

Test Method for Antibacterial Activity of Textile Materials: Agar Plate

Developed in 1958 by AATCC Committee RA31; revised 1962, 1965, 2010; reaffirmed 1970, 1974, 1977, 1982, 2016; editorially revised 1971, 1972, 1974, 1982, 1985, 1986, 2019 (with title change); removed 1989; reactivated/revised 2011.

Foreword

The Agar Plate Method is a qualitative method to determine antibacterial activity of diffusible antimicrobial agents on treated textile materials. This method is an adaptation of a test method described by Ruehle and Brewer (see 12.1) and was originally adopted as an AATCC tentative test method in 1958. RA31 revised this method in 1962 in order to clearly define the objectives and limitations of this method. In 1989, the method was discontinued and removed from the *Manual of International Test Methods and Procedures*. The reason cited for removal was that pertinent aspects of the agar plate method were incorporated in the re-write of AATCC TM147, Test Method for Antibacterial Activity Assessment of Textile Materials: Parallel Streak Method. In 2011, RA31 updated and reactivated the method in part because ASTM cites this method in their standard ASTM E1115, Standard Test Method for Evaluation of Surgical Hand Scrub Formulations, and because the agar plate method was found to be beneficial when testing odd shape test samples, batting, fiber fill, etc. This method is used to demonstrate bacteriostatic activity by the diffusion of the antibacterial agent through agar. Molten agar is seeded with the test organisms and allowed to become semi-solid or solid depending on the test specimen to be tested. The test specimen is then placed in intimate contact with the agar surface. An advantage of the Agar Plate Method is that a non-embedded test specimen can be removed to determine inhibition in the contact area without fear of removing the test organism with the test sample. Both Gram positive and Gram negative bacteria are tested.

1. Purpose and Scope

1.1 The objective is to qualitatively detect bacteriostatic activity on products that are treated with antimicrobials and are capable of producing a zone of inhibition. Some test samples do not have a flat

surface, therefore, this method provides the advantage of testing odd shaped and non-flat surfaces by partially embedding them into molten agar prior to solidification. This provides the intimate contact required between the test sample and the seeded agar.

2. Principle

2.1 Specimens of the test material, including corresponding untreated controls of the same material if available, are placed in intimate contact with agar which has been previously seeded with an inoculum of a test bacterium. After incubation, a clear area of interrupted growth underneath and/or adjacent to the test material indicates antibacterial activity of the specimen. A standard strain of bacteria is used that is specific to the requirements of the material under test. If no other bacterial species is specified, *Staphylococcus aureus* and *Klebsiella pneumoniae* may be used as representative Gram positive and Gram negative organisms. Other recommended strains can be used.

3. Terminology

3.1 **activity**, n.—of an antibacterial agent, a measure of effectiveness of the agent.

3.2 **antibacterial agent**, n.—any chemical which kills bacteria (bactericide) or interferes with the multiplication, growth or activity of bacteria (bacteriostat).

3.3 **zone of inhibition**, n.—clear area of no growth of a microorganism, cultured onto the surface of an agar growth medium, in proximity to the borders of a specimen placed in direct contact with this agar surface.

NOTE: A zone of inhibition occurs as a result of the diffusion of an antimicrobial agent from the specimen.

4. Safety Precautions

NOTE: These safety precautions are for information purposes only. The precautions are ancillary to the testing procedures and are not intended to be all inclusive. It is the user's responsibility to use safe and proper techniques in handling materials in this test method. Manufacturers MUST be consulted for specific details such as material safety data sheets

and other manufacturer's recommendations. All OSHA standards and rules must also be consulted and followed.

4.1 This test should be performed only by trained personnel. The U.S. Department of Health and Human services publication *Biosafety in Microbiological and Biomedical Laboratories* should be consulted (see 12.2).

4.2 CAUTION: Some of the bacteria used in this test are pathogenic; i.e., capable of infecting humans and producing disease. Therefore, every necessary and reasonable precaution must be taken to eliminate this risk to the laboratory personnel and to personnel in the associated environment. Wear protective clothing and respiratory protection that prevents penetration by the bacteria.

4.3 Good laboratory practices should be followed. Wear safety glasses in all laboratory areas.

4.4 All chemicals should be handled with care.

4.5 An eyewash/safety shower should be located nearby for emergency use.

4.6 Sterilize all contaminated samples and test materials prior to disposal.

4.7 Exposure to chemicals used in this procedure must be controlled at or below levels set by government authorities (e.g., Occupational Safety and Health Administrations [OSHA] permissible exposure limits [PEL] as found in 29 CFR 1910.1000 (see web site: www.osha.gov for latest version). In addition, the American Conference of Governmental Industrial Hygienists (ACGIH) Threshold Limit Values (TLVs) comprised of time weighted averages (TLV-TWA), short term exposure limits (TLV-STEL) and ceiling limits (TLV-C) are recommended as a general guide for air contaminant exposure which should be met (see 12.3).

5. Uses and Limitations

5.1 The method is not suitable for materials which tend to encapsulate and prevent the diffusion of the antibacterial agent or contain antibacterial-neutralizing substances.

6. Test Organisms

6.1 Test bacteria:

6.1.1 *Staphylococcus aureus*, ATCC 6538, CIP 4.83, and DSM 799, NBRC 13276, NCIMB 9518 or equivalent strain (see 12.4)

6.1.2 *Klebsiella pneumoniae*, ATCC

4352, CIP 104216, DSM 789, NBRC 13277, NCIMB 10341 or equivalent strain (see 12.4)

6.1.3 Other suitable species can also be used.

6.2 Maintain test organisms according to good laboratory practice standard (see 12.5).

6.3 Whenever possible, test the activity of the culture to be used against a standard control specimen (a positive control) with known antibacterial activity.

6.4 To determine whether the antibacterial activity is due to the antibacterial agent, test a specimen of the same material treated in exactly the same way with whatever other finishing agents were used, but without the antibacterial agent. Many standard textile finishing chemicals, especially crease resistant and permanent press reagents, will often give strong antibacterial activity even after many washes.

7. Materials, Media and Reagents

7.1 Media and Reagents. Suitable broth/agar media are:

7.1.1 Nutrient broth/agar.

7.1.2 Trypticase Soy broth/agar.

7.1.3 Brain-Heart Infusion broth/agar.

7.1.4 Mueller Hinton broth/agar.

7.1.5 Other appropriate broths/agars can be used depending on test organisms used.

7.2 Materials.

7.2.1 Incubator maintained at $37 \pm 2^\circ\text{C}$ ($99 \pm 4^\circ\text{F}$).

7.2.2 Inoculating loop.

7.2.3 Bunsen burner or equivalent.

7.2.4 Water bath maintained at $45\text{--}50^\circ\text{C}$ ($113\text{--}122^\circ\text{F}$).

7.2.5 Pipets, 1 mL, sterile.

7.2.6 Culture Tubes with caps; minimum 10 mL capacity.

7.2.7 Petri dishes, 100 mm diam. $\times$ 15 mm deep, sterile.

7.2.8 Forceps, sterile.

7.2.9 Stereomicroscope, minimum 40X magnification.

7.2.10 Ruler.

8. Test Specimens

8.1 Test specimens are cut by hand or with a die. Standard samples are circular with a diameter of 2.5 cm (1 in.); however, any convenient size can be used.

9. Procedure

9.1 Prepare the test organism by taking

a cooled flame sterilized inoculation loop and inoculating separate culture tubes of the appropriate broth with the organism to be used. Incubate the cultures at $37 \pm 2^\circ\text{C}$ ($99 \pm 4^\circ\text{F}$) for 18–24 h.

9.2 Pipette 1.0 mL of each challenge organism that has been incubated for 18–24 h into separate 150 mL portions of sterile, molten agar maintained in a $45\text{--}50^\circ\text{C}$ ($113\text{--}122^\circ\text{F}$) water bath. An equivalent ratio would be 0.1 mL of test organism to 15 mL of molten agar. For each organism, aseptically transfer 15 mL of the inoculated agar into separate 100×15 mm petri dishes. Allow the agar to cool to a semi-gelatinous state for irregular test specimens that need to be embedded in the agar for intimate contact and to a solid state for flat surface regular samples.

9.3 Using flame sterilized or autoclave sterilized forceps that have been cooled, gently press the sample into the agar for embedded test specimens and on top of the agar for non-embedded test specimens. For embedded test samples aseptically pour additional seeded agar containing the same organism into the plate to achieve a depth of approximately 6 mm.

9.4 Cover the plate with the Petri dish lid and allow the agar to harden. Incubate at $37 \pm 2^\circ\text{C}$ ($99 \pm 4^\circ\text{F}$) for 18–24 h.

NOTE: Care must be taken to use sufficient agar to contact the sample, but not to deeply embed the sample and obscure the observation of any zone of inhibition.

10. Evaluation

10.1 Examine the incubated plates for a growth free zone around the test sample and for clearing directly beneath the test sample. Avoid parallax by looking directly down on the plate bottom. If a zone is not observed with the naked eye then the test samples should be placed under a stereomicroscope at a minimum magnification of 40X to detect any small zones. The average width of a zone of inhibition on either side of the test specimen may be calculated using the following equation:

$$W = (T - D)/2$$

where:

W = width of clear zone of inhibition in mm;

T = total diameter of test specimen and clear zone in mm; and

D = diameter of the test specimen in mm.

10.2 The size of the zone cannot be

construed as a quantitative evaluation of antibacterial activity. Treated materials should be compared to an untreated corresponding material and a material specimen with known bacteriostat activity. Report of results will include an observation of zones of inhibition and growth under the specimen if present. The criterion for passing the test must be agreed upon by the interested parties. To constitute acceptable antibacterial activity, there must be no bacterial colonies directly under the sample in the contact area.

11. Precision and Bias

11.1 Precision for this test method has not been established. Until a precision statement is generated for this method, use standard statistical techniques in making any comparisons of test results for either *within-laboratory* or *between-laboratory* averages.

12. Notes and References

12.1 Reuhle, G. G. A and Brewer, C. M., United States Food and Drug Administration Methods of Testing Antiseptics and Disinfectants. *U.S. Dept. Agric. Circular*, 198, 20pp (1931).

12.2 Publication available from U.S. Department of Health and Human Services, CDC/NIH-HHS Publication No. (CDC) 84-8395; web site: www.hhs.gov.

12.3 Available from Publications Office, ACGIH, Kemper Woods Center, 1330 Kemper Meadow Dr., Cincinnati OH 45240; tel: +1.513.742.2020; web site: www.acgih.org.

12.4 ATCC is the American Type Culture Collection (USA), P.O. Box 1549, Manassas VA 20108; tel: +1.703.365.2700; fax: +1.703.365.2701; web site: www.atcc.org; CIP is the Pasteur Institute Collection (France); DSM is the German Collection of Microorganisms and Cell Cultures (Germany); NBRC is the NITE Biological Resource Center (Japan); and NCIMB is the National Collection of Industrial Bacteria (UK). Equivalent bacteria strains obtained from agencies of the World Federation of Culture Collection (WFCC) may be used by agreement between the interested parties. The strains used in the test shall be documented with their supply source.

12.5 Consistent and accurate testing requires maintenance of a pure, uncontaminated, non-mutant test culture. Avoid contamination by using good sterile technique in plating and transferring. Avoid mutation by strict adherence to monthly stock transfers. Check culture purity by making streak plates periodically and observing for a single species-characteristic type of colonies.