C-024

Differentiation of *Mycobacterium chelonae* and *M. abscessus* using SmartCycler™ PCR and MGB Eclipse™ Probes

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ABSTRACT

The use of partial 16S rDNA sequencing (first one-third of the gene) for the identification of *Mycobacterium* species from cultured isolates has become recognized as a very accurate method for identification. The distinction between *M. chelonae* and *M. abscessus*, however, is not possible with partial 16S rDNA sequencing and therefore requires additional testing. Conventional tests that have been used for the distinction include growth on 5% sodium chloride and growth on citrate medium, both of which have been problematic with test inaccuracies and prolonged turnaround time. Restriction analysis of an amplified fragment of the *hsp*65 gene can distinguish *M. chelonae* from *M. abscessus*, but this requires gel analysis and interpretation of fragment sizes and is sometimes difficult because of the similarity of restriction patterns. In this study we developed a simple and rapid method using real-time PCR with the SmartCycler™ (Cepheid, Sunnyvale, CA) to distinguish *M. abscessus* and *M. chelonae*. The PCR assay is designed with 1 set of primers targeting a small portion of the 16S-23S ITS region and 2 separately labeled MGB Eclipse™ probes (Nanogen, Inc., Bothell, WA); 1 specific for *M. chelonae* and 1 specific for *M. abscessus*. We compared the PCR results of 50 isolates (25 *M. chelonae* and 25 *M. abscessus*) with results obtained using restriction patterns of the hsp65 gene and showed 100% agreement. Additionally we performed PCR, in a blinded fashion, on 90 routine isolates submitted for 16S rDNA sequencing for identification. The samples included 26 isolates of *M. chelonae-M. abscessus* complex and 64 remaining isolates including *Mycobacterium* species, *Nocardia* species, other actinomycetes and culture contaminants (*Staphylococcus epidermidis*, *Microbacterium* species). Specificity and sensitivity were both 100% using the PCR assay compared to 16S rDNA sequencing. The described PCR assay using MGB Eclipse™ Probes is very accurate and specific, is easy to perform, and is a good supplemental test when using 16S rDNA sequencing for the identification of *Mycobacterium* sp.

METHODS

Design of Primers and Probes

- - Obtained sequences from the 16S-23S ITS region
- - Sequences aligned (Lasergene® v6.0, DNASTAR, Inc., Madison, WI)
- - Conserved regions for primers determined
- - Variable regions for probes determined
- - Custom oligonucleotides selected for the MGB Eclipse™ Probe System (Table 1)

MGB Eclipse™ Probe System

- - MGB Eclipse™ Design Software (Nanogen, Inc., Bothell, WA).
- - Primer mix:
  - forward primer (5'-CATTTCCCAGTCGAATGAACT)
  - reverse primer (5'-CAGG[A*]TTTA[A*]AAA[A*]CAT[A*]TTCACCAAG)
- - Probe mix:
  - *M. chelonae* probe (TET (5'-AG[N*]G[A*]GTTTC[T*]GT[A*]G[T*]GG)
  - *M. abscessus* probe (FAM (5'-AGTAGGCATCTGTAGTGG)

- [A*] designating a proprietary modified base, Super A™
- [N*] designating a universal proprietary modified base, Super N™
- [T*] designating proprietary modified bases Super T™

DNA Extraction

DNA was extracted from isolated bacterial organisms using the PrepMan Ultra reagent (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions.

PCR

The PCR master mix was prepared according to Table 2 using OmniMix® beads (Cepheid, Sunnyvale, CA). PCR amplification was performed using the SmartCycler™ (Cepheid, Sunnyvale, CA) with the parameters described in Table 3.

Validation

25 *M. chelonae* and 25 *M. abscessus* were identified by PCR-restriction analysis (PRA) of a 441 bp (Telenti) fragment of the *hsp*65 according to Telenti et al. (1993, J. Clin. Microbiol. 31:175-178). The ability of the assay to differentiate between *M. chelonae* and *M. abscessus* was evaluated by testing these 50 species in a blinded fashion.

PCR specificity was determined by testing 90 consecutive samples of bacteria, identified by partial 16S rDNA sequencing (Table 4).

Table 1. Probe sequences showing positions of ambiguity among various strains of *M. chelonae* and *M. abscessus*.

Species	Source	Probe Target Sequence
PCR Probe *M. chelonae*	Current Assay	AGNGAGTTTCTGTAGTGG
M. chelonae	ATCC 35752[T]	AGCGAGTTTCTGTAGTGG
M. chelonae	GenBank AJ291582, AJ291583	AGCGAGTTTCTGTAGTGG
M. chelonae	GenBank AJ291584	AGCGAGTTTCTGTAGTGG
PCR Probe *M. abscessus*	Current Assay	AGTAGGCATCTGTAGTGG
M. abscessus	ATCC 19977[T]	AGTAGGCATCTGTAGTGG
M. abscessus, type 2	ATCC 700868, 700869	AGTAGGCATCTGTAGTGG

[T] - type strain

Table 2. PCR Master Mix.

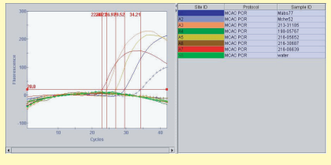
Omni-Mix Beads	1 bead (2 samples)	Final Conc.
Water	27 ul	
Mg++	0	4 mM
Primer Mix (5 uM ea)	10 ul	1.0 uM
FAM Probe (4 uM)	2.5 ul	0.2 uM
TET Probe (4 uM)	2.5 ul	0.2 uM
DNA sample: 4 ul		

Table 3. PCR Amplification Protocol.

Initial	95C – 120 sec
42 Cycles	95C – 20 sec
	55C – 20 sec
	76C – 20 sec
Final	76C – 120 sec

Figure 1

M. CAC PCR using the SmartCycler. Solid lines are *M. abscessus* and slashed lines are *M. chelonae*. Vertical red lines show the relative crossing threshold.



RESULTS

Table 4. Validation of MCAC PCR assay: comparison with partial (5') 16S rDNA sequencing.

Partial 16S rDNA Identification	No. tested	MCAC PCR
M. chelonae-M. abscessus	22	Pos-*M. abscessus*
M. chelonae-M. abscessus	4	Pos-*M. chelonae*
M. branderi	1	Neg
M. fortuitum Complex (sqv I)	1	Neg
M. fortuitum Complex (sqv II)	1	Neg
M. fortuitum Complex (sqv III)	4	Neg
M. fortuitum Complex (sqv IV)	1	Neg
M. goodii	1	Neg
M. gordonae (sqv I)	9	Neg
M. gordonae (sqv II)	1	Neg
M. gordonae (sqv III)	1	Neg
M. gordonae (sqv V)	2	Neg
M. immunogenum	1	Neg
M. intracellulare	3	Neg
M. kansasii [a]	1	Neg
M. kansasii (sqv III or VI-2)	1	Neg
M. kubicae	2	Neg
M. lentiflavum	2	Neg
M. marinum [a]	3	Neg
M. mucogenicum	3	Neg
M. neoaurum	1	Neg
"M. paraffinicum"	1	Neg
M. parascrofulaceum	1	Neg
M. shimoidei	1	Neg
M. simiae	3	Neg
M. szulgai	1	Neg
M. terrae	1	Neg
M. terrae Complex (MCRO6 [b])	1	Neg
M. triplex	1	Neg
M. xenopi	2	Neg
"M. lacticola"	2	Neg
Microbacterium species	1	Neg
Nocardia asteroides (Drug group IV)	1	Neg
Nocardia cyriacigeorgica	1	Neg
Nocardia transvalensis	1	Neg
Nocardia cyriacigeorgica	1	Neg
Nocardia farcinica	1	Neg
Nocardia veterana	1	Neg
Arcobacter butzleri	1	Neg
Gordonia bronchialis	1	Neg
Rhodococcus sp.	1	Neg
Staphylococcus epidermidis	1	Neg

sqv, sequevar
[a] Sequence identification confirmed by colony morphology and pigmentation studies.
[b] Matching sequence retrieved from GenBank.

Figure 2

Crossreactivity of the M. CAC PCR assay. The solid line is the positive control for *M. abscessus* and the slashed line is the positive control for *M. chelonae*.

Crossreactivity

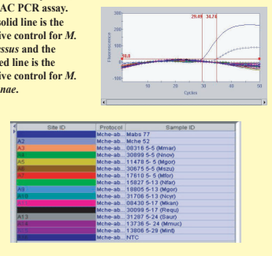


Figure 3

100% accuracy was achieved for the differentiation of *M. abscessus* and *M. chelonae* using PCR compared to *hsp*65 PRA.

PCR vs. *hsp*65 PRA

Differentiation	*M. abscessus* (PRA)	*M. chelonae* (PRA)
M. abscessus (PCR)	25	0
M. chelonae (PCR)	0	25

Figure 4

100% sensitivity and 100% specificity were obtaind for the detection of *M. chelonae-M. abscessus* complex using PCR compared to partial 16S rDNA sequencing.

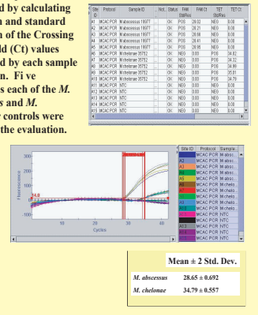
PCR vs. 5' 16S rDNA Sequencing

Detection	MCAC 16S rDNA	Not MCAC 16S rDNA	
Positive PCR	26	0	100% Sensitivity
Negative PCR	0	39	100% Specificity

Figure 5

Within run precision was evaluated by calculating the mean and standard deviation of the Crossing Threshold (Ct) values generated by each sample in the run. Fi ve replicates each of the *M. abscessus* and *M. chelonae* controls were used for the evaluation.

Within Run Precision

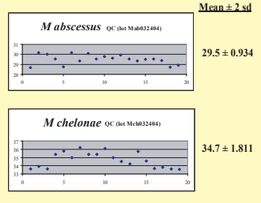


	Mean ± 2 Std. Dev.
M. abscessus	28.65 ± 0.692
M. chelonae	34.79 ± 0.557

Figure 6

Realtime PCR allows for the use of Levy-Jennings plots to evaluate between-run precision using day-to-day quality control (QC). The Crossing Threshold (Ct) values of daily QC are plotted. The mean and standard deviation are calculated from the Ct values.

Between Run Precision



	Mean ± 2 sd
M abscessus QC (lot Mab032404)	29.5 ± 0.934
M chelonae QC (lot Mch032404)	34.7 ± 1.811

CONCLUSIONS

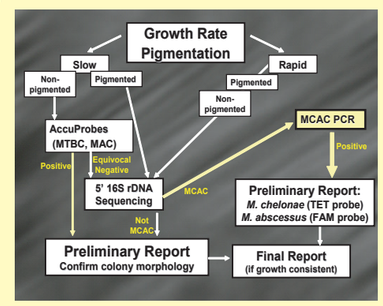
The MCAC SmartCycler™ PCR assay described was designed for use in the microbiology laboratory as a component of the culture algorithm for the identification of *Mycobacterium* species.

The MGB Eclipse™ Probe Systems proved to be an acceptable design of the assay and performed well using the SmartCycler instrument.

The described PCR assay is accurate and specific, is easy to perform, and is a good supplemental test when using 16S rDNA sequencing for the identification of *Mycobacterium* species.

Figure 7

New culture algorithm for the identification of *Mycobacterium* species.



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