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An American National Standard

Standard Test Method for Enumeration of *Mycobacteria* in Metalworking Fluids by Direct Microscopic Counting (DMC) Method[1]

This standard is issued under the fixed designation E2564; 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 test method describes a direct microscopic counting method (DMC) for the enumeration of the acid fast stained mycobacteria population in metalworking fluids. It can be used to detect levels of total mycobacteria population, including culturable as well as non-culturable (possibly dead or moribund) bacterial cells. This test method is recommended for all water-based metalworking fluids.

1.2 *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.* For additional safety information, see *Laboratory Safety: Principle and Practices, 4th Edition*[2]

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D2881 Classification for Metal Working Fluids and Related Materials

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *acid-fast bacteria*, *n*—a distinctive staining property of *Mycobacteria* due to their lipid-rich cell walls.

3.1.1.1 *Discussion*—Once stained, mycobacterium resist decolorization when exposed to acidified organic solvents, and are therefore, informally designated acid-fast.

3.1.2 *non-tuberculous Mycobacteria (NTM)*—environmental mycobacteria, not associated with tuberculosis.

3.1.3 *microscopic factor (MF)*, *n*—a calibrated conversion factor for calculating the Mycobacterium count per mL sample.

3.1.3.1 *Discussion*—The average number of mycobacterium cells per one microscopic field (or oil field, OIF) is multiplied by the MF to give the concentration of mycobacterium per mL of sample.

3.1.4 *oil immersion field (OIF)*, *n*—the circular area of a microscopic field visible in the eye piece of the microscope using oil immersion objective.

4. Summary of Test Method

4.1 The method describes a semi quantitative test for enumerating acid fast stained environmental mycobacterium (AFB) from metal working fluids by direct microscopic counting (DMC) method[4]. It is used to determine total mycobacterium counts including culturable and possibly dead or moribund cells in the sample. This test method cannot be used to determine the total viable mycobacterium population in the sample. A known sample volume (centrifuged or direct) is spread over a known area (1 cm^2 or similar) on a microscope slide (marked by frosted or painted circles). Following differential acid-fast staining[5], the acid-fast cells are counted in several microscopic fields over the designated area. The calculation is based on using a calibrated microscope with a known Microscopic Factor (MF). The MF is determined by the microscopic area over which a known amount of sample was spread, the number of microscopic fields in the marked circle, and the volume of sample examined. The number of acid fast stained mycobacterium cells per microscopic field multiplied by the MF gives the mycobacterium number per mL of sample.

5. Significance and Use

5.1 During the past decade, it has become increasingly apparent that non-tuberculous mycobacteria are common members of the indigenous MWF bacterial population. Measurement of mycobacterial cell count densities is an important step in establishing a possible relationship between mycobacteria and occupational health related allergic responses, for example,

[1] This test method 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] Mary J. R. Gilchrist: Biosafety Precautions for Airborne Pathogens, in *Laboratory Safety Principles and Practices*, pp. 67-76, 1995, ASM Press

[3] 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.

[4] *Standard Methods for the Examination of Dairy Products*, Chapter: 10: Direct Microscopic Methods for Bacteria or Somatic Cells, 16th ed. America Public Health Association, Inc., Washington, DC, 1978.

[5] Ebersole L.L.: Acid-fast stain procedures, pp. 3.5.1-3.5.11. In *Clinical Microbiology Procedures Handbook*, Vol. 1. American Society for Microbiology, 1994 , Washington ,D.C.

Hypersensitivity Pneumonitis (HP) in persons exposed to aerosols of metalworking fluids. It is known that the viable mycobacteria count underestimates the total mycobacterial levels by not counting the non-culturable, possibly dead or moribund population that is potentially equally important in the investigation of occupational health related problems. The Direct Microscopic Counting Method (DMC) described here gives a quantitative assessment of the total numbers of acid-fast bacilli. It involves using acid-fast staining to selectively identify mycobacteria from other bacteria, followed by enumeration or direct microscopic counting of a known volume over a known area. Although other microbes—particularly the Actinomycetes—also stain acid fast, they are differentiated from the mycobacteria because of their morphology and size. Non-mycobacteria, acid-fast microbes are 50-100 times larger than mycobacteria. The method provides quantitative information on the total (culturable and non-culturable viable, and non-viable) mycobacteria populations. The results are expressed quantitatively as mycobacteria per mL of metalworking fluid sample.

5.2 The DMC method using the acid-fast staining technique is a semi- quantitative method with a relatively fast turnaround time.

5.3 The DMC method can also be employed in field survey studies to characterize the changes in total mycobacteria densities of metalworking fluid systems over a long period of time.

5.4 The sensitivity detection limit of the DMC method depends on the MF and the sample volume (direct or centrifuged, etc.) examined.

6. Interferences

6.1 Some metalworking fluid formulations fail to completely dry or provide an uneven film on the microscope slide (for example, synthetic fluids and metalworking fluids with high trap tramp oil content and debris). For these samples the results can be difficult to interpret as heat fixing may not provide full adherence. These samples should be re-stained or a new slide may be prepared.

6.2 A negative acid fast staining reaction does not necessarily indicate that a sample will be culturally negative for *Mycobacteria* since the culture method has a lower detection limit (1 cell/mL) than the DMC method.

7. Apparatus

7.1 *Centrifuge*, (“microfuge”) 14,000 relative gravities.

7.2 *Centrifuge tubes with caps*, disposable, 1 mL-2 mL capacity, such as Eppendorf Safe–Lock Tube or any other suitable centrifuge tubes.

7.3 *Calibrated variable pipet*, with sterile tips: 5 µL, 10 µL, 1.0 mL, 5 mL.

7.4 *Microscope slides*, with 100 mm^2 or similar areas marked by frosted or painted circles and frosted labeling ends.

7.5 *Calibrated stage micrometer*, 0.01 mm or similar divisions.

7.6 *Compound microscope*, with oil immersion lens.

7.7 *Microscope eye pieces*, 10× magnification, equipped with a net micrometer (10 mm by 10 mm) or similar.

7.8 *Slide drying apparatus*, (box) 50-60°C with level drying rack.

7.9 *Staining hood*

7.10 *Staining rack and running water*

7.11 *Hand tally or electrical counter*

7.12 *Kinyoun Acid-Fast Stain Kit*, (see 8.1).

7.13 *Analytical balance*

8. Reagents and Materials

8.1 *Staining Reagents for Acid-Fast Staining Procedure for Staining Mycobacteria by the Kinyoun (Cold) Acid-Fast Procedure*:

8.1.1 *TB Quick Stain Carbol-Fuchsin, Reagent A:* Basic Fuchsin (alcoholic) 17.0g, Aqueous Phenol 1000.0 mL

8.1.2 *TB-Decolorizer:* Hydrochloric Acid, 30.0 mL, Denatured Ethanol/Methanol: 970 mL

8.1.3 *TB Quick Stain Methylene Blue Reagent B:* Methylene Blue (alcoholic) 2.0 g, acid-alcohol 1000.0 mL; (acid-alcohol: 30 mL HCl 970 mL, 90-95 % Ethanol) or Brilliant Green 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 plastic containers (100-200 mL) for microbiological sampling of metalworking fluids. The sample should be a random representative portion of 50-100 mL that is from the circulating tank opposed to a pooled, spillover or stagnant hose contents. Refrigerate samples 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. Dispense 1 mL directly into the centrifuge tube. In the case of very viscous fluids, a 1-g sample should be weighed on an analytical balance.

11.2 Centrifuge samples at 13.000 relative gravities for 30 minutes at 22°C.

11.3 Remove supernatant gently using a disposable micropipet end.

11.4 Remove oily residues completely from the tube using a sterile cotton swab. Gently remove the whole pellet with a sterile loop or a micropipet end without disturbing the sediment.

11.5 Transfer the whole amount of sediment to the 1-cm^2 designated area on the microscope slide and spread it evenly using a disposable pipet end.

11.6 Dry slides over a level drying box at 50-60°C for minimum of one hour. Some fluid formulations require longer drying time. These samples can be dried as long as overnight on the drying box. The slides that remain oily even after the