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Effect of Methodology, Dilution, and Exposure Time on the Tuberculocidal Activity of Glutaraldehyde-Based Disinfectants

EUGENE C. COLE,[1]† WILLIAM A. RUTALA,[1,2]* LEONARD NESSEN,[3] NANCY S. WANNAMAKER,[2] AND DAVID J. WEBER[1,2]

Division of Infectious Diseases, Department of Medicine, University of North Carolina at Chapel Hill, Chapel Hill, North Carolina 27599-7030[1]; Department of Hospital Epidemiology, University of North Carolina Hospitals, Chapel Hill, North Carolina 27514[2]; and The Food and Drug Administration, Rockville, Maryland 20850[3]

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The Association of Official Analytical Chemists (AOAC) test for assessing the tuberculocidal activity of disinfectants has been shown to be variable. A modified AOAC test, which substituted Middlebrook 7H9 broth as the primary subculture medium and used neutralization by dilution, was compared with the standard AOAC method to assess the mycobactericidal activity of three glutaraldehyde-based disinfectants at 20°C and various exposure times. These changes had a marked effect on results, with the modified AOAC test providing more positive penicylinders per 10 replicates in 12 of the 13 comparisons that provided positive results. These differences were observed with both *Mycobacterium bovis* (ATCC 35743) and a clinical isolate of *Mycobacterium tuberculosis*. The effects of various exposure times to and dilutions of the glutaraldehyde-based disinfectants were also examined. The minimum exposure time needed to inactivate reliably *M. bovis* or *M. tuberculosis* with 2% glutaraldehyde was 20 min at 20°C. Diluting 2% glutaraldehyde caused a significant decline in mycobactericidal activity. Modification of the standard AOAC test to improve its sensitivity in detecting the failure of disinfectants to inactivate mycobacteria is indicated.

Since 1966, the tuberculocidal activity of chemical disinfectants has been assessed by using a method published by the Association of Official Analytical Chemists (AOAC) (4). This qualitative carrier method, like the AOAC use-dilution method for bactericidal activity of disinfectants (1, 7–12, 24), has been shown to have a high degree of variability (2, 3). Interestingly, variable results with the AOAC tuberculocidal test method were reported over 20 years ago, even before the method was adopted as the official method (20, 21, 25, 26). In the past few years, studies have demonstrated several problems associated with the current AOAC tuberculocidal test, including variability associated with the number of cells that remain attached to the carrier and the effect of the temperature at which the test is conducted (2, 3). These and other deficiencies have caused significant differences in intralaboratory results (3).

This study was initiated to further define and minimize sources of variability in the standard AOAC method. Previous studies have suggested other causes of variation in results, including the inability of modified Proskauer-Beck (MPB) medium to serve as an initial subculture medium (21, 25, 26). A modified AOAC method, which substituted Middlebrook 7H9 broth as the initial subculture medium and used neutralization by dilution, was compared with the standard AOAC method in order to assess the mycobactericidal activity of three glutaraldehyde-based disinfectants.

MATERIALS AND METHODS

Organisms, media, and growth conditions. *Mycobacterium bovis* BCG ATCC 35743 (Tice strain), the strain recommended by the AOAC for tuberculocidal activity testing, was obtained from the American Type Culture Collection (Rockville, Md.). The test organism as received marginally exceeded (1 of 10 replicates at the 1:50 dilution of phenol yielded growth) the AOAC phenol resistance requirements (no growth on all 10 replicates at 1:50 dilution of phenol). The lyophilized cells were reconstituted with MPB medium and maintained at 37°C as specified by the AOAC tuberculocidal activity test (4). A clinical isolate of *Mycobacterium tuberculosis* was obtained from the Mycobacteriology Laboratory at the University of North Carolina Hospitals, Chapel Hill. This isolate was sensitive to the first-line antituberculous drugs: isoniazid, rifampin, ethambutol, and streptomycin. The isolate was maintained and prepared for testing as described for *M. bovis* (4).

Middlebrook 7H9 broth (Difco Laboratories, Detroit, Mich.) with albumin-glucose-catalase enrichment was prepared according to the AOAC procedure (4). Other media employed included D/E neutralizing broth (Difco), Letheen broth (Difco) with 0.22% sodium bisulfite, D/E neutralizing broth base (Difco), and Mycobacteria 7H11 agar (Difco). Sterile distilled water, USP (Travenol Laboratories, Deerfield, Ill.), was used for medium preparation and disinfectant dilution unless tap water was specified for dilutions.

Disinfectants. Three Environmental Protection Agency-registered, glutaraldehyde-based disinfectants were purchased for this study. All required activation prior to use. The type of product, use life after activation, recommended use-dilution, and tuberculocidal label claim (for 100% kill) were 2% alkaline glutaraldehyde, 14 days, undiluted, and 45 min at 25°C, respectively, for disinfectant A; 2% alkaline glutaraldehyde, 14 days, undiluted, and 20 min at 20°C, respectively, for disinfectant B; and 2% alkaline glutaraldehyde–7.05% phenol–1.2% sodium phenate, 30 days, diluted 1:16, and 10 min at 20°C, respectively, for disinfectant C. All products were used within their specified use life. Dilutions of disinfectant C were freshly prepared each day.

Neutralizer evaluation. In order to determine the potential for cell toxicity caused by chemical neutralizers, the following experiment was performed. To stimulate carry-over of

* Corresponding author.
† Present address: Research Triangle Institute, Research Triangle Park, NC 27709.

disinfectant, sterile uninoculated porcelain penicylinders (Fisher Scientific Co., Pittsburgh, Pa.) were placed in individual tubes containing 10 ml of disinfectant. The penicylinders were then removed and placed into tubes containing 10 ml of neutralizing broth (D/E neutralizing broth or Letheen broth with bisulfite). The tubes were shaken by hand, and samples of the neutralizer-disinfectant mixture were added in increasing amounts (i.e., 0.005, 0.01, 0.025, 0.05, 0.1, 0.5, 1.0, 2.0, and 2.5 ml) to tubes containing 10 ml of MPB or 7H9 medium. Thus, four panels of nine dilution tubes each were created. In panel I the penicylinder was placed in D/E neutralizing broth, and samples were then placed into MPB broth; in panel II the penicylinder was placed in D/E neutralizing broth, and samples were then placed into 7H9 broth; in panel III the penicylinder was placed in Letheen broth, and samples were then placed into MPB broth, and in panel IV the penicylinder was placed in Letheen broth, and samples were then placed into 7H9 broth. Control panels, panels V and VI, were constructed by adding portions of D/E neutralizing broth base, which contains no neutralizers, to either MPB medium or 7H9 broth.

A cell suspension of *M. bovis* was prepared by placing an inoculated penicylinder into 10 ml of 0.1% Tween 80–saline, sonicating it in an ultrasonic cleaner (Health Sonics Corp., Pleasanton, Calif.) for 10 min, and vortexing it (Vortex-Genie; Fisher) at setting 5 for 2 min. A 1:10 dilution of the suspended *M. bovis* was prepared, and 10-μl samples were added to each series of 10 tubes. After tubes 3, 7, and 10 were prepared, 7H11 agar plates were also inoculated with 10 μl to quantify the number of CFU added to the tubes.

Neutralization by dilution was evaluated by placing four uninoculated porcelain penicylinders into 10 ml of disinfectant A and four penicylinders into 10 ml of a 1:16 dilution of disinfectant C. Two of the four penicylinders were transferred individually into 20-ml tubes of MPB medium, and the other two penicylinders were transferred into 20-ml tubes of 7H9 broth. Since approximately 0.01 ml of disinfectant is carried over to the recovery medium, there is a 1:2,000 dilution of the disinfectant. Low numbers of *M. bovis* were added to all tubes, as described above.

All tubes were capped with permeable membrane cap closures (Biomedical Polymers Inc., Leominster, Mass.), which allowed gas exchange but retarded evaporation, and were incubated at 37°C for 60 days, at which time they were examined for growth. Plates were inverted and enclosed in 0.5-mil plastic bags at 37°C for 28 days, at which time the number of CFU per plate were counted.

AOAC tuberculocidal activity test. The AOAC tuberculocidal activity operating technique (technique 4.041) was used as the standard method (4); exposure times were varied, as required by experimental design and as noted below. Following disinfectant exposure, each penicylinder was removed, placed into 10 ml of D/E neutralizing broth, shaken, and transferred into a tube containing 20 ml of MPB broth. One 2-ml sample of the D/E neutralizing broth-disinfectant mixture was transferred to each of two tubes of 7H9 broth. 7H9 broth is the only one of the three recommended subculture media specified by the method that is currently available. All tubes were capped and incubated at 37°C for 60 days. Tubes showing questionable growth and a sampling of positive tubes were vortexed for 15 s at setting 4, and 0.5 ml of the solution was inoculated onto 7H11 agar. Plates were sealed in 0.5-mil plastic bags and incubated at 37°C for 28 days. Colonies were microscopically examined for acid-fast bacilli by using the Kinyoun stain. Controls for growth, media, and neutralization were employed.

Modified AOAC tuberculocidal activity test. The AOAC tuberculocidal activity test (4) was employed with two changes. First, 7H9 broth was substituted for MPB broth as the initial recovery medium. Second, disinfectant neutralization was accomplished by dilution rather than by chemical neutralization (i.e., D/E neutralizing broth). That is, following disinfectant exposure, each penicylinder was placed into a 20-ml tube of 7H9 broth, removed, and transferred to a second 20-ml tube of 7H9 broth. This resulted in a 1: 4,000,000 dilution of the disinfectant in the second 7H9 broth tube (assuming that 0.01 ml is transferred each time). All tubes were capped and incubated as stated above.

Comparative evaluation of two disinfectant tests. The three disinfectants were evaluated by the standard and modified AOAC methods using *M. bovis* at various exposure times. Each disinfectant was tested by both methods with 10 inoculated porcelain penicylinders prepared from the same standardized cell suspension. Disinfectants A and B were used undiluted, as suggested by the manufacturer, while disinfectant C was diluted 1:16 with either distilled water (as specified in the AOAC method) or tap water (as specified by the label). The disinfectants were tested at each exposure time (i.e., 10, 20, 30, and 45 min) on the same day. All tests were conducted at 20 ± 0.2°C. The temperature was monitored with a thermometer with calibration traceable to a National Bureau of Standards thermometer. A clinical isolate of *M. tuberculosis* was also tested at 20°C for 20 min.

Quantitation of cells on the penicylinders was done at the beginning and end of each test day. Penicylinders were placed into a tube containing 10 ml of 0.1% Tween 80–saline, sonicated in an ultrasonic cleaning bath for 10 min, and vortexed at setting 5 for 2 min. From the 10^{-2} and 10^{-3} dilutions, 1-ml portions were removed, and 0.5 ml of each portion was inoculated onto each of two 7H11 agar plates. The inoculum was streaked with a flamed glass rod, and the plates were incubated at 37°C for 28 days.

Comparative evaluation of diluted disinfectants. The effect of dilution on the mycobactericidal activity of glutaraldehyde-based disinfectants was also evaluated. Disinfectants A and C were diluted and tested against *M. bovis* and *M. tuberculosis* by using the modified AOAC method. Each organism and five concentrations of the glutaraldehyde-based disinfectants (undiluted and 1:2, 1:4, 1:8, 1:16) were evaluated using 10 penicylinders per concentration. Both disinfectants were tested simultaneously for each dilution at 20°C and with a 20-min exposure. Quantitation of cells on penicylinders was performed four times throughout each test day. All 7H9 broth tubes were incubated at 37°C for 60 days, and 7H11 plates were incubated at 37°C for 28 days. Subculture of known and suspected positive tubes was performed as described above.

RESULTS

Neutralization toxicity. When MPB medium tubes (which held various concentrations of D/E neutralizing broth, D/E neutralizing broth base, and Letheen broth) were used to recover low numbers of mycobacteria, growth was not observed in any tube. The control tubes (i.e., MPB medium only) were also negative. In contrast, only the tubes containing 7H9 broth with >0.05 ml of D/E neutralizing broth or >0.1 ml of Letheen broth were negative. All other tubes demonstrated mycobacterial growth.

Neutralization by dilution was demonstrated to be effective, since mycobacterial growth was observed in all 7H9 broth tubes but not in the MPB medium tubes. The mean

TABLE 1. Abilities of standard and modified AOAC tests to measure inactivation of *M. bovis*[a]

Exposure time (min)	No. of positive penicylinders/10 replicates							
	Disinfectant A		Disinfectant B		Disinfectant C diluted with:			
					Distilled water		Tap water	
	AOAC	Modified AOAC	AOAC	Modified AOAC	AOAC	Modified AOAC	AOAC	Modified AOAC
10	0	8	0	7	0	10	0	10
20	2	0	0	0	5	10	0	10
30	0	0	0	0	2	10	7	10
45	0	0	0	0	1	10	4	10

[a] Disinfectants were tested at 20°C (for details, see text). Mean numbers of CFU per penicylinder were 3×10^5 before and 1.5×10^5 after testing; passing score for AOAC test is 0 positive penicylinders per 10 replicates.

number of *M. bovis* CFU added to the tubes was 17 (standard deviation, ±11.5).

Comparative evaluation of two disinfectant tests. Table 1 compares the abilities of the standard and modified AOAC methods to measure the inactivation of *M. bovis* with three glutaraldehyde-based disinfectants at various exposure times. The data demonstrate that, with one exception, no growth was observed with disinfectants A and B when the standard or modified AOAC tests with exposure times of 20 min or longer were used. When the exposure time was 10 min, growth was not observed with disinfectants A and B when the standard AOAC test was used, but it was observed when the modified AOAC test was used. In the standard AOAC test, growth was observed with disinfectant C in three of four trials (75%) when distilled water was used as a diluent and in two of four trials (50%) when tap water was employed. The data also show that when disinfectant C was diluted with either distilled or tap water, each replicate yielded growth at every exposure time when the modified AOAC test was employed. With only one exception (disinfectant A for 20 min), all positives by the standard AOAC test were in the MPB medium tubes but not the 7H9 broth tubes which contained 2 ml of D/E neutralizing broth.

Table 2 shows the activity of three glutaraldehyde-based disinfectants against *M. tuberculosis* in the standard and modified AOAC tests. Disinfectants A and B inactivated *M. tuberculosis* in both disinfectant tests when a 20-min exposure was used, whereas disinfectant C failed in both tests.

The effect of dilution on the ability of two glutaraldehyde-based disinfectants to inactivate *M. bovis* and *M. tuberculosis* in the modified AOAC test (20-min exposure) is shown in Table 3. The 2% alkaline glutaraldehyde was effective against *M. bovis* but demonstrated 1 positive penicylinder per 10 replicates against *M. tuberculosis*. As disinfectant A was diluted, the product demonstrated reduced mycobactericidal activity. The glutaraldehyde-phenol disinfectant inactivated *M. bovis* and *M. tuberculosis* (with one exception) when the concentration was 0.5% glutaraldehyde–1.76% phenol or greater. When the product was diluted 1:8 (0.25% glutaraldehyde–0.88% phenol) or 1:16 (0.13% glutaraldehyde–0.44% phenol), it was unable to demonstrate consistent mycobactericidal activity.

TABLE 2. Abilities of standard and modified AOAC test to measure inactivation of *M. tuberculosis*[a]

Disinfectant	No. of positive penicylinders/10 replicates	
	AOAC	Modified AOAC
A	0	0
B	0	0
C[b]	7	10

[a] Temperature, 20°C; exposure time, 20 min; mean number of cells per penicylinder, 1.4×10^6. Disinfectants were used at the recommended use-dilution of the manufacturer. Passing score for AOAC test is 0 positive penicylinders per 10 replicates.

[b] Results were the same whether tap or distilled water was used as a diluent.

DISCUSSION

The AOAC tuberculocidal activity test has been criticized because it produces results which are neither accurate nor reproducible (2, 3, 14). Many deficiencies have been identified by investigators (2, 3) (e.g., cell wash off, effect of temperature changes, variability in number of cells on penicylinders), but the two deficiencies tested in our study were the recovery media and neutralization. Previous studies have shown that Middlebrook 7H9 broth yields significantly improved recovery of *M. bovis* compared with *M. bovis* recovery with MPB broth (21, 25, 26). In one study that compared several recovery media by using the AOAC test, the investigators found 17 of 110 penicylinders positive with 7H9 broth and 0 of 110 positive with MPB broth when a 1:60 dilution of phenol was tested, 23 of 40 penicylinders positive with 7H9 broth and 0 of 40 positive with MPB medium when isopropyl alcohol was evaluated, and 49 of 50 penicylinders positive with 7H9 broth and 6 of 50 positive with MPB medium when ethyl alcohol was tested (25). Thus, in considering deficiencies of the test, it was apparent that 7H9

TABLE 3. Effect of dilution on mycobactericidal activity of glutaraldehyde-based disinfectants in a modified AOAC method[a]

Active ingredient (disinfectant) and concn (%)	No. of positive penicylinders/10 replicates	
	M. bovis	*M. tuberculosis*
Glutaraldehyde (A)		
2.0[b]	0	1
1.0	1	5
0.5	9	7
0.25	10	10
0.13	10	10
Glutaraldehyde-phenol (C)		
2.0–7.05	0	1
1.0–3.53	0	0
0.5–1.76	0	0
0.25–0.88	3	10
0.13–0.44[b]	9	10

[a] Temperature, 20°C; exposure time, 20 min; mean numbers of CFU per penicylinder, 3.8×10^5 for *M. bovis* and 5.7×10^5 for *M. tuberculosis*.

[b] Manufacturer-recommended use-dilution.

broth should be substituted for MPB medium as a primary recovery medium. This change was supported by the observation that low numbers (about 20 CFU) of non-disinfectant-exposed *M. bovis* will not grow in MPB medium. Obviously, the use of a recovery medium that can neither efficiently support the growth of healthy mycobacteria nor resuscitate sublethally damaged cells can provide misleading results. The only other change was the elimination of chemical neutralizers. Since data demonstrated that transferring 2 ml of D/E neutralizing broth into the 7H9 subculture medium exerted a mycobacteriostatic effect, neutralization by dilution was employed. Dilution is a recommended procedure in the evaluation of the mycobactericidal activity of glutaraldehydes (6, 22) and phenols (22).

These changes had a dramatic effect on results, as evidenced by the modified AOAC test, which provided significantly more positive penicylinders per 10 replicates in 12 of 13 comparisons that yielded growth. In a number of trials, the standard AOAC test provided no positive penicylinders per 10 replicates, and the modified AOAC test provided 10 positive penicylinders per 10 replicates. These differences were observed for both *M. bovis* and the human pathogen *M. tuberculosis*. These data confirm the results of Shelanski and Karras, who in 1965 stated that the use of MPB medium in the AOAC test was undesirable because it passed samples which failed with other media (26). Thus, there are several problems associated with the standard AOAC tuberculocidal activity test, and claims about *M. tuberculosis* based on this method should be considered suspect. These concerns were recognized by the AOAC in 1987 when it was stated that the "method has not been validated for glutaraldehyde-based products" (5).

Other aspects of our study were the measurement of mycobactericidal activity at various exposure times and with various concentrations of active ingredients. These data and the results of other studies (3, 14) support the recommendation that a 20-min exposure at room temperature is the minimum needed to reliably kill *M. tuberculosis* with 2% alkaline glutaraldehyde (23).

It has also been observed that diluting 2% glutaraldehyde causes a significant decline in mycobactericidal activity (15). This is important because glutaraldehyde-based disinfectants are used for up to 30 days, with dilution occurring during use (19). Our data revealed that a 0.5% glutaraldehyde concentration of disinfectant A demonstrated little mycobactericidal activity, while a 0.5% glutaraldehyde concentration of disinfectant C, which contains 1.76% phenol, demonstrated excellent mycobactericidal activity. This activity is presumably attributable to the presence of 1.76% phenol, a mycobactericidal agent (13), in disinfectant C.

We are concerned because exaggerated germicidal label claims are not restricted to low-level disinfectants (12, 24); they also appear on chemical sterilants and high-level disinfectants (which destroy all microorganisms, with the exception of high numbers of bacterial endospores) such as glutaraldehyde-based disinfectants (3, 17). Disinfectant C (0.13% glutaraldehyde–0.44% phenol), tested by the standard AOAC method with a 20-min exposure, failed to inactivate a clinical strain of *M. tuberculosis* (Table 2). This observation was made when either distilled water (as specified in the AOAC method) or tap water (as specified on the label) was employed as a diluent. The inability of the 1:16 dilution of disinfectant C to inactivate all mycobacteria on the carrier is attributable to the high dilution of the product. This same disinfectant did not inactivate the *M. bovis* strain that is recommended for use in the standard AOAC test; however, this strain was found to be more phenol resistant than recommended (1 of 10 replicates positive rather than 0 of 10 replicates positive with a 1:50 dilution). This, in part, may have led to the high failure rate of disinfectant C in both the standard and modified tests, since phenol provides added mycobactericidal activity to disinfectant C. Nevertheless, this is clinically irrelevant because disinfectant C at its recommended use-dilution was unable to inactivate a clinical isolate of *M. tuberculosis*. These data corroborate the results of Ascenzi et al. (3) as well as Isenberg et al., who used a suspension test and found that 0.13% glutaraldehyde–0.44% phenol produced only a 90% kill against *M. bovis* in 10 and 60 min (17). In contrast, Leach found 0 positive penicylinders in 10 replicates by using the standard AOAC test and *M. bovis* (18).

High-level disinfectants (e.g., glutaraldehyde based) are used on semicritical patient care items such as endoscopes, and nosocomial infections that are secondary to the use of ineffective disinfectants or ineffective disinfection procedures on endoscopes or bronchoscopes continue to occur (16, 28). Much of the confusion surrounding the inability to verify claims of tuberculocidal activity for glutaraldehyde-based disinfectants resides with the standard AOAC test, which provides results which are neither accurate nor reproducible. This supposition is supported by other investigators (2, 3), including the Environmental Protection Agency microbiological testing facility in Beltsville, Md., which obtained inconsistent test results from preregistration tuberculocidal tests on disinfectants in the 1970s by using the standard AOAC test (27).

Recognizing that there is controversy concerning the standard AOAC method and that there is a need for alternate tuberculocidal activity testing options, the Environmental Protection Agency published a notice of policy on testing methods which allows three testing options: a new quantitative suspension method, the standard AOAC test with 20°C and a 10-min exposure, and the standard AOAC test with a substantial modification of exposure time and temperature (27). As a follow-up action, the Environmental Protection Agency issued a data call-in requiring all affected registrants to provide data by using any one of the three testing options.

Our data suggest that one can modify the standard AOAC test in order to improve its sensitivity in detecting the failure of disinfectants to inactivate mycobacteria.

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