MULTIPLEX PCR FOR DETECTION OF CLARITHROMYCIN RESISTANCE AND SIMULTANEOUS SPECIES IDENTIFICATION OF *MYCOBACTERIUM AVIUM* COMPLEX

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Abstract. Multiplex PCR (mPCR) was established for the simultaneous detection of clarithromycin (CLR) resistance and species identification of *Mycobacterium avium* complex (MAC). mPCR was tested on 218 MAC clinical isolates. CLR-resistance was detected by mPCR in 31 of 35 isolates identified by a microdilution method. Of the remaining 187 susceptible isolates identified by mPCR, 183 isolates had MIC ≤8 μg/ml (susceptible), 3 with MIC of 16 (intermediate resistant) and 1 with MIC of ≥ 32 μg/ml (resistant). Comparing with the PCR-restriction enzyme analysis, mPCR concordantly identified 185 isolates either as being *M. avium* or *M. intracellulare,* whereas one isolate was misidentified and 32 isolates could not be identified. Comparing with reference methods, the mPCR showed the sensitivity, specificity, positive predictive and negative predictive value of 89, 100, 100, and 98% for detection of CLR resistance; 92, 98, 99, and 78% for identification of *M. avium*; and 57, 100, 100, and 89% for identification of *M. intracellulare*, respectively.

Key words: PCR, *Mycobacterium*, clarithromycin resistance, *M. avium* complex

INTRODUCTION

Mycobacterium avium complex (MAC) comprises two closely related but genetically distinct species including *M. avium* and *M. intracellulare*. Disseminated MAC infection is the most common opportunistic infection in HIV-infected patients, particularly patients with CD4 cell counts less than 200/μl and is an independent predictor of mortality (Chaisson, 1992; Moore and Chaisson, 1996). Recently, the annual rate of disseminated MAC has dramatically decreased as a result of the introduction of highly active antiretroviral therapy (HAART) (Karakousis *et al*, 2004). However, HIV-infected patients with low CD4 cell counts and patients who are not receiving or are unable to tolerate HAART continue to be at risk for disseminated MAC infection.

Macrolides, particularly clarithromycin (CLR) and azithromycin (AZM) have been shown to be effective drugs for both localized and disseminated MAC in-

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fections (Heifets, 1996). *In vitro* susceptibility testing of CLR and AZM has been demonstrated to be correlated with clinical response and should be performed particularly in AIDS patients with disseminated MAC infection (Chaisson *et al*, 1994).

Susceptibility testing by standard phenotypic methods either on solid or in liquid medium is time-consuming (Kent and Kubica, 1985; NCCLS, 2002). It requires 3-4 weeks. Several studies demonstrated that the major cause of macrolide resistance (>95%) is associated with point mutations in the 23S rRNA gene, mostly at position 2274 and 2275 (corresponding to *E. coli* 23S rRNA gene positions 2058 and 2059, respectively) (Meier *et al*, 1994; Nash and Inderlied, 1995; Meier *et al*, 1996). Rapid methods, therefore, enabling detection of such mutations would be useful for rapid identification of resistant strains.

Similarly, conventional identification of MAC is dependent on a time-consuming and laborious biochemical method (Kent and Kubica, 1985). Since the method relies on bacterial growth, it requires 3-4 weeks before interpreting the results. Genotypic identification by multiplex PCR, PCR-restriction enzyme analysis, DNA probe, and DNA sequencing has been introduced for identifying MAC (Kiehn and Edwards, 1987; Frothingham and Wilson, 1993; Telenti *et al*, 1993; Thierry *et al*, 1993; Kulski *et al*, 1995). However, these genotypic methods still have a limitation due to high diversity of the sequences among MAC species, resulting in an inability to detect some strains.

However, a PCR-based method is the most cost-effective, is simple to perform and requires only standard equipments generally available in molecular biology laboratories. To shorten the time for both CLR susceptibility testing and species identification, the present study aimed to establish a simple multiplex PCR (mPCR) for detecting CLR-resistant MAC and simultaneous identifying MAC species.

MATERIALS AND METHODS

Mycobacterial strains and growth condition

Twenty mycobacterial reference strains used in the study are listed in Table 1. Two spontaneous CLR-resistant *M. avium* ATCC 700898 strains carrying the A2274G mutation were generated in this study and used as CLR-resistant reference strains. A total of 218 MAC clinical isolates were obtained from the Mycobacteriology Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University during 2003 to 2008. All isolates were derived from individual patients. Mycobacteria were cultured on Löwenstein-Jensen (LJ) medium (Becton, Dickinson and Company, Sparks, MD) and incubated at 37ºC for 3-4 weeks or until colonies appeared.

Preparation of mycobacterial DNA from pure culture

Mycobacterial DNA was prepared by using the heat lysis method. In brief, one loopful of mycobacterial cells from LJ medium was resuspended in 100 μl of TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0) buffer. The suspension was heated at 95ºC for 15 minutes and centrifuged at 13,000*g* for 3 minutes to remove cell debris and supernatant was stored at -20ºC until use.

mPCR

mPCR was developed based on the combination of *M. avium*-specific primers (Kulski *et al*, 1995), *M. intracellulare*-specific primers (Kulski *et al*, 1995) and the 23S rDNA allele-specific primers as shown in Fig 1. The primer sequences are listed in

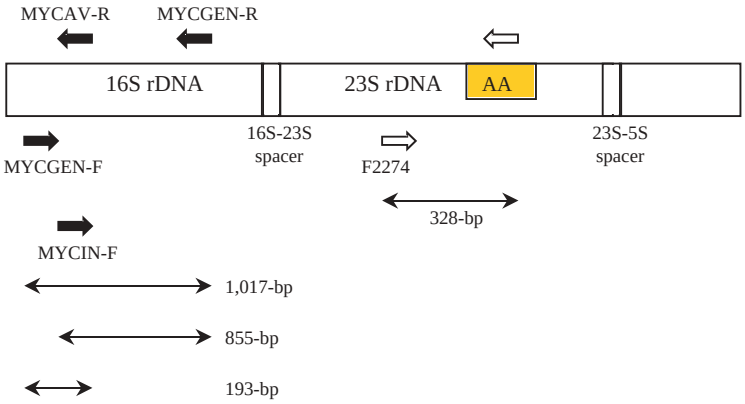

Fig 1–Location of primers used in mPCR. Wild type nucleotides at positions 2274 and 2275 of the 23S rRNA gene are shown in the shaded box. Short arrows depict the primers; black and white arrows are primers used for identification and detection of mutations respectively. Long double-headed arrows represent PCR products.

Table 1
Mycobacterial reference strains used in the study.

Mycobacterial species	Strain	Source
M. avium	ATCC 700898	Dr S Foongladda
M. austroafricanum	3005	MCC
M. bovis BCG	ATCC 35735	Dr P Palittapongarnpim
M. chelonae	ATCC 23016	MCC
M. duvalii	MNC 442	TB Division, Thailand
M. flavescens	ATCC 23035	TB Division, Thailand
M. gordonae	ATCC 144701	Dr C Abe
M. intracellulare	ATCC 13950	Dr C Abe
M. kansasii	ATCC 12478	Dr C Abe
M. marinum	ATCC 927	Dr C Abe
M. nonchromogenicum	ATCC 19530	Dr C Abe
M. phlei	ATCC 23042	TB Division, Thailand
M. scrofulaceum	ATCC 19981	Dr C Abe
M. simiae	ATCC 25275	MCC
M. smegmatis	ATCC 16941	MCC
M. szulgai	JATA 3201	Dr C Abe
M. terrae	ATCC 15775	TB Division, Thailand
M. tuberculosis H37Rv	ATCC 27294	TB Division, Thailand
M. tuberculosis H37Ra	ATCC 25177	Dr P Palittapongarnpim
M. xeopi	ATCC 19250	Dr C Abe

ATCC, American Type Culture Collection (Rocksville, MD); JATA, Japan Anti-Tuberculosis Association (Kiyose-shi, Tokyo); MNC, Mycobacteria Nocardia Culture Collection (Copenhagen, Denmark); MCC, Mycobacterial Culture Collection (Bangkok, Thailand)

Table 2
Nucleotide sequences of primers used in this study.

	Primer	Sequence (5'→3')	Reference
Multiplex PCR	MYCGEN-F	AGAGTTTGATCCTGGCTCAG	Kulski, 1995
	MYCGEN-R	TGCACACAGGCCACAAGGGA	Kulski, 1995
	MYCIN-F	CCTTTAGGCGCATGTCTTTA	Kulski, 1995
	MYCAV-R	ACCAGAAGACATGCGTCTTG	Kulski, 1995
	F2274	TGGGAGCGGGATTCGGCC	This study
	R2274	AAAGACCCCGGGACCTTC	This study
23S rDNA sequencing	F2274	TGGGAGCGGGATTCGGCC	This study
	R74	ACAGACGCCAGTTTGTGTGG	This study

Table 2. mPCR was carried out in a total volume of 20 μl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each dNTP (SibEnzyme, Academtown, Russia), 0.4% (w/v) bovine serum albumin, 0.2 μM each of MYCGEN-F, MYCGEN-R and MYCAV-R primers, 0.5 μM of MYCIN-F primer, 0.1 μM each of F2274 and R2274 primers, 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), and 5 μl of template DNA. The PCR reaction was performed in Touchgene Gradient thermocycler (Techne, Princeton, NJ) with the following condition: 94ºC for 5 minutes; 35 cycles of 94ºC for 1 minute, 60ºC for 1 minute, and 72ºC for 1 minute; and a final heating at 72ºC for 10 minutes. Then, 5 μl aliquot of PCR products was analyzed by 2% agarose gel-electrophoresis (Research Organics, Cleveland, OH). The sizes of amplicons were estimated by comparing with the 100-bp plus 1.5-kb DNA ladder (SibEnzyme, Academtown, Russia).

Amplification of partial 23S rRNA gene and DNA sequencing

PCR was performed in a total volume of 50 μl reaction mixture containing 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 2 mM $MgCl_2$, 200 μM of each dNTP (SibEnzyme, Academtown, Russia), 0.1 μM each of F2274 and R74 primers, 1 U *Taq* DNA polymerase (Invitrogen, Carlsbad, CA), and 5 μl of template DNA. Thermocycling and analysis of PCR products were as described above. The obtained PCR product (410 bp) was subsequently purified using NucleoSpin® Extraction II kit (Macherey-Nagel, Düren, Germany) according to the manufacturer's instruction before submitting to DNA sequencing.

A partial sequence of the 23S rRNA gene covering the nucleotide positions 2274 and 2275 was determined in both directions using primers F2274 and R74. Sequencing was performed in an Applied Biosystems Prism 3700 automated DNA sequencer (Applied Biosystems, Foster City, CA) by MACROGEN (Seoul, Korea). All sequence data were aligned with the published sequences for *M. avium* 23S rRNA gene (GenBank accession number X74494) using CLUSTAL X 1.81 program.

Polymerase chain reaction and restriction enzyme analysis (PCR-REA)

PCR-REA of the *hsp65* and/or *rpoB* gene was performed as previously described (Cheunoy *et al*, 2005). PCR was carried out in a total volume of 50 μl reaction mixture containing 10 mM Tris-HCl

(pH 8.0), 50 mM KCl, 2.5 mM $MgCl_2$, 0.001% gelatin, 200 μM of each dNTP, 10 μM each of primers (Tb11/Tb12 primers and RPO5'/RPO3' primers for *hsp65* and *rpoB* amplification, respectively), 1 U *Taq* DNA polymerase, and 5 μl of template DNA. Amplification was performed in Touchgene Gradient thermocycler (Techne, Princeton, NJ) with the following condition: 94ºC for 5 minutes; 35 cycles of 94ºC for 1 minute, 60ºC for 1 minute, and 72ºC for 1 minute; and a final heating at 72ºC for 10 minutes.

The 439-bp PCR product of *hsp65* and 360-bp of *rpoB* was then digested separately with 2 restriction enzymes, *Bst*EII (Amersham Pharmacia, Cleveland, OH) and *Hae*III (New England Biolabs, Beverly, MA) for *hsp65*-PCR products or *Msp*I (Amersham Pharmacia) and *Hae*III for *rpoB*-PCR products. The digestion reaction was achieved in a total volume of 15 μl, consisting of 12.5 μl of PCR products, 1.5 μl of 10x reaction buffer, and 2 units of restriction enzyme. After incubation at 37ºC overnight, the digested products were separated using 4% agarose gel-electrophoresis (~2.5 V/cm). The sizes of digested products were estimated by comparing with the 25-bp DNA ladder (Invitrogen) and the digested products of *M. tuberculosis* were used as internal marker. The mycobacterial species were identified using the published algorithms previously described (Devallois *et al*, 1997; Lee *et al*, 2000).

Clarithromycin susceptibility test

Minimum inhibitory concentration (MIC) of CLR (Abbott Laboratories, Abbott Park, Illinois) was determined using the broth microdilution method in Nunc U96 MicroWell™ plate (Nunc, Roskilde, Denmark) according to the Clinical and Laboratory Standards Institute (CLSI) (former NCCLS) recommended protocols (NCCLS, 2002). *M. avium* ATCC 700898 was used in parallel with test isolates as a control. The test was performed in Middlebrook 7H9 broth (pH 7.4) (Becton, Dickinson and Company, Sparks, MD). One hundred μl aliquots of the inoculum (5 $x10^3$ cfu/ml) were inoculated into wells containing 100 μl of either CLR-containing or CLR-free medium and incubated at 37ºC. After 7 days of incubation, if growth in the control well was sufficient and the reference strain had a correct MIC value (0.5-2 μg/ml), the MICs of test isolates were recorded. If growth in the control well was insufficient, the plate was incubated for an additional 7 days. MIC value ≥ 32 μg/ml was interpreted as being resistant, whereas MIC value of 16 and ≤8 μg/ml was interpreted as intermediate resistant and susceptible, respectively.

Assessment of mPCR

mPCR was evaluated in terms of its sensitivity, specificity, positive predictive values (PPV) and negative predictive values (NPV) by comparing the identification and susceptibility results with those obtained from PCR-REA and the broth microdilution method, respectively. Additionally, in order to evaluate the use of mPCR for direct detection of CLR-resistant strains in clinical samples, the method was tested with mixed populations of susceptible and resistant strains in different ratios. The simulated samples were prepared by mixing cell suspensions of susceptible and resistant *M. avium* or *M. intracellulare* ranging from 100% susceptible to 100% resistant strain.

RESULTS

CLR susceptibility test by mPCR

The specificity of mPCR to detect CLR-resistant strains was determined by

Table 3
CLR susceptibility of 218 MAC clinical isolates determined by mPCR compared with broth microdilution method.

mPCR	No. (%) of isolates and MIC (μg/ml) by broth microdilution method		
	≤ 8 (Susceptible)	16 (Intermediate)	≥ 32 (Resistant)
+	0	17	14
-	183	3	1
Total	183	20	15

+, resistant; -, susceptible

testing with CLR-susceptible mycobacterial and two CLR-resistant reference strains. The 328-bp allele-specific amplicon was obtained only from wild-type (susceptible) strains (data not shown). In contrast, amplification of two CLR-resistant strains, which harbored the A2274G mutation, did not generate the specific amplicon due to a mismatch at the 3'-end of the allele-specific primer R2274. Notably, *M. scrofulaceum* ATCC 19981 that did not generate either *M. avium* or *M. intracellulare*-specific PCR product could generate the 328-bp allele-specific product.

MIC values of CLR of 218 MAC clinical isolates demonstrated 15 isolates with MIC ≥32 μg/ml (resistant), 20 isolates with MIC of 16 μg/ml (intermediate resistant), and 183 isolates with MIC ≤8 μg/ml (susceptible) (Table 3). The mPCR correctly identified 14 of 15 isolates with MIC ≥32 μg/ml and 17 of 20 isolates with MIC of 16 μg/ml as CLR-resistant isolates whereas all 183 susceptible isolates were interpreted as CLR-susceptible isolates. Of these discordant isolates, two isolates (DS10934 and DS16056) were submitted for partial 23S rDNA sequencing and both of them showed wild type nucleotide sequences at position 2274 and 2275 (Table 4).

Species identification by mPCR

The specificity of mPCR to identify *M. avium* and *M. intracellulare* was determined by using DNA prepared from 19 different mycobacterial species. All mycobacterial strains generated the 1,017-bp *Mycobacterium* genus-specific product (data not shown). This product was designed as an internal control for determining PCR inhibitors. The 193-bp *M. avium*-specific amplicon and the 855-bp *M. intracellulare*-specific amplicon was obtained only from *M. avium* ATCC 700898 and *M. intracellulare* ATCC 13950, respectively (data not shown). Of 218 MAC clinical isolates, 171 and 47 isolates were identified as *M. avium* and *M. intracellulare*, respectively, by PCR-REA. mPCR could identify 185 (85%) isolates either as *M. avium* or *M. intracellulare*, whereas one (0.4%) isolate was misidentified as *M. avium* and the remaining 32 (15%) isolates could not be identified at the species level (see Fig 2 for representative results). Correlation of identification results between mPCR and PCR-REA is summarized in Table 5.

Assessment of mPCR

The sensitivity, specificity, positive predictive value and negative predictive value of mPCR were calculated by com-

Table 4
Partial 23S rDNA sequencing of 33 CLR-resistant MAC isolates covering positions 2274 and 2275.

Isolate no.	MIC (μg/ml)	mPCR			Mutation of 23S rDNA at position 2274 or 2275
		Mav	Mint	CLR	
M. avium					
S14	≥32	+	-	R	A2275C
S34	≥32	+	-	R	A2274C
S93	≥32	+	-	R	A2275C
S143	≥32	+	-	R	A2274G
DS10934	≥32	+	-	S	No mutation
DS12643	16	+	-	R	A2275G
DS10922	16	+	-	R	A2275C
DS17062	16	+	-	R	A2274G
DS16056	16	+	-	S	No mutation
DS10602	16	+	-	R	A2274G
DS11028	≥32	+	-	R	A2275G
DS10380	16	+	-	R	A2274G
DS13851	≥32	+	-	R	A2274C
DS17063	16	+	-	R	A2274G
DS16656	16	+	-	R	A2274G
DS19388	≥32	+	-	R	A2275G
DS13844	≥32	+	-	R	A2274G
DS13766	16	+	-	R	A2274G
DS13511	≥32	+	-	R	A2274C
DS18427	≥32	+	-	R	A2274G
M. intracellulare					
S68	≥32	-	+	R	A2275G
DS8488	16	-	+	R	A2274C
DS10967	16	-	+	R	A2274C
DS10693	16	-	+	R	A2274G
DS16848	16	-	-	R	A2275C
DS16434	16	-	+	R	A2274G
DS17326	16	-	-	R	A2275G
DS9324	16	-	-	R	A2275G
DS7715	≥32	+	-	R	A2275G
DS10035	16	-	+	R	A2275C
DS18837	≥32	-	+	R	A2275C
DS10968	16	-	+	R	A2274G
DS16985	≥32	-	+	R	A2275G

Mav: *M. avium*; Mint: *M. intracellulare*; R: resistant; S: susceptible

paring the susceptibility and identification results with those obtained from the broth microdilution and PCR-REA, respectively (Table 6). mPCR method might be more useful if it could detect CLR-resistant strains directly from clinical samples. The

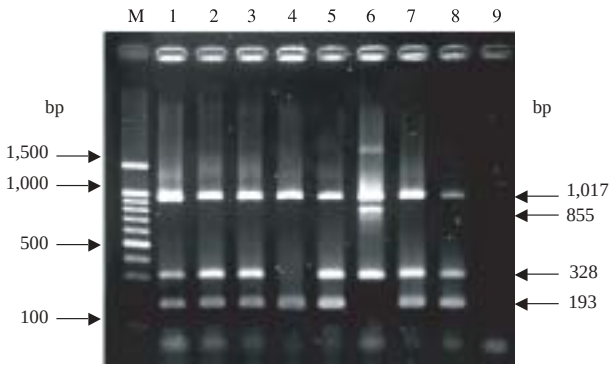

Fig 2–2% agarose gel-electrophoresis of PCR products amplified by mPCR of 8 MAC clinical isolates. The 1,017-bp, 855-bp, 328-bp, and 193-bp is Genus-, *M. intracellulare*-, allele-, and *M. avium*-specific amplicon, respectively. Lane M, 100-bp ladder; lane 1, S130 (CLR-susceptible *M. avium*); lane 2, S131 (CLR-susceptible *M. avium*); lane 3, S132 (CLR-susceptible *M. avium*); lane 4, S143 (CLR-resistant *M. avium*); lane 5, S135 (CLR-susceptible *M. avium*); lane 6, S147 (CLR-susceptible *M. intracellulare*); lane 7, S148 (CLR-susceptible *M. avium*); lane 8, S150 (CLR-susceptible *M. avium*); lane 9, negative control (without DNA template).

simulated samples consisting of susceptible and resistant subpopulations were prepared and tested by mPCR. The 328-bp amplicon was able to be detected until the resistant subpopulation reached ≥ 90% (data not shown).

DISCUSSION

Although the treatment and prophylaxis of MAC diseases have been improved by the macrolide antibiotics (CLR and AZM), the success has been hindered somewhat by the development of macrolide resistance. For MAC clinical isolates, the major cause (>95%) of macrolide resistance is associated with a point mutation at position 2274 or 2275 of the 23S rRNA gene, and at least five distinct point mutations (A2274→G, C, T and A2275→G, C) have been reported (Meier *et al*, 1994, 1996; Nash and Inderlied, 1996; Jamal *et al*, 2000; Thiermann *et al*, 2002). The ability to detect rapidly and accurately macrolide resistance would be useful in the management of MAC diseases, especially in HIV-infected patients. Recent study has supported this conclusion as it was shown that even though the rate of HIV-associated MAC patients in the era of potent antiretroviral therapy has decreased, the rate of macrolide resistance among these cases has increased (Gardner *et al*, 2005). Therefore, the macrolide susceptibility testing of all MAC clinical isolates recovered from HIV-infected patients should be performed.

In the present study, an inexpensive and simple PCR-based method was established not only for detection of the point mutations associated with CLR resistance but also for identification of MAC species in a single reaction. Species differentiation of MAC isolates into *M. avium* and *M. intracellulare* by mPCR showed 98% and 100% concordant results, respectively with PCR-REA. However, approximately 15% of the MAC clinical isolates could not be identified by this PCR method, although all generated the mycobacterial genus-specific amplicon. This result was not surprising because MAC organisms contain heterogeneity of their 16S rRNA gene sequence (Harmsen *et al*, 2003; Koivula *et al*, 2004; Lebrun *et al*, 2005). Previous studies using various genotypic methods, such as DNA probe assay (Kiehn and Edwards, 1987; Peterson *et al*, 1989; Goto *et al*, 1991; Lim *et al*, 1991), PCR-REA of *hsp65* and/or *rpoB* (Cheunoy *et al*, 2005), DNA sequencing of the 16S-23S rRNA internal transcribed spacer (Frothingham and Wilson,

Table 5
Comparison of identification results of 218 MAC clinical isolates by mPCR with PCR-REA.

PCR-REA result	No. (%) of isolates by mPCR		
	M. avium	*M. intracellulare*	Unidentified mycobacteria
M. avium	158	0	13
M. intracellulare	1	27	19
Total	159	27	32

Table 6
Diagnostic values of mPCR.

Value (%)	Identification of species		Identification of CLR resistance
	M. avium	*M. intracellulare*	
Sensitivity	92	57	89
Specificity	98	100	100
Positive predictive	99	100	100
Negative predictive	78	89	98

1993) and *hsp65* (Swanson *et al*, 1998; McNabb *et al*, 2004), to identify MAC isolates reported unidentified MAC at different percentages. This false-negative result may be reduced by combining many different molecular targets to increase the sensitivity of the method.

Genotypic identification of CLR-resistant MAC clinical isolates by mPCR showed a concordant result with the microdilution method for CLR susceptible (MIC ≤ 8 μg/ml) cases. DNA sequencing indicated that 31 of 33 resistant isolates had a mutation in the 23S rRNA either at position 2274 or 2275. Two remaining isolates showed a wild type sequence, resulting in mPCR identifying both of them as susceptible isolates. These findings suggested that the mPCR could differentiate between wild type and mutated bases, even if the point mutation occurred either at position 2274 or 2275. However, a limitation of this method was demonstrated in the strains having other resistant mechanisms, such as modification of macrolide target by methyltransferase (Weisblum, 1995) and/or active macrolide efflux pump (Clancy *et al*, 1996; Daly *et al*, 2004; Marimon *et al*, 2005) as reported from other pathogenic bacteria. Therefore, these mechanisms need to be investigated whether they are present in MAC organisms. Another limitation was found when the mPCR was performed directly with simulated samples. mPCR could detect the drug-resistant subpopulation only when they are present in ≥90% of the whole population, otherwise mPCR identified them as CLR-susceptible.

For CLR-resistant MAC isolates that

could not be identified (only *Mycobacterium* genus-specific PCR product was generated), this might lead to the misinterpretation that these MAC isolates as being other mycobacterial species. However, colony morphology and pigmentation should be considered in combination with the mPCR result. In addition, some other molecular targets, such as DT1, DT6 and IS*1245* (Thierry *et al*, 1993; Guerrero *et al*, 1995), could be combined into the PCR reaction in order to maximize the sensitivity of the method for identifying MAC species.

In summary, the mPCR developed is a rapid, inexpensive and more practical method for use in resource-limited countries. This method showed a potential use for identifying CLR-resistant MAC isolates and simultaneous differentiation of the species.

ACKNOWLEDGEMENTS

This study was supported by Siriraj Graduate Thesis Scholarship, Thesis Grant of Faculty of Graduate Studies, Mahidol University and King Prajadhipok and Queen Rambhai Barni Memorial Foundation. The authors thank all staff of the Mycobacteriology Laboratory, Department of Microbiology, Faculty of Medicine Siriraj Hospital, Mahidol University and Molecular Mycology and Mycobacteriology Laboratory, Drug-Resistant Tuberculosis Research Fund under the Patronage of the late Her Royal Highness Princess Galyani Vadhana. All authors have no conflict of interest.

REFERENCES

Chaisson R. Clarithromycin is effective against *M. avium* complex in AIDS. *Am Fam Physician* 1992; 46: 1784.

Chaisson RE, Benson CA, Dube MP, *et al*. Clarithromycin therapy for bacteremic *Mycobacterium avium* complex disease. A randomized, double-blind, dose-ranging study in patients with AIDS. AIDS Clinical Trials Group Protocol 157 Study Team. *Ann Intern Med* 1994; 121: 905-11.

Cheunoy W, Prammananan T, Chaiprasert A, Foongladda S. Comparative evaluation of polymerase chain reaction and restriction enzyme analysis: two amplified targets, *hsp65* and *rpoB*, for identification of cultured mycobacteria. *Diagn Microbiol Infect Dis* 2005; 51: 165-71.

Clancy J, Petitpas J, Dib-Hajj F, *et al*. Molecular cloning and functional analysis of a novel macrolide-resistance determinant, *mefA*, from *Streptococcus pyogenes*. *Mol Microbiol* 1996; 22: 867-79.

Daly, MM, Doktor S, Flamm R, Shortridge D. Characterization and prevalence of *MefA*, *MefE*, and the associated *msr(D)* gene in *Streptococcus pneumoniae* clinical isolates. *J Clin Microbiol* 2004; 42: 3570-4.

Devallois A, Goh KS, Rastogi N. Rapid identification of mycobacteria to species level by PCR-restriction fragment length polymorphism analysis of the *hsp65* gene and proposition of algorithm to differentiate 34 mycobacterial species. *J Clin Microbiol* 1997; 35: 2969-73.

Fabry W, Schmid EN, Ansorg R. Comparison of the E test and a proportion dilution method for susceptibility testing of *Mycobacterium avium* complex. *J Med Microbiol* 1996; 44: 227-30.

Frothingham R, Wilson KH. Sequence-based differentiation of strains in the *Mycobacterium avium* complex. *J Bacteriol* 1993; 175: 2818-25.

Gardner EM, Burman WJ, DeGroote MA, Hildred G, Pace NR. Conventional and molecular epidemiology of macrolide resistance among new *Mycobacterium avium* complex isolates recovered from HIV-infected patients. *Clin Infect Dis* 2005; 41: 1041-4.

Goto M, Oka S, Okuzumi K, Kimura S,

Shimada K. Evaluation of acridinium-ester-labeled DNA probes for identification of *Mycobacterium tuberculosis* and *Mycobacterium avium-Mycobacterium intracellulare* complex in culture. *J Clin Microbiol* 1991; 29: 2473-6.

Guerrero C, Bernasconi C, Burki D, Bodmer T, Telenti A. A novel insertion element from *Mycobacterium avium*, IS*1245*, is a specific target for analysis of strain relatedness. *J Clin Microbiol* 1995; 33: 304-7.

Harmsen D, Dostal S, Roth A, *et al*. RIDOM: comprehensive and public sequence database for identification of *Mycobacterium* species. *BMC Infect Dis* 2003; 3: 26.

Heifets LB. Clarithromycin against *Mycobacterium avium* complex infections. *Tuber Lung Dis* 1996; 77: 19-26.

Heifets LB, Lindholm-Levy PJ, Comstock RD. Clarithromycin minimal inhibitory and bactericidal concentrations against *Mycobacterium avium*. *Am Rev Respir Dis* 1992; 145: 856-8.

Jamal MA, Maeda S, Nakata N, Kai M, Fukuchi K, Kashiwabara Y. Molecular basis of clarithromycin-resistance in *Mycobacterium avium-intracellulare* complex. *Tuber Lung Dis* 2000; 80: 1-4.

Jarboe E, Stone BL, Burman WJ, *et al*. Evaluation of a disk diffusion method for determining susceptibility of *Mycobacterium avium* complex to clarithromycin. *Diagn Microbiol Infect Dis* 1998; 30: 197-203.

Karakousis PC, Moore RD, Chaisson RE. *Mycobacterium avium* complex in patients with HIV infection in the era of highly active antiretroviral therapy. *Lancet Infect Dis* 2004; 4: 557-65.

Kent PT, Kubica GP. Public health mycobacteriology. A guide for the level III laboratory. Atlanta: US Department of Health and Human Service, 1985.

Kiehn TE, Edwards FF. Rapid identification using a specific DNA probe of *Mycobacterium avium* complex from patients with acquired immunodeficiency syndrome. *J Clin Microbiol* 1987; 25: 1551-2.

Koivula T, Cristea-Fernstrom M, Chryssanthou E, Petrini B, Kallenius G. Genetic diversity in clinical isolates of *Mycobacterium avium* complex from Guinea-Bissau, West Africa. *Microbes Infect* 2004; 6: 1320-5.

Kulski JK, Khinsoe C, Pryce T, Christiansen K. Use of a multiplex PCR to detect and identify *Mycobacterium avium* and *M. intracellulare* in blood culture fluids of AIDS patients. *J Clin Microbiol* 1995; 33: 668-74.

Lebrun L, Weill FX, Lafendi L, *et al*. Use of the INNO-LiPA-MYCOBACTERIA assay (version 2) for identification of *Mycobacterium avium-Mycobacterium intracellulare-Mycobacterium scrofulaceum* complex isolates. *J Clin Microbiol* 2005; 43: 2567-74.

Lee H, Park HJ, Cho SN, Bai GH, Kim SJ. Species identification of mycobacteria by PCR-restriction fragment length polymorphism of the *rpoB* gene. *J Clin Microbiol* 2000; 38: 2966-71.

Lim SD, Todd J, Lopez J, Ford E, Janda JM. Genotypic identification of pathogenic *Mycobacterium* species by using a nonradioactive oligonucleotide probe. *J Clin Microbiol* 1991; 29: 1276-8.

Marimon JM, Valiente A, Ercibengoa M, Garcia-Arenzana JM, Perez-Trallero E. Erythromycin resistance and genetic elements carrying macrolide efflux genes in *Streptococcus agalactiae*. *Antimicrob Agents Chemother* 2005; 49: 5069-74.

McNabb A, Eisler D, Adie K, *et al*. Assessment of partial sequencing of the 65-kilodalton heat shock protein gene (*hsp65*) for routine identification of *Mycobacterium* species isolated from clinical sources. *J Clin Microbiol* 2004; 42: 3000-11.

Meier A, Heifets L, Wallace RJ Jr, *et al*. Molecular mechanisms of clarithromycin resistance in *Mycobacterium avium*: observation of multiple 23S rDNA mutations in a clonal population. *J Infect Dis* 1996; 174: 354-60.

Meier A, Kirschner P, Springer B, *et al*. Identification of mutations in 23S rRNA gene

of clarithromycin-resistant *Mycobacterium intracellulare*. *Antimicrob Agents Chemother* 1994; 38: 381-4.

Moore RD, Chaisson RE. Natural history of opportunistic disease in an HIV-infected urban clinical cohort. *Ann Intern Med* 1996; 124: 633-42.

Nash KA, Inderlied CB. Genetic basis of macrolide resistance in *Mycobacterium avium* isolated from patients with disseminated disease. *Antimicrob Agents Chemother* 1995; 39: 2625-30.

Nash KA, Inderlied CB. Rapid detection of mutations associated with macrolide resistance in *Mycobacterium avium* complex. *Antimicrob Agents Chemother* 1996; 40: 1748-50.

National Committee for Clinical Laboratory Standards (NCCLS). Susceptibility testing of Mycobacteria, Nocardiae, and other aerobic Actinomycetes. 2nd ed. Tentative standard M24-A. Wayne, PA: NCCLS, 2002.

Peterson EM, Lu R, Floyd C, Nakasone A, Friedly G, de la Maza LM. Direct identification of *Mycobacterium tuberculosis, Mycobacterium avium,* and *Mycobacterium intracellulare* from amplified primary cultures in BACTEC media using DNA probes. *J Clin Microbiol* 1989; 27: 1543-7.

Swanson DS, Pan X, Kline MW, *et al*. Genetic diversity among *Mycobacterium avium* complex strains recovered from children with and without human immunodeficiency virus infection. *J Infect Dis* 1998; 178: 776-82.

Telenti A, Marchesi F, Balz M, Bally F, Böttger 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.

Thiermann S, Munzinger J, Bodmer T. Comparison of phenotypic and genotypic methods for the detection of clarithromycin resistance in *Mycobacterium avium*. *J Antimicrob Chemother* 2002; 49: 679-81.

Thierry D, Vincent V, Clement F, Guesdon JL. Isolation of specific DNA fragments of *Mycobacterium avium* and their possible use in diagnosis. *J Clin Microbiol* 1993; 31: 1048-54.

Weisblum B. Erythromycin resistance by ribosome modification. *Antimicrob Agents Chemother* 1995; 39: 577-85.