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An American National Standard

Standard Practice for Enumeration of Non-Tuberculosis *Mycobacteria* in Aqueous Metalworking Fluids by Plate Count Method[1]

This standard is issued under the fixed designation E2563; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This practice covers the detection and enumeration of viable and culturable rapidly growing *Mycobacteria* (RGM), or non-tuberculosis *Mycobacteria* (NTM) in aqueous metalworking fluids (MWF) in the presence of high non-mycobacterial background population using standard microbiological culture methods.

1.2 The detection limit is one colony forming unit (CFU)/mL metalworking fluid.

1.3 This practice involves culture of organisms classified as Level 2 pathogens, and should be undertaken by a trained microbiologist in an appropriately equipped facility. The microbiologist should also be capable of distinguishing the diverse colonies of *Mycobacteria* from other microorganism colonies on a Petri dish and capable of confirming *Mycobacteria* by acid fast staining method

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D5465 Practice for Determining Microbial Colony Counts from Waters Analyzed by Plating Methods

E1326 Guide for Evaluating Nonconventional Microbiological Tests Used for Enumerating Bacteria

2.2 *Other Documents:*[3]

Kinyuon Acid-Fast Staining Procedure

3. Terminology

3.1 *Definitions:*

3.1.1 *rapidly growing mycobacteria (RGM)*—non-tuberculous *Mycobacteria* that grow and produce visible colonies in four to seven days.

4. Summary of Practice

4.1 For recovery and enumeration of viable and culturable *Mycobacteria* population in metalworking fluid field samples selective culture medium containing antimicrobial agents to suppress bacterial and fungal contamination is recommended. (See Section 8). Standard microbiological spread and droplet plating techniques are used for the enumeration of *Mycobacteria*. After a minimum of 14 days incubation at 30°C, the *Mycobacteria* colonies are counted and confirmed by acid-fast staining technique specific for *Mycobacteria*.

5. Significance and Use

5.1 This practice allows for the recovery and enumeration of viable and culturable, non-tuberculosis, rapidly growing *Mycobacteria* (*M.immunogenum*, *M.chelonae*, *M. absessus*, *M. fortuitum*, and *M.smegmatis*) in the presence of high gram negative background populations in metalworking fluid field samples. During the past decade it has become increasingly apparent that non-tuberculous *Mycobacteria* are common members of the indigenous MWF bacterial population. This population is predominantly comprised of gram negative bacteria and fungi. Mycobacterial contamination of metalworking fluids has been putatively associated with hypersensitivity pneumonitis (HP) amongst metal grinding machinists. The detection and enumeration of these organisms will aid in better understanding of occupational health related problems and a better assessment of antimicrobial pesticide efficacy.

5.2 The measurement of viable and culturable mycobacterial densities combined with the total mycobacterial counts (including viable culturable (VC), viable-non culturable (VNC) and non viable (NV) counts) is usually the first step in establishing any possible relationship between *Mycobacteria* and occupational health concerns (for example, HP).

5.3 The practice can be employed in survey studies to characterize the viable-culturable mycobacterial population densities of metal working fluid field samples.

[1] This practice is under the jurisdiction of ASTM Committee E34 on Occupational Health and Safety and is the direct responsibility of Subcommittee E34.50 on Health and Safety Standards for Metal Working Fluids.

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[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] *Public Heatlth Microbiology*: A Guide for the Level III Laboratory. Centers for Disease Control, U.S. Department of Health and Human Services, Atlanta, GA, 1985.

5.4 This practice is also applicable for establishing the mycobacterial resistance of metalworking fluid formulations by determining mycobacterium survival by means of plate count technique.

5.5 This practice can also be used to evaluate the relative efficacy of microbicides against *Mycobacteria* in metalworking fluids.

6. Interferences

6.1 In some metal working fluid samples very high ($>10^6$/mL) microbial background population levels; mainly gram negative pseudomonads and fungi can interfere the enumeration of *Mycobacteria* by "overgrowth" on the agar surface.

6.2 Sample dilution or smaller sample size can be used to minimize interference of non-target bacterial and fungal densities. Replicates of sample dilutions could be also plated and the results combined.

6.3 In some metalworking fluid samples chemicals (antimicrobial pesticides, functional additives, and other components) can interfere with the culturability of total viable *Mycobacteria* count in the sample. If interference by chemicals is suspected, sample dilution may also overcome this interference but will reduce sensitivity.

7. Apparatus

7.1 Laboratory Incubator, 30 ± 2°C.

7.2 Microscope with oil immersion lens, magnification 1000×.

7.3 Staining tray or sink with running water and drying rack.

8. Reagents and Materials

8.1 *Test Tubes,* with close fitting or airtight caps, 20 by 150 mm, sterile.

8.2 *Test Tube Racks,* sufficient size to hold 20 by 150–mm test tubes.

8.3 *Sterile Spreaders.*

8.4 *Sterile Loops.*

8.5 *Sterile 1–mL Pipets,* with 0.01–mL divisions.

8.6 *Dilution Water Blanks,* sterile, 9 mL.

8.7 *Selective Mitchison Modified 7H11 Agar.*

8.8 *Microscope Slides.*

8.9 *Paraffinic Laboratory Film,* 2.54 cm wide.

8.10 *Staining Reagents* for Acid Fast Staining procedure for staining *Mycobacteria* by the Kinyoun (cold) acid-fast procedure.

8.10.1 *TB Quick Stain Reagents:*

8.10.1.1 *Carbolfuchsin Reagent A*—Basic Fuchsin 17.0 g, Aqueous Phenol 1000 mL (aqueous solution of Phenol containing approximately 10 % water).

8.10.1.2 *TB-Decolorizer:* Hydrochloric Acid (37 %) 30.0 mL, Alcohol (denatured 95 % Ethanol or Methanol) 970 mL.

8.10.1.3 *Methylene Blue Reagent B:* Methylene Blue 2.0 g, acid alcohol 1000 mL (acid/alcohol: 3 mL 37 % HCl 97 mL in 90 to 95 % Ethanol).

NOTE 1—Brilliant Green stain can be used instead of the Methylene Blue stain (Brilliant Green 2.0 g, Sodium Hydroxide 0.02 g, Distilled water 1000 mL).

9. Hazards

9.1 The analyst must know and observe good laboratory practices and safety procedures required in the microbiology laboratory in preparing, using and disposing of cultures, reagents and materials.

10. Sampling, Test Specimens, and Test Units

10.1 Use sterile screw-capped, non-breakable plastic screw-capped containers (100-200 mL) for metalworking fluid sampling for microbiological analysis. The sample should be a random representative portion that is from the circulating tank as opposed to a pooled spillover or stagnant hose content. Collect approximately 100–mL size samples. Analyze samples immediately if possible, or refrigerate until analyzed. Maximum sample storage time is 24 h at refrigeration temperatures. Follow sample documentation procedure in accordance with Good Laboratory Practices.

11. Procedure

11.1 Gently agitate sample to re-suspend any sediment.

11.2 Using a sterile disposable pipet, distribute 2 × 0.5–mL aliquots (1 mL) directly from the liquid sample onto two Selective Mitchison Modified 7H11 Agar plate. Spread sample evenly using a sterile spreader. The total count following incubation for these two plates combined is the 10^0 dilution.

11.3 Spread plate 0.1–mL sample over the agar surface, (10^{-1} dilution).

11.4 Make decimal dilutions of the sample by diluting 1 mL of sample in 9–mL sterile water dilution blank and plate 10^{-2} to 10^{-7} dilutions using the Standard Droplet Plate dilution technique.

11.5 Allow plates to dry before inverting and sealing with paraffinic laboratory film to prevent media from drying out during the long term (minimum two weeks) incubation period.

11.6 Incubate plates at 30°C ± 2°C for 14 days. Examine the plates daily for growth. Plates with zero colonies after 14 days may be evaluated as having <1 CFU.

11.7 When colonies appear on plates verify at least ten colonies per plate as Acid Fast Bacilli using the Kinyuon Acid Fast Staining method before reporting the results.

11.8 Confirm *Mycobacteria* colonies by means of Acid-Fast Staining Method.

11.8.1 Use a sterile loop to transfer a small amount of a suspicious *Mycobacteria* colony to a drop of water on the microscope slide and spread it evenly with the loop.

11.8.2 Heat-fix the slides over flame by gently passing the slide through the flame fast once or twice. The heat fixed slide should be warm, not hot after flaming. In order to avoid over heating the slides the flaming can be substituted by a standard temperature heat block at 75°C for 10 to 20 minutes.

11.8.3 Acid-Fast Stain the slides by using the modified Kinyuon Acid Fast Staining Kit.

NOTE 2—Any other Acid-Fast Staining method can be used.

11.8.4 If the Modified Kinyuon Acid-Fast Staining Method is used follow the directions for staining:

11.8.4.1 Flood the slides with Carbol Fuchsin TB quick stain Reagent A for 4 to 5 minutes.

11.8.4.2 Rinse slide gently with water.

11.8.4.3 Flood slide with TB Acid Alcohol Decolorizer for 15 to 30 seconds.

11.8.4.4 Rinse slide gently with water until the rinse water is mostly clear.

11.8.4.5 Gently remove excess water.

11.8.4.6 Counter-stain slide with Methylene Blue/Quick Stain Reagent B for 4 to 5 minutes. (Staining with Brilliant Green for 30 seconds can replace Methylene Blue).

11.8.4.7 Rinse slide under running water and dry slide completely before examining it under oil immersion objective at 1000× magnification. *Mycobacteria* cells will retain the stain and will show characteristic morphology. Control slide with stained *Mycobacteria* can also be used if the examiner is inexperienced in recognizing mycobacterial morphology.

12. Calculation or Interpretation of Results

12.1 Use the following equation to calculate the viable mycobacterium count per ml of metalworking fluid sample:

$$\textit{Mycobacterium per mL Metalworking Fluid} = \textit{Number of Mycobacteria Colonies multiplied by the sample dilution} \quad (1)$$

13. Report

13.1 Report results as number of viable and culturable *Mycobacteria* per ml of metal working fluid sample.

14. Precision and Bias

14.1 *Precision*—Since precision will depend on the fluids, challenge microbes, and microbicide treatments used to perform individual investigations, no statement on precision is made.

14.2 *Bias*—Since there is no accepted reference material suitable for the bias in this practice, no statement on bias is made.

15. Keywords

15.1 acid-fast bacteria; hypersensitivity pneumonitis; metalworking fluid; non-tuberculous *Mycobacteria*; rapidly growing *Mycobacteria*