Infection, Genetics and Evolution 76 (2019) 104040

Contents lists available at ScienceDirect

Infection, Genetics and Evolution

journal homepage: www.elsevier.com/locate/meegid

Short communication

Discovery of a novel *Mycobacterium asiaticum* PRA-*hsp65* pattern

William Marco Vicente da Silva*, Mayara Henrique Duarte, Luciana Distásio de Carvalho, Paulo Cesar de Souza Caldas, Carlos Eduardo Dias Campos, Paulo Redner, Jesus Pais Ramos

National Reference Laboratory for Tuberculosis, Centro de Referência Professor Hélio Fraga, Escola Nacional de Saúde Pública, Fiocruz, RJ, Brazil

ARTICLE INFO

Keywords:
PRA-*hsp65*
Identification
M. asiaticum

ABSTRACT

Twenty-one pulmonary sputum samples from nine Brazilian patients were analyzed by the PRA-hsp65 method for identification of *Mycobacterium* species and the results were compared by sequencing. We reported a mutation at the position 381, that generates a suppression cutting site in the *Bst*EII enzyme, thus leading to a new PRA-*hsp65* pattern for *M. asiaticum* identification.

Nontuberculous mycobacteria (NTM) are opportunistic human pathogens. NTM are widespread in nature and are found in environmental sources, including water, soil, and aerosols. They are resistant to most disinfectants, including those used in treated water. More than 170 NTM species have been described (http://www.bacterio.net/mycobacterium.html), however, the knowledge about NTM infections is still limited (Chin'ombe et al., 2016; Tortoli, 2014).

Mycobacterium asiaticum is a slow-growing and photochromogenic species (Kubica and Wayne, 1984). Strains isolated from primate specimens were described in 1965 (Karassova et al., 1965), but only in 1971 these strains were classified as *M. asiaticum* (Weiszfeiler et al., 1971). The first report of human disease appeared in 1983 in five patients with lung infection in Australia (Blacklock et al., 1983). There are few cases of *M. asiaticum* infections, but it has already been found in many countries, such as the United States (Ford, 1998), Australia (Grech et al., 2010), Thailand (Wongwatana and Sriyabhaya, 1992), Saudi Arabia (Varghese et al., 2013), Zimbabwe (Chin'ombe et al., 2016), Uganda (Muwonge et al., 2012), and Zambia (Mwikuma et al., 2015). This species was also reported in several infection sites, which increases the clinical relevance (Ford, 1998). In addition, Leysen et al. (1989) described that *M. asiaticum* as more resistant to ciprofloxacin and ofloxacin than other mycobacteria.

The rapid identification through molecular approaches is extremely important from a clinical perspective, because it reduces both morbidity and mortality of NTM infections (Baldwin et al., 2019). The NTM identification in many laboratories is still based on time-consuming phenotypic approaches. Furthermore, these methods are not specific for many NTM species (Springer et al., 1996). The standard PCR restriction analysis (PRA-*hsp65*) described by Telenti et al. (1993) is a fast method widely used for identification of *Mycobacterium* species. The PRA-*hsp65* methodology consists of restriction analysis of a 441 bp PCR fragment of the *hsp65* gene with enzymes *Bst*EII and *Hae*III (Campos et al., 2012; Devulder, 2005; Tamura et al., 2011; Verma et al., 2017). Nowadays, *M. asiaticum* has a single pattern described as type 1 in the PRAsite database (http://app.chuv.ch/prasite/index.html) based on the following fragments: *Bst*EII (210/235) and *Hae*III (105/115). The aim of this work is to report a new *M. asiaticum* PRA-hsp65 pattern, which is an important methodology for NTM identification.

We used twenty-one isolates from pulmonary samples of nine different patients which were collected at the National Reference Laboratory for Tuberculosis of the Centro de Referência Professor Hélio Fraga/ENSP/FIOCRUZ from 2014 to 2016. All patients had pulmonary *M. asiaticum* disease based on American Thoracic Society criteria (Griffith et al., 2007).

This study was based on molecular biology methods. Therefore, we do not use any microbiological tests. The PRA-hsp65 method was performed with these samples according to the protocol described by Telenti et al. (1993). The partial 16s rDNA, ITS, and *hsp65* fragments were amplified by PCR as described by Hall et al. (2003), Roth et al. (2000) and Telenti et al. (1993), respectively.

The analysis revealed that all isolates obtained from the same patient were identical, and only the oldest isolate from each patient was further analyzed. The nine strains were named as follows: HF199910, HF72263, HF81080, HF193326, HF184811, HF77911, HF102944, HF56166, and HF109245.

DNA sequencing of the 16s rDNA, ITS, and hsp65 was carried out for all samples. The acquired sequences were compared by similarity in the GenBank database using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.

* Corresponding author at: National Reference Laboratory for Tuberculosis, Centro de Referência Prof. Hélio Fraga, ENSP/FIOCRUZ, Estrada da Curicica 2000, Rio de Janeiro, RJ 22780192, Brazil.

E-mail addresses: william.marco@outlook.com, williammvs@yahoo.com.br (W.M.V. da Silva).

https://doi.org/10.1016/j.meegid.2019.104040
Received 30 January 2019; Received in revised form 7 September 2019; Accepted 12 September 2019
Available online 15 September 2019

cgi). The 16s rDNA, ITS, and *hsp65* sequences were used to create a concatenated neighbor-joining tree with MEGA program version 7 (Devulder, 2005; Tamura et al., 2011). Kimura's 2-parameter was used as the substitution model and 1000 bootstrap replications were implemented.

All *hsp65* sequencing fragments were also submitted to *in silico* digestion, performed through manual analysis to evaluate the size of fragments to confirm the previously PRA-*hsp65* results.

The *hsp65* PCR product obtained from the nine strains showed the same pattern as follows: *Hae*III – 105 and 115 bp, *Bst*EII – 440 bp (Fig. A.1). This new pattern was not found in PRAsite database.

To perform a more accurate identification, the sequences were compared for similarity matches in the BLAST databases. The *hsp65* sequences were identical in all strains, presenting 98% of identity with *M. asiaticum* type strain ATCC25276 (8 differences). The 16s rDNA sequences were identical in all strains, presenting 100% of identity with *M. asiaticum* type strain. The ITS sequences of strains HF56166, HF102944, HF77911, and HF109245 were identical and presenting 99,64% of identity with *M. asiaticum* type strain (2 differences). The ITS sequences of strains HF184811, HF193326, HF199910, HF72263, and HF81080 are also identical, but they have two more mismatches, with 99,28% of identity when it was compared with *M. asiaticum* type strain.

All *hsp65*, ITS and 16s rDNA sequences were used to create a concatenated neighbor-joining tree. The tree showed that *M. asiaticum* type strain and all strains studied are in the same branch (with a bootstrap value of 100) and distinct from the other analyzed species. The result confirms the identification of strains as *M. asiaticum* (Fig. A.2).

The *hsp65* sequences obtained from strains analyzed *in silico* presented a profile with nine fragments (21/23/33/34/36/36/40/106/112 bp) corresponding to *Hae*III and only one fragment (441 bp) without any restriction site corresponding to *Bst*EII digestion enzyme. Due to the sensitivity of PRA-*hsp65* methodology, fragment sizes are approximated and values less than 50 bp are not considered in the PRAsite database. According to these criteria, the values of HaeIII obtained were considered the same ones as those of *M. asiaticum* type 1 (105/115). However, the result obtained from *Bst*EII was different (Fig. A.1) due to a suppression of the cleavage site of this enzyme, caused by a mutation characterized as a C to G substitution (Table A.1).

We were unable to access much clinical information from the patients from which the samples were obtained. The only information available from our samples (*M. asiaticum* type 1) is that they are all collected from pulmonary site. Although *M. asiaticum* causes pulmonary and extrapulmonary diseases, the majority of the samples described to date are also pulmonary (Blacklock et al., 1983; Chin'ombe et al., 2016., Grech et al., 2010), thus we do not consider that there are significant clinical differences between the samples of *M. asiaticum* type 1 and 2, at least in relation to the infection site. Therefore, the importance of the discovery of new restriction PRA-*hsp65* patterns lies in the diagnosis of infecting species, since the use of the methodology in a sample with an unknown pattern will result in an unidentified species.

Although there have been few reported cases of *M. asiaticum* infection and little is known about their genetic diversity and clinical differences, there are other NTM species with higher genetic diversity, such as *M. kansasii*, whose the clinical aspects are better defined. This species presents seven different patterns of PRA-*hsp65*. Genotypes I and II are the most prevalent genotypes and are more associated with human disease, while the other types only sporadically cause disease, being environmental contaminants (Bakuła et al., 2016). Other common mycobacteria also have much more patterns, such as *M. gordonae*, which presents ten genotypes, *M. intracellulare*, presenting five, and *M. fortuitum*, presenting three as available in the PRAsite database.

We described a new PRA-*hps65* pattern for *M. asiaticum* species, classified as type 2. This new profile will be of special relevance for the accurate identification of *M. asiaticum* in laboratories worldwide that use the PRA-*hsp65* methodology.

Declaratin of Competing Interest

None.

Appendix A. Appendix

Table A.1
The mutation C to G (position 381) suppressed the enzyme cleavage site of *Bst*EII (5′ GGTNACC 3).

Strains	
HF199910	GTCGAGAAGGTCAC**G**CAGACCCTGCTCAGCTC
HF72263	GTCGAGAAGGTCAC**G**CAGACCCTGCTCAGCTC
HF81080	GTCGAGAAGGTCAC**G**CAGACCCTGCTCAGCTC
HF193326	GTCGAGAAGGTCAC**G**CAGACCCTGCTCAGCTC
HF184811	GTCGAGAAGGTCAC**G**CAGACCCTGCTCAGCTC
HF77911	GTCGAGAAGGTCAC**G**CAGACCCTGCTCAGCTC
HF102944	GTCGAGAAGGTCAC**G**CAGACCCTGCTCAGCTC
HF56166	GTCGAGAAGGTCAC**G**CAGACCCTGCTCAGCTC
HF109245	GTCGAGAAGGTCAC**G**CAGACCCTGCTCAGCTC
M. asiaticum[a]	GTCGAGAAGGTCAC**C**CAGACCCTGCTCAGCTC

[a] Type strain.

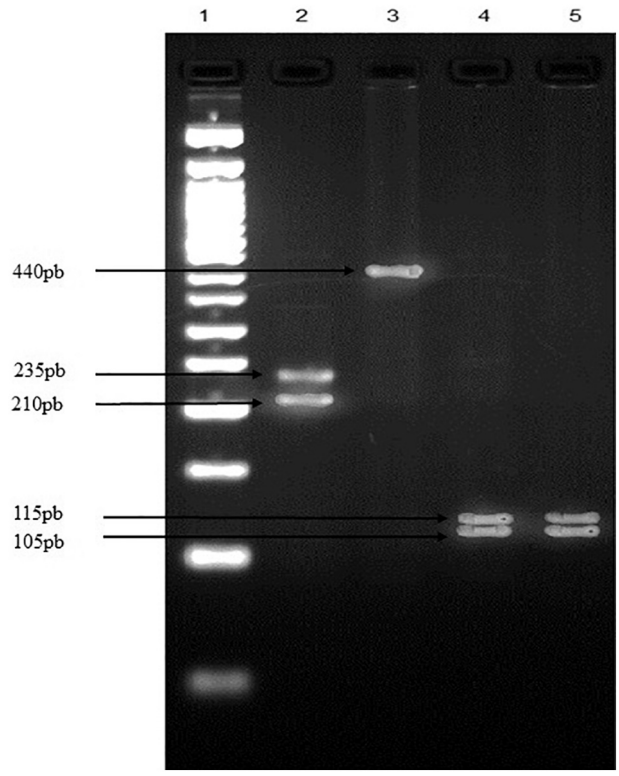

Fig A.1. The M. asiaticum type strain (PRA-hsp65 type 1) and the new PRA-hsp65 M. asiaticum pattern in 3.5% agarose gel. Lane 1–100 bp DNA ladder marker. Lane 2 - hsp65 restriction fragments of M. asiaticum type strain produced by BstEII. Lane 3 - hsp65 restriction fragments of strain HF56166 produced by BstEII. Lane 4 - hsp65 restriction fragments of M. asiaticum type strain produced by HaeIII. Lane 5 - hsp65 restriction fragments of strain HF56166 produced by *Hae*III.

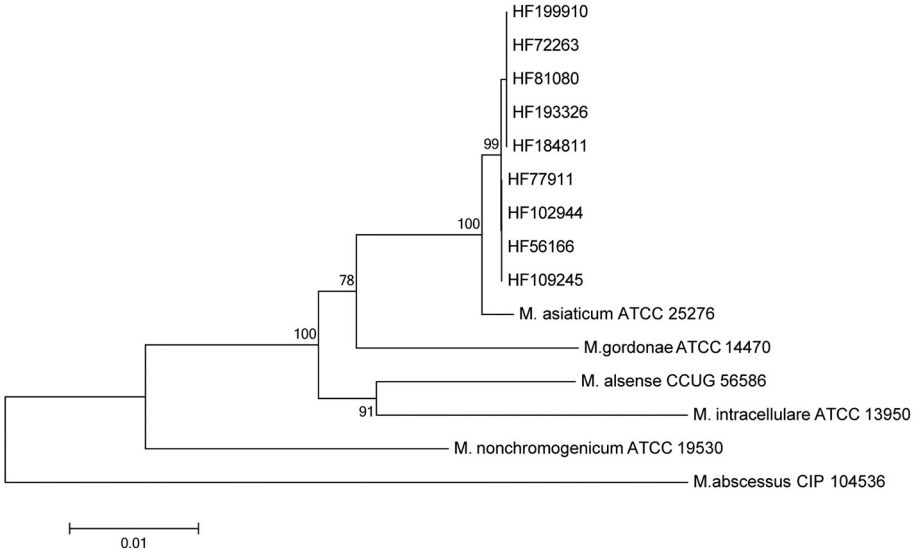

Fig A.2. Phylogenetic concatenated tree computed from 16S rDNA, *hsp65* gene, and ITS sequences *Mycobacterium abscessus* type strain was used as outgroup. Bootstrap values are indicated on the branch.

Appendix B. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.meegid.2019.104040.

References

Bakuła, Z., Modrzejewska, M., Safianowska, A., van Ingen, J., Proboszcz, M., Bielecki, J., Jagielski, T., 2016. Proposal of a new method for subtyping of *Mycobacterium kansasii* based upon PCR restriction enzyme analysis of the tuf gene. Diagn. Microbiol. Infect. Dis. 84 (4), 318–321 (Dec).

Baldwin, S.L., Larsen, S.E., Ordway, D., Cassell, G., Coler, R.N., 2019. The complexities and challenges of preventing and treating nontuberculous mycobacterial diseases. PLoS Negl. Trop. Dis. 13 (2), e0007083 (Feb).

Blacklock, Z.M., Dawson, D.J., Kane, D.W., McEvoy, D., 1983. *Mycobacterium asiaticum* as a potential pulmonary pathogen for humans. Am. Rev. Respir. 127, 241.

Campos, C.E.D., Caldas, P.C., Ohnishi, H., Watanabe, T., Ohtsuka, K., Matsushima, S., et al., 2012. First isolation of *Mycobacterium kyorinense* from clinical specimens in Brazil. J. Clin. Microbiol. 50 (7), 2477–2478 (Jul 1).

Chin'ombe, N., Muzividzi, B., Munemo, E., Nziramasanga, P., 2016. Molecular

identification of Nontuberculous mycobacteria in humans in Zimbabwe using 16S Ribosequencing. Open Microbiol. J. 10 (1), 113–123 (May 26).
Devulder, G., 2005. A multigene approach to phylogenetic analysis using the genus *Mycobacterium* as a model. Int. J. Syst. Evol. Microbiol. 55 (1), 293–302 (Jan 1).
Ford, J., 1998. Nontuberculous mycobacterial keratitis in South Florida. Ophthalmology 105 (9), 1652–1658 (Sep 1).
Grech, M., Carter, R., Thomson, R., 2010. Clinical significance of *Mycobacterium asiaticum* isolates in Queensland, Australia. J. Clin. Microbiol. 48 (1), 162–167 (Jan).
Griffith, D.E., et al., 2007. An official ATS/IDSA statement: diagnosis, treatment, and prevention of Nontuberculous mycobacterial diseases. Am. J. Respir. Crit. Care Med. 175 (4), 367–416 (Sep).
Hall, L., Doerr, K.A., Wohlfiel, S.L., Roberts, G.D., 2003. Evaluation of the MicroSeq system for identification of mycobacteria by 16S ribosomal DNA sequencing and its integration into a routine clinical Mycobacteriology laboratory. J. Clin. Microbiol. 41 (4), 1447–1453 (Apr 1).
Karassova, V., Weissfeiler, J., Krasznay, E., 1965. Occurrence of atypical mycobacteria in Macacus rhesus. Acta Microbiol. Acad. Sci. Hung. 12 (3), 275–282.
Kubica, G.P., Wayne, L.G., 1984. The Mycobacteria: A Sourcebook. Dekker, New York.
Leysen, D.C., Haemers, A., Pattyn, S.R., 1989. Mycobacteria and the new quinolones. Antimicrob. Agents Chemother. 33 (1), 1–5 (Jan 1).
Muwonge, A., Kankya, C., Johansen, T.B., Djonne, B., Godfroid, J., Biffa, D., et al., 2012. Non-tuberculous mycobacteria isolated from slaughter pigs in Mubende district, Uganda. BMC Vet. Res. 8 (1), 52.
Mwikuma, G., Kwenda, G., Hang'ombe, B.M., Simulundu, E., Kaile, T., Nzala, S., et al., 2015. Molecular identification of non-tuberculous mycobacteria isolated from clinical specimens in Zambia. Ann. Clin. Microbiol. Antimicrob. 14 (1), 1.
Roth, A., Reischl, U., Streubel, A., Naumann, L., Kroppenstedt, R.M., Habicht, M., et al., 2000. Novel diagnostic algorithm for identification of mycobacteria using genus-specific amplification of the 16S-23S rRNA gene spacer and restriction endonucleases. J. Clin. Microbiol. 38, 11.
Springer, B., Stockman, L., Teschner, K., Roberts, G.D., Bottger, E.C., 1996. Two-laboratory collaborative study on identification of mycobacteria: molecular versus phenotypic methods. J. Clin. Microbiol. 34, 8.
Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28 (10), 2731–2739 (Oct 1).
Telenti, A., Marchesi, F., Balz, M., Bally, F., Bottger, E.C., Bodmer, T., 1993. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. J. Clin. Microbiol. 4.
Tortoli, E., 2014 Oct. Microbiological features and clinical relevance of new species of the genus *Mycobacterium*. Clin. Microbiol. Rev. 27 (4), 727–752.
Varghese, B., Memish, Z., Abuljadayel, N., Al-Hakeem, R., Alrabiah, F., Al-Hajoj, S.A., 2013. Emergence of clinically relevant non-tuberculous mycobacterial infections in Saudi Arabia. Small PLC, editor. PLoS Negl. Trop. Dis. 7 (5), e2234 (May 30).
Verma, A.K., Sarin, R., Arora, V.K., Kumar, G., Arora, J., Singh, P., et al., 2017 Jan. Amplification of *Hsp65* gene and usage of restriction endonuclease for identification of non-tuberculous rapid grower *Mycobacterium*. Ind. J. Tuberc. 65 (1), 57–62.
Weiszfeiler, G., Karasseva, V., Karczag, E., 1971. A new *mycobacterium* species: *Mycobacterium asiaticum* n. sp. Acta Microbiol. Acad. Sci. Hung. 18 (4), 247–252.
Wongwatana, S., Sriyabhaya, N., 1992 Jan. Nontuberculous mycobacterial infection of the lung in a chest hospital in Thailand. J. Med. Assoc. Thail. 75, 1):1–10.