 5 min and 4 °C for 1 min) using GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA). The tube containing the crude DNA-lysate was used directly for the subsequent PCR reaction.

A new pair of primers was designed based on the conserved end regions of a 667-bp variable region, identified by alignment of the available *hsp65* gene sequences for different mycobacteria (DNASTAR, Inc., Madison, WI). The newly designed primers had the following sequences: hsp667-forward (5′-GGC CAA GAC AAT TGC GTA CG-3′) and hsp667-reverse (5′-GGA GCT GAC CAG CAG GAT G-3′). The amplification was performed using GeneAmp PCR system 9700 (Applied Biosystems, Foster City, CA) and a 50-μl reaction mixture containing 50 ng of template DNA, 1.25 units of *Ex Taq* DNA polymerase (Panvera, Madison, WI), 1× *Ex Taq* buffer with $MgCl_2$, 200 μM of each of the four dNTPs and 100 ng each of the forward and reverse primers. Reaction mixtures were subjected to amplification for 30 cycles (each cycle using 94 °C for 45 s, 57 °C for 45 s and 72 °C for 45 s) followed by 5 min incubation at 72 °C. The PCR products were electrophoresed on a 1% Trevigel gel matrix (Trevigen, Gaithersburg, MD) with 1× TAE buffer containing ethidium bromide (0.5 μg ml^{-1}) and using 5 μl of a 100 bp DNA size marker (Invitrogen Corp., Carlsbad, CA).

2.3. Comparative amplifiability of 441-bp versus 667-bp hsp65 regions

In order to compare amplifiability of 441 versus 667 bp segments of the *hsp65* gene, extracted genomic DNA was quantified and equalized to 50 ng/μl. Equal amount (50 ng) of DNA was used for amplification of the two target regions. Amplification of 16S rDNA region was used as a control for amplifiability. The 441-bp fragment was amplified with primers TB11 and TB12 as recommended [9] and the 16S rDNA region were amplified by using the previously standardized method [16].

2.4. Development and validation of the Amplified hsp65 Restriction Analysis (AHSPRA) assay

The 667-bp amplicons generated based on direct lysis-PCR were clarified to remove cell debris, salts and detergents of the lysis solution to facilitate the restriction digestion. The PCR tubes with amplicons were centrifuged

at 13,000 rpm for 2 min to pellet the cell debris and the supernatants were clarified using Montage PCR Centrifugal Filters (Millipore Corp., Bedford, MA) per the manufacturer's recommendations.

Each of the purified 667-bp PCR products (8 μl) was digested in a 20 μl reaction mixture using 10 units of one of the three identified restriction enzymes, *Bbv*I or *Eco*0109I or *Nar*I (New England Biolabs, Beverly, MA) using appropriate reaction buffer supplied by the manufacturer. The resulting restriction fragments for each enzyme reaction were resolved by electrophoresis on 1.5% Trevigel gel matrix at 70 V for 2 h using 100 bp DNA ladder (Invitrogen Corp., Carlsbad, CA). Fragment sizes were estimated visually by comparison with appropriate controls run in parallel (positive controls included ATCC strains of *M. immunogenum*, *M. chelonae* and *M. abscessus*; negative controls included ATCC strains of non-MCC species).

2.5. Sequencing of 667-bp amplicons of MCC species

The 667-bp PCR products for the ATCC reference strains of *M. immunogenum*, *M. chelonae* and *M. abscessus* were cloned using TOPO Cloning Kit (Invitrogen Corp., Carlsbad, CA) following manufacturer's recommendations. The plasmid inserts were sequenced at the University's DNA core facility. Amplicons for the field isolates were directly sequenced. Deduced restriction maps of the sequences for the three MCC reference species were prepared using Gene Runner program (Hastings Software, Inc., Hudson, NY) to determine the appropriate species-specific restriction sites and to select appropriate commercial restriction enzymes.

2.6. Nucleotide sequence accession numbers

Sequences of the 667-bp amplicons for the three MCC-member species (ATCC strains) were submitted to GenBank with the accession numbers AY498741, AY498742 and AY498743 for *M. immunogenum*, *M. chelonae* and *M. abscessus*, respectively.

3. Results and discussion

3.1. Relative amplifiability of 667-bp versus 441-bp hsp65 fragments in M. immunogenum

In our recent efforts to identify individual member species of the *M. chelonae* complex, the classical 441-bp *hsp65*-PCR method yielded low or no amplicon signals for *M. immunogenum* using its reference strains (15). In the present study, field isolates of *M. immunogenum* were tested to confirm the low amplifiability of the 441-bp region. This included comparison of the amplifiability of the *hsp65* target region by the classical versus the new method, developed in this study.

The *hsp65* PCR protocol based on the expanded (667-bp) variable region, developed as described under Section 2, yielded an apparently same size (667-bp) amplicon in all 14 MWF isolates and the 12 ATCC reference species of mycobacteria (data not shown). This showed broader applicability of the designed primers for different species and strains of rapidly growing mycobacteria.

Amplicon intensities for 441 and 667-bp fragments of *hsp65* gene of mycobacterial isolates and reference strains (MCC and non-MCC) were compared using equal quantity of genomic DNA template (50 ng). The 441-bp based PCR reaction yielded a weak amplification in *M. immunogenum* reference strain and its MWF isolates M-JY3, M-JY4, M-JY10, M-JY12, M-JY13, and M-JY14 (Fig. 1A) as compared to the other reference species. In contrast, strong amplicon intensity was observed for the 667-bp target in all the reference strains and the isolates (Fig. 1B). The control target 16S rDNA (924 bp) also yielded equally good amplification (Fig. 1C) indicating that the poor amplifiability of the 441-bp *hsp65* target region in *M. immunogenum* is not due to poor DNA quality in *M. immunogenum* and its isolates. Instead, it is due to non-optimal factors such as nucleotide sequence variation in the primer-binding region (15) as confirmed by 667-bp amplicon sequencing. The poor amplification interferes with the detection limit of the assay as well as yields an insufficient amplicon quantity for subsequent restriction analysis for species identification and differentiation.

3.2. hsp667-bp based PCR-restriction analysis

In the original PRA method proposed by Telenti et al. [9] based on 441-bp region, the recommended restriction enzyme of the method, *Bst*EII, was not applicable to *M. immunogenum* because of lack of restriction site in its 441-bp region and the other enzyme, *Hae*III did not provide unique restriction pattern for *M. immunogenum* [4]. Consequently, a modified version of the classical 441-bp-based restriction analysis method was developed based on a combination of restriction enzymes to differentiate species of *M. chelonae* complex; however, it involved a two-step restriction analysis in which the individual enzyme patterns could differentiate only two of the three species of the MCC group [4]. Furthermore, intraspecies sequence polymorphism in the targeted restriction sites of this modified 441-bp method yielded different restriction patterns within the same species such as *M. chelonae* as observed by Wilson et al. [4] and in our studies (data not shown). Similar intraspecies polymorphism was also reported for *M. peregrinum* [18]. Likewise, the 441-bp PRA method was found to be unsuitable in discriminating several mycobacterial species in other studies as well [11,13,17]. As a result, further standardization of the 441-bp based PRA method has been recommended by earlier reports to differentiate the rapidly growing mycobacteria [13,19].

In the developed Amplified *hsp65* gene Restriction Analysis (AHSPRA) protocol, the 667-bp amplicons for

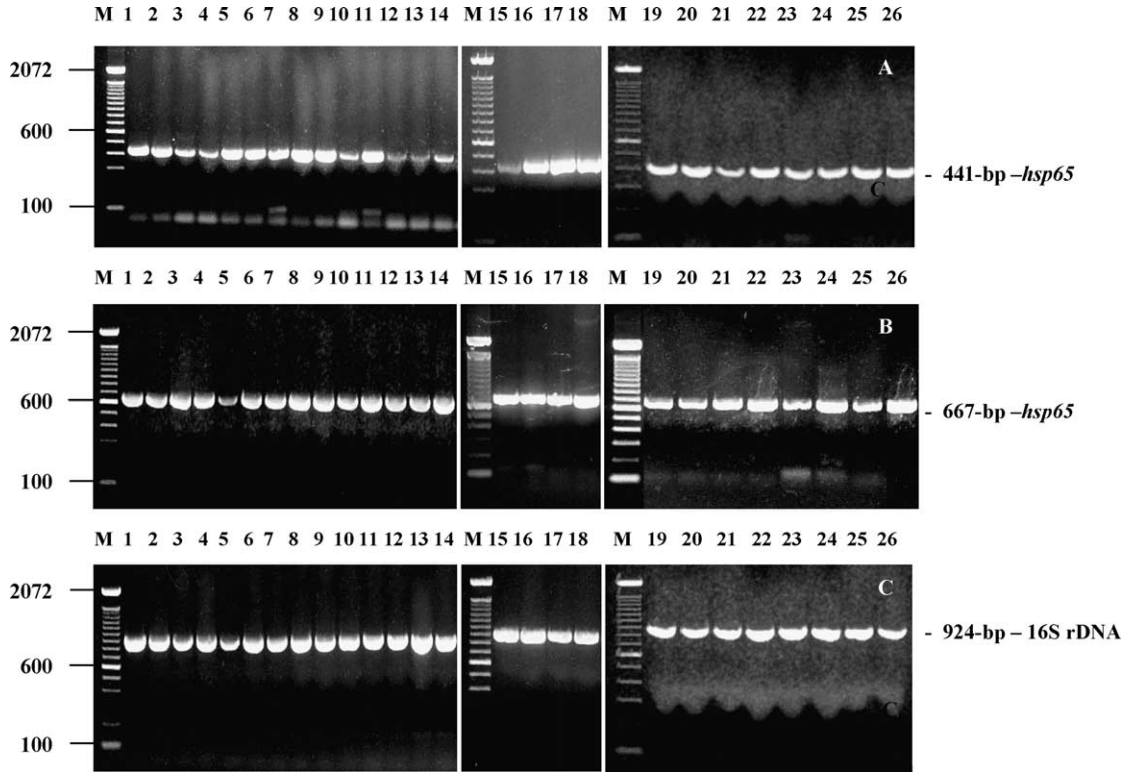

Fig. 1. Comparison of amplification signals for 441-bp and 667-bp regions of 65-kDa-heat shock protein gene *hsp65* and 16S rDNA region using equal quantity of genomic DNA template of *M. chelonae* complex (MCC) and non-MCC reference species and MCC isolates. Panel A, B and C: amplification of 441-bp *hsp65* region, 667-bp *hsp65* region and 16 S rDNA region, respectively. Lanes 1–14: MCC isolates (M-JY1–M-JY14) from metalworking fluid samples. Lanes 15–26: ATCC reference strains of *M. immunogenum*, *M. chelonae*, *M. abscessus*, *M. smegmatis*, *M. fortuitum*, *M. mageritense*, *M. mucogenicum*, *M. peregrinum*, *M. phlei*, *M. senegalense*, *M. vaccae* and *M. wolinskyi*. Lane M: 100 bp DNA size marker (Invitrogen Corp., Carlsbad, CA).

different reference species and the isolates were subjected to restriction analysis using any one of the *in silico* selected endonucleases, *Bbv*I or *Eco*0109I or *Nar*I, as described in Section 2. The estimated sizes of all the restriction fragments generated with each of these enzymes are listed in Table 1. Only fragments >70 bp were included in the actual analysis considering the resolving power of the gel separation conditions used.

The three reference species of the MCC group, *M. immunogenum*, *M. chelonae* and *M. abscessus*, yielded unique species-specific restriction patterns with either *Bbv*I or *Eco*0109I enzyme; however, *Nar*I yielded a distinct pattern for *M. immunogenum* but a common pattern for *M. chelonae* and *M. abscessus*. AHSPRA analysis using *Bbv*I digestion yielded the following fragment sizes: *M. immunogenum* (two fragments of 559 and 108 bp), *M. chelonae* (three fragments of 304, 255 and 108 bp) and *M. abscessus* (three fragments of 372, 187 and 108 bp) (Fig. 2A). In *Eco*0109I restriction analysis, *M. chelonae* was discriminated by the presence of two fragments, 483 and 95 bp instead of single fragment of 578 bp in *M. immunogenum*; *M. abscessus* differed from the other two species by the presence of an additional restriction site yielding unique 381 and 102 bp fragments (Fig. 2B). In *Nar*I restriction analysis, *M. immunogenum* was differentiated from the other two species by the presence of a 75 bp fragment (Fig. 2C).

AHSPRA patterns of the 14 MWF isolates using each of the three enzymes showed that seven isolates (M-JY1, M-JY2, M-JY6, M-JY7, M-JY8, M-JY9, and M-JY11) belonged to *M. chelonae* and the remaining isolates (M-JY3, M-JY4, M-JY10, M-JY12, M-JY13, and M-JY14) belonged to *M. immunogenum* (Fig. 2A, B and C). M-JY5, which was previously identified as *M. diernhoferi* [16], yielded a different pattern on restriction with any of the three enzymes (Fig. 2A, B and C). Likewise, the non-MCC reference

Table 1
Restriction fragments[a] of the *hsp65* gene amplicon (667-bp) for *M. chelonae* complex member species generated by *Bbv*I, *Eco*0109I and *Nar*I enzymes

MCC species	Restriction fragment sizes (bp)		
	*Bbv*I	*Eco*0109I	*Nar*I
M. immunogenum (ATCC 700506)	559, 108	578, 49, 40	324, 139, 129, 75
M. chelonae (ATCC 35752)	304, 255, 108	483, 95, 49, 40	343, 195, 129
M. abscessus (ATCC 19977)	372, 187, 108	381, 102, 95, 49, 40	343, 195, 129

[a] AHSPRA patterns were based on >70 bp fragments in gel analysis.

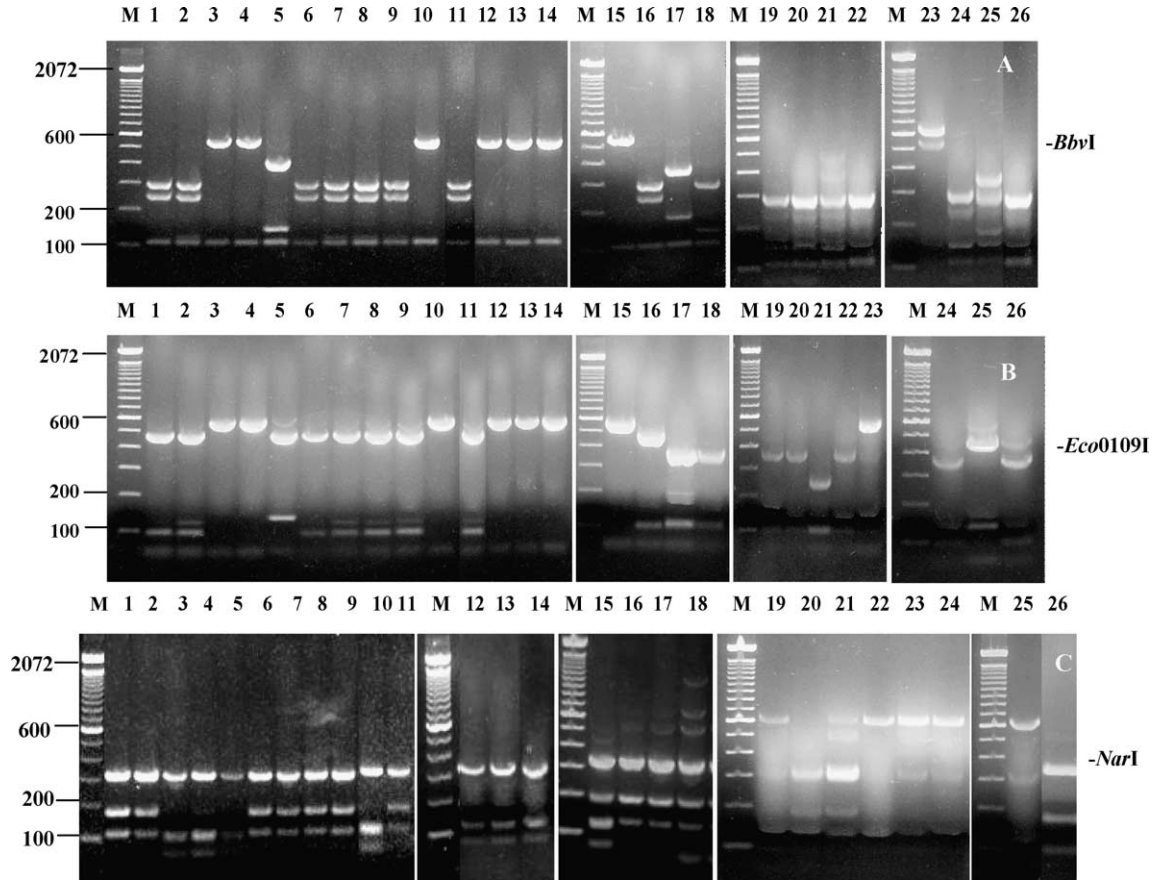

Fig. 2. Comparison of Amplified *hsp65* Restriction Analysis (AHSPRA) patterns for *M. chelonae* complex member species (*M. immunogenum*, *M. chelonae* and *M. abscessus*), MCC isolates from metalworking fluids, and non-MCC species, based on the target 667-bp variable sequence region of the *hsp65* gene. Panels A, B and C: *Bbv*I, *Eco*0109I and *Nar*I restriction patterns, respectively, of 12 ATCC reference strains of MCC and non-MCC species and 14 MCC-isolates. Lanes 1–14: *M. chelonae* complex isolates (M-JY1–M-JY14) from metalworking fluids. Lanes 15–26: ATCC reference strains of *M. immunogenum*, *M. chelonae*, *M. abscessus*, *M. smegmatis*, *M. fortuitum*, *M. mageritense*, *M. mucogenicum*, *M. peregrinum*, *M. phlei*, *M. senegalense*, *M. vaccae* and *M. wolinskyi*. Lane M: 100 bp DNA size marker (Invitrogen Corp., Carlsbad, CA).

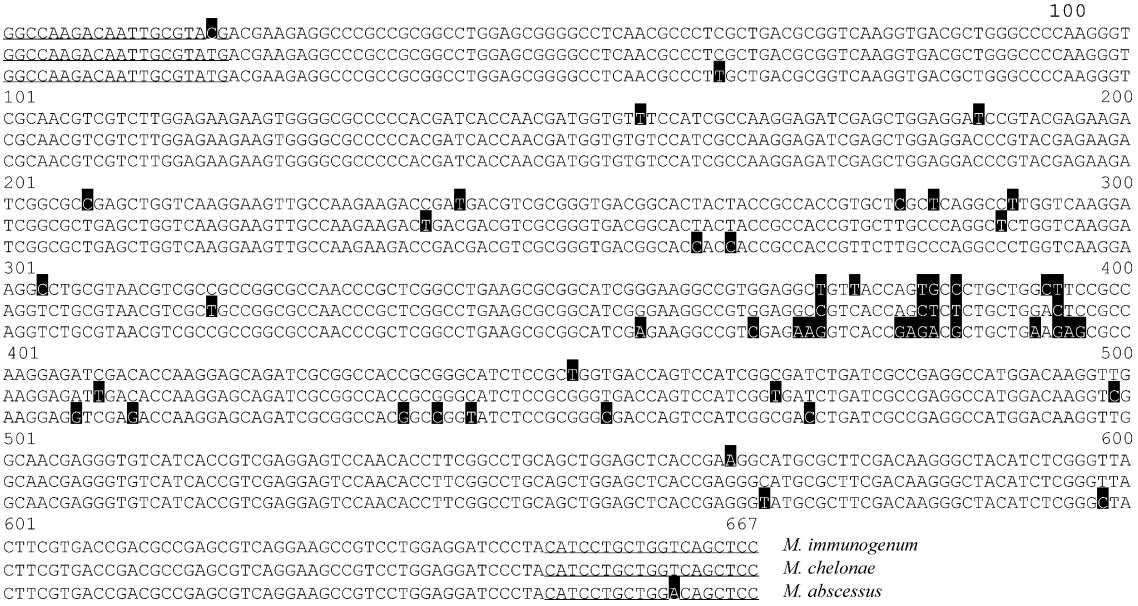

Fig. 3. Sequence alignment of the amplified 667-bp *hsp65* regions of the MCC member species, *M. immunogenum*, *M. chelonae* and *M. abscessus*. Terminal sequences corresponding to the primers are underlined at both ends and the shaded areas indicate the nucleotide base differences in the aligned sequences.

species yielded either entirely different restriction patterns or did not show AHSPRA pattern due to lack of any restriction site unlike the MCC species (Fig. 2A, B and C). The above results clearly indicate that the 667-bp based AHSPRA method will be practically useful for an unambiguous identification and differentiation of MCC species from other fast growing nontuberculous mycobacteria and from one another. Hence this method provides an improved alternative to the classical *hsp*441-bp method for MCC species.

Multiple alignment of the sequences for the three *hsp65* amplicons (667-bp) showed that *M. immunogenum*, *M. chelonae* and *M. abscessus* differed from one another in several bases underlying the unique restriction patterns for the individual species. *M. immunogenum* differed from the other two members in 18 bases; the corresponding numbers for *M. chelonae* and *M. abscessus* were 11 and 27 bases, respectively (Fig. 3). In order to verify the AHSPRA-based species identification, amplicon sequences from the 14 isolates were compared with those of the MCC reference species, *M. immunogenum* and *M. chelonae*; this led to similar species assignment for the corresponding isolates by the two methods (AHSPRA and amplicon sequencing). Sequencing technique is an effective differentiation tool for closely related organisms [20]. However, it is not very adaptable for routine practical diagnostic or analytical purposes in terms of rapidity and expenses.

Collectively, the results on reference species (MCC and non-MCC) as well as MCC field isolates proved that AHSPRA method could efficiently differentiate the individual *M. chelonae* complex species isolated from diverse sources. Moreover, the method is rapid as it involves single-tube-direct cell-lysis for template preparation bypassing the conventional time-consuming culturing step and long DNA extraction protocol. The developed method can be completed in about 4.5 h, including template preparation (6 min), PCR amplification (90 min), amplicons clarification (14 min), restriction digestion (60 min) and gel separation of restricted fragments (90 min). The 667-bp based AHSPRA typing offers an advantage over 441-bp based PRA method because of its improved specificity for species as well as strains of the *M. chelonae* complex and involvement of a single-step restriction digestion versus the two-step digestion. Moreover, the AHSPRA method could be easily adapted as a stand-alone technique in diagnostic or reference laboratories to detect and differentiate the MCC species, as it is based on unique restriction patterns for the individual MCC-members and is simpler, rapid and more specific as compared to the existing methods.

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