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hsp65 PCR-restriction analysis (PRA) with capillary electrophoresis for species identification and differentiation of *Mycobacterium kansasii* and *Mycobacterium chelonae–Mycobacterium abscessus* group

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SUMMARY

Objectives: The aim of the present study was to identify and differentiate *Mycobacterium kansasii* and *Mycobacterium chelonae–Mycobacterium abscessus* group strains isolated from clinical and environmental sources in different countries.

Methods: PCR-restriction analysis of the *hsp65* gene (PRA) with automated capillary electrophoresis was applied to the isolates previously identified by conventional biochemical testing and the molecular INNO-LiPA MYCOBACTERIA assay.

Results: PRA performed very well in comparison with the two other methods (96.4% concordance). Among 27 *M. kansasii* isolates, this method detected five genetic types, of which type 1 represented the most common clinical isolate, as it is worldwide. PRA differentiated 29 *M. chelonae–M. abscessus* group isolates into *Mycobacterium immunogenum* type 2 ($n = 13$), *M. chelonae* ($n = 12$), and *M. abscessus* types 1 ($n = 1$) and 2 ($n = 1$). *M. immunogenum* was the most frequent (69%) isolate from humans, but only one of 11 cases was clinically significant. *M. chelonae* was the most commonly (83%) recovered from water. PRA also identified two isolates with *hsp65* alleles representing previously unreported patterns.

Conclusions: PRA based on automated capillary electrophoresis is a rapid, simple, and reliable method for the identification and differentiation of both clinically relevant and environmental isolates of *M. kansasii* and *M. chelonae–M. abscessus* group.

1. Introduction

Among the most frequently isolated clinical strains of non-tuberculous mycobacteria (NTM) are *Mycobacterium kansasii* and *Mycobacterium chelonae–Mycobacterium abscessus* group.[1] *M. kansasii* is best known as a cause of lung disease in immunocompetent patients, although it may also produce pulmonary or disseminated infections in HIV-infected patients.[2,3] *M. chelonae* has been implicated in localized or disseminated infections and health care-associated disease, and *M. abscessus* can cause chronic lung disease.[4,5] *Mycobacterium immunogenum* is related to metalworking fluid-associated hypersensitivity pneumonitis,[6] and the newly described *Mycobacterium massiliense*[7] and *Mycobacterium bolletii*[8] to skin and soft tissue infections.[9,10]

Identification of mycobacteria to the species level is necessary for the application of adequate drug therapy and to address epidemiological questions. Conventional identification techniques based on the culture and biochemical characteristics of acid-fast isolates are the most commonly used methods for the determination of mycobacterial species, but these procedures are time-consuming and labor-intensive. The development of molecular tests has speeded up diagnosis, but most methods suffer from specific drawbacks. The INNO-LiPA MYCOBACTERIA assay (LiPA; Innogenetics, Ghent, Belgium), a commercially available assay targeting the 16S–23S internal transcribed spacer (ITS) region, was developed for the current identification of 16 species (including *M. kansasii* and *M. chelonae*). The assay is highly specific and sensitive, but its cost remains high and it cannot differentiate the members of the *M. chelonae–M. abscessus* group.[11] Moreover, the correlation of each of the three possible hybridization patterns (MCH-1, MCH-2, and MCH-3) with the members of this group has not yet been fully evaluated. Partial sequencing of conserved genes and DNA regions such as the 16S rRNA gene, ITS, *rpoB*, *hsp65*, *sodA*, and the new target *secA*, is the most sensitive and accurate, but still expensive and technically demanding, procedure for the identification of mycobacterial species.[9,10,12–14]

Restriction enzyme analysis of PCR products of specific genes generates mostly species-specific DNA patterns, and provides a comparatively cheap alternative over DNA sequencing. In particular, PCR-restriction analysis (PRA) of part of the gene encoding the

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65-kDa heat shock protein (*hsp65*)[15] has been widely used for diagnostic purposes.[9,10,16–18] This method is based on the amplification of a 441-bp fragment of the *hsp65* gene present in all mycobacteria, followed by digestion of the PCR product with the restriction enzymes *Bst*EII and *Hae*III. By combining both restriction patterns a species assignment is possible based on comparison with patterns described in published algorithms[15,16] or available from an Internet database (http://app.chuv.ch/prasite).

The aim of this study was to evaluate the *hsp65* PRA with an automated capillary electrophoresis in the identification and differentiation of *M. kansasii* and *M. chelonae–M. abscessus* group in a reference laboratory. The results were compared to identification by conventional methods and the commercial LiPA assay.

2. Materials and methods

2.1. Mycobacterial strains

A total of 56 cultured isolates studied here were selected on the basis of their origins from different sources and geographical areas. They included 27 *M. kansasii* isolates from Belgium ($n = 13$), Germany ($n = 7$), and the Czech Republic ($n = 7$). These isolates were recovered from human ($n = 15$), environmental ($n = 11$), and animal ($n = 1$) sources. All of the environmental *M. kansasii* strains were isolated from tap water. Twenty-nine strains of *M. chelonae–M. abscessus* group were isolated in Belgium ($n = 15$), Germany ($n = 13$), and Colombia ($n = 1$). These strains were of human ($n = 16$) and environmental ($n = 13$) origin. Two of the environmental isolates were recovered from river surface water, while the remaining strains were isolated from tap water. All of the strains had previously been identified as *M. kansasii* or *M. chelonae–M. abscessus* by conventional phenotypic methods and the LiPA assay (INNO-LiPA MYCOBACTERIA, Innogenetics, Ghent, Belgium).[19]

Reference strains of *M. kansasii* (ATCC 12478), *M. chelonae* (ATCC 35752), and *M. abscessus* (ATCC 19977) were used. The bacterial strains were stored at −70 °C in Dubos broth with 9.5% glycerol. Prior to use, the strains were subcultured onto Löwenstein–Jensen medium slants.

2.2. hsp65 PRA

PCR-restriction enzyme analysis of the *hsp65* gene (*hsp65* PRA) was performed for all isolates and reference strains as previously described by Telenti et al.,[15] with minor modifications.[20]

Restriction products were electrophoresed both on a 3% agarose gel prepared in 1 × TBE (Tris–borate–EDTA) buffer and sized with 50- and 25-bp DNA ladders (Promega, Madison, WI, USA) and by automated fluorescence capillary electrophoresis using the Agilent 2100 bioanalyzer (Agilent Technologies, Waldbronn, Germany) for more accurate determination of fragment sizes. For the latter purpose, aliquots of restriction digests were ethanol-precipitated and 1-μl samples were then electrophoresed and analyzed using the DNA 1000 LabChip® kit in accordance with the manufacturer's instructions.

The observed PRA patterns were interpreted first by comparison with patterns in our own identification table based on automated capillary electrophoresis.[20] Strains with PRA patterns not matching any of those included in our bioanalyzer system-based interpretative scheme were identified using the latest algorithm published by Chimara et al.[16] and also the PRA site query.

2.3. DNA sequence analysis

For those isolates for which PRA gave inconclusive results due to patterns unreported so far, the partial *hsp65* and *rpoB* gene sequences were obtained. A 441-bp fragment of the *hsp65* gene was amplified as described previously.[20] Amplification of the 723-bp *rpoB* variable region was performed as described by Adékambi et al.[14] The PCR primers were also used for the direct sequencing of both strands of the amplification products. Amplicons were sequenced by VIB Genetic Service Facility, University of Antwerp, Wilrijk, Belgium with the use of dye terminator reactions followed by electrophoresis on an automated ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA). The sequences obtained were compared with those deposited in the GenBank database using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

3. Results

3.1. Identification and genotypic characterization of M. kansasii strains

There was complete agreement of identification results between all three methods used: (1) conventional phenotypic methods, (2) the commercial LiPA assay, and (3) *hsp65* PRA. The sizes of restriction fragments produced by the latter method and measured by the Agilent 2100 bioanalyzer were consistently smaller than those estimated on the basis of agarose gel electrophoresis (Tables 1 and 2). Despite those differences, it provided highly reproducible and sufficiently polymorphic patterns necessary for the differentiation of species and PRA types of species, consistent with our previous observations.[20]

Restriction enzyme analysis of the *hsp65* gene from 27 isolates and the reference strain detected five of the six recognized genetic types of *M. kansasii*[16] (Table 1). Eleven clinical isolates and the reference strain presented the type 1 PRA pattern, while this genotype was isolated only once from tap water (shower). Of the remaining 15 isolates, most ($n = 7$) presented PRA type 2 pattern, and this was the most ubiquitous, being recovered four times from humans, twice from tap water, and also from the single animal

Table 1
Genotypic characterization of *Mycobacterium kansasii* by PRA

PRA type	No. of isolates			Restriction fragments (bp) by *hsp65* PRA[a]	
	Human ($n = 16$)	Environmental ($n = 11$)	Animal ($n = 1$)	*Bst*EII	*Hae*III
1	12[b]	1	0	226 (235), 193 (210)	122 (130), 93 (105), 69 (80)
2	4	2	1	227 (235), 127 (130), 78 (85)	127 (130), 93 (105)
3	0	5	0	230 (235), 127 (130), 78 (85)	124 (130), 88 (95), 62 (70)
4	0	2	0	227 (235), 112 (120), 78 (85)	127 (130), 111 (115), 66 (75), 52 (60)
5	0	1	0	306 (320), 111 (115)	127 (130), 88 (95), 66 (75), 52 (60)

PRA, PCR-restriction analysis.
[a] Fragment sizes determined by agarose gel electrophoresis are in parentheses, whereas those estimated by the capillary electrophoresis instrument (Agilent 2100 Bioanalyzer) are without them. Sizes are average of the results obtained for *M. kansasii* strains presenting a given type of PRA pattern, except for type 5, which was found only in a single strain.
[b] Including the *M. kansasii* reference strain.

Table 2
Genetic differentiation of *Mycobacterium chelonae–Mycobacterium abscessus* group by PRA

Strain and type	No. of isolates			Restriction fragments (bp) by *hsp65* PRA[a]	
	Clinical (n = 18)	Tap water (n = 11)	River water (n = 2)	*Bst*EII	*Hae*III
M. abscessus 1	2[b]	0	0	224 (235), 197 (210)	141 (145), 61 (70), 50 (60), 43 (55)
M. abscessus 2	1	0	0	227 (235), 199 (210)	190 (200), 62 (70), 51 (60), 39 (50)
M. chelonae	3[c]	9	1	306 (320), 127 (130)	192 (200), 52 (60), 46 (55), 38 (50)
M. immunogenum 2	11	2	0	304 (320), 127 (130)	190 (200), 63 (70), 52 (60), 46 (55)
New pattern 1	1	0	0	305 (320), 127 (130)	157 (170), 62 (70), 50 (60), 38 (50)
New pattern 2	0	0	1	307 (320), 127 (130)	190 (200), 107 (115), 51 (60), 39 (50)

PRA, PCR-restriction analysis.
[a] Fragment sizes determined by agarose gel electrophoresis are in parentheses, while those measured by the capillary electrophoresis instrument (Agilent 2100 bioanalyzer) are without them. Sizes are average of the results obtained for the strains presenting a given type of PRA pattern, except for a single isolate of *M. abscessus* type 2 and two isolates with new patterns.
[b] Including the *M. abscessus* reference strain.
[c] Including the *M. chelonae* reference strain.

specimen examined here. Isolates with PRA patterns 3 (n = 5), 4 (n = 2), and 5 (n = 1) were all isolated from tap water.

3.2. Identification and differentiation of M. chelonae–M. abscessus group

Of the 29 strains previously identified as *M. chelonae–M. abscessus* by conventional testing and the molecular LiPA assay, 27 (93%) isolates also yielded concordant results in the *hsp65* PRA. Moreover, PRA enabled further differentiation of these strains to member species and PRA types of species, which was not possible with the other methods. The results of identification of 29 *M. chelonae–M. abscessus* group isolates and the two reference strains are presented in Figure 1 and Table 2. Most of the clinical isolates (n = 11) gave the *hsp65* PRA patterns corresponding to *M. immunogenum* type 2. All these strains were isolated from respiratory specimens. *M. immunogenum* type 2 was only isolated twice from tap water. On the other hand, *M. chelonae* was the most frequently recovered from the environment (nine times from tap water and once from river surface water) and was only recovered twice from human respiratory samples. Of the remaining two clinical isolates from patient sputa, one had a pattern that matched *M. abscessus* type 1, and the other was identified as *M. abscessus* type 2. These two genotypes were not found in environmental samples. The reference strain of *M. abscessus* presented the type 1 PRA pattern.

For two (7%) of the isolates studied, PRA patterns did not match any of those in available databases (Figure 1 and Table 2). The two isolates with inconclusive results were subjected to partial sequence determination of the *hsp65* and *rpoB* genes, which are widely studied molecular targets for identification purposes, using the BLAST search against the GenBank database (Table 3). The isolate with new PRA pattern 1 was recovered from a bronchial aspirate from a patient in Belgium. Following sequencing of its *rpoB* amplicon, an unambiguous species assignment could not be achieved since that sequence yielded the highest (97%) match to

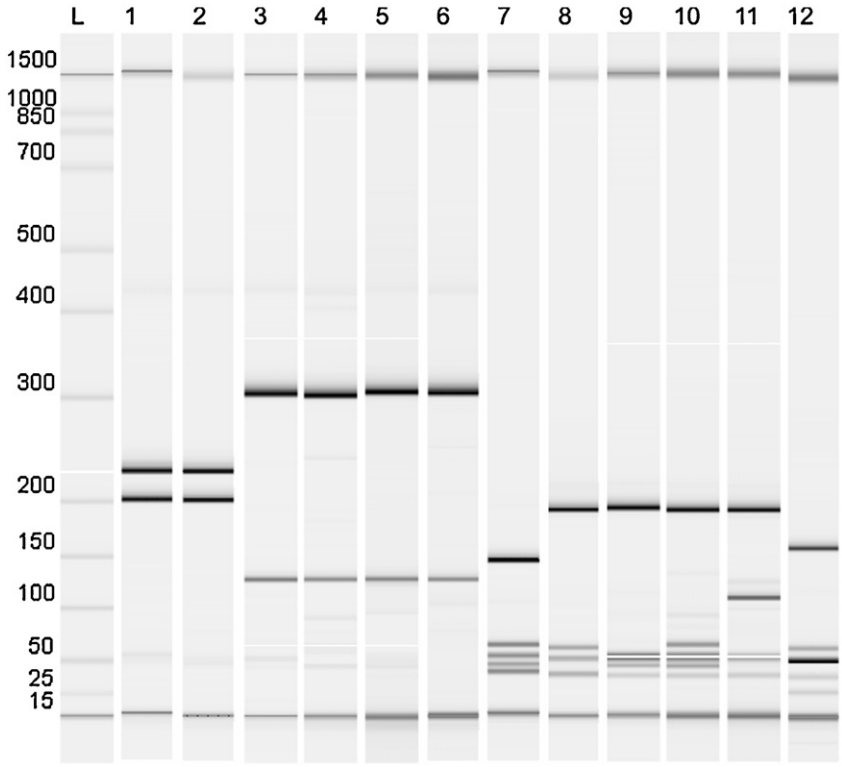

Figure 1. Electrophoretic gel image of PRA patterns resulting from *Bst*EII (lanes 1–6) and *Hae*III (lanes 7–12) digestion of the *hsp65* gene of *M. chelonae–M. abscessus* group representative isolates obtained by using the bioanalyzer system. Lanes: L, DNA ladder; 1 and 7, *M. abscessus* 1; 2 and 8, *M. abscessus* 2; 3 and 9, *M. chelonae*; 4 and 10, *M. immunogenum* 2; 5 and 11, new pattern 2; 6 and 12, new pattern 1. The 15- and 1500-bp bands in all lanes represent lower and upper internal size markers.

Table 3
Evaluation of two inconclusive PRA identification results using sequence analysis of the *hsp65* and *rpoB* genes with the GenBank database[a]

Conventional and LiPA identification	*hsp65* PRA[b]	*hsp65*		*rpoB*	
		Species	% Identity	Species	% Identity
M. chelonae–M. abscessus	New pattern 1 (320/130–170/70/60/50)	*M. immunogenum*	99.0	*M. immunogenum/M. chelonae*	97.0
	New pattern 2 (320/130–200/115/60/50)	*M. salmoniphilum*	99.0	*M. salmoniphilum*	100

[a] The data for the final identification by sequencing of the *hsp65* and *rpoB* genes are provided along with the values indicating the percentage of identity observed for the best matching sequence available in the database.
[b] Fragment sizes (bp) after digestion (*Bst*EII–*Hae*III), obtained with the use of an agarose gel electrophoresis, are presented in parentheses.

those of both *M. immunogenum* and *M. chelonae*, whereas *hsp65* shared the highest identity (99%) with the *M. immunogenum* sequence. For the remaining isolate, which exhibited new PRA pattern 2, DNA sequencing gave concordant results. The isolate showed 100% identity with *rpoB* sequence and 99% identity with *hsp65* sequence of *Mycobacterium salmoniphilum*. This isolate originated from river surface water in Germany.

4. Discussion

In recent years the identification of mycobacterial isolates to the species level has been speeded up and simplified by the use of molecular techniques, of which *hsp65* PRA has the greatest potential for being widely used as a quick method for identifying all mycobacteria.[2,15–17] Specifically, PRA with capillary electrophoresis can be used to increase the accuracy and rapidity of the identification process.[21,22] In this study, we aimed to identify and differentiate *M. kansasii* and *M. chelonae–M. abscessus* group isolates with the use of automated capillary electrophoresis-based PRA and our previously constructed diagnostic scheme.[20]

The results obtained using the automated fluorescence capillary electrophoresis instrument agreed with the results obtained with the same strains using conventional agarose gel electrophoresis. The three reference strains and 54 of 56 (96.4%) test isolates of *M. kansasii* ($n = 27$) and *M. chelonae–M. abscessus* group ($n = 27$) were correctly identified by PRA despite differences (average −8.5 bp) in band sizes measured automatically by the bioanalyzer system and estimated on the basis of 3% agarose gel electrophoresis. The obtained PRA patterns were highly reproducible and allowed accurate and fast identification of the *Mycobacterium* species studied. All isolates investigated here had previously been assigned *M. kansasii* ($n = 27$) or *M. chelonae–M. abscessus* ($n = 29$) by phenotypic methods and a DNA-based commercial LiPA assay.[19] For all isolates of *M. kansasii* the three methods provided identical species identification (100% concordance), whereas 27 out of 29 (93%) *M. chelonae–M. abscessus* group isolates were correctly identified by PRA. For two (7%) isolates, PRA could not yield a species assignment since their patterns have not been reported so far. No discrepancies were observed between the results of the three methods used. With the overall agreement of 96.4%, PRA has proven to be similarly effective, as found in other studies.[16–18] Moreover, PRA proved superior to both conventional tests and the molecular LiPA assay in enabling differentiation of the *M. chelonae–M. abscessus* group member species. Our bioanalyzer system-based PRA currently identifies 35 different *Mycobacterium* species, and this number will likely increase in the future as new reference strains are included.[20]

PRA analysis of *M. kansasii* isolates allowed us to detect five out of six currently defined genetic types within this species,[16] originally described by Picardeau et al. in 1997.[23] Thus, our data confirm and extend prior reports on the heterogeneity within *M. kansasii* and the distribution of different types.[2,18,23–25]

Members of the *M. chelonae–M. abscessus* group are often not distinguished by clinical laboratories, despite the fact that they cause diseases requiring different treatment regimens. In this study, isolates of the individual species and genotypes within species were easily identified by PRA. Unexpectedly, the vast majority – 11 out of 16 (69%) – of the isolates from humans were identified as *M. immunogenum*. However, only one of them was clinically significant causing pulmonary disease (isolated from a sputum specimen), whereas the other 10 isolates were recovered from respiratory specimens obtained by bronchoscopes and were considered to be contaminants. Our results indicate that the occurrence of *M. immunogenum*, one of the new member species of the *M. chelonae–M. abscessus* group,[6] in culture collections previously classified as *M. chelonae*/*M. abscessus* is likely underestimated. In contrast, *M. chelonae* were the most frequent (83%) environmental (water) isolates in the present study. In contrast to previously published data,[17,26] in this study *M. chelonae* and *M. abscessus* were rarely associated with pulmonary infections, but the observed difference was most likely due to a lower number of isolates examined here and/or the sample bias.

Among 29 *M. chelonae–M. abscessus* group isolates studied here, two (7%) yielded PRA patterns not previously reported. Sequence analysis of the variable regions of the *hsp65* and *rpoB* genes, which proved highly effective for the differentiation of mycobacteria,[14,27] identified one of these isolates as *M. salmoniphilum*, a species closely related to *M. chelonae*.[28] The other isolate showed inconclusive results: *M. immunogenum* by *hsp65* sequence analysis and *M. immunogenum* or *M. chelonae* by *rpoB* sequence analysis. Several recent studies have reported similar problems with the discrimination of *M. abscessus*, *M. massiliense*, and *M. bolletii*. The authors reported a number of isolates showing ambiguous identification by partial sequencing of *rpoB*, *hsp65*, and *sodA*[29,30] or *secA*.[12] Very recently, Macheras et al. reported 15 out of 59 clinical isolates with discordant nucleotide sequences of *rpoB*, *hsp65*, and *sodA*. Even sequence analysis of an additional five housekeeping genes did not result in unambiguous identification of two of the isolates studied. The authors hypothesized that such a composite genetic structure of certain *M. abscessus* group isolates most probably resulted from genetic exchange among the member species.[13]

Our results on reference strains as well as test isolates of *M. kansasii* and *M. chelonae–M. abscessus* group proved that the PRA method based on fluorescence capillary electrophoresis could efficiently identify and differentiate the individual species and genetic types within species isolated from diverse sources and geographical areas. Although relatively expensive, which might limit its wide use, this molecular method is simple, fast, and reliable and thus appears a suitable format for performance of both PCR-based identification and typing of NTM. This is of particular importance on the background of the predicted increasing incidence of interactions between humans and mycobacteria in coming years, which will likely result in more clinical cases of NTM.

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