

Rapid Identification of Pathogenic Rapidly Growing Mycobacteria by PCR-Restriction Endonuclease Analysis

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Abstract

Introduction: The accuracy and practicality of PCR-restriction endonuclease analysis (PRA) for rapid identification of pathogenic rapidly growing mycobacteria (RGM) isolates were evaluated. **Materials and Method:** PRA identification using an amplified 439-bp segment (amplicon) of the 65-kDa heat shock protein gene was compared to identification by conventional methods, for 39 clinically significant RGM isolates. **Results:** The accuracy of PRA in the identification of RGM isolates was comparable to that of conventional methods. Moreover, PRA was able to identify RGM faster, within 2 to 3 working days compared to conventional methods which require 2 to 4 weeks to perform and complete different tests. **Conclusion:** PRA methodology could be easily incorporated into the clinical laboratory setting. This would be beneficial for the management of patients with infections due to pathogenic RGM.

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Key words: Diagnosis, PRA, RGM

Introduction

The pathogenic rapidly growing mycobacteria (RGM) capable of producing disease in humans consist primarily of the *Mycobacterium abscessus* (*M. abscessus*), *Mycobacterium chelonae* (*M. chelonae*), *Mycobacterium fortuitum* species group (*M. fortuitum* group), and the *M. smegmatis* group. Clinical features of RGM infections include post-traumatic wound infections, disseminated cutaneous disease, bone and joint infections, catheter-related infections and chronic pulmonary disease.¹ Conventional methods for identification of RGM isolates are laborious and time-consuming, and require 2 to 4 weeks for final results.² In recent years, increasing numbers of clinically significant RGM have been isolated and diagnostic tests with faster turnaround time are required. Successful applications of PCR-restriction endonuclease analysis (PRA) for rapid identification of mycobacterial species have been reported³ and have gained wide acceptance.^{1,3-6} This study aims to apply PRA methodology for rapid identification of pathogenic RGM and to explore the feasibility of incorporating PRA test into routine laboratory service.

Materials and Methods

Organisms

A total of 39 RGM isolates from 36 patients with suspected infections caused by pathogenic RGM were included in this study. One was referred by an overseas laboratory, 5 were isolated in this laboratory and assessed as clinically significant by the clinicians, and the rest were isolated in the clinical laboratories of various hospitals in Singapore and referred to this laboratory for further identification. Reference control strains of *M. fortuitum* ATCC 6841, *M. chelonae* CAP E10/2000 (ATCC 35752), *M. smegmatis* ATCC 19420 and *M. abscessus* CAP E11/2001 were utilised as controls for PRA.

Identification of RGM Isolates

The culture isolates were differentiated using the commercial DNA probe (AccuProbe, Gen-Probe, San Diego, Calif., USA) for *M. tuberculosis* complex (MTBC) and conventional methods consisted of optimum growth temperature, growth rate and pigmentation. Selected biochemical tests including 3-day arylsulfatase, iron uptake,

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Table 1. Identification of 39 RGM Isolates by Conventional Methods and PCR-Restriction Endonuclease Analysis (PRA)

Isolate No.	Source	Conventional method	PRA
**02MC19552	Cerebrospinal fluid	<i>M. fortuitum</i> group	<i>M. fortuitum</i>
02MC27041	Blood	<i>M. fortuitum</i> group	<i>M. fortuitum</i>
02MC29135	Seroma	<i>M. fortuitum</i> group	<i>M. fortuitum</i>
02MC31302	Buttock abscess	<i>M. fortuitum</i> group	<i>M. fortuitum</i>
**02MC32176	Cerebrospinal fluid	<i>M. fortuitum</i> group	<i>M. fortuitum</i>
02MC32153	Tip of catheter	<i>M. fortuitum</i> group	<i>M. fortuitum</i>
02MC41754	Blood	<i>M. fortuitum</i> group	<i>M. fortuitum</i>
03MC3316	Foot abscess	<i>M. fortuitum</i> group	<i>M. fortuitum</i>
03MC19375	Tip of catheter	<i>M. fortuitum</i> group	<i>M. fortuitum</i>
03MC24322	Hand abscess	<i>M. fortuitum</i> group	<i>M. fortuitum</i>
03MC33622	Swab from abdomen	<i>M. fortuitum</i> group	<i>M. fortuitum</i>
02MC29542	Blood	<i>M. abscessus</i>	<i>M. abscessus</i>
02MC29847	Wound swab	<i>M. abscessus</i>	<i>M. abscessus</i>
02MC36767	Blood	<i>M. abscessus</i>	<i>M. abscessus</i>
**02MC38584	Lymph node	<i>M. abscessus</i>	<i>M. abscessus</i>
02MC38811	Wound swab	<i>M. abscessus</i>	<i>M. abscessus</i>
* 02MC39128	Sputum	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC1488	Blood	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC8699	Conjunctiva lesion	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC9112	Peritoneal dialysis fluid	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC9663	Peritoneal dialysis fluid	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC12207	Finger tissue	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC19115	Cornea ulcer	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC22322	Wound tissue	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC23323	Peritoneal dialysis fluid	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC23691	Eye ulcer	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC23837	Hand tissue	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC24184	Cornea ulcer	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC24596	Breast abscess	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC29294	Peritoneal dialysis fluid	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC32683	Bronchial lavage	<i>M. abscessus</i>	<i>M. abscessus</i>
03MC33501	Bronchial lavage	<i>M. abscessus</i>	<i>M. abscessus</i>
04MAFB-ID1	Swab from left ear	<i>M. abscessus</i>	<i>M. abscessus</i>
04MAFB-ID2	Swab from left thigh	<i>M. abscessus</i>	<i>M. abscessus</i>
**02MC32221	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>
**02MC37949	Sputum	<i>M. chelonae</i>	<i>M. chelonae</i>
03MC30742	Peritoneal dialysis fluid	<i>M. smegmatis</i> group	<i>M. smegmatis</i> group
03MC31439	Peritoneal dialysis fluid	<i>M. smegmatis</i> group	<i>M. smegmatis</i> group
03MC7168	Wound swab	Non-tuberculous mycobacteria	<i>M. fortuitum</i>

* Isolated and referred by overseas clinical laboratory for further identification.

** Isolated in this laboratory, PRA confirmation requested by clinician.

The rest were isolated and referred by clinical laboratories from various hospitals in Singapore for further identification.

nitrate reduction, growth on Löwenstein-Jensen (L-J) medium slant containing 5% sodium chloride, Tween-80 hydrolysis, tellurite reduction, urease, growth on MacConkey agar and utilisation of carbon sources (citrate, mannitol, inositol) were also carried out to further identify the RGM isolates.

PCR Amplification

Bacteria were harvested by centrifugation at 6000 x g for 5 minutes. DNA extracts were prepared using QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden) according to the manufacturer's instructions. 10 µL of purified target DNA

was added to 60 µL of PCR SuperMix, (Invitrogen Life Technologies, San Diego, Calif., USA) 2 µL each of primers TB11 (5'-ACCAACGATGGTGTGTCCAT) and TB12 (5'-CTTGTCGAACCGCATAACCCT). The mixture was amplified at 94°C, 55°C, and 72°C for 1 min each for 38 cycles, then held for a 10-minute extension period at 72°C.

Restriction Enzyme Digestions

BstEII and *HaeIII* (New England Biolabs, Beverly, Mass., USA) were used in the production of PRA band size patterns. Restriction digestions were carried out according to recommendation by the manufacturer. Restriction

fragments were electrophoresed using 3% Metaphor agarose (BioWhittaker Molecular Application, Rockland, Maine, USA), in a Mini-Sub-Cell electrophoresis system (Scie-Plas, Warwickshire, UK) at 100 V for 1.5 to 2.0 hours.

Pattern Analysis and Isolate Identification

PRA band sizes in base pairs (bp) were estimated visually by comparison with 2 molecular size markers, a 50 bp ladder (MBI Fermentas'), a 100-bp ladder (BioWhittaker Molecular Application, Rockland, Maine, USA), and the PRA patterns obtained for the control strains on each gel. Visual PRA isolate identifications were made prior to, and without knowledge of, identification results by conventional methods.

Results and Discussion

Conventional and PRA techniques both identified 23 *M. abscessus*, 11 *M. fortuitum* group, 2 *M. chelonae* (from same patient) and 2 *M. smegmatis* group (from the same patient) isolates (Table 1). Thirty-eight of 39 (97.4%) identifiable RGM by conventional methods produced PRA patterns that matched species-specific patterns of *M. fortuitum*, *M. abscessus*, *M. chelonae* and *M. smegmatis*. However, 1 isolate (03MC7168) isolated from a wound swab was not speciated by conventional methods. Failure of conventional methods in identification of this isolate was due to repeated negative nitrate reduction and indistinct iron uptake results. The difference in phenotypic derivations

was likely attributable to intra-species variation in enzymatic activity and phenotypic characteristics.⁷⁻¹⁰ In contrast, this isolate was easily identified by PRA as *M. fortuitum* (Fig. 1). PRA correctly identified 39 of 39 (100%) clinically significant RGM isolates in this study. The predominant pathogenic RGM isolates in this study were *M. abscessus* and *M. fortuitum* group (89.7% of total 39 RGM isolates). As the current PRA database has been well-developed for these species of non-pigmented rapidly growing mycobacteria,⁴ there was no difficulty in interpreting the PRA results in this study. Identification of RGM isolates by the PRA technique provided identification within 2 to 3 working days upon receiving the isolate, which was significantly faster than conventional methods, which required 2 to 4 weeks (data not shown). The time difference was largely accounted for by the 10 to 13 biochemical tests needed for RGM identification by conventional methods.² The high accuracy of the PRA technique was comparable with other investigators' findings.^{3,4,6,8} Importantly, there is no requirement for costly equipment in implementing PRA methodology. In contrast, other identification methods^{1,7} such as 16S rRNA, *hsp65* gene sequencing and high-performance liquid chromatography (HPLC) require expensive equipment. Our data clearly documented the accuracy of the PRA technique in identifying RGM compared to conventional techniques. The faster turnaround time will enable clinicians to provide a more rapid diagnosis and enable them to administer the appropriate therapy.

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REFERENCES

1. Brown-Elliott BA, Wallace RJ Jr. Clinical and taxonomic status of pathogenic nonpigmented or late-pigmenting rapidly growing mycobacteria. *Clin Microbiol Rev* 2002;15:716-46.
2. Kent PT, Kubica GP. *Public Health Mycobacteriology: A guide for the Level III Laboratory*. Atlanta, USA: Center for Disease Control, Department of Health and Human Service, 1985.
3. Telenti A, Marchesi F, Balz M, Bally F, Bottger EC, Bodmer T. Rapid identification of mycobacteria to the species level by polymerase chain reaction and restriction enzyme analysis. *J Clin Microbiol* 1993;31:175-8.
4. Steingrube VA, Gibson JL, Brown BA, Zhang Y, Wilson RW, Rajagopalan M, et al. PCR amplification and restriction endonuclease analysis of a 65-kilodalton heat shock protein gene sequence for taxonomic separation of rapidly growing mycobacteria. *J Clin Microbiol* 1995;33:149-53. Erratum in: *J Clin Microbiol* 1995;33:1686.

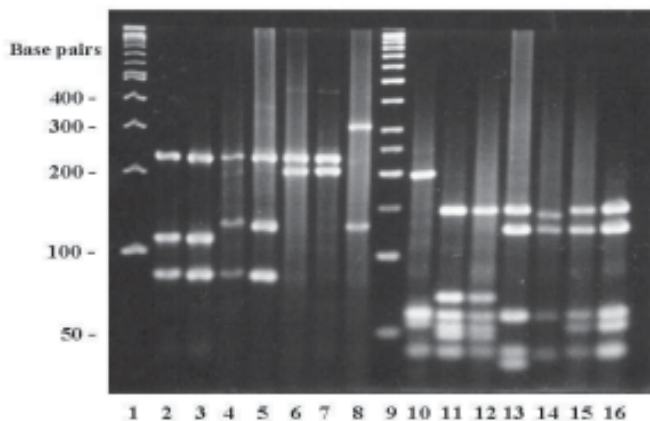


Fig 1. PRA patterns from RGM isolates and reference control strains. From left to right: 1, 100-bp markers; 2, MC7168; 3, *M. fortuitum* ATCC 6841; 4, MC30742; 5, *M. smegmatis* ATCC 19420; 6, MC24184; 7, *M. abscessus* CAP E11/2001; 8, *M. chelonae* ATCC 35752; lanes 2 to 8 were *BstEII* digested amplicons. 9, 50-bp markers; 10, *M. chelonae* ATCC 35752; 11, *M. abscessus* CAP E11/2001; 12, MC24184; 13, *M. smegmatis* ATCC 19420; 14, MC30742; 15, *M. fortuitum* ATCC 6841; 16, MC7168; lanes 10 to 16 were *HaeIII* digested amplicons. MC24184, MC30742, and MC7168 were identified as *M. abscessus*, *M. smegmatis* group and *M. fortuitum* by matching each of their PRA band size patterns with reference control strains.

5. Taylor TB, Patterson C, Hale Y, Safranek WW. Routine use of PCR-restriction fragment length polymorphism analysis for identification of mycobacteria growing in liquid media. *J Clin Microbiol* 1997;35:79-85.
 6. Brown BA, Springer B, Steingrube VA, Wilson RW, Pfyffer GE, Garcia MJ, et al. *Mycobacterium wolinskyi* sp. nov. and *Mycobacterium goodii* sp. nov., two new rapidly growing species related to *Mycobacterium smegmatis* and associated with human wound infections: a cooperative study from the International Working Group on Mycobacterial Taxonomy. *Int J Syst Bacteriol* 1999;49(Pt 4):1493-511.
 7. Ringuet H, Akoua-Koffi C, Honore S, Varnerot A, Vincent V, Berche P, et al. *hsp65* sequencing for identification of rapidly growing mycobacteria. *J Clin Microbiol* 1999;37:852-7.
 8. Mondragon-Barreto M, Vazquez-Chacon CA, Barron-Rivero C, Acosta-Blanco P, Jost KC Jr, Balandrano S, et al. Comparison among three methods for mycobacteria identification. *Salud Publica Mex* 2000;42:484-9.
 9. Conville PS, Witebsky FG. Variables affecting results of sodium chloride tolerance test for identification of rapidly growing mycobacteria. *J Clin Microbiol* 1998;36:1555-9.
 10. Conville PS, Witebsky FG. Lack of usefulness of carbon utilization tests for identification of *Mycobacterium mucogenicum*. *J Clin Microbiol* 2001;39:2725-8.
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