Journal of Microbiological Methods 80 (2010) 190–197

Contents lists available at ScienceDirect

Journal of Microbiological Methods

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

hsp65 PCR-restriction analysis (PRA) with capillary electrophoresis in comparison to three other methods for identification of *Mycobacterium* species

Anna Sajduda [1], Anandi Martin, Françoise Portaels, Juan Carlos Palomino *

Mycobacteriology Unit, Department of Microbiology, Institute of Tropical Medicine, Nationalestraat 155, B-2000 Antwerp, Belgium

ARTICLE INFO

Article history:
Received 10 November 2009
Received in revised form 15 December 2009
Accepted 16 December 2009
Available online 4 January 2010

Keywords:
Capillary electrophoresis
hsp65
Non-tuberculous mycobacteria
PRA
Species identification

ABSTRACT

We developed a scheme for rapid identification of *Mycobacterium* species using an automated fluorescence capillary electrophoresis instrument. A 441-bp region of the *hsp65* gene was examined using PCR-restriction analysis (PRA). The assay was initially evaluated on 38 reference strains. The observed sizes of restriction fragments were consistently smaller than the real sizes for each of the species as deduced from the sequence analysis (mean variance = 7 bp). Nevertheless, the obtained PRA patterns were highly reproducible and resulted in correct species identifications. A blind test was then successfully performed on 64 test isolates previously characterized by conventional biochemical methods, a commercial INNO-LiPA Mycobacteria assay and/or sequence determination of the 5′ end of 16 S rRNA gene. A total of 14 of 64 isolates were erroneously identified by conventional methods (78% accuracy). In contrast, PRA performed very well in comparison with the LiPA (89% concordance) and especially with DNA sequencing (93.3% of concordant results). Also, PRA identified seven isolates representing five previously unreported *hsp65* alleles. We conclude that *hsp65* PRA based on automated capillary electrophoresis is a rapid, simple and reliable method for identification of mycobacteria.

1. Introduction

The genus *Mycobacterium* contains more than 100 species, including organisms that cause serious human and animal diseases (Tortoli, 2003). Identification of mycobacteria up to the species level is necessary for application of adequate drug therapy and to address epidemiological questions. Conventional identification techniques based on the cultural and biochemical characteristics of acid-fast isolates have been the most commonly used methods for the determination of mycobacterial species, but these procedures are time-consuming and sometimes fail to produce a precise identification.

During the last decade, nucleic acid sequence-based identification tests have been developed, and commercially available systems such as AccuProbe (Gen-Probe, San Diego, CA) and INNO-LiPA Mycobacteria (LiPA; Innogenetics, Ghent, Belgium) are important new acquisitions for the diagnostic laboratory. These assays are highly specific and sensitive, but characterize a limited number of species and their costs remain high (Scarparo et al., 2001; Tortoli et al., 2003). The most sensitive and accurate, but still expensive and technically demanding, procedure for identification of a large number of mycobacterial species is sequencing of a fragment of conserved genes, of which the 16 S rDNA analysis is now regarded as the "gold standard" (Cloud et al., 2002). In the last years, several studies have been performed using other sequences such as *recA* (Blackwood et al., 2000), *rpoB* (Kim et al., 1999), 16 S–23 S internal transcribed spacer (ITS, (Mohamed et al., 2005)), *sod* (Zolg and Philippi-Schultz, 1994), *gyrB* (Kasai et al., 2000) or *hsp65* (Häfner et al., 2004; Senna et al., 2008), and a combination of sequences of several genes that gives the possibility of increasing discriminatory power (Devulder et al., 2005).

Restriction enzyme analysis of *Mycobacterium*-specific PCR products generates mostly species-specific DNA patterns, and provides a comparatively cheap alternative over sequencing regions of the 16 S rRNA gene. One such approach, analysis of a region of the gene coding for the 65-kDa heat shock protein (*hsp65*) by PCR-restriction analysis (PRA), was described by Telenti et al., 1993. 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 BstEII and HaeIII. By combining both restriction patterns a species assignment is possible based on the comparison with patterns described in published algorithms (Telenti et al., 1993; Devallois et al., 1997; Taylor et al., 1997; Brunello et al., 2001; Chimara et al., 2008) or available from an Internet database (http://app.chuv.ch/prasite). PRA has been used for diagnostic (Taylor et al., 1997; Wong et al., 2003; Chimara et al., 2008) and taxonomic purposes (Ferdinand et al., 2004), and characterization of isolates with novel characteristics (da Silva Rocha et al., 2002).

The aim of this study was to evaluate the *hsp65* PRA with an automated capillary electrophoresis in identification of mycobacteria isolated from clinical and environmental sources in a reference

* Corresponding author. Tel.: +32 3 247 6334; fax: +32 3 247 6333.
E-mail address: palomino@itg.be (J.C. Palomino).
[1] Present address: Department of Genetics of Microorganisms, University of Łódź, Banacha 12/16, 90-237 Łódź, Poland.

0167-7012/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.mimet.2009.12.012

laboratory, and its comparison with conventional and molecular methods (LiPA and 16 S rDNA sequencing).

2. Materials and methods

2.1. Study strains

Thirty-eight *Mycobacterium* reference strains (Table 1) were investigated to standardize PRA in our laboratory. Thereafter, the method was used in a blind test to determine the species of 64 cultured isolates from human ($n=29$), animal ($n=22$) and environmental ($n=5$) origin (the sources of 8 isolates were unknown). All the isolates had been previously submitted to classical biochemical identification procedures. Thirty-six (56%) strains were also identified by the INNO-LiPA Mycobacteria v2 assay (Innogenetics, Ghent, Belgium), and 26 (41%) strains were subjected to sequencing of the hypervariable region of PCR-amplified 16 S rDNA. Selection of test species for this study was based on both clinical significance and difficulties with identification by conventional biochemical tests.

2.2. DNA preparation

Crude DNA preparations were obtained from Löwenstein–Jensen medium cultures by suspending a loopful of each strain in 400 µl of TET buffer (10 mM Tris–HCl, 1 mM EDTA, 1% Triton X-100) and boiling once for 10 min. Each sample was then centrifuged at 12,000×*g* for 5 min to remove cell debris. The supernatant containing the extracted DNA was transferred to a clean microcentrifuge tube and frozen at −20 °C for at least 18 h. The supernatant was used as a template for amplification.

Table 1
Reference *Mycobacterium* strains used in the present study.

Species	Strain[a]
M. tuberculosis	ITM M8613
M. africanum	ITM 01-12
M. microti	ITM 99-2426
M. abscessus	ATCC 19977
M. avium	ITM 96-1091
M. avium s. *silvaticum*	ITM 2668
M. celatum	ITM 95-143
M. chelonae	ATCC 35752
M. chitae	ATCC 19627
M. duvalii	NCTC 348
M. fallax	IPP 301585
M. flavescens	ATCC 14474
M. fortuitum	ITM 97-461
M. frederiksbergense	ITM 04-361
M. genavense	ITM 96-0109
M. haemophilum	ITM 3065
M. interjectum	ITM 96-116
M. intermedium	ITM 96-117
M. intracellulare	ITM 4199
M. kansasii	ATCC 12478
M. lentiflavum	ITM 96-190
M. malmoense	ITM 96-1635
M. mucogenicum	ITM 98-1288
M. nebraskiae	ITM 03-2889
M. neoaurum	ITM 98-1357
M. nonchromogenicum	ATCC 19530
M. parafortuitum	ATCC 25808
M. peregrinum	ATCC 14467
M. phlei	NCTC 8151
M. scrofulaceum	CIPT 140220031
M. smegmatis	ATCC 607
M. szulgai	CIPT 14024003
M. terrae	CIPT 14032001
M. triplex	ITM 97-966
M. triviale	ATCC 23292
M. ulcerans	NCTC 10417
M. vaccae	ATCC 15483
M. xenopi	ITM 9741

[a] ATCC, American Type Culture Collection; NCTC, National Collection of Type Cultures; CIPT, Collection Instituut Pasteur de Paris-Tuberculose; IPP, Instituut Pasteur de Paris; and ITM, Institute of Tropical Medicine, Antwerp, Belgium.

2.3. DNA amplifications

Ten µl of DNA extract was used to amplify a 441-bp fragment of the *hsp65* gene with primers Tb11 (5′-ACCAACGATGGTGTGTCCAT-3′) and Tb12 (5′-CTTGTCGAACCGC ATACCCT-3′) (Telenti et al., 1993). The PCR reaction mixture (50 µl) contained 50 mM KCl, 10 mM Tris–HCl (pH 8.6), 1.65 mM $MgCl_2$, 0.1% Triton X-100, 160 µM each deoxynucleotide triphosphate, 20 pM of each primer, and 1 U of AmpliTaq DNA polymerase (Roche Molecular Systems, Brussels, Belgium) under mineral oil. PCR amplification was performed using the following protocol: initial denaturation at 95 °C for 5 min, 45 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, extension at 72 °C for 1 min, and a final extension at 72 °C for 7 min.

Primers P7 (5′-CATGCAAGTCGAACGGAAAGG-3′) and P16 (5′-CGAACAACG CGACAAACCA-3′) were used in the amplification of about 500-bp 5′ end fragment of the 16 S rRNA gene. Two µl of crude bacterial lysate was used as a template in PCR together with the remaining reaction components listed above. The PCR conditions consisted of initial denaturation at 94 °C for 5 min followed by 40 cycles of denaturation at 94 °C for 45 s, annealing at 58 °C for 45 s, and extension at 72 °C for 45 s with additional 10 min of elongation step after the last cycle.

The presence of amplified products was confirmed by agarose gel electrophoresis.

2.4. Restriction enzyme analysis and interpretation of hsp65 PRA patterns

Fifteen µl of PCR product was digested with 10 U of BstEII or HaeIII (Promega, Madison, WI) in a total reaction volume of 25 µl, according to the manufacturer's instructions. BstEII digestion was incubated at 60 °C under mineral oil and HaeIII digestion at 37 °C for 4 h.

Digestion products were electrophoresed on both a 3% agarose gel prepared in 1×Tris-borate EDTA (TBE) and sized with 50- and 25-bp ladders (Promega), and by 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 sample was then electrophoresed and analyzed using the DNA 1000 LabChip® kit according to the manufacturer's instructions. Samples were combined in disposable labchip wells with a sieving polymer (gel matrix) containing fluorescent dye and internal size markers, vortex mixed, and electrophoresed. Twelve samples and a molecular size ladder in individual wells moved through the microchannels, and were then injected into a separation channel where components were electrophoretically separated and detected by fluorescence. The bioanalyzer system software displayed data as both simulated bands on gel images (Fig. 1) and peaks in electrophoregrams. Results also included band migration time (s), size (bp), concentration (ng/µl), corrected peak area, and molarity (nM/l) of DNA fragments.

The observed PRA patterns were compared to the patterns reported in publications (Telenti et al., 1993; Devallois et al., 1997; Taylor et al., 1997; Brunello et al., 2001; Chimara et al., 2008) and on the PRA site or calculated from *hsp65* gene sequences deposited in the National Center for Biotechnology Information GenBank database (http://www.ncbi.nlm.nih.gov).

2.5. DNA sequence analysis

For those isolates for which conventional and PRA methods gave discordant or inconclusive results, sequences of about 500-bp hypervariable region of 16 S rRNA or *hsp65* gene amplicons were determined with the primers used for PCR amplifications. The PCR products were