**Abstract**

**Background:** The MYChrOme™ Culture Plate is a chromogenic media for the detection and differentiation of rapid-growing nontuberculous mycobacteria (NTM) in water, aided by MYCOn™ decontamination to reduce background microbiota. **Objective:** Evaluate the MYChrOme Culture Plates for the detection of rapid-growing NTM in potable and non-potable water as part of the AOAC Performance Tested Method (PTM) program. **Methods:** Inclusivity and exclusivity of MYChrOme were evaluated with 50 target and 30 non-target organisms. Method robustness and lot stability of MYChrOme were analyzed. The candidate method was compared to a modified US Food and Drug Administration (FDA) Method: U.S. FDA—Isolation and Identification of Nontuberculous Mycobacteria in Tattoo Inks using an equivalency test. The matrix study consisted of artificially contaminated potable water and naturally contaminated non-potable water. Independent laboratory testing was conducted to verify method performance in non-potable water. **Results:** The inclusivity of MYChrOme was 94% within one week, and 98% within two weeks. The exclusivity was 96% for untreated samples and 100% for treated samples. The candidate method remained statistically equivalent for robustness and a three-month shelf-life was confirmed. For both matrixes, the candidate and reference methods were not equivalent, with more colonies enumerated on the candidate method except for one contamination level of the potable matrix. **Conclusion:** The MYChrOme culture method can successfully detect and differentiate rapid-growing NTM in the matrixes tested, with sensitivity equivalent or higher than the reference method. **Highlights:** The MYChrOme culture plate offers differentiation of rapid-growing NTM colonies, improved detection in non-potable samples with MYCOn decontamination, and results within 7 days.

**Principle of the Method**

The MYChrOme™ Culture Plate includes a unique formula for the detection and differentiation of rapid-growing nontuberculous mycobacteria (NTM) in environmental water samples. On this media, rapid-growing NTM colonies remain white/non-colorizer and other bacteria are colorized by crystal violet dye (usually purple).
An example of the differentiation is shown in Figure 1 where Mycobacterium porcinum (Phigenics Culture Collection) forms white colonies and Klebsiella pneumoniae (NCTC 13340) forms purple colonies on the MYChrOme Culture Plate. This is due to the Mycobacterium genus’s ability to metabolize and decolorize the crystal violet. The high level of crystal violet in the media also inhibits the growth of most gram-positive bacteria. In order to further decontaminate the sample, a novel decontamination step is used. The MYCOnTM decontamination reagent relies on low pH and sodium dodecyl sulfate (SDS) to kill the non-mycobacteria. The strong cell wall and hardy nature of mycobacterial cells allow them to survive the decontamination step. Non-potable water samples are plated with the decontamination step and potable water samples with <1000 colony-forming units (CFU)/mL of background microbiota are plated without the decontamination step. After 7 days of incubation at 30 ± 1°C, the white colonies are counted and confirmed to be NTM using serological or molecular methods, such as acid-fast staining or real-time PCR.

General Information

NTM are a category of the genus Mycobacterium that excludes the species that cause tuberculosis (Mycobacterium tuberculosis complex) and leprosy (Mycobacterium leprae complex). NTM are acid-fast, non-motile, and have a unique and complex cell wall structure made up of mycolic acids. This waxy, hydrophobic, and thick cell wall make NTM extremely hardy and thus difficult to disinfect (1). There are two different types of NTM, rapid-growing NTM, which take less than two weeks to form colonies, and slow-growing NTM, which take 2–8 weeks to form colonies.

NTM can cause serious infections, such as skin/soft tissue infections, lymphadenitis, disseminated disease, and most commonly, lung disease presenting similarly to tuberculosis. Exposure to NTM is most common through water systems, especially in monochloraminated systems. NTM can grow and proliferate in biofilms and tend to aggregate in water. This is especially a problem in facilities such as hospitals with large populations of immunocompromised individuals that can potentially be exposed to NTM through sinks and showers (1).

Current methods for detecting NTM are adapted from methods for the isolation of M. tuberculosis and involve spread-plating a sample onto a limited nutrient agar such as Middlebrook 7H10 or Middlebrook 7H11 Selective (7H11S). An additional decontamination step with cetylpyridinium chloride (CPC) is often utilized before plating but has been noted to be overly harsh to several species of NTM (2). Differentiation of NTM from other bacteria is not possible on these agars.

Scope of Method

(a) Analyte.—Rapid-growing nontuberculous Mycobacterium spp.
(b) Matrixes.—Potable and non-potable water (200 mL).
(c) Summary of validated performance claims.—The MYChrOme Culture Plate method was found to be as equivalent or better than the US Food and Drug Administration (FDA).—Isolation and Identification of Nontuberculous Mycobacteria in Tattoo Inks (3), modified for water samples.

Definitions

(a) Repeatability (s_r).—Standard deviation of replicates for each analyte at each concentration of each matrix for each method.
(b) Mean difference between candidate and reference methods.—Mean difference between candidate and reference method transformed results with 90% confidence interval for each analyte at each concentration of each matrix.
(c) Selectivity.—Ability of the method to detect analyte without interference from matrix or other components of similar behavior.
(d) Bias.—Bias is the difference between the candidate method mean result and the true value or reference method value, mean_{cand} − known spike or mean_{cand} − mean_{ref}.
(e) Standard deviation of repeatability.—s_r = \sqrt{\frac{\sum_{i=1}^{n} (x_i - \bar{x})^2}{n-1}}
Materials and Methods

Test Kit Information

(a) Kit name.—MYChrOme Culture Plate.
(b) Product number.—MB-2650.
(c) Ordering information.—Contact Phigenics, LLC 3S701 West Ave. Suite 100 Warrenville, IL 60555; Tel: 1.844.850.4087. Website: info.phigenics.com, for more information.

Test Kit Components

(a) MYChrOme Culture Plate.
(b) MYCON decontamination reagent.

Additional Supplies and Reagents

(a) Water collection bottles.—Sterile with 100 mg/L sodium thiosulfate.
(b) Reusable filter unit.—Thermo Scientific Cat. No. 09-740-23A, or equivalent.
(c) 0.22 μm filters.—47 mm, track-etched polycarbonate.
(d) 15 mL conical tubes.
(e) Sterile forceps.
(f) 1:40 Ringer’s Solution.—Diluted from 1:4 Ringer’s (Sigma-Aldrich, Cat. No. 96724-100TAB).
(g) Disposable plastic cell spreaders.—Sterile.
(h) Disposable plastic loops.—Sterile.
(i) Micropipet and tips.—2–20 μL.
(j) Micropipet and tips.—100–1000 μL.
(k) Microcentrifuge tubes.—1.5 mL.
(l) Mycobacterium genus-specific real-time colony PCR.—For colony confirmation.

Apparatus

(a) Incubator.—Set to 30 ± 1°C.
(b) Real-time PCR Thermocycler.—Bio-Rad, CFX96 Deep Well Touch, or equivalent.
(c) Vortex.

Reference Materials

Bacterial strains used in this study were sourced from the following institutions:

(a) American Type Culture Collection (ATCC).—Manassas, VA.
(b) National Collection of Type Cultures (NCTC).—Porton Down, UK.
(c) Phigenics Culture Collection (Phigenics).—Porton Down, UK.
(d) Culture Collection University of Gothenburg (CCUG).—Gothenburg, Sweden.
(e) US FDA.—Irvine, CA.
(f) Public Health England (PHE).—London, UK.
(g) National Collection of Industrial, Food, and Marine Bacteria (NCIMB).—Aberdeen, UK.

Safety Precautions

Follow biosafety level 2 precautions and wear appropriate personal protective equipment. All work should be conducted in properly equipped facilities utilizing the appropriate safety equipment (for example, physical containment devices). Biological samples have the potential to transmit infectious diseases.

(a) Decontaminate all surfaces and equipment prior to and after use.
(b) It is the responsibility of each laboratory to handle waste and effluents processed according to their nature and degree of hazardness and to treat and dispose of them in accordance with applicable local, state, and federal regulations.

Sample Preparation

(a) Potable and non-potable water.—Collect water samples in sterile bottles with sodium thiosulfate (100 mg/L) to neutralize the residual oxidant. Filter concentrates 200 mL of the water sample on a 0.22 μm track-etched 47 mm polycarbonate membrane. Testing must be performed within 36 h of sample collection.

Analysis

(a) Resuspend the filter in 10 mL of one of the following: Sterile 1:40 Ringer’s solution, filtrate, sterile deionized (DI) water, or sterile phosphate buffered saline (PBS).

(b) Vortex the sample for 30 s.

(1) For potable samples, 100 μL of the concentrated sample is spread plated onto a MYChrOme Culture Plate.

(2) For non-potable water, transfer 981 mL of concentrated sample to 19 mL of MYCon decontamination reagent, pipet up and down to mix, and incubate at room temperature for 5 min. After the incubation period, 100 μL of the decontaminated sample is spread plated onto a MYChrOme Culture Plate.

(c) Incubate the MYChrOme plates at 30 ± 1°C for 7 days.

Calculations, Interpretation, and Test Result Report

(a) After 7 days of incubation, enumerate all white colonies, very light purple, grey colonies, or bright yellow colonies (some NTM form yellow colonies). These are the suspect rapid-growing NTM colonies that require confirmation.

(b) All purple, brown, or pink colonies are not NTM and do not need to be analyzed further.

Confirmation

(a) Confirm that the suspect colonies identified are NTM using serological (acid-fast) or molecular methods (Mycobacterium genus-specific real-time PCR).

(b) Recommended confirmation method.—Real-time PCR for NTM that targets the atpE gene using the primers and probe from Radomski et al. (4). The following thermocycling conditions were used: The activation step (1 cycle) is 95°C for 3 min, followed by 40 cycles of 95°C for 10 s, and 60°C for 30 s. The suspect colony is picked and directly inoculated into 40 μL of PCR master mix.
Validation Study

This validation study was conducted under the AOAC Research Institute Performance Tested Method(s)™ program and the AOAC INTERNATIONAL Methods Committee Guidelines for Validation of Microbiological Methods for Food and Environmental Surfaces (5).

Method developer studies were conducted in the laboratory of Phigenics, LLC (Reno, NV), and included the inclusivity/exclusivity study, matrix studies for potable and non-potable, product consistency and stability studies, and robustness testing. The independent laboratory study was conducted by Silliker Food Science Center: Mérieux NutriSciences (Crete, IL), and included a matrix study for non-potable water.

Method Developer Studies

Inclusivity testing.—Methodology.—A total of 50 rapid-growing NTM strains were used for inclusivity testing (Table 1). Each strain was cultured in 1395 Middlebrook 7H9 broth (Hardy Diagnostics, Santa Maria, CA) for 5–7 days at 30 ± 1°C. Each culture was diluted in sterile PBS and inoculated into 200 mL of sterile DI water to achieve a concentration of approximately 1–100 CFU/mL. This 200 mL sample was filter-concentrated through a 0.22 μm track-etched 47 mm polycarbonate membrane. The filter was placed into a 15 mL conical tube, resuspended in 10 mL of sterile DI water, and vortexed for 30 s. Each sample was inoculated onto MYChrOme media by spread plating 100 μL. This protocol, in addition to five replicates of the non-target organism (Mycobacterium abscessus, Mycobacterium neoaurum, Mycobacterium aurum, Mycobacterium murale, Mycobacterium moriokaense) was not detected (n = 19) on MYChrOme. The one strain with an off-white colony color was Nocardia brasiliensis which had 31 colonies on the untreated plate and zero colonies on the treated plate (see discussion).

Results.—Results are shown in Table 1. Out of 50 non-mycobacterial strains (100%) were not detected (n = 28) or had purple colonies (n = 2) for the treated samples. The two strains with purple colony growth after the treatment were Chryseobacterium sihigense and Psedomonas aeruginosa, with 46 and 3 colonies, respectively. For the untreated samples, 29 (96.6%) strains were either not detected (n = 11) or had purple colonies (n = 19) on MYChrOme. The one strain with an off-white colony color was Nocardia brasiliensis which had 31 colonies on the untreated plate and zero colonies on the treated plate (see discussion).

Robustness study.—Methodology.—The MYChrOme protocol, including sample concentration, decontamination, and plating, was analyzed for its ability to remain unaffected by small variations in method parameters. The parameters analyzed in a factorial design were sample volume, filtrate volume, and decontamination incubation time (Table 3). Sterile DI water artificially contaminated with 1 × 10^6 CFU/mL of Acinetobacter baumannii (NCIMB 12457) was used for the non-target organism samples and spiked with two levels of Mycobacterium chelonae (CCUG 37827) for the target organism samples. The low-level target organism samples were spiked with 25–50 CFU/mL and the high-level target organism samples were spiked with approximately 5000 CFU/mL. Five replicates per sample type were analyzed in a randomized, blind-coded fashion by each of the nine factorial methods by plating 100 μL of treated sample onto MYChrOme. The plates were incubated at 30 ± 1°C for 7 days and colonies were counted. Each modified method was compared to the standard MYChrOme method (number 9) using an unpaired equivalency test assuming equal variances with a 90% confidence interval.

Results.—Robustness results are shown in Table 3. For the low-level target organism, the colony counts for the eight modified methods were statistically equivalent to the nominal method. For the high-level target organism, all nine methods resulted in too numerous to count MYChrOme plates with all white colonies. For the non-target organism, all nine methods resulted in zero colonies on the MYChrOme plates.

Product consistency (lot-to-lot) and stability study.—Methodology.—Three lots of MYChrOme plates were analyzed for consistency, stability, bias, and repeatability over three months at 4°C. Lot 1 (end of shelf-life) was manufactured on September 1st, 2020; lot 2 (middle of shelf-life) was manufactured on October 10th, 2020; and lot 3 (beginning of shelf-life) was manufactured on November 15th, 2020. All three lots were manufactured by the Phigenics Analytical Services Laboratory in Fayetteville, AR. This study was performed on December 2nd, 2020. To evaluate the product consistency five replicates of high and low-level target organism (Mycobacterium fortuitum, ATCC 6841) were analyzed in addition to five replicates of the non-target organism (A. baumannii, NCIMB 12457). The M. fortuitum stock was cultured on MYChrOme for 5 days at 30 ± 1°C and used to inoculate sterile PBS and diluted in order to plate approximately 5000 CFU/mL.
The cell suspension for the high-level target organism was diluted to 10^{-2} and plated in quintuplicate in order to recover countable plates. For the non-target organism, A. baumannii was grown in non-selective broth for 24 h at 30 ± 1°C and was not diluted. 100 μL of each cell suspension was plated onto five replicates of each MYChOme lot in a randomized, blind-coded fashion. After 7 days of incubation at 30 ± 1°C, the plates were counted and colony color was recorded. Each lot was compared to the other with an unpaired equivalency test assuming equal variances and using a 90% confidence interval.

**Results.**—The stability study results are summarized in Table 4. All MYChOme lots were statistically equivalent for the high- and low-level target organism samples. All of the high-level and approximately 50 CFU/mL for the low-level.

| Genus                  | Species                      | Source            | Origin          | Untreated, Log_{10} CFU/plate | Treated, Log_{10} CFU/plate |
|------------------------|------------------------------|-------------------|-----------------|-------------------------------|-----------------------------|
| Mycobacterium          | abscessus                    | FDA 858508-1      | Not available   | 2.48                          | 2.48                        |
| Mycobacterium          | abscessus                    | FDA 923093-1075   | Not available   | 2.10                          | 2.10                        |
| Mycobacterium          | abscessus subsp. abscessus   | CCUG 71636        | Human blood     | 0.48                          | 0.04                        |
| Mycobacterium          | abscessus subsp. bolletii    | CCUG 50184        | Human bronchial lavage | 4.00                      | 0.48                        |
| Mycobacterium          | abscessus subsp. massiliense | CCUG 48898        | Human sputum    | 0.48                          | 0.30                        |
| Mycobacterium          | agri                         | CCUG 37673        | Soil            | 2.48                          | 2.48                        |
| Mycobacterium          | aubagnense                   | CCUG 50186        | Human bronchial aspirate | 0.04                      | 0.04                        |
| Mycobacterium          | aurum                        | CCUG 70546        | Soil            | 2.48                          | 2.48                        |
| Mycobacterium          | barrassiae                   | CCUG 50398        | Human bronchial lavage | 2.48                      | 2.48                        |
| Mycobacterium          | boenickei                    | CCUG 47580        | Human wound     | 1.08                          | 0.70                        |
| Mycobacterium          | brisbanense                  | CCUG 47584        | Antral sinus    | 0.30                          | 0.60                        |
| Mycobacterium          | canariensis                  | CCUG 47953        | Human blood     | 0.85                          | 0.04                        |
| Mycobacterium          | chelonae                     | PHE               | Not available   | 1.18                          | 0.90                        |
| Mycobacterium          | chelonae                     | Pheigenics        | Env. isolate    | 2.00                          | 1.90                        |
| Mycobacterium          | chelonae                     | CCUG 72969        | Human eye       | 0.48                          | 0.48                        |
| Mycobacterium          | chelonae                     | CCUG 37827        | Human wound     | 0.60                          | 0.04                        |
| Mycobacterium          | chelonae                     | FDA 858509-1-1-1  | Not available   | 2.48                          | 2.48                        |
| Mycobacterium          | chelonae                     | FDA 858509-2-3-2  | Not available   | 2.54                          | 2.48                        |
| Mycobacterium          | chelonae                     | CCUG 39181        | Soil            | 1.15                          | 0.04                        |
| Mycobacterium          | chelonae                     | CCUG 57579        | Human feces     | 0.04                          | 0.04                        |
| Mycobacterium          | chelonae                     | CCUG 5204         | Human blood     | 0.95                          | 0.90                        |
| Mycobacterium          | chelonae                     | CCUG 52935        | Cold abscess    | 0.30                          | 0.04                        |
| Mycobacterium          | chelonae                     | FDA 858508-10     | Not available   | 2.48                          | 2.48                        |
| Mycobacterium          | chelonae                     | FDA 923093-1278   | Not available   | 2.48                          | 2.48                        |
| Mycobacterium          | fortuitum                    | CCUG 46694        | Human blood     | 0.48                          | 0.48                        |
| Mycobacterium          | fortuitum                    | CCUG 55442        | Human feces     | 2.30                          | 1.93                        |
| Mycobacterium          | fortuitum                    | CCUG 5204         | Human blood     | 0.60                          | 0.30                        |
| Mycobacterium          | fortuitum                    | CCUG 38151        | Chemical contaminate | 1.34                      | 1.20                        |
| Mycobacterium          | fortuitum                    | PHE               | Not available   | 0.78                          | 0.78                        |
| Mycobacterium          | fortuitum                    | CCUG 52935        | Water for injection | 0.60                      | 0.04                        |
| Mycobacterium          | iranicum                     | CCUG 52927        | Human sputum    | 1.89                          | 1.04                        |
| Mycobacterium          | lagenitens                   | CCUG 51275        | Human calf      | 0.95                          | 1.11                        |
| Mycobacterium          | mariolaense                  | CCUG 37671        | Soil            | 1.74                          | 0.48                        |
| Mycobacterium          | mucogenicum                  | Pheigenics        | Env. isolate    | 2.00                          | 0.48                        |
| Mycobacterium          | mucogenicum                  | FDA 858510-2      | Not available   | 0.30                          | 0.04                        |
| Mycobacterium          | mucogenicum                  | FDA 858510-4      | Not available   | 1.81                          | 1.60                        |
| Mycobacterium          | mucogenicum                  | FDA 858510-9      | Not available   | 2.46                          | 2.12                        |
| Mycobacterium          | murale                       | CCUG 57579        | Wall material   | ND                            | ND                          |
| Mycobacterium          | neaurum                      | Pheigenics        | Env. isolate    | 2.16                          | 1.23                        |
| Mycobacterium          | peregrinum                   | CCUG 41354        | Human bronchial aspiration | 2.00                      | 1.38                        |
| Mycobacterium          | phocicum                     | Pheigenics        | Env. isolate    | 1.23                          | 0.48                        |
| Mycobacterium          | phocicum                     | CCUG 50185        | Human bronchial aspirate | 0.30                      | 0.04                        |
| Mycobacterium          | phocicum                     | FDA 858510-1      | Not available   | 2.20                          | 2.10                        |
| Mycobacterium          | porcinum                     | Pheigenics        | Env. isolate    | 1.91                          | 1.28                        |
| Mycobacterium          | porcinum                     | CCUG 37674        | Swine lymph node | 0.70                          | 0.30                        |
| Mycobacterium          | porcinum                     | CCUG 59339        | Human sputum    | 1.70                          | 0.04                        |
| Mycobacterium          | septicum                     | CCUG 47583        | Not available   | 0.78                          | 0.04                        |
| Mycobacterium          | smegnatis                    | ATCC 14468        | Not available   | 1.23                          | 0.48                        |
| Mycobacterium          | woliniskyi                   | CCUG 47168        | Human abscess   | 1.57                          | 1.15                        |
level target organism plates had too numerous to count white colonies, so the dilution plates were used for accurate colony counts. One dilution plate of the high-level target organism was contaminated on lot 1 and could not be counted. All lots of the non-target organism were too numerous to count with purple colonies, as expected.

Table 2. Exclusivity testing results for the MyChrOme culture plate method

| Genus          | Species            | Source     | Origin                  | Untreated, CFU/mL-color | Treated, CFU/mL-color |
|---------------|--------------------|------------|-------------------------|-------------------------|-----------------------|
| Acinetobacter | baumannii          | NCIMB 12457 | Urine                   | Lawn-purple             | ND*                   |
| Aeromonas     | hydrophila         | ATCC 35654 | Not available           | Lawn-purple             | ND                    |
| Alcaligenes   | faecalis           | ATCC 35655 | Not available           | Lawn-purple             | ND                    |
| Bacillus      | subtilis           | ATCC 14990 | Nose                    | ND                      | ND                    |
| Burkholderia  | cepacia            | ATCC 25608 | Incision wound          | Lawn-purple             | ND                    |
| Chryseobacterium | shigense         | ATCC 51823 | Milk                    | Lawn-purple             | 23-dark purple        |
| Elizabethkingia | meningoseptica   | ATCC 13253 | Spinal fluid            | Lawn-purple             | ND                    |
| Escherichia   | coli               | ATCC 10536 | Not available           | Lawn-purple             | ND                    |
| Klebsiella    | aerogenes          | ATCC 13048 | Sputum                  | Lawn-purple             | ND                    |
| Klebsiella    | pneumonia          | NCTC 13340 | Not available           | Lawn-purple             | ND                    |
| Legionella    | Anisa              | Phigenics  | Env. isolate            | ND                      | ND                    |
| Legionella    | birminghamensis    | CCUG 31233 | Human lung biopsy       | 0.5-purple              | ND                    |
| Legionella    | bozemanii          | CCUG 16416 | Lung aspirate           | ND                      | ND                    |
| Legionella    | Feei               | CCUG 29668 | Human lung tissue       | 3-slate gray            | ND                    |
| Legionella    | Jordansis          | CCUG 16413 | Jordan river            | ND                      | ND                    |
| Legionella    | longbeachae        | ATCC 33462 | Human lung              | ND                      | ND                    |
| Legionella    | pneumophilia        | CCUG 9568T | Human lung              | ND                      | ND                    |
| Legionella    | pneumophilia sg 1  | CCUG 33823 | Human lung              | ND                      | ND                    |
| Legionella    | sainthelenesi      | CCUG 29672T| Stream water            | ND                      | ND                    |
| Legionella    | uadswothrii        | CCUG 16415T| Human sputum            | ND                      | ND                    |
| Methylobacterium | spp.              | Phigenics  | Env. isolate            | ND                      | ND                    |
| Microbacterium | oxydans/maritipicu | Phigenics  | Env. isolate            | ND                      | ND                    |
| Nocardia      | brasiliensis       | ATCC 19296 | Not available           | 15.5-off white          | ND                    |
| Pseudomonas   | aeruginosa         | ATCC 27853 | Blood                   | Lawn-purple             | 1.5-purple            |
| Pseudomonas   | Fragi              | ATCC 51821 | Milk                    | Lawn-purple             | ND                    |
| Pseudomonas   | Masseli            | ATCC 49838 | Not available           | Lawn-purple             | ND                    |
| Pseudomonas   | Stutzeri           | ATCC 17588 | Spinal fluid            | Lawn-purple             | ND                    |
| Sphingomonas  | paucimobilis       | ATCC 29837 | Hospital respirator     | Lawn-purple             | ND                    |
| Staphylococcus | Aureus            | ATCC 25923 | Clinical                | 200-purple              | ND                    |
| Stenotrophomonas | maltophilia       | ATCC 17666 | Tissue culture          | Lawn-purple             | ND                    |

*ND – No detection.

Table 3. Robustness of the MYChrOme culture plate, bias, and repeatability

| Parameters | Sample volume, mL | Filtrate volume, mL | Decontamination incubation time, min | N | Mean | s | r | Nominal condition | Mean | s | Mean difference | LCL | UCL |
|------------|-------------------|---------------------|--------------------------------------|---|------|---|---|-------------------|------|---|-----------------|-----|-----|
| Non-potable water with Mycobacterium—low level |                  |                     |                                      |   |      |   |   |                   |      |   |                 |     |     |
| 1          | 175               | 9                   | 4                                   | 5 | 1.598| 0.231| 9 | 5 | 1.763 | 0.05 | –0.165 | –0.362 | 0.032 |
| 2          | 175               | 9                   | 6                                   | 5 | 1.525| 0.234| 9 | 5 | 1.763 | 0.05 | –0.238 | –0.437 | –0.04 |
| 3          | 175               | 11                  | 4                                   | 5 | 1.745| 0.204| 9 | 5 | 1.763 | 0.05 | –0.018 | –0.193 | 0.156 |
| 4          | 175               | 11                  | 6                                   | 5 | 1.726| 0.19  | 9 | 5 | 1.763 | 0.05 | –0.037 | –0.2   | 0.126 |
| 5          | 225               | 9                   | 4                                   | 5 | 1.662| 0.282| 9 | 5 | 1.763 | 0.05 | –0.101 | –0.339 | 0.136 |
| 6          | 225               | 9                   | 6                                   | 5 | 1.652| 0.147| 9 | 5 | 1.763 | 0.05 | –0.11  | –0.24  | 0.018 |
| 7          | 225               | 11                  | 4                                   | 5 | 1.956| 0.218| 9 | 5 | 1.763 | 0.05 | 0.193  | 0.007  | 0.379 |
| 8          | 225               | 11                  | 6                                   | 5 | 1.747| 0.189| 9 | 5 | 1.763 | 0.05 | –0.016 | –0.179 | 0.146 |

*Each parameter test combination is being compared to the nominal test condition—9–200 mL sample volume, 10 mL filtrate volume, 5 min decontamination incubation.

N – Number of test portions.

Mean of five replicate portions after logarithmic transformation: Log_{10}[CFU/g ± (0.10)].

Repeatability standard deviation.

Mean difference between the candidate and reference methods.

90% Lower confidence limit for difference of means.

90% Upper confidence limit for difference of means.
Table 4. Product consistency (lot-to-lot) and stability of MYChrOme culture plate, bias, and repeatability

| Level | N° | Mean | s | Lot | Mean | s | Mean difference | LCL | UCL |
|-------|----|------|---|-----|------|---|----------------|-----|-----|
| 1—High | 5  | 4.105 0.015 | 2 | 4.076 0.034 | 0.792 | –0.063 0.006 |
| 2—High | 5  | 4.036 0.034 | 3 | 4.024 0.015 | –0.052 | –0.083 –0.022 |
| 3—High | 5  | 4.024 0.015 | 1 | 4.105 0.015 | 0.740 | –0.1 –0.062 |
| 1—Low | 5 | 2.493 0.051 | 2 | 2.411 0.063 | –0.082 | –0.15 –0.015 |
| 2—Low | 5 | 2.411 0.063 | 3 | 2.351 0.052 | –0.06 | –0.128 0.008 |
| 3—Low | 5 | 2.351 0.052 | 1 | 2.493 0.051 | –0.142 | –0.203 –0.082 |

N = Number of test portions.
Mean of five replicate portions after logarithmic transformation: Log$_{10}$[CFU/g ± (0.1)].
Repeatability standard deviation.
Mean difference between the candidate and reference methods.
90% Lower confidence limit for difference of means.
90% Upper confidence limit for difference of means.
Lot 1 was beginning of expiration period. Lot 2 was middle of expiration period. Lot 3 was end of expiration period.

Matrix study.—Methodology.—Two matrixes, potable and non-potable water, were evaluated for NTM detection by the MYChrOme Culture Plate and a modified FDA method (larger test portion size validated to accommodate water sample). The study included five replicate test portions at each contamination level. There were low, medium, and high levels of contamination for each matrix, with each level being approximately 1 log$_{10}$ higher than the previous so that the range of samples covered at least two log$_{10}$ units. Potable sink water (e.g., tap water) was inoculated at three contamination levels (10–100, 100–1000, and 1000–10 000 CFU/mL) with an untested liquid culture of M. fortuitum (ATCC 6841). An uncontaminated control was also analyzed. Samples were equilibrated at 4°C for 48 h prior to testing. Potable outdoor fountain water was evaluated containing natural contamination of NTM and was tested without an equilibration phase. The candidate method and reference method were performed on the same day for each contamination level on split 400 mL samples. The candidate and reference methods were evaluated using unpaired test portions. Modified US FDA—Isolation and Identification of Nontuberculous Mycobacteria in Tattoo Inks.—Methodology.—To each 200 mL test portion, 8 mL of 0.04% (w/v) CPC was added and left at room temperature for 30 min. A 200 mL test portion was vacuum filtered using a 0.45 μm black grid HABG 47 mm filter. The filter was rinsed with sterile phosphate buffer, placed in separate 15 mL conical tubes, and resuspended with 10 mL of sterile PBS. The concentrated sample was vortexed for 30 s. Each sample was spread plated using 1.0 mL (0.33, 0.33, and 0.34 mL) onto three separate 7H10 and 7H11S agar plates. Each replicate was plated in duplicate. A 0.1 mL sample was plated in duplicate onto separate 7H10 and 7H11S agar plates. Two 10-fold serial dilutions were prepared by transferring 1.0 mL into 9.0 mL of PBS of the previous dilution. From each dilution, 0.1 mL was spread plated in duplicate onto separate M7H10 and M7H11S agar plates. Two 10-fold dilutions were prepared by transferring 1.0 mL into 9.0 mL of PBS of the previous dilution. From each dilution, 0.1 mL was spread plated in duplicate onto separate M7H10 and M7H11S agar plates. Plates were allowed to dry and sealed in a gas permeable container. The plates were incubated at 37°C ± 1°C. The plates were examined every 7 days for up to 2 weeks. One to two isolated colonies were selected from each test portion and resuspended in 100 μL of sterile water in a 1.5 mL microcentrifuge tube. A 50 μL aliquot was transferred into a 1.5 mL microcentrifuge tube containing 100 μL of InstaGene Matrix (Bio-Rad, Hercules, CA) for DNA extraction. The microcentrifuge tubes were vortexed for 10 s and incubated at 56°C for 15 min. The microcentrifuge tubes were vortexed for 10 s, then heated at 100°C for 8 min. The tubes were centrifuged at 12 000 rpm for 2 min, then analyzed by PCR.

The instructions in the FDA method were followed for setting up the PCR thermocycler—5700 Fast—parameters. The activation step was set (1 cycle) to 95°C 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 ramped from 60°C for 1 min to 95°C for 15 s at a 1% ramp rate. The master mix was prepared with n + 4 reactions, with each reaction containing 1.25 μL of 10 μM (acid-fast bacilli) AFB primer mix, 12.5 μL 2x FastStart Universal SYBR green master mix, and 9.25 μL molecular-grade water. Aliquots of 23 μL of the master mixes were dispensed into each PCR tube. Two microliters of extracted DNA or positive/negative control were added. The caps of the PCR tubes were closed, mixed, centrifuged briefly, then placed into the thermocycler and analyzed.

MYChrOme Culture Plate.—Methodology.—A 200 mL test portion was filter-concentrated using a 0.22 μm pore size track-etched 47 mm polycarbonate membrane. The filter was placed in a separate 15 mL conical tube, resuspended with 10 mL of 1:40 sterile Kinger’s solution, and vortexed for 30 s. For the non-potable matrix, a 981 μL aliquot of the concentrated sample was added to 19 μL of MYCOn in a microcentrifuge tube. This sample was mixed twice by pipetting up and down, then incubated at room temperature (20–25°C) for 5 min. A 100 μL aliquot of treated or untreated sample was plated onto a MYChrOme culture plate. Serial dilutions were prepared in sterile PBS and plated in the same manner. Plates were incubated at 30°C ± 1°C for 7 days after which typical colonies were enumerated. White colonies were confirmed as NTM with a Mycobacterium genus-specific real-time PCR (see Confirmation section).

Results.—Matrix study results are shown in Table 5. Each contamination level of the candidate method was compared to the modified FDA method using an unpaired equivalency test assuming equal variances with a 90% confidence interval. For the potable water matrix study, in comparison to both 7H10 and 7H11S plates used in the reference method, MYChrOme had consistently higher mean log$_{10}$ results; therefore, the candidate method is not equivalent to the reference method but demonstrated higher recovery of the target organisms. The one exception being the medium contamination level in comparison to the 7H10 plate, where the reference method had a slightly higher average enumeration of NTM colonies.

For the non-potable matrix study, the candidate method was not equivalent to the reference method, with significantly more NTM colonies enumerated on MYChrOme. The relative standard deviation for both reference and candidate method were low (<0.4 and <0.3, respectively).

Independent Laboratory Studies

Methodology.—Non-potable water was evaluated for rapidly growing NTM by the MYChrOme Culture Plate and modified US FDA method. The same matrix study design was followed as above. Non-potable water (residential laundry rinse water) was inoculated with M. chelonae (CCUG 37827) in liquid form. The samples were allowed to equilibrate at 4°C for 72 h after contamination. The candidate method and reference method were
performed on the same day for each contamination level. The candidate and reference methods were evaluated using unpaired test portions.

The same methods used in the method developer matrix study for the reference method and candidate method were followed with the following modifications. Suspect colonies from both methods were confirmed using the US FDA AFB PCR method. After concentration of the MYChrOme protocol test portion, the filter was resuspended in 10 mL of filtrate.

Results.—The results are summarized in Table 5. The MYChrOme Culture Plate method and modified US FDA method were not equivalent with results favoring the candidate method for each contamination level in comparison to 7H11S plates. The same was observed in comparison to the 7H10 plates with the exception of the low contamination level that had a difference of means of –0.042. The MYCOn decontamination reagent appeared to decrease background microflora on the plates for some samples but not all samples. Naturally occurring Mycobacterium was detected in the uninoculated non-potable water which is indicative of real-world samples.

Discussion

The results of this validation study indicate that the MYChrOme method is equivalent, and in some areas, an improvement upon the reference method for the detection of NTM. These results also validate that the MYChrOme method is successful in differentiating NTM from other bacteria.

Fifty rapid-growing NTM strains were tested in the inclusivity portion of this study. Ninety-four percent of the strains grew on MYChrOme within 7 days and 98% of the strains grew within 14 days. The only NTM strain that did not grow on MYChrOme was M. murale, a rare species of NTM that has only been isolated from the walls of a daycare in Finland. This species was included to be able to evaluate 50 diverse strains and would not be expected to be found in product testing. All inclusivity strains that grew on MYChrOme were not colorized by the crystal violet dye and thus were easily distinguishable on the media except for M. abscessus subsp. massiliense, which was white with a slight purple pigment.

There were 30 non-mycobacteria species that were tested for exclusivity and 29 were either not detected or grew purple colonies on MYChrOme (treated and untreated).

Nocardia brasiliensis grew only 31 off-white colonies after being plated at >10^6 CFU/mL. This high concentration of the bacteria indicates a worst-case scenario and typically would not be found in real-world samples. N. brasiliensis is an acid-fast bacterium, so it was expected to produce white colonies on the MYChrOme culture plate if any cells could survive on the crystal violet. There was approximately a 4 log_10 reduction in N. brasiliensis colonies on the untreated MYChrOme Culture Plate, and all colonies were eliminated on the treated plate.
Colony confirmation with colony PCR eliminated this false-positive result.

The robustness study showed that the MYChrOme Culture Plate method can withstand modest variation in three critical parameters simultaneously. The product consistency study supports a 3-month shelf-life at 4°C from the time of manufacturing.

For both method developer and independent laboratory non-potable matrix studies, the candidate method achieved higher plate counts than the reference method. One exception being the low contamination level for the independent laboratory study in comparison to the 7H10 plates, where slight differences were observed. All non-potable samples analyzed by the method developer with the MYChrOme method had white colonies with all non-mycobacteria eliminated by the decontamination step. On 7H10 and 7H11S plates, some background microbiota were present that increased analysis time. The MYChrOme protocol was innovated specifically to address the issue of high background microbiota in non-potable water samples. Similar results were observed in the potable water matrix study, with a difference of means in favor of the candidate method, in all but one sample set. A slightly higher difference of means in favor of the 7H10 data set for the medium contamination level was noted. The disparities in colony counts between the two methods could be due to the fact that the FDA method is meant for tattoo ink analysis; however, minor modifications were included in the approved study outline to make the reference method more applicable to water samples. Overall, the same number of matrix study samples had positive NTM detections by the candidate method and the 7H10 reference method. Additionally, the MYChrOme method achieved results in 7 days versus 14 days. These results indicate that the MYChrOme Culture Plate is a comparable method to current standards for the detection of NTM in water samples.

Conclusions

Based on the results of this validation study, it is recommended that the MYChrOme Culture Plate be granted Performance Tested Method status for detection of rapid-growing NTM in potable and non-potable water sources.

Credit Author Statement

Katherine Fisher: Conceptualization, Investigation, Formal Analysis, Writing—original draft. Avneet Chhabra: Conceptualization, Investigation, Methodology, Writing—original draft. Leah Wickenberg: Resources, Project Administration, Writing—review & editing. William McCoy: Supervision, Funding acquisition.

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Conflict of Interest
During this validation study, all authors were employed by Phigenics, LLC.

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