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Journal of Regulatory Science

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Regulatory Science

Journal of Regulatory Science 01 (2015) 9–15

Isolation and Identification of Nontuberculous Mycobacteria Associated with Tattoo-related Outbreaks

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Abstract

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 an investigation to determine the source of the contamination. During the investigation, environmental and water samples were collected from tattoo parlors and manufacturers of tattoo ink. These samples were subjected to isolation of mycobacteria at Wadsworth Center of the New York State Department of Health and at FDA PRLSW followed by species identification of the isolates at PRLSW. 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 screened using multiplex real- time PCR coupled with melting curve analyses specific for the genus *Mycobacterium* and for differentiating the species within the *M. chelonae-M. abscessus* group. Mycobacterial isolates were subsequently identified via sequencing analysis within the coding regions of both 16s rRNA and RNA polymerase subunit beta. In total, 45 colonies of Mycobacterium were isolated and identified as *M. chelonae*, *M. immunogenum*, and *M. mucogenicum*. The isolates from each set of samples contained the corresponding species of Mycobacterium recovered from outbreak patients. Our results suggested that both unsanitary manufacturing processes during production of tattoo ink and the use of non-sterile water for dilution of tattoo ink were possible causes for outbreaks of skin infection in clients of the affected parlors. In addition, the two-step approach taken for screening and identifying mycobacterial colonies in the current study facilitated rapid investigation of tattoo-related outbreaks of nontuberculous mycobacterial infection, thereby enhancing FDAs ability to better protect public health.

Keywords:
Nontuberculous Mycobacteria, Tattoo, Skin Infection, Outbreaks

1. Introduction

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 [1] . More well-known species within the genus include *M. tuberculosis* and *M. leprae* which cause tuberculosis and Hansens 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 [2]. 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* [3–9].

Mycobacteria are slow growing, and may require up to 8 weeks of incubation under optimal conditions to produce visible colonies, depending upon the species [1]. 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 [10, 11].

Traditional methods for identification of mycobacteria rely on traits such as rate of growth, colony morphology, pigmentation, and biochemical profiles [1]. Although these methods are

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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 [12, 13]. 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 a report on our participation in the investigation of recent tattoo-related outbreaks of NTM infection in the United States, in which environmental and water samples were collected for analysis from tattoo parlors and manufacturers of tattoo ink. Our goal was to recover and identify NTM from the samples in a time-sensitive manner. To achieve the goal, we devised a two-step screening and classification procedure. The screening step involves two separate real-time PCR reactions coupled with melting curve analyses that are specific for the genus *Mycobacterium* and for differentiating the species within the *M. chelonae-M. abscessus* group (MCAG). Mycobacterial isolates were then classified via sequencing analysis targeting the coding regions of both 16S rRNA and RNA polymerase subunit beta, rpoB.

2. Materials and methods

2.1. Equipment/Supplies

Microfiltration assembly (Kontes* Ultra-Ware* 47mm Microfiltration Assembly), Membrane filter (Black gridded 0.45 um 47mm, Millipore) Incubator, 30^{o}C, Biological safety cabinet, Micro-centrifuge, Cepheid Smartcycler (Cepheid, Sunnyvale, CA), Cepheid Smartcycler PCR tubes, 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, Micropipettors (P10, P20, P200, P1000), Filter-barrier, aerosol resistant pipette tips.

2.2. Reagents

Cetylpyridinium chloride (CPC, Sigma-Aldrich), 0.5% stock solution, Phosphate buffered saline (PBS, Sigma-Aldrich) with 0.02% Tween 80 (Polyethylene glycol sorbitan monooleate, Sigma-Aldrich) (pH 7.4), Middlebrook 7H10 Agar (Becton Dickinson), Instagene Matrix (Bio-Rad), 2x FastStart Sybr green master mixture (Roche Diagnostics), PCR and sequencing primers (see Table 1 [14, 15]), HotStarTaq Master Mix Kit (Qiagen), DNA ladder (DNA Ladder 100bp TRACKIT, Invitrogen), ExoSAP-IT (Affymetrix), BigDye Terminator Cycle Sequencing Kit (Life Technologies), Agencourt CleanSEQ (Beckman Coulter).

2.3. NTM strains and isolates

Reference NTM strains of *M. chelonae* (ATCC 35752) and *M. abscessus*(ATCC 19977) were purchased from American Type Culture Collection, ATCC. Some of the isolates analyzed in the current study were provided by Dr. Ellen Braun-Howland and were selectively recovered from environmental swabs and water samples at Wadsworth Center, New York State Department of Health [8].

2.4. Sample Processing

Water samples collected by ORA Kansas City Districts Investigations Branch were processed according to a CDC protocol [16]. Specifically, each sub-sample of water was mixed thoroughly by shaking, and divided into two equal parts. One of the aliquots was untreated and the other was decontaminated by adding cetylpyridinium chloride, CPC, to a final concentration of 0.005% followed by incubation at room temperature for 30 minutes. Each aliquot was separately filtered through a black 0.45 μm pore-size membrane filter. The membrane was rinsed by filtration of 5 ml PBS with Tween 80, and placed onto a plate of Middlebrook 7H10 agar followed by incubation at 30^{o}C for up to 10 days, checking for growth daily.

2.5. 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, followed by addition of 50 to 200 μl of InstaGene Matrix, depending on the size of the bacterial pellet. The mixture was incubated at 56^o C for 15 min, vortexed at high speed for 10 second, and heated at 100^{o}C for 8 min. Before using the resulting DNA preparation, the suspension was vortexed for 10 seconds and centrifuged at 12, 000 rpm for 2 min. The remaining DNA preparation was stored at -20^{o}C.

2.6. 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^{o}C activation step for 5 min, followed by 40 cycles of 95^{o}C for 15 s, 60^{o}C for 30 s, and 72^{o}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^{o}C to 95^{o}C at 0.2^{o}C/s and the fluorescence was continuously measured. The melting curves were generated by using the instruments software and displaying the First Derivative and the Melt Temperature.

2.7. DNA Sequencing

Mycobacterial isolates were classified via sequencing analysis targeting the coding regions of both 16S rRNA and RNA polymerase subunit beta, rpoB, using primers shown in Table 1. Two μl of bacterial DNA was used for amplification of the 16S rRNA or the rpoB 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^{o}C activation step for 5 min, followed by 35 cycles of 95^{o}C for 40 s, 60^{o}C for 30 s, and 72^{o}C for 2 min

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		
5F-PCR	TTGRAGAGTTTGATYMTGGCT	16S	Guarin N. et al. and personal communication
531R-PCR	GTATTACCGCGGCKGCTG		
MycobF	GGCAAGGTCACCCCGAAGGG	rpoB	Adékambi T. et al.
MycobR	AGCGGCTGCTGGGTGATCATC		

Table 2. Characterization of isolates using PCR and melting curve analyses

Isolate	*Mycobacterium* genus		MCAG	
	PCR [a]	T_m (^{o}C)	PCR [b]	T_m (^{o}C)
347+CPC-1	–	n/a	–	n/a
373+CPC-1	–	n/a	–	n/a
376++-2	–	n/a	–	n/a
CPC 2-1	+	82.77	–	n/a
CPC 4-1	+	82.96	–	n/a
CPC 4-2	+	83.03	–	n/a
CPC 5-1	+	80.85	+	76.28
CPC 5-2	+	81.06	+	76.37
S2-1	+	80.71	+	76.89
CPC 5-3	+	80.67	+	82.18
M. abscessus	+	80.91	+	81.92
M. chelonae	+	81.10	+	77.40
H_2O	–	n/a	–	n/a

[a] Primer set used was AFB genus FWD-06 and AFB genus REV-01.
[b] Primer set used was *M. chelonae* FWD, *M. abscessus/M. immunogenum* FWD, and MCAG REV.

and a final 72^{o}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^{o}C for 15 min and then 80^{o}C for 15 min. Two μl of the resulting mixture was used in each of two otherwise identical cycle sequencing reactions containing 2 pmoles 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^{o}C for 1 min, followed by 25 cycles at 96^{o}C for 10 s, 50^{o}C for 5 s, and 60^{o}C for 75 s. The sequencing products were purified with an Agencourt CleanSEQ kit following the manufacturers protocol, and were analyzed on a 3500xL Genetic Analyzer. For some of the mycobacterial isolates, the 16S rRNA coding regions were sequenced using a Fast MicroSeq 500 16S rDNA Bacterial Identification PCR Kit. The sequencing results were queried against the BLAST database for significant alignments at http://blast.ncbi.nlm.nih.gov/Blast.cgi.

3. Results and discussion

To identify the bacterial colonies recovered from both the environmental and the water samples, the isolates were first subjected to a screening procedure consisting of two separate real-time PCR reactions coupled with melting curve analyses specific for the genus *Mycobacterium* and for differentiating the species within the MCAG. Representative results of this screening procedure are shown below in Table 2 and Figure 1.

Combined results of the aforementioned assays unambiguously concluded whether an isolate belonged to the MCAG and/or the genus *Mycobacterium* or not (Table 2). However, as depicted in Figure 1, although isolate CPC 5-2 and reference strain *M. chelonae* had similar melting curves, their Tm values had a large difference of 1.03^{o}C. Also, the Tm values for isolate CPC 5-3 and reference strain *M. abscessus* were similar. But their melting curves contained a significant difference in the shoulder peaks. These results exemplified the need for additional data in order to definitively classify the isolates.

To identify the isolates with certainty, the coding regions of both 16S rRNA and RNA polymerase subunit beta, rpoB, were sequenced and queried against the BLAST database for the most significant alignments (Table 3). The rpoB gene sequences were also used to generate sequence alignments and a phylogenetic tree (Figure 2). Consistent with previous findings, 16S rRNA sequence analysis served as a rapid alternative for bacterial identification. Also, for mycobacterial isolates, sequence analysis of the rpoB gene provided more differentiation

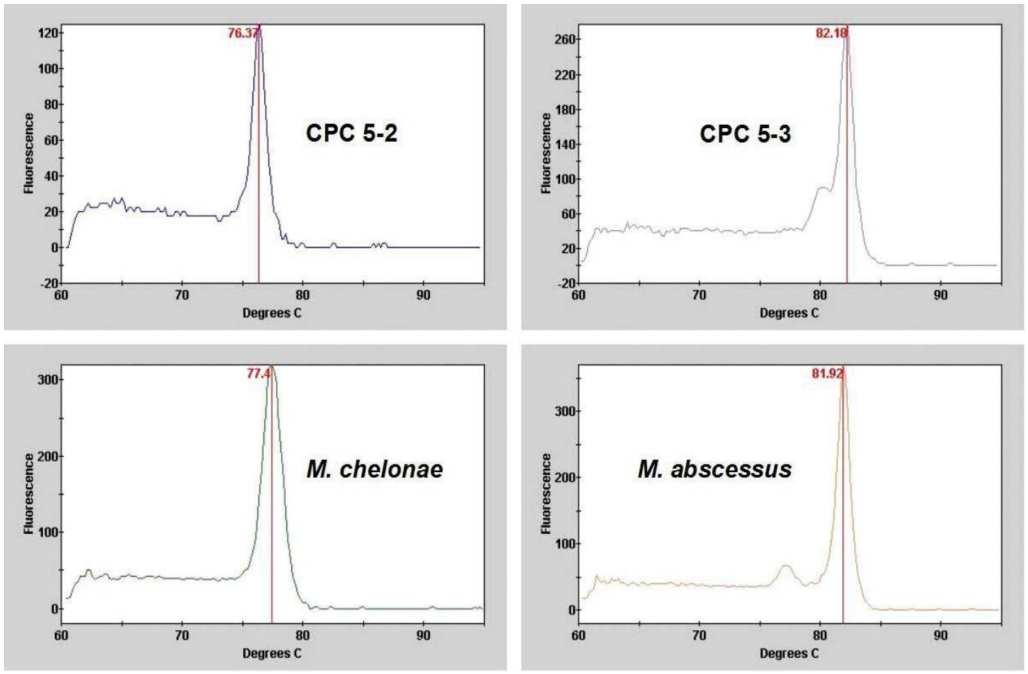

Figure 1. Differentiation and identification of isolates using MCAG specific PCR coupled with melting curve analysis. Suspect mycobacterial isolates were subjected to a PCR based screening procedure as described in the EXPERIMENTAL section and under Table 2. The numbers in red near the peaks represented Tm values for the amplicons.

Table 3. Characterization of isolates using DNA sequencing analysis

Isolate	16S rRNA*	rpoB*
347+CPC-1	*Sphingomonas sp.* and uncultured bacterium clones	N.D.
373+CPC-1	*Methylobacterium populi*	N.D.
376++-2	*Methylococcaceae bacterium*	N.D.
CPC 2-1	*Mycobacterium phocaicum, Mycobacterium mucogenicum, Actinobacterium,* and others	*Mycobacterium mucogenicum*
CPC 4-1	*Mycobacterium phocaicum, Mycobacterium mucogenicum, Actinobacterium,* and others	*Mycobacterium mucogenicum*
CPC 4-2	*Mycobacterium phocaicum, Mycobacterium mucogenicum, Actinobacterium,* and others	*Mycobacterium mucogenicum*
CPC 5-1	*Mycobacterium fuerth*	*Mycobacterium chelonae*
CPC 5-2	*Mycobacterium chelonae*	*Mycobacterium chelonae*
S2-1	N.D.	*Mycobacterium chelonae*
CPC 5-3	*Mycobacterium sp. 28, Mycobacterium immunogenum,* and uncultured bacterium clones	*Mycobacterium immunogenum*
M. abscessus	*Mycobacterium abscessus, Mycobacterium massiliense, Mycobacterium chelonae,* and others	*Mycobacterium abscessus*
M. chelonae	*Mycobacterium chelonae, Mycobacterium fuerth, Mycobacterium sp. E34,* and others	*Mycobacterium chelonae*

* DNA sequencing was performed as described in the EXPERIMENTAL section, and each result was queried against the BLAST database for the most significant alignment(s).
N.D.: not done

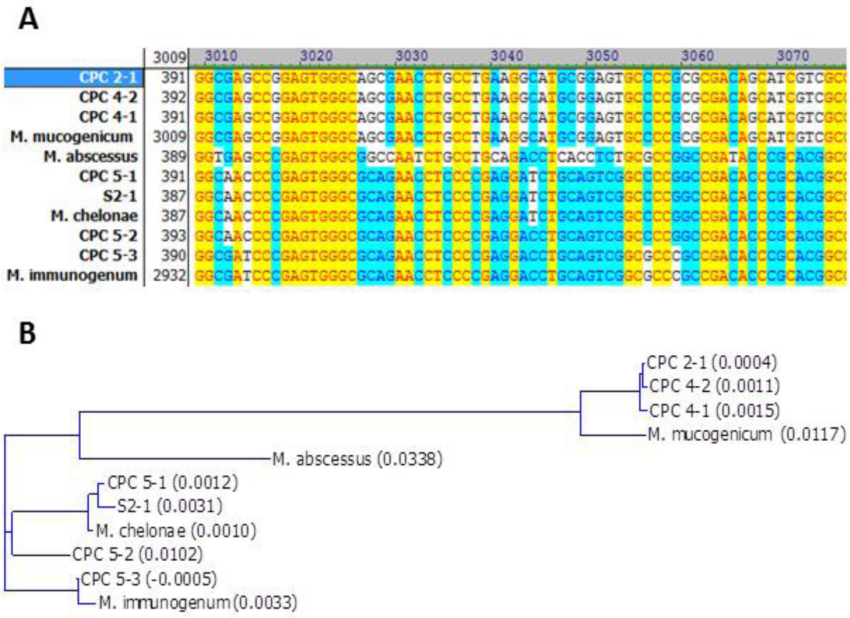

Figure 2. Analyses based on the rpoB genes of the mycobacterial isolates. (A) Sequence alignments of the rpoB genes of the isolates highlighting one of the most variable regions of the target. The multiple sequences were aligned by using AlignX of Vector NTI Advance 11.0 that employs the ClustalW algorithm for pairwise alignments. Identical residues were highlighted in yellow, conservative residues in light blue, and non-similar residues without any highlight. (B) A phylogenetic tree resulted from the sequence alignments. The phylogenetic tree was drawn by Neighbor Joining method in AlignX of the Vector NTI Advance 11.0. Calculated relative distance values were shown in parentheses following the names of the isolates or strains.

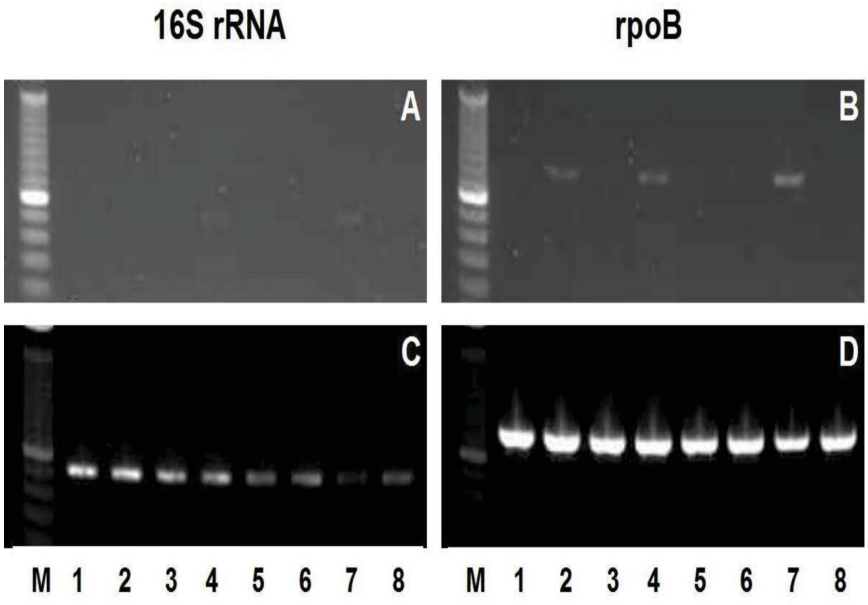

Figure 3. Effect of DNA purification on PCR amplification. Mycobacterial DNA was extracted using a boiling method (panels A and B) or purified using Instagene Matrix (panels C and D). The resulting DNA preparations were used in PCR reactions of sequencing analyses.

among species of *mycobacteria*, and therefore more accurate classification [17].

Following the aforementioned two-step screening and classification procedure, a total of 45 colonies of *Mycobacterium* were isolated and identified as *M. chelonae*, *M. immunogenum*, or *M. mucogenicum*. The isolates from each set of samples contained the matching species of *Mycobacterium* recovered from outbreak patients [8](data not shown). Our findings suggested that both unsanitary manufacturing processes during production of tattoo ink and the use of non-sterile water for dilution of tattoo ink were possible causes for outbreaks of skin infection in clients of the affected parlors.

During the course of this investigational study, two key experimental conditions for the current methodology were identified and optimized. The first key condition was related to the purity of extracted DNA from the mycobacterial isolates. DNA extracted using a boiling method was inhibitory for the PCR step of the sequencing analysis (Figure 3, panels A and B). Use of Instagene Matrix for DNA purification removed the contaminating inhibitor and consistently resulted in acceptable levels of PCR amplification (Figure 3, panels C and D).

The second key experimental condition was the choice of sequencing primer. According to the original method, internal sequencing primers were used for sequencing of the rpoB gene to ensure the heterogeneity of the isolates [17]. However, the internal sequencing primers repeatedly produced low quality reads for all 8 isolates in the initial analysis. On the contrary, use of the PCR primers, especially the MycobF, consistently resulted in high quality sequencing data (results not shown).

In the current investigational study to isolate and identify NTM associated with tattoo-related outbreaks of skin infection, we devised a two-step screening and classification procedure for identifying the isolates. The PCR based screening step allowed fast and unambiguous determination of whether an isolate belonged to the MCAG and/or the genus *Mycobacterium* or not (Table 2). In contrast to the traditional screening method of acid fast staining, minimum amount of bacterial growth is sufficient for performing the PCR based analyses, thus enabling early screening of suspect colonies when they just became visible to the naked eyes. More importantly, the PCR based analysis is capable of determining if suspect colonies belong to the MCAG, which is implicated in most tattoo-related skin infection. Also, in contrast to the widely used PCR-restriction fragment length pattern analysis (PRA) [18], our two-step screening and classification procedure does not involve restriction enzyme digestion nor gel electrophoresis, and is therefore more time saving, less labor intensive, and easily adaptable for handling large number of samples. Although in theory the PRA may provide more data for classification of mycobacteria, such data, however, are obsolete when 16S rRNA and rpoB sequences are available (Table 3). Therefore, we concluded that our two-step screening and classification procedure is highly efficient and effective, especially in facilitating rapid investigation of tattoo-related outbreaks of NTM infection.

4. Declaration of conflicting interest

The authors declare that there is no conflict of interest. Research was funded by U.S. Food and Drug Administration.

5. Disclaimer

The views expressed are those of the authors and should not be construed to represent the views or policies of the U.S. Food and Drug Administration. Any reference to a specific commercial product, manufacturer, or otherwise, is for the information and convenience of the public and does not constitute an endorsement, recommendation or favoring by the U.S. Food and Drug Administration.

6. Acknowledgments

The current study is part of the outbreak investigations coordinated by FDA Core Group I, and conducted by ORA Kansas City Districts Investigations Branch. Dr. Ellen Braun-Howland provided some of the isolates analyzed in the current study that were selectively recovered from environmental swabs and water samples at Wadsworth Center, New York State Department of Health. We thank the staff of the PRLSW Microbiology Branch, especially Geraldine Peregrin and the Media/QC Group, for their technical assistance.

7. Article information

The article was received on July the 2nd, 2015 and available on-line August the 12th, 2015.

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