# Isolation and Identification of Nontuberculous Mycobacteria Associated with Tattoo-related Skin Infections

## Contents

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Introduction</td>
<td>2</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>2</td>
</tr>
<tr>
<td>Method Overview</td>
<td>2</td>
</tr>
<tr>
<td>Equipment and Supplies</td>
<td>3</td>
</tr>
<tr>
<td>Materials and reagents</td>
<td>3</td>
</tr>
<tr>
<td>Selective Recovery of NTM from Tattoo Inks</td>
<td>3</td>
</tr>
<tr>
<td>Extraction and Purification of Bacterial DNA</td>
<td>4</td>
</tr>
<tr>
<td>PCR Coupled with Melting Curve Analyses</td>
<td>4</td>
</tr>
<tr>
<td>DNA Sequencing</td>
<td>5</td>
</tr>
<tr>
<td>References</td>
<td>6</td>
</tr>
<tr>
<td>Appendix 1. Work Instruction for using the AB7500 Fast Real-Time PCR System</td>
<td>7</td>
</tr>
<tr>
<td>Appendix 2. Work Instruction for Microbial Identification by MicroSeq® System</td>
<td>11</td>
</tr>
</tbody>
</table>
Introduction

There have been several tattoo-related outbreaks of nontuberculous mycobacterial infection in the US in recent years. In response to the outbreaks, FDA PSFFL developed a two-step approach for screening and identifying suspect mycobacterial colonies to facilitate rapid investigation of such incidents (1). The method developed performed successfully in several emergency usages and underwent a single and independent laboratory validation study in 2015 as well as a multi-laboratory validation study in 2021 (1-3). The multi-laboratory validation report was approved by the FDA Microbiological Methods Validation Subcommittee in 2022.

Materials and Methods

Method Overview

An overview and a flowchart (Figure 1) of the method are as follows: NTM in tattoo inks are selectively recovered using both Selective Middlebrook 7H11 and Middlebrook 7H10 agars. Typical colonies are then screened morphologically followed by 2 different PCRs coupled with melting curve analyses: one specific for detecting acid-fast bacteria (AFB) and the other for differentiating the species within the M. chelonae–M. abscessus group (MCAG). Isolates positive for the AFB PCR are subsequently identified and classified via DNA sequencing analyses targeting the coding regions of both 16S rRNA and RNA polymerase subunit beta.

Figure 1. A flowchart of the method for isolation and identification of nontuberculous mycobacteria.
Equipment and Supplies

- Incubator, 30 ± 2°C
- Biological safety cabinet
- Micro-centrifuge
- Mini plate spinner or equivalent
- Adjustable heat block or equivalent
- Applied Biosystems 7500 Fast Real-Time PCR System
- Latex or nitrile gloves
- Vortex mixer
- Micro-pipettors (P10, P20, P200, P1000)
- Filter-barrier aerosol resistant pipette tips
- Other routine lab equipment and supplies

Materials and reagents

- Eppendorf DNA LoBind Microcentrifuge Tubes
- Applied Biosystems MicroAmp Optical 96-Well Reaction Plate
- Applied Biosystems MicroAmp Optical Adhesive Film
- Middlebrook 7H10 Agar
- Selective Middlebrook 7H11 Agar
- Instagene Matrix (Bio-Rad)
- PCR primers (see Table 1)
- FastStart Universal SYBR Green Master (ROX)

Selective Recovery of NTM from Tattoo Inks

1. Thoroughly mix tattoo inks by shaking the containers.
2. Wipe the exteriors of the containers with 70% alcohol prior to opening.
3. Remove from each container an amount of 0.1 ml tattoo ink per plate using a P1000 micro-pipettor, for direct plating onto one each of Selective Middlebrook 7H11 and Middlebrook 7H10 agars.
4. Immediately spread the tattoo ink evenly on each of the plate, followed by plating of culture controls.
5. Label and incubate the plates at 30 ± 2°C for up to 10 days.
6. Visually screen for typical colonies daily whenever possible starting on Day 3. Rapid growing NTM colonies may be seen starting from Day 3 after plating.
7. Upon sufficient growth, isolate typical colonies (see below) or if necessary sub-culture them onto a corresponding Selective Middlebrook 7H11 or Middlebrook 7H10 agar plate for purity.
8. Keep a working culture or storage stock for each isolate and perform PCR screening followed by DNA sequencing analyses as appropriate for typical colonies as detailed below.
9. Record growth data for each sub-sample at the end of the 10-day incubation or when isolated typical colonies are picked.
Extraction and Purification of Bacterial DNA

1. Pick suspect bacterial colonies using 1000 µl micropipette tips (as the typical colonies may not stick to bacterial inoculation loops), and resuspend the colony in 100 µl of sterile water in a 1.5-ml micro-centrifuge tube (e.g. using a P1000 pipette with setting at 100 µl). Close and vortex the micro-centrifuge tubes.

2. Transfer 50 µl of each bacterial suspension to a 1.5-ml micro-centrifuge tube containing 100 µl of InstaGene Matrix for DNA extraction. Use the remaining bacterial suspension to prepare a working culture or storage stock.

3. Vortex the tubes at top speed for 10 seconds, and incubate at 56°C for 15 min.

4. Vortex the tubes at top speed for 10 seconds, and heat at 100°C for 8 min.

5. Centrifuge the tubes at 12,000 rpm for 2 min. The extracted DNA in the supernatant is used for PCR and sequencing analyses (see below).

6. Store the remaining DNA preparations at -20°C.

PCR Coupled with Melting Curve Analyses

Each suspect colony is screened with two different PCR reactions coupled with melting curve analyses using the AB7500 Fast Real-Time PCR System. The two PCR reactions utilize primers either specific for the acid-fast bacteria (AFB) or for differentiating the species within the M. chelonae–M. abscessus group (MCAG) (Table 1).

1. Program the AB7500 Fast Real-Time PCR System with the following parameters, which are the same for both AFB and MCAG PCR reactions: 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 “collect data on hold”. Following the last cycle of the PCR reaction, the temperature is ramped from 60°C for 1 min to 95°C for 15 s at 1% ramp rate. [Note: For a more detailed Work Instruction for using the AB7500 Fast Real-Time PCR System, see Appendix 1.]

2. Tally the total number of typical colonies (n) for setting up the two AFB and MCAG PCR assays. For each run of AFB or MCAG PCR assay, add 2 additional reactions -- one negative H2O control and one positive NTM control. Because of the 96-well format of the AB7500 Fast Real-Time PCR System, each run should not exceed the maximum limit of 96 reactions. [Note: If n > 94, i.e. (n+2) > (94+2) = 96, at least 2 separate runs will be necessary -- one for AFB and the other for MCAG PCR. If n = 46 or less, the two PCR assays can be performed in the same run since the run parameters are identical.]

3. Prepare a master mix of (n+4) reactions for each run of AFB or MCAG PCR, each reaction containing 1.25 µl of 10 µM primer mix (AFB or MCAG specific, as appropriate), 12.5 µl FastStart Universal SYBR Green Master (ROX), and 9.25 µl molecular-grade water.

4. Dispense 23 µl of the master mix into each well designated for the PCR assay(s) in a MicroAmp Optical 96-Well Reaction Plate.

5. Add 2 µl of the corresponding extracted bacterial DNA or negative/positive control to each of the designated wells.

6. Seal the plate with a MicroAmp Optical Adhesive Film, then mix and spin briefly.

7. Run the PCR assay(s) using the program specified above in Step 1.
8. Save the run file(s).

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

<table>
<thead>
<tr>
<th>PRIMER</th>
<th>NUCLEOTIDE SEQUENCE (5’→3’)</th>
<th>TARGET</th>
<th>ANALYSIS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td>AFB genus FWD-06</td>
<td>CCGCAAGRCTAAACTCAAA</td>
<td>16S</td>
<td>AFB PCR</td>
<td>4</td>
</tr>
<tr>
<td>AFB genus REV-01</td>
<td>TGCACACAGGCCACAAAGGA</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>M. chelonae FWD</td>
<td>ACGGGGTGGACAGGATTATAT</td>
<td>ITS</td>
<td>MCAG PCR</td>
<td>5</td>
</tr>
<tr>
<td>M. abscessus/M. immunogenenum FWD</td>
<td>TGCTCGCAACCACTATTCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MCAG REV</td>
<td>TAAGGAGCACCATTCCCAG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MycobF</td>
<td>GGCAAGGTCACCSCGAAGGG</td>
<td>rpoB</td>
<td>Sequencing</td>
<td>6</td>
</tr>
<tr>
<td>MycobR</td>
<td>AGCGGCTGCTGGTGATCATC</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

DNA Sequencing

If a suspect colony is positive for the AFB PCR reaction, then it needs to be identified and classified via sequencing analyses targeting the coding regions of 16S rRNA and RNA polymerase subunit beta, rpoB. For the 16S sequencing analysis, commercially available MicroSeq 500 16S rDNA kits are used. SOPs and links to the manufacturer’s protocols are listed in Appendix 2. For rpoB sequencing reactions, use the BigDye Terminator Cycle Sequencing Kit and follow the manufacturer’s protocol. A modified manufacturer’s protocol is detailed below for your reference. Resulting rpoB sequences are queried against the BLAST database for significant alignments, and up to 3 top matches are reported.

1. Set up PCR reactions each containing 1.25 µL of 10 µM rpoB primer mix (Table 1), 12.5 µL HotStarTaq Master Mix, 9.25 µL molecular-grade water, and 2 µL of extracted bacterial DNA or negative/positive control.
2. Run the PCR reactions using the following program: a 95°C activation step for 5 min, 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.
3. To ensure target amplification and quality control, take 5 µL of PCR mixture from each reaction for electrophoresis and subsequent visualization (e.g. on an 1% agarose gel).
4. If target amplification and quality control are satisfactory, take 10 µL of PCR mixture from each reaction, mix with 2 µL ExoSAP-IT, and incubate at 37°C for 15 min and then 80°C for 15 min using a PCR instrument.
5. For each of the PCR reactions, set up two otherwise identical cycle sequencing reactions each containing a forward or reverse primer: 2 µL of ExoSAP-IT treated PCR mixture, 2 µL of BigDye Terminator mixture, 3 µL of 5x BigDye Terminator buffer, and 7.8 µL of PCR grade water, and 0.2 µL of 10 µM MycobF or MycobR primer. (Note: two otherwise identical master mixes without the ExoSAP-IT treated PCR mixture should be prepared first each containing one of the primers, followed by aliquoting of the master mixes and then addition of ExoSAP-IT treated PCR mixture for each cycle sequencing reaction.)
6. Run the cycle sequencing reactions using the following program: 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.

7. The sequencing reaction products are purified using an Agencourt CleanSEQ kit or equivalent following the manufacturer’s protocol (see also Appendix 2, MicroSEQ ID Test Method, 6.9. Alternative Clean Up of Cycle Sequencing Products Using Agencourt CleanSEQ® kit and a magnetic plate), and then analyzed on a DNA sequencing instrument.

References


Appendix 1. Work Instruction for using the AB7500 Fast Real-Time PCR System

1. Turn on the AB7500 Fast Real-Time PCR System.
2. On the screen of the connected computer, open the 7500 Software (v2.3 is illustrated in this Work Instruction) and then click on the icon for “Advanced Setup”.
3. The “Experiment Menu” is found on the left side of the computer screen. Under the “Setup” and on the “Experiment Properties” page, fill out the field for “Experiment Name”.
4. Select “7500 FAST (96 Wells)”.  
5. Select “Quantitation - Standard Curve”.  
6. Select “SYBR Green Reagents”.  
7. Select “Fast (~40 minutes to complete run)”  
8. Click “Plate Setup”, which is below the "Experiment Properties".
9. Under the “Define Targets” on the left side of the page, click on the “Add New Target” until you have 2 targets

10. Change Target 1 to AFB, and Target 2 to MCAG.

11. Make sure the reporter dye is SYBR for both AFB and MCAG, and Quencher is None.

12. Under the “Define Samples” one the right side of the page, click “Add New Sample” until you have the requisite number of reactions, i.e. n+2 (where n = total number of typical colonies, see SOP for details).

13. Change the sample names from “Sample 1” etc. to more descriptive names.

14. Click the “Assign Targets and Samples” tab next to the “Define Targets and Samples” tab near the top of the page.

15. One reaction at a time, click on the well position where you want it to be, and then assign Target (AFB or MCAG) and Sample (your descriptive sample name) by checking the appropriate boxes on the left side of the page under the “Assign target(s) to the selected wells” and “Assign sample(s) to the selected wells”.

8
16. Under “Select the dye to use as the passive reference”, scroll to ROX.
17. Repeat the above two steps until all samples on the plate are defined.
18. Select “Run Method” under “Setup” on the left side of the screen.

19. Choose either the “Graphical View” or the “Tabular View” tab near the top of the page.
20. Enter 25 μl for “Reaction Volume Per Well”
21. You will need one "Holding Stage", one "Cycling Stage" with 3 steps, and one "Melt Curve Stage" with 2 steps. Add or "Delete Selected" stages and steps as needed by using the buttons right under the “Reaction Volume Per Well”.
22. Under the "Holding Stage", set the parameters at 95°C, 5 min.
23. Under the "Cycling Stage", set the “Number of Cycles” to 40, Step 1 at 95°C for 15 s, Step 2 at 60°C for 30 s, Step 3 at 72°C for 30 s. Click to highlight Step 3, then click on the "Collect Data" button to choose “Collect Data On Hold”.
24. Under the "Melt Curve Stage", click on the button for "Continuous", set Step 1 at 60°C for 1 min, Step 2 at 95°C for 30 s, and the ramp rate from 60°C to 95°C at 1%.
25. Name and save your experiment setup as a "eds" file.
26. Click the “Run” tab near the upper left side of the screen, and then the green “START RUN” button.
27. Make sure that the run is started successfully by checking the Run Status, which should show the Estimated Time Remaining. You can also click on the "Temperature Plot" under the "Run" tab to verify that the temperature is as programmed.
Appendix 2. Work Instruction for Microbial Identification by MicroSeq® System

Document title: MicroSEQ ID Test Method
Document number: WEAC-AB-TM.004

Document title: AB MicroSEQ Identification System
Document number: ORA-LAB.018

Document title: Supplemental Information for ORA-LAB.018 AB MicroSEQ Identification System
Document number: JA-000140