Single and Independent Laboratory Validations of a Method for Isolation and Identification of Nontuberculous *Mycobacteria* Associated with Tattoo-related Outbreaks

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SUMMARY

The current study is single and independent laboratory validations of a method for isolation and identification of nontuberculous mycobacteria associated with tattoo-related outbreaks. The validations were conducted following the "Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods" and the 2nd Edition of the Guidelines from the Office of Foods Science and Research Steering Committee. Results of the validations demonstrate that the performance of the test method fulfilled the requirements specified, and suggests that collaborative validation study of the method is warranted. Development of FDA validated methodology for isolation and identification of nontuberculous mycobacteria would help increase the ability of the Agency to better conduct surveillance activities, trace-back analyses, and response to disease outbreaks of infections by nontuberculous mycobacteria.

INTRODUCTION

Recently, there have been several tattoo-related outbreaks of nontuberculous mycobacterial skin infections in the United States. In an effort to halt the outbreaks and to prevent similar events from occurring, FDA conducted investigations to determine the source of the contamination. During the investigations, environmental and water samples were collected from tattoo parlors and manufacturers of tattoo ink. These samples were subjected to selective recovery of mycobacteria followed by species identification of the isolates (2014: Chou et al.). In order to conduct the investigational studies in a time-sensitive manner, a two-step screening and classification procedure was devised. In this scheme, suspect mycobacterial colonies were isolated from both environmental and water samples on selective media. Isolates obtained were then screened using multiplex PCR coupled with melting curve analyses specific for the genus *Mycobacterium* and for differentiating the species within the *M. chelonae–M. abscessus* group (MCAG). Mycobacterial isolates were subsequently identified and classified via sequencing

analysis within the coding regions of both 16S rRNA and RNA polymerase subunit beta. In the investigational study, a total of 45 colonies of *Mycobacterium* were isolated and classified as *M. chelonae, M. immunogenum*, and *M. mucogenicum.* Furthermore, the isolates from each set of samples contained the corresponding species of *Mycobacterium* recovered from outbreak patients.

Nontuberculous mycobacteria (NTM) belong to the genus *Mycobacterium*, a family of Gram-positive bacilli with cell walls high in lipid content and containing characteristic mycolic acids with long branched chains (1999: Metchock et al.). More well-known species within the genus include *M. tuberculosis* and *M. leprae* which cause tuberculosis and Hansen's disease or leprosy, respectively. NTM are widely distributed in the environment, particularly in natural and municipal water. Recently, a group of rapidly growing NTM emerged as important causes of localized cutaneous infections resulted from procedures including Mohs micrographic surgery, cutaneous surgery, breast reconstruction, facial plastic surgery, laser resurfacing, liposuction, body piercing, and pedicures (2010: Drage et al.). Sporadic cases and outbreaks of skin infection associated with tattooing have also been reported identifying the causative pathogens as *M. chelonae*, *M. abscessus, M. immunogenum, M. fortuitum,* and *M. haemophilum* (2009: Preda et al.; 2010: Bechara et al.; 2011: Mitchell et al.; 2011: Kay et al.; 2012: Suvanasuthi et al.; 2012: Kennedy et al.; 2012: Centers for Disease Control and Prevention).

Mycobacteria are slow growing, and may require up to 8 weeks of incubation under optimal conditions to produce visible colonies, depending upon the species (1999: Metchock et al.). Thus, procedures for isolation of mycobacteria from samples containing other faster growing micro organisms typically involve a chemical treatment step prior to plating and incubation of the samples in order to facilitate selective recovery of the mycobacteria. Optimal outcomes of selection and recovery are dependent on choices of chemical treatment, culture media, and incubation conditions. Methods for isolation of mycobacteria from environmental samples have been developed previously by others (1999: Covert et al.; 1997: Neumann et al.).

Traditional methods for identification of mycobacteria rely on traits such as rate of growth, colony morphology, pigmentation, and biochemical profiles (1999: Metchock et al.). Although these methods are well established and relatively inexpensive, they lack the speed and power of strain differentiation and identification. Newer identification methods employ a large array of molecular techniques and are superior to the phenotypic based methods (2009: Behr et al.; 2008: Neonakis et al.). However, each of the newer typing method is designed to provide a particular level of discrimination but not all the requisite data for differentiation and identification.

The present study is single and independent laboratory validations of the aforementioned method used in the outbreak investigational study for isolation and identification of nontuberculous mycobacteria. The goal was to determine whether the method meets the validation criteria set forth by the Office of Foods Science and Research Steering Committee and the suitability of the method for supporting FDA's regulatory and compliance roles.

EXPERIMENTAL

Equipment/Supplies

Incubator, 30ºC
Biological safety cabinet
Micro-centrifuge
Mini plate spinner or equivalent
7500Fast Real-Time PCR System
Cepheid Smartcycler (Cepheid, Sunnyvale, CA)
Cepheid Smartcycler PCR tubes
Applied Biosystem MicroAmp Optical 96-Well Reaction Plate or equivalent
Applied Biosystem MicroAmp Optical Adhesive Film or equivalent
Thermal cycler (Veriti 96 Well Thermalcycler, Life Technologies)
Agarose gel electrophoresis
Gel imaging device (Gel Doc XR, Bio-Rad)
DNA sequencer (3500xL Genetic Analyzer, Life Technologies) with consumables
Latex or nitrile gloves
Vortex mixer
Micro-pipettors (P10, P20, P200, P1000)
Filter-barrier aerosol resistant pipette tips

Reagents

Middlebrook 7H10 Agar (Becton Dickinson)
Middlebrook 7H11 Agar (Remel)
Middlebrook OADC Enrichment (BBL)
Trimethoprim lactate (RPI Research)
Amphotericin B (Fisher Scientific)
Carbenicillin (Mediatech Inc.)
Polymixin B (Fisher Scientific)
Instagene Matrix (Bio-Rad)
2x FastStart Sybr green master mixture (Roche Diagnostics)
FastStart Universal SYBR Green Master (Rox) (Sigma)
PCR and sequencing primers (see Table 1)
HotStarTaq Master Mix Kit (Qiagen)
DNA ladder (DNA Ladder 100bp TRACKIT, Invitrogen)
ExoSAP-IT (Affymetrix)
BigDye Terminator Cycle Sequencing Kit (Life Technologies)
MicroSeq 500 16S rDNA PCR Kit (Life Technologies)
MicroSeq 500 16S rDNA Sequencing Kit (Life Technologies)
Agencourt CleanSEQ (Beckman Coulter)
Tattoo inks, Greywash Set (Fusion, www.fusiontattooinks.com)

Bacterial Strains and Media Preparation

Strains of inclusivity and exclusivity were obtained from American Type Culture Collection (ATCC) or BEI Resources (www.beiresources.org), which is funded by the National Institute of Allergy and Infectious Diseases and managed by ATCC (Table 2). Middlebrook 7H10 agar plates were prepared according to the manufacturer's instruction. Middlebrook 7H11 Selective agar plates were prepared using the following procedure: Suspend 20 grams of Middlebrook 7H11 agar base in 800 ml of water containing 5 ml of glycerol. Heat the suspension to a boil to dissolve completely followed by autoclaving. Cool media to 45-50ºC and aseptically add 100 ml of OADC enrichment, 50 ml of 0.4 mg/ml trimethoprim lactate, 50 ml of 0.2 mg/ml amphotericin B, 1 ml of 0.2 mg/ml carbenicillin and 1 ml of 33.3 mg/ml polymixin B. Mix thoroughly and pour plates.

Sample Processing

Tattoo inks were thoroughly mixed by shaking the container bottles and the exteriors of the bottles were sanitized with 70% alcohol prior to opening. An amount of 0.1 ml ink per plate was removed from the bottle and spiked with 30 colonies forming units of each of the inclusivity or exclusivity stains. Twenty replicates of each 0.1 ml spiked inks were then directly plated onto Middlebrook 7H10 or Middlebrook 7H11 Selective agars followed by incubation at 30˚C for up to 10 days, checking for growth daily. Suspect colonies were isolated or sub-cultured onto a second plate for purity if necessary. Upon sufficient growth, PCR screening and DNA sequencing analyses were performed.

Extraction and Purification of Bacterial DNA

To extract and purify bacterial DNA, a modification of the InstaGene Matrix (Bio-Rad) protocol provided by the manufacturer was used. Specifically, bacterial growth from each isolate was transferred using a pipette tip and re-suspended in 200 µl sterile distilled water in a 1.5-ml micro-centrifuge tube, then pelleted by centrifugation at 12,000 rpm for 1 minute to remove the supernatant, followed by addition of 100 µl of InstaGene Matrix and vortexing. The mixture was incubated at 56ºC for 15 min, vortexed at high speed for 10 second, and heated at 100ºC for 8 min. Before using the resulting DNA preparation, the suspension was vortexed and centrifuged at 12,000 rpm for 2 min. The remaining DNA preparation was stored at -20ºC.

Real-Time PCR Assays

In the current study, two different real-time PCR reactions coupled with melting curve analyses were employed. The assays utilized primers either specific for the genus *Mycobacterium* or for differentiating the species within the MCAG (Table 1). Each PCR reaction contained 1.25 µl of 10 µM primer mix, 12.5 µl 2x FastStart Sybr green master mixture, 9.25 µl molecular-grade water, and 2 µl extracted bacterial DNA. The assays were performed using a SmartCycler real-time PCR instrument. The PCR program included a 95°C activation step for 5 min, followed by 40 cycles of 95°C for 15 s, 60°C for 30 s, and 72°C for 30 s with measurement of the Sybr green fluorescence. Following the last cycle of the PCR reaction, the temperature was ramped from 60°C to 95°C at 0.2°C/s and the fluorescence was continuously measured. The melting curves

were generated by using the instrument's software and displaying the First Derivative and the Melt Temperature.

Table 1. Primers used for PCR amplification and sequencing in the current study

Primer	Nucleotide sequence (5' → 3')	Target gene	Reference
AFB genus FWD-06	CCGCAAGRCTAAAACTCAAA	16S	Richardson E.T. et al.
AFB genus REV-01	TGCACACAGGCCACAAGGGA		
M. chelonae FWD	ACGGGGTGGACAGGATTTAT	ITS	Guarin N. et al.
M. abscessus/M. immunogenum FWD	TGCTCGCAACCACTATTCAG		
MCAG REV	TAAGGAGCACCATTTCCCAG		
MycobF	GGCAAGGTCACCCCGAAGGG	rpoB	Adékambi T. et al.
MycobR	AGCGGCTGCTGGGTGATCATC		

The above real-time PCR assays were extended recently to include use of the 7500Fast Real-Time PCR System. In the platform extension study, both the reaction conditions and the run method remained the same, except that following the last cycle of the PCR reaction, the temperature was ramped from 60°C for 1 min to 95°C for 15s at 1% ramp rate.

DNA Sequencing

Mycobacterial isolates were classified via sequencing analysis targeting the coding regions of both 16S rRNA and RNA polymerase subunit beta, rpoB. For 16S sequencing, commercially available kits were used and manufacture's protocols were followed. For rpoB sequencing, primers shown in Table 1 together with 2 µl of bacterial DNA were used for amplification of the target gene in a 25-µl reaction mixture consisting of 0.5 µM of primers and 1x HotStarTaq Master Mix. The PCR program included a 95°C activation step for 5 min, followed by 35 cycles of 95°C for 40 s, 60°C for 30 s, and 72°C for 2 min and a final 72°C elongation step for 10 min. Five µl of the PCR product was visualized on a 1% agarose gel to ensure amplification of the targets.

To sequence the resulting amplicon, 10 µl of the PCR product was treated with 2 µl ExoSAP-IT at 37°C for 15 min and then 80°C for 15 min. Two µl of the resulting mixture was used in each of two otherwise identical cycle sequencing reactions containing 2 pM of one of the two proceeding PCR primers, 2 µl of BigDye Terminator mixture, 3 µl of 5x BigDye Terminator buffer, and 8 µl of PCR grade water. The cycling conditions included an initial denaturation step of 96°C for 1 min, followed by 25 cycles at 96°C for 10 s, 50°C for 5 s, and 60°C for 75 s. The sequencing products were purified with an Agencourt CleanSEQ kit following the manufacturer's protocol, and were analyzed on a 3500xL Genetic Analyzer. The sequencing results were queried against the BLAST database for significant alignments at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

RESULTS AND DISCUSSION

Regarding the selection of exclusivity organisms, the "Guidelines for the Validation of Analytical Methods for the Detection of Microbial Pathogens in Foods" states that "The choice of exclusivity strains should closely reflect related, potentially cross-reactive organisms". Since the family *Mycobacteriaceae* belongs to the order *Actinomycetales*, and is the most similar to the family *Actinomycetaceae* characteristically (1974: Buchanan et al.), 2 strains each from the 5 genus of the family *Actinomycetaceae* were selected as exclusivity organisms (Table 2).

Regarding the level of background flora in the matrix, the Guidelines specify the need for inclusion of competitive microflora at normal background level and adherence to the AOAC-established parameter, i.e. 1 log greater than microbial analyte being tested for matrices that exhibit low naturally-occurring microflora background. To test the robustness of the current validation method, we chose to use a commercially available tattoo ink as the study matrix, which contains 2.4×10^4 colony forming units per millimeter on Middlebrook 7H10 agars. The medium is commonly used for selective recovery and cultivation of mycobacteria, and was used in our outbreak investigational study to successfully isolate mycobacteria from water samples (2014: Chou et al.). The microflora in the tattoo ink was found to contain the following micro-organisms with different morphologies than those typical for mycobacteria: *Agrobacterium tumefaciens, Alcaligenes faecalis, Alpha proteobacterium, Bradyrhizobium elkanii, Brevundimonas vesicularis, Methylobacterium sp., Microbacterium arthrosphaerae, Microbacterium testaceum, Pseudomonas stutzeri,* and *Rhodopseudomonas sp.* via isolation on Middlebrook 7H10 and Middlebrook 7H11 Selective agars as well as subsequent 16S rDNA sequencing (data not shown).

Considering the close biological relatedness of the inclusivity and exclusivity organisms, it is surprising that all of the exclusivity strains failed to grow on Middlebrook 7H11 Selective agars, and only 2 of them, *Corynebacterium striatum* and *Corynebacterium renale*, had minimum growth on Middlebrook 7H10 agars. Plates of Middlebrook 7H11 Selective agars for inks spiked with the inclusivity organisms supported recovery of the mycobacteria, while Middlebrook 7H10 agars failed due to over growth of the high level of background flora. Therefore, only the Middlebrook 7H11 Selective agar plates were used for the remaining validation study. For each strain of inclusivity organisms spiked into tattoo inks and plated onto Middlebrook 7H11 Selective agars, one colony of suspect mycobacteria from each of the 20 replicates of plates was isolated for further study.

Since no exclusivity organisms could be recovered from spiked tattoo inks, pure cultures of the exclusivity as well as the inclusivity organisms, together with the 20 isolates per inclusivity strain were used for validating the molecular analyses, which consist of a two-step screening and classification procedure (2014: Chou et al., submitted for publication in LIB). The screening step involves two different multiplex PCRs coupled with melting curve analyses specific for the genus *Mycobacterium* (AFB PCR/Tm) or for differentiating the species within the *M. chelonae–M. abscessus* group (MCAG PCR/Tm). Suspect mycobacterial isolates were subsequently identified and classified via sequencing analyses within the coding regions of both 16S rRNA and RNA polymerase subunit beta, rpoB.

For the AFB PCR/Tm analysis, all of the pure cultures of inclusivity strains were positive (Table 2). However, 5 or 7 out of the 10 exclusivity strains, depending on the PCR systems used, were also positive likely due to the close biological relatedness of the inclusivity and exclusivity organisms. For the MCAG PCR/Tm analysis, only species within this group were positive, which does not include *M. fortuitum* (Table 2). To conduct the subsequent DNA sequencing analyses, PCRs for the 16S and rpoB genes were carried out. As expected, all of the strains were positive for the 16S PCR. Meanwhile, all of the inclusivity strains in addition to 3 of the exclusivity strains were positive for the rpoB PCR, again likely due to the close biological relatedness of the inclusivity and exclusivity organisms (Table 2). Results of the DNA sequencing demonstrate that both 16S rDNA and rpoB sequencing analyses together accurately identified all of the inclusivity and exclusivity organisms. It is interesting to note that 16S rDNA sequencing was successful for identifying organisms negative for the rpoB PCR amplification, and that rpoB sequencing was more specific than 16S rDNA sequencing in classifying organisms positive for the rpoB PCR amplification (Table 2). For the 20 isolates per inclusivity strain, nearly identical results were obtained corresponding to those for the pure cultures (data not shown). Taken together, the results of the molecular analyses demonstrated that screening via AFB PCR/Tm analysis resulted in successful inclusion of the entire inclusivity panel though with false positive for some of the nearest neighbor non-target organisms. Subsequent analyses of MCAG PCR/Tm as well as 16S rDNA and rpoB PCR/sequencing accurately identified all of the inclusivity and exclusivity strains.

In the independent laboratory validation study, the inclusivity and exclusivity strains used were *M. chelonae* (ATCC 35752) and *Corynebacterium renale* (ATCC 19412), respectively. Results were consistent with those observed in the single laboratory validation (Appendix 1).

In summary, combination of the selectivity of the Middlebrook 7H11 Selective agars with the specificity of the a two-step screening and classification molecular analyses resulted in the successful recovery and accurate identification of 10 mycobacterial strains spiked into tattoo inks containing a high level of complex background flora in the current validation study. The results indicate that the performance of the test method meets the validation criteria set forth by the Office of Foods Science and Research Steering Committee, and suggest that collaborative validation study of the method is warranted. Development of FDA validated methods for isolation and identification of nontuberculous mycobacteria would help increase the ability of the Agency to better conduct surveillance activities, trace-back analyses, and response to disease outbreaks of infections by nontuberculous mycobacteria.

Table 2. Summary results of method validation for isolation and identification of mycobacteria.

Inclusivity / Exclusivity	Organism	Source	Growth on 7H10	Growth on 7H11	AFB PCR/Tm (SmartCycler)		AFB PCR/Tm (7500)		MCAG PCR/Tm (SmartCycler)		MCAG PCR/Tm (7500)		PCR for sequencing		DNA sequencing	
					PCR	Tm	PCR	Tm	PCR	Tm	PCR	Tm	16S	rpoB	16S	rpoB
inclusivity	M. abscessus	ATCC 700869	+	+	+	80.5	+	79.6	+	81.0	+	80.8	+	+	Mycobacterium abscessus Mycobacterium chelonae	Mycobacterium massiliense*
inclusivity	M. abscessus	ATCC 19977	+	+	+	80.7	+	80.1	+	81.6	+	80.8	+	+	Mycobacterium abscessus Mycobacterium chelonae	Mycobacterium abscessus
inclusivity	M. abscessus	ATCC 203018	+	+	+	81.0	+	80.2	+	82.7	+	80.9	+	+	Mycobacterium abscessus Mycobacterium sp. SR35	Mycobacterium (abscessus subsp.) bolletii
inclusivity	M. fortuitum	ATCC 9820	+	+	+	81.7	+	81.2	–	N/A	–	N/A	+	+	Mycobacterium fortuitum Mycobacterium sp. SY-1-26	Mycobacterium fortuitum
inclusivity	M. fortuitum	ATCC 6841	+	+	+	81.8	+	81.2	–	N/A	–	N/A	+	+	Mycobacterium fortuitum Mycobacterium sp. SY-1-26	Mycobacterium fortuitum
inclusivity	M. immunogenum	ATCC 700505	+	+	+	80.5	+	79.8	+	82.0	+	81.0	+	+	Uncultured bacterium Mycobacterium	Mycobacterium immunogenum
inclusivity	M. immunogenum	ATCC 700506	+	+	+	80.4	+	79.9	+	83.0	+	81.0	+	+	Uncultured bacterium Mycobacterium	Mycobacterium immunogenum
inclusivity	M. chelonae	ATCC 35752	+	+	+	81.4	+	80.2	+	77.3	+	76.2	+	+	Mycobacterium abscessus Mycobacterium chelonae	Mycobacterium chelonae
inclusivity	M. chelonae	ATCC 19235	+	+	+	80.2	+	80.1	+	76.9	+	76.2	+	+	Mycobacterium chelonae Mycobacterium abscessus	Mycobacterium chelonae
inclusivity	M. chelonae	ATCC BAA-2245	+	+	+	80.7	+	80.0	+	76.5	+	75.6	+	+	Mycobacterium chelonae Mycobacterium abscessus	Mycobacterium chelonae
exclusivity	Rothia dentocariosa	BEI HM-245	–	–	+	82.2	+	80.6	–	N/A	–	N/A	+	–	Rothia dentocariosa	N.D.
exclusivity	Rothia mucilaginosa	ATCC 25296	–	–	+	81.6	+	80.7	–	N/A	–	N/A	+	±**	Rothia mucilaginosa	Rothia mucilaginosa
exclusivity	Corynebacterium striatum	ATCC BAA-1293	±	–	+	81.3	+	80.5	–	N/A	–	N/A	+	–	Corynebacterium striatum	N.D.
exclusivity	Corynebacterium renale	ATCC 19412	±	–	+	82.2	+	81.2	–	N/A	–	N/A	+	–	Corynebacterium renale	N.D.
exclusivity	Bifidobacterium breve	BEI HM-412	–	–	+	83.0	+ (2/3)	81.9	–	N/A	–	N/A	+	–	Bifidobacterium breve	N.D.
exclusivity	Bifidobacterium spp.	ATCC BAA-718	–	–	–	N/A	+ (2/3)	81.9	–	N/A	–	N/A	+	–	Bifidobacterium longum	N.D.
exclusivity	Propionibacterium acnes	BEI HM-523	–	–	–	N/A	+ (1/3)	82.1	–	N/A	–	N/A	+	+	Uncultured Propionibacterium sp.	Propionibacterium acnes
exclusivity	Propionibacterim avidum	ATCC 25577	–	–	–	N/A	–	N/A	–	N/A	–	N/A	+	+	Propionibacterium avidum	Propionibacterium avidum
exclusivity	Actinomyces odontolyticus	ATCC 17929	–	–	–	N/A	–	N/A	–	N/A	–	N/A	+	–	Actinomyces odontolyticus	N.D.
exclusivity	Actinomyces isrealii	ATCC 12102	–	–	–	N/A	–	N/A	–	N/A	–	N/A	+	–	Actinomyces israelii	N.D.

* Mycobacterium massiliense is a subspecies of Mycobacterium abscessus

** A low level of amplicon produced

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Appendix 1. Results of independent laboratory validation

Mycobacteria verification-FCC 8/18/2015

Organisms received-*Mycobacterium chelonae* and *Corynebacterium*

ORGANISM CULTURE/DNA PREPARATION

1. 100 µl of tattoo ink was spiked with ~30 cfu of each organism.
2. 100 µl of the spiked ink was plated to both Middlebrook 7H10 and 7H11.
3. The plates were incubated for 4 days at 30ºC.

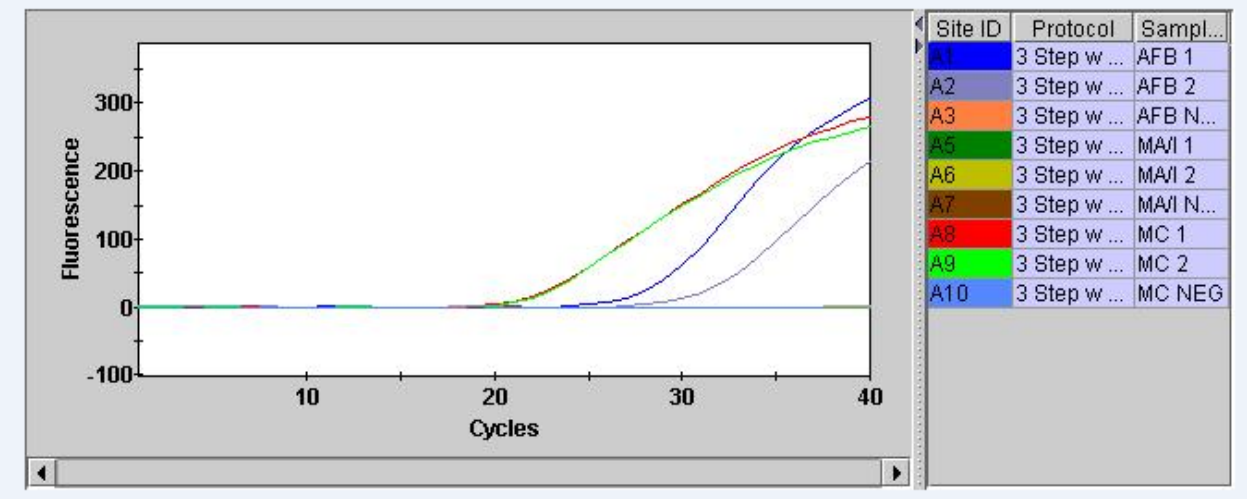
RESULT-Middlebrook 7H10 was not sufficiently selective; multiple colony types were present on 7H10 plates spiked with either organism. Only one apparent colony type was observed on the Middlebrook 7H11 plates spiked with *Mycobacterium*. No growth was observed on the 7H11 plates spiked with *Corynebacterium* after 4 days of incubation.

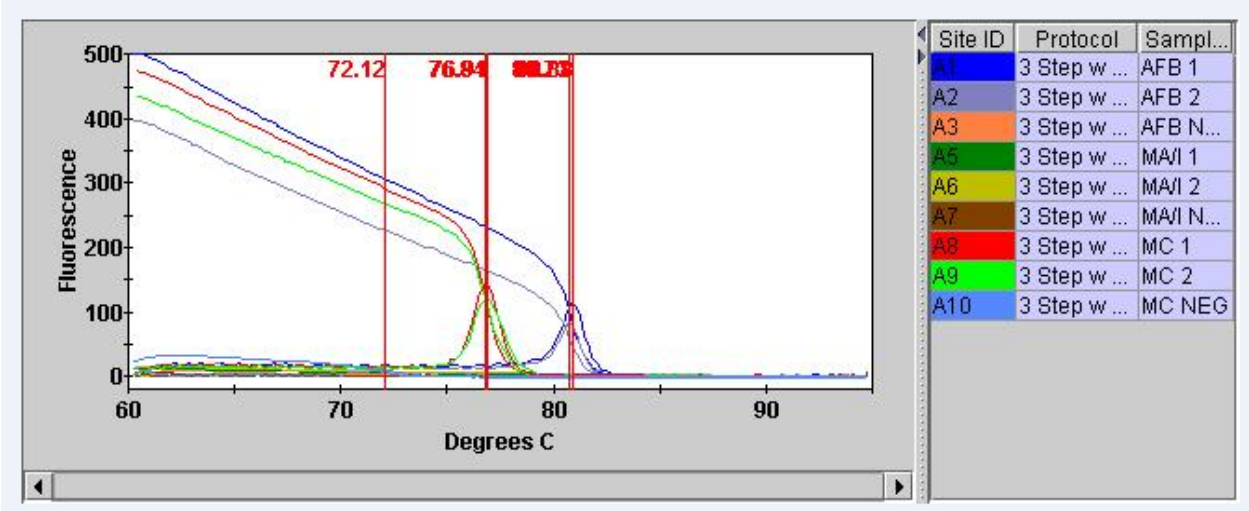
4. Two colonies from the Middlebrook 7H11 plates were streaked for isolation to two new 7H11 plates and incubated at 30ºC for 5 days.
5. DNA was isolated from two colonies from the isolation plates using the provided InstaGene Matrix.

REAL-TIME PCR

1. SYBR Green PCR was performed according to the directions

Mix	Colony #1 Ct	Colony #2 Ct	No Template Ct
AFB genus	28.39	31.69	NEG
M. chel/MCAG REV	23.20	23.37	NEG
M. abs/MCAG REV	NEG	NEG	NEG





RESULT-The *Mycobacteria* genus and *Mycobacteria chelonae* products are amplified with DNA from the *Mycobacteria* colonies. The DNA from the colonies is negative using the *Mycobacteria abscessus* primer set as expected.

MICROSEQ 16S SEQUENCING

1. Isolated DNA was diluted 1:50 in molecular biology grade water, and processed according to the manufacturer's instructions.

Project : PRLSW_MYCO_8_19_15 Specimen : Specimen1 Specimen2 Specimen3 Specimen4 Kit : Bacterial500Kit

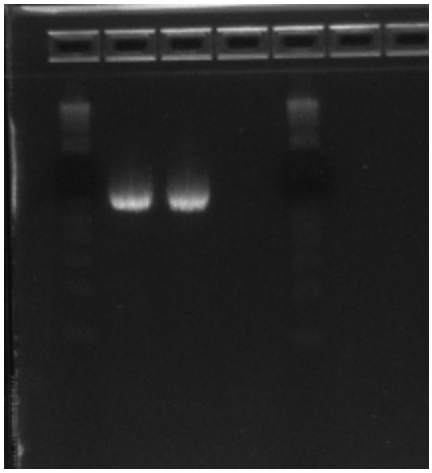
Specimen	% Match	Sequence Entry	Library
COLONY_1	100.00	Mycobacterium abscessus abscessus" (ATCC=19977)	AB_Bacterial500Lib_2013
	100.00	Mycobacterium chelonae chelonae (ATCC=35752)	AB_Bacterial500Lib_2013
	97.84	Mycobacterium farcinogenes (ATCC=35753)	AB_Bacterial500Lib_2013
	97.72	Mycobacterium senegalense (ATCC=35796)	AB_Bacterial500Lib_2013
	97.37	Mycobacterium fortuitum acetamidolyticum (ATCC=359	AB_Bacterial500Lib_2013
COLONY_2	100.00	Mycobacterium abscessus abscessus" (ATCC=19977)	AB_Bacterial500Lib_2013
	100.00	Mycobacterium chelonae chelonae (ATCC=35752)	AB_Bacterial500Lib_2013
	97.78	Mycobacterium farcinogenes (ATCC=35753)	AB_Bacterial500Lib_2013
	97.67	Mycobacterium senegalense (ATCC=35796)	AB_Bacterial500Lib_2013
	97.33	Mycobacterium fortuitum acetamidolyticum (ATCC=359	AB_Bacterial500Lib_2013
NEG_CTRL			No Libraries Searched Against
POS_CTRL	100.00	Escherichia coli (ATCC=11303)	AB_Bacterial500Lib_2013
	100.00	Escherichia coli W3110 (Sigma=W3110)	AB_Bacterial500Lib_2013
	99.90	Escherichia coli (ATCC=35382)	AB_Bacterial500Lib_2013
	99.78	Escherichia coli (ATCC=53503)	AB_Bacterial500Lib_2013
	99.78	Shigella flexneri (ATCC=29903)	AB_Bacterial500Lib_2013

Specimen	# Samples	Base calling	Filter	Assembly	Specimen Score	Top Match	% Match	Consensus Length	Library Entry Length
COLONY_1	2				43	Mycobacterium abscessus abscessus* (ATCC=19977)	100.00	462	461
COLONY_2	2				44	Mycobacterium abscessus abscessus* (ATCC=19977)	100.00	462	461
NEG_CTRL	2				0				
POS_CTRL	2				42	Escherichia coli (ATCC=11303)	100.00	488	489

RESULT The sequence quality is good, but 16S rDNA 500 bp sequencing is insufficient to separate *M. chelonae* from *M. abscessus*.

rpoB Sequencing

1. The initial PCR was conducted according to the work instruction. Lanes 1 and 5-marker; Lanes 2 and 3-colony 1 and 2 DNA; Lane 4-negative control.



2. The sequencing reaction was as follows: (the math in the work instruction doesn't seem to account for the addition of primer volume).

1.5 µl F or R primer (diluted to 20 pM)
3 µl -5X buffer
6.5 µl water
2 µl Big Dye
13 µl + 2 µl DNA-15 µl total volume

1. After sequencing and purification, 10 µl of sample was mixed with 10 µl Hi-Di formamide and sequenced. (3130xl with POP-7).
2. The sequences were entered into Geneious Pro, a consensus sequence derived, and a nucleotide BLAST performed.

Description	Max score	Total score	Query cover	E value	Ident	Accession
Mycobacterium chelonae genome	1074	1074	98%	0.0	99%	CP010946.1
Mycobacterium chelonae strain CIP 104535T RpoB (rpoB) gene, complete cds	1074	1074	98%	0.0	99%	AY147163.1
Mycobacterium chelonae strain FI-08088 RNA polymerase subunit B (rpoB) gene, partial cds	1068	1068	98%	0.0	99%	FJ418059.1
Mycobacterium chelonae strain ATCC 19237 RpoB gene, complete cds	1068	1068	98%	0.0	99%	AY262740.1
Mycobacterium sp. MG7 partial rpoB gene, strain MG7	1064	1064	97%	0.0	99%	AM421385.1
Mycobacterium sp. MG1 partial rpoB gene, strain MG1	1064	1064	97%	0.0	99%	AM421379.1
Mycobacterium sp. MG6 partial rpoB gene, strain MG6	1062	1062	97%	0.0	99%	AM421384.1
Mycobacterium sp. MG2 partial rpoB gene, strain MG2	1062	1062	97%	0.0	99%	AM421380.1
Mycobacterium sp. MG8 partial rpoB gene, strain MG8	1059	1059	97%	0.0	99%	AM421386.1
Mycobacterium chelonae strain PCH-038 RNA polymerase subunit B (rpoB) gene, partial cds	1055	1055	97%	0.0	99%	JN400396.1

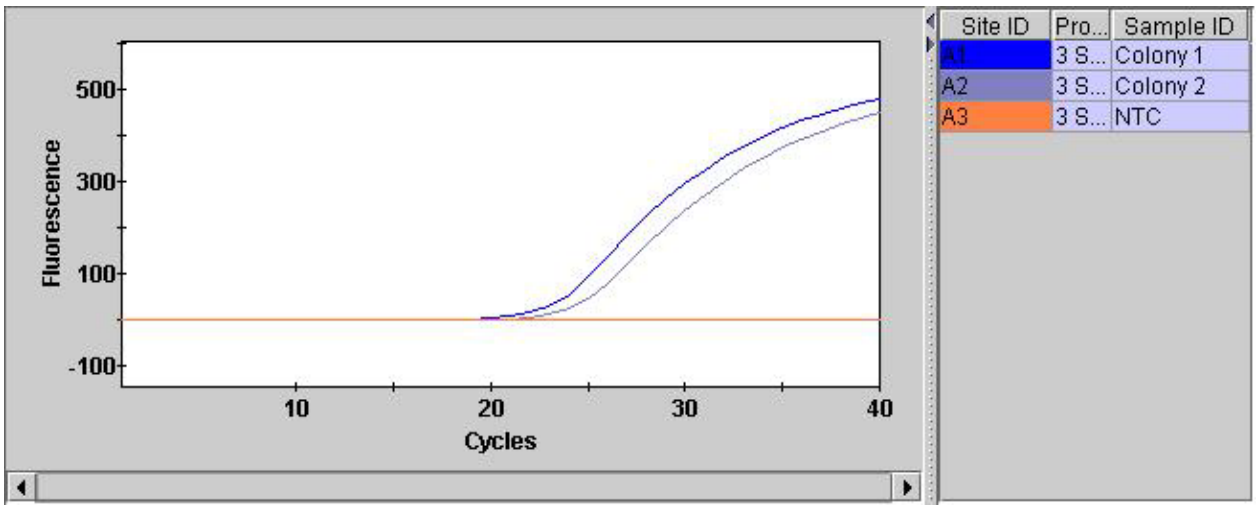
RESULT- the top 50 GenBank hits were *M. chenolae*.

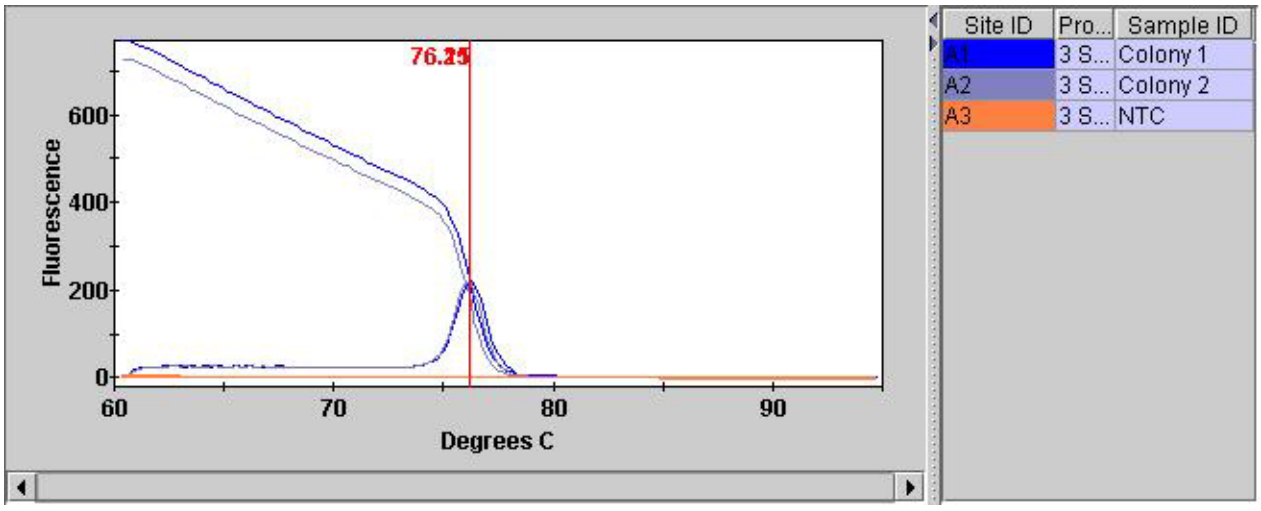
SUMMARY-The assay seems to work as described.

REAL-TIME PCR, experiment 2, performed on 8/20/2015

Note: MCAG PCR was performed with all 3 primers mixed in one reaction

Mix	Colony #1 Ct	Colony #2 Ct	No Template Ct
M. chel/M. abs/MCAG REV	22.95	24.25	NEG


Fluorescence
500
300
100
-100
10
20
30
40
Cycles
Site ID
Pro...
Sample ID
A1
3 S...
Colony 1
A2
3 S...
Colony 2
A3
3 S...
NTC


76.25
Fluorescence
600
400
200
0
60
70
80
90
Degrees C
Site ID
Pro...
Sample ID
A1
3 S...
Colony 1
A2
3 S...
Colony 2
A3
3 S...
NTC