

# A PROCEDURE FOR EVALUATING THE EFFICIENCY OF BACTERICIDAL AGENTS

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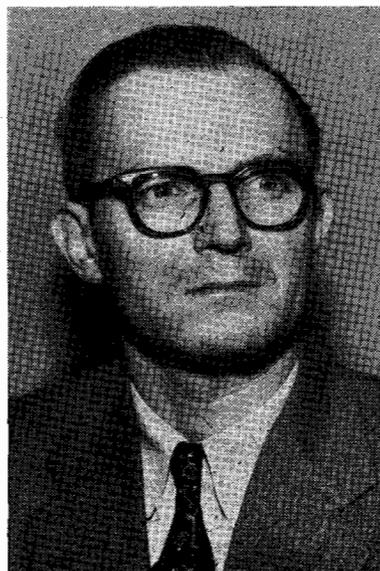
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An up to date revision of a germicide test which has been used at the U. S. Public Health Service, Robert A. Taft Sanitary Engineering Center since 1942. This method combines flexibility with control including methods for determining neutralizer efficiency and toxicity. Test organisms are grown on agar, suspended in buffered water, and filtered through sterile Whatman No. 2 paper. Sufficient test solution is available for related chemical tests. 10 refs.

The test described here has evolved from a method originally reported by Butterfield *et al.* (2) and has been extensively used in germicide research at the Robert A. Taft Sanitary Engineering Center. In continued use it has been necessary to revise and elaborate the procedure as originally employed. This has been particularly true with regard to the sections pertaining to neutralizers and the preparation of cultures and test suspensions. This method combines flexibility with a considerable degree of bacteriological control, and provides a test volume sufficiently large to permit withdrawal of a reasonable number of aliquots for chemical examination. The procedure is a revision of the information recently presented as Germicide Memorandum 1. (1)

## A. Preparation of Glassware

1. Erlenmeyer Flasks (250-ml wide-mouth)
  - (a) Strong, fresh dichromate cleaning solution prepared with concentrated  $H_2SO_4$ . Use finely powdered dichromate (to secure maximum dichromate concentration). The saturated aqueous solutions generally specified in cleaning solution formulae are unsatisfactory for this purpose.
  - (b) *Fill* and drain completely at least 3 times with both tap and distilled water.
  - (c) *Fill* with distilled water and autoclave (15 lbs., 15 min.).
  - (d) Drain completely, cap with paper, and sterilize in hot air oven. For most tests, sterilization can be in the autoclave if desired. In tests with low concentrations of chlorine, autoclaving imparts a chlorine demand. Procedures for preparing equipment for use in studies dealing with low concentrations of chlorine have been published (2).
2. Preparation Equipment — containers for collecting test water, including those in which the test concentration of the germicide is prepared



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as well as graduates or pipettes used in these measurements or in measuring the germicide-water mixture into the test flasks — Same treatment as A - 1 (a).

3. Other Glassware — any good routine laboratory cleaning procedure.
4. General — In the preparation of all glassware other less laborious methods may be used where controls have proven the absence of residual toxicity and/or interference with the activity of the germicide being investigated.

## B. Preparation of Culture Suspension

1. Use *Escherichia coli* ATCC-11229 or *Micrococcus pyogenes* var. *aureus* F. D. A.-209. Other species may be used for special purposes.

- (a) Nutrient agar<sup>1</sup> shall be used as the growth medium.
- (b) Transfer the cultures monthly to an agar slant and incubate 6 to 8 hours at 35°C.
- (c) Store at 5° - 10°C for *not more than 1 month* and then transfer the test cultures directly to a fresh agar slant and incubate 6 to 8 hours at 35°C as in Item B-1 (b).
2. Preparation of Stored Culture for Routine Use.
- (a) Remove a stock culture from 5 - 10°C storage.
- (b) Make daily transfers for 3 successive days to nutrient agar slants incubating the transfers 20 to 24 hours at 35°C.
- (c) The third daily transfer (Item B-2 (b)) may be used as a source of inoculum for culture to be used in the germicidal tests (Item B-5).
- (d) The culture may then be used continuously, so long as daily agar slant transfers are made, subject to the following:
- (1) Do not continue to use a culture for more than 30 days without starting anew from a stored stock culture (Item B-1 (c)).
- (2) If only 1 daily transfer has been missed, no special procedures are required. Two daily transfers may be missed if a transfer is incubated 6 - 8 hours at 35°C and then stored at 5 - 10°C, not to exceed 48 hours, otherwise repeat the four daily transfers as noted in Item B-2 (b).
3. As an added safeguard, it is recommended that the resistance of the test cultures to phenol be determined at least every three months by the AOAC method. (5) It may also be desirable to conduct culture resistance tests using the active agent of a particular germicide.
4. Inoculate into a 175-ml. Pyrex French square bottle (borosilicate glass) containing 20 ml. of nutrient agar which has been fortified by the addition of 1½ percent (15 g. per liter) of extra agar and allowed to solidify with the bottle resting in a horizontal position.
5. Culture bottles are inoculated by washing the growth from an agar slant into a 99-ml. phosphate buffer dilution blank, (8) and adding 2 ml. of this suspension to each culture bottle which is tilted back and forth to distribute the suspension after which excess liquid is drained off. Incubation is for 18 to 24 hours at 35°C, agar side down.
6. Culture is removed from the agar surface of 5 culture bottles of the type described in Item B-5 above, using 4 ml. of phosphate buffered dilution water (8) to suspend the growth from each bottle. This is accomplished by gently shaking the suspending fluid back and forth over the agar surface. Suspension so prepared is transferred to a screw cap tube and violently agitated, following which it is filtered through a sterile No. 2 Whatman paper, the filtrate being collected in a sterile screw cap tube. Removing culture by rubbing the agar surface with an inoculating needle or stirring rod is to be avoided due to increasing the amount of agar suspended. The number of bottles of culture used may be varied according to the volume of suspension needed. With *E. coli* ATCC 11229, suspension prepared in the manner described should contain approximately 10,000,000 organisms per ml. When 1 ml. of this suspension is added to 99 ml. of test water the density of organisms in the germicide test mixture should be approximately 100,000,000 per ml. (4) Any lower suspension density desired may be obtained by dilution.
7. During the filtration the surface of the filter should be periodically cleaned by gently rubbing with a sterile policeman or stirring rod. Contact with the tip of the filter should be avoided and reasonable caution must be exercised at all times to avoid rupturing the filter. However, these filters are sufficiently strong to withstand a considerable amount of such rubbing. This treatment should be initiated whenever the liquid in the funnel stem is obviously beginning to clear.
7. During an experiment the tube containing the filtered suspension is stored in a beaker containing cracked ice.

### C. Neutralizer

An effective non-toxic neutralizer for instantly stopping the bactericidal action shall be used and neutralizing effectiveness and toxicity shall be determined as follows:

#### 1. Toxicity:

Using suspension prepared in Item B-6, make a dilution using phosphate buffered water (8) which contains from 750 to 1250 organisms per ml.

<sup>1</sup>Containing per liter: 15 g. agar, 3 g. beef extract, 5 g. peptone (Bacto or equivalent - *special grades not to be used*). Other, non-specified, media may be used if it has been determined that nutrient agar is not a satisfactory medium when test organisms other than *E. coli* or *M. pyogenes* var. *aureus* are used.

Add 1 ml. of this dilution to 9 ml. of neutralizer in a screw cap test tube. Mix quickly and *immediately* plant and *immediately* pour with agar a set of 3 plates, each containing a 1-ml. portion from this neutralizer tube. Plant a second such set of plates from this tube at a time equal to the maximum interval between neutralization and plating in a germicide test. The temperature of the material in the neutralizer tubes should not be less than 25°C during these tests. Any significant reduction in count between the first and second planting indicates that the neutralizer is toxic to the test organisms. This test should be repeated a sufficient number of times to leave no doubt as to the absence of toxicity and should also be repeated whenever a new lot of test tube caps or cap liners is put into service.

#### 2. Neutralizer Effectiveness:

Prepare a dilution of the bactericide (at least as strong as the test concentration) in *distilled* water. Add 1 ml. of the bactericide to a tube containing 9 ml. of neutralizer, followed in not more than 15 seconds by 1 ml. of the dilution of test organisms previously described in Item C-1. Mix quickly and plant as in Item C-1. Any significant reduction in count between the first and second planting indicates inadequate neutralization of the bactericide.

3. Neutralizer blanks are prepared by dispensing neutralizer solution in 16 x 125 mm. screw cap test tubes, 9 ml. per tube. The method of sterilization is not stipulated due to the fact that the stability of the particular neutralizer used may have a marked bearing on the method of sterilization necessary.

4. Tween 80 Asolectin (lecithin) (6,10) is a satisfactory neutralizer for testing quarternary ammonium compounds. However, the required concentration of neutralizer varies with different test organisms and neutralizing effectiveness should be determined when test organisms other than *E. coli* and *M. pyogenes* var. *aureus* are used. Sodium thiosulfate is a satisfactory neutralizer for hypochlorite type compounds and should be used at a concentration which provides an excess of neutralizing capacity. Tween 80 Asolectin and sodium thiosulfate neutralizers may be sterilized at 121°C for 15 minutes.

#### D. Agar for Plating

Tryptone glucose extract agar or other agar medium of proven productivity for the test organism used.

#### E. Test Water

Selection of a test water is governed by the particular application for which the germicide is being investigated. Accordingly, the water may be either natural, tap, or synthetic, including organic additives, if desired. When tap waters are used, the following precautions should be taken:

Test tap waters for chlorine and, if present, dechlorinate by the following procedure: Add, in small increments, 0.2 percent sodium sulfite (freshly prepared in water which is boiled, cooled and immediately used to prepare the solution) determining the chlorine concentration, using the orthotolodine test after each addition of sodium sulfite. When a point is reached where only a trace (less than 0.01 ppm) of chlorine remains, add enough sodium sulfite to leave a slight excess after all of the chlorine is destroyed. Determine by test that no chlorine remains. If the water is then shaken vigorously and allowed to stand for a few hours, the excess sodium sulfite will be oxidized. Dechlorination may also be accomplished by means of sunlight or ultraviolet lamp.

An alternate dechlorination method can frequently be used. In this method a measured amount of sodium sulfite solution, more than adequate to neutralize the chlorine in the volume of water to which it is added, is introduced into a measured volume of water. Elimination of chlorine is verified by test. The applicability of this method is determined by establishing, by means of a sufficient number of control tests, that the amount of sodium sulfite added does not in any way affect the action of the germicide being tested.

In critical work involving the germicidal effect of low concentrations of halogens, a chlorine-free chlorine-demand-free water should be used. (3)

Where tap water is treated by processes other than chlorination, residual germicide must be eliminated by an appropriate method.

References to some synthetic test waters are included. (7,9)

#### F. Performance of Test

1. Measure 99 ml. of test water, containing bactericide at the concentration to be tested, into chemically clean, sterile, 250-ml. *wide mouth* Erlenmeyer flasks and place in constant temperature bath until temperature becomes stabilized at 25°C., or whatever temperature is appropriate for the application considered. Prepare duplicate or triplicate flasks for each germicide to be tested. Also, prepare a similar flask, using distilled water, for each germicide tested and an "initial numbers" control con-

taining 99 ml. of test water to which no germicide has been added. If more material is needed for related chemical tests, the volume may be increased by using larger flasks and maintaining a ratio of 1 ml. of test suspension for each 99 ml. of test water.

2. Add 1 ml. of culture suspension to each test flask as follows: whirl flask, stopping just before suspension is added, creating sufficient residual motion of liquid to prevent pooling of suspension at point of contact with test water. Add suspension midway between the center and edge of the liquid surface with tip of pipette slightly immersed in test solution. *Avoid touching pipette to neck or side of test flask during addition of suspension.* Transfer 1 ml. portions to neutralizer blanks at exactly 15 and 30 seconds, 1, 2, 5, 10, 20 and 30 minutes or whatever time interval may be appropriate for the application anticipated, and mix well immediately after transfer. In the case of the "numbers control", plants need only be made immediately after adding culture and again after an interval equal to the longest time used in an actual test with germicide, *e.g.*, if the maximum exposure in a germicide test flask is 20 minutes then the "numbers control" would be planted initially and again after 20 minutes. In the "numbers control" there should be no significant change in count between the initial and final planting.

In the performance of the test when short time intervals are used, it has been found advantageous to use milk pipettes for adding the culture and withdrawing samples (due to their fast drainage rate.)

3. Plate from neutralizer tube to agar. Where 1/10 ml. portions are planted a 1 ml. pipette graduated at 1/10 ml. intervals is suggested.

For necessary dilutions to give countable plates use phosphate buffer dilution water prepared according to Standard Methods. (8)

4. Incubate plates at 35°C for 48 hours before counting. In initial tests with a given germicide, if plates counted at 24 hours show no increase in count after reincubation for an additional 24 hours incubation time may be shortened to 24 hours in subsequent work with that germicide.
5. Sterility Controls: (To be included in each experiment) All sterility controls are poured agar plate determinations.
  - (a) Neutralizer — plant 1 ml. from a previously unopened tube of neutralizer,

(b) Each type of test water used — 1 ml. quantity in a plate. Certain raw or other unsterilized natural or tap waters may not be entirely sterile. Therefore, sterility must be determined insofar as presence of the test organism is concerned. In practice, the problem of small numbers of extraneous contaminants generally resolves itself because these organisms are usually killed by contact with the germicide prior to adding the test organisms.

(c) Sterile distilled water used for germicide testing — 1 ml. in 1 plate.

(d) Control on each bottle of agar.

(e) Culture survival control — Immediately after last test flask as been dosed, add 1 ml. of "culture suspension" (Item F-2) to 99 ml. of dilution water and plant dilutions to determine whether original bacterial concentration of suspension has remained constant throughout the elapsed time of the entire experiment.

6. After counting plates confirm that surviving organisms were *E. coli* by transfer to brilliant green bile broth fermentation tubes. (8) Usually, it is only necessary to pick from representative colonies, if many survivors are present. However, where only a few colonies survive there is more justification for confirming all colonies. The same procedure should be used with other test organisms using a satisfactory confirmatory procedure or medium. Confirmation by staining is suggested when *M. pyogenes* var. *aureus* is the test organism. *In all instances sufficient colonies should be confirmed to leave no question that survivors were actually the test organism rather than a contaminant.*
7. Determine pH initially on each type of test water used and on each individual test flask immediately after the last portion is removed for a bacteriological determination.

#### REFERENCES

1. A Procedure for Evaluating the Efficiency of Bactericidal Agents. U. S. Public Health Service. R. A. Taft San. Eng. Center, Germicide Memorandum No. 1: 1-6, 1955.
2. Butterfield, C. T., Wattie, E., Megregian, S., and Chambers, C. W. Influence of pH and Temperature on the Survival of Coliforms and Enteric Pathogens when Exposed to Free Chlorine. Public Health Repts. (U.S.), 58: 1837-1866. 1943.
3. Megregian, S. The Preparation of Chlorine Demand Free Water. Public Health Repts. (U.S.), 63: 137-141. 1948.
4. Milk Ordinance and Code, 1953 Recommendations of the Public Health Service (U.S.) Appendix F, Reprint in Press. 1956.
5. Official Methods of Analysis of the Association of Of-

ficial Agricultural Chemists. 7th Ed., Assoc. Offic. Agr. Chemists, Washington, D. C., 1950.

6. Quisno, R., Gibbey, I. W., and Foter, M. J. A. Neutralizing Medium for Evaluating the Germicidal Potency of the Quaternary Ammonium Salts. *Am. J. Pharm.*, 118: 320-323. 1946.

7. Report to the Sub Committee on Food Supply of the Committee on Sanitary Engineering. Germicidal Rinse Compound for Field Use of the Armed Forces. National Reserch Council

Division of Medical Sciences, Washington, D. C. 15-3. 1952.

8. Standard Methods for the Examination of Dairy Products. 10th Ed. Am. Public Health Assoc. New York, 1953.

9. U. S. Navy Bureau of Ships Manual 51 -- I-19. 1946.

10. Weber, G. R., and Black, L. A. Laboratory Procedure for Evaluating Practical Performance of Quaternary Ammonium and other Germicides Proposed for Sanitizing Food Utensils. *Am. J. Public Health*, 38: 1405-1417. 1948.