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Modified Quantitative Association of Official Analytical Chemists Sporicidal Test for Liquid Chemical Germicides

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The Association of Official Analytical Chemists (AOAC) test for sporicidal activity of disinfectants (966.04) is used in the United States as the legal criteria for classifying a liquid as a chemical sterilant and also as the indicator of the highest level of disinfectant. This qualitative test contains procedures that may cause inaccurate results. A modified, quantitative version of the AOAC sporicidal test has been developed that uses a specified minimum number of spores (2.0×10^5) of *Bacillus subtilis* or *Clostridium sporogenes* dried onto porcelain penicylinders. This modified test was validated with three commercial sterilant chemicals tested on three separate groups of spore-labeled cylinders.

In the United States, a liquid chemical germicide may be labeled as a sterilant if it passes the Association of Official Analytical Chemists (AOAC) test for sporicidal activity of disinfectants (test 966.04) (1, 14). In this test, unglazed porcelain penicylinders or silk suture loops are soaked (labeled) in cultures of *Bacillus subtilis* grown in soil extract nutrient broth or of *Clostridium sporogenes* grown in soil extract-egg-meat medium (2). After being dried for a minimum of 24 h at an ambient temperature in a desiccator, these spore-labeled cylinders or silk loops (carriers) are exposed to the test disinfectant for a certain time period and at a specified temperature. The carriers are then transferred to a medium that is able to neutralize the disinfectant and support the growth of low numbers of spores, and surviving spores are cultured.

The AOAC sporicidal test 966.04 defines a chemical sterilant, and it also defines the highest level of a disinfectant. A high-level disinfectant is a chemical sterilant that is always used at the same concentration and temperature, except a shorter exposure time is required to kill all types of microbes except spores (7).

Many authors have written on the variables, deficiencies, and inconsistent results of the AOAC sporicidal test (3, 4, 6, 8–10, 12–16). Cylinders that are new or have been previously used with 2.5 N HCl may have deep open pores in which medium and spores can collect and dry as a plug. Test disinfectants may be unable to reach spores occluded within this plug. The porous surfaces of used cylinders may be different from those of new cylinders in that the pores of used cylinders are filled with medium from previous uses and spores remain on the surface, accessible to disinfectants. Forty-three percent of new cylinders failed to be sterilized under conditions identical to those with which 100% of used cylinders were sterilized (9).

Sporicidal test 966.04 uses resistance to 2.5 N HCl as a standard for minimum spore-carrier resistance and does not require a measurement of the number of spores per carrier. A cylinder may carry as few as 500 to 1,000 spores and still meet the minimum 2.5 N HCl resistance standard (5). The performance of a test chemical, however, will vary depending on the number of spores per carrier. In test 966.04, an extract of garden soil is added to spore culture media. Since the soil type, location, and fertilizers and pesticides used can vary, the garden soil is an uncontrolled variable. The protein of silk suture material reacts with chlorine dioxide and halogen-type sterilants and eliminates their sporicidal chemistry before it can function. The procedures of the AOAC sporicidal test are technique sensitive. Spore-labeled carriers are manually placed into a 25- by 150-mm test tube with 10.0 ml of test disinfectant. If any spores accidentally contaminate the walls of the test tube during placement, they may recontaminate the sterile cylinder as it is manually removed from the test tube. The presence of even one nonsterile carrier in a total of 240 leads to failure of the test disinfectant, and it is difficult to determine if the nonsterile carrier was a true failure of the test chemical or a technical accident. The test uses thick egg-meat medium, coupled with filtration through cotton. Any particles of egg-meat medium that pass through the cotton filter can occlude and dry on the spores, thereby shielding them from contact with the test disinfectant. The reasoning behind this sporicidal test is that it tests dry spores, which are known to be much more resistant than wet spores. However, the drying time varies from a minimum of 24 h to more than 7 days.

We have modified the AOAC sporicidal test, retaining the basic concept of spores of *B. subtilis* and *C. sporogenes* dried onto carriers but eliminating the variable histories of porcelain cylinder surfaces, eliminating silk suture loops entirely, substituting $MnSO_4$ for the variable soil extract, eliminating egg-meat medium and filtration through cotton, specifying drying of the spore-labeled carriers for 72 h for a uniform standard of dryness, requiring measurement of the average number of spores on carriers, and setting a minimum average number of spores per carrier as a standard.

The test is made quantitative by exposing eight test tubes per exposure time period, each tube containing five spore-labeled cylinders with an average of $\geq 2.0 \times 10^5$ dry spores/cylinder to provide $\geq 10^6$ spores dried onto cylinder surfaces per test tube, to the test chemical over a range of exposure times at 20°C. The results are plotted on a graph as the percentage of the 40 spore-labeled cylinders sterilized per exposure time period as a function of increasing exposure time.

The exposure time required to repeatedly (three times) sterilize 40 cylinders, i.e., $40 \times (\geq 2.0 \times 10^5)$ or $\geq 8.0 \times 10^6$ dry spores, is considered a half-cycle, and twice the half-cycle exposure time is the full-cycle sterilization time. If the half-cycle

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sterilization time kills at least 10^6 spores, assuming a linear rate of kill, then twice the half-cycle time, or the full-cycle sterilization time, kills 10^{12} spores, a sterility assurance level (SAL) of 10^{-6}.

Cultures. *B. subtilis* ATCC 19659 was grown on slants of nutrient agar in 25- by 150-mm screw-capped test tubes for 72 h at 35°C. This stock culture of *B. subtilis* was stored at 0 to 5°C until used and was transferred monthly. A 100.0-ml volume of trypticase soy broth containing 5 ppm of $MnSO_4 \cdot H_2O$ in a 1.0-liter Erlenmeyer flask was inoculated with *B. subtilis* from the stock culture. This culture was incubated for 96 h at 35°C. The culture was shaken by hand and then homogenized in a 40.0-ml Ten-Broeck tissue homogenizer by three to five slow strokes to break up the pellicle, filtered through sterile VWR Scientific Products filter paper (grade 113V) supported by a sterile glass funnel, and then used immediately to label cylinders.

C. sporogenes ATCC 3584 was inoculated into 20.0 ml of fluid thioglycolate medium (FTM) in a 25- by 150-mm screw-capped test tube and incubated for 72 h at 35°C. This stock culture of *C. sporogenes* was stored at 0 to 5°C until used and was transferred monthly. A 100.0-ml volume of Columbia broth (Difco) in a 200-ml-capacity French square bottle was inoculated with *C. sporogenes* from the stock culture and incubated for 96 h at 35°C in a BBL GasPak anaerobic chamber. This culture was shaken vigorously to disrupt clumps of cells and then used immediately to label cylinders.

Preparation of uniform cylinder surfaces. Unglazed porcelain penicylinders (henceforth called cylinders; Fisher Scientific) were placed in a glass beaker and covered with FTM. After 30 min, the cylinders were transferred to 15- by 100-mm petri plates matted with two layers of P5 filter paper (Fisherbrand) and dried for 24 h over $CaSO_4$ (Drierite) in a desiccator. These cylinders were then placed in a glass beaker and covered with 2% glutaraldehyde, buffered with Na_2HPO_4 to pH 7.2, for 2 h at an ambient temperature. The cylinders were then removed from the glutaraldehyde, immersed in 0.01% Triton X-100 in USP purified water in a glass beaker, and swirled gently to wash them. The Triton X-100 was drained off, and the cylinders were rinsed four times with an excess volume of USP purified water. These cylinders were then sterilized in a steam sterilizer for 20 min at 121°C and dried in an oven at 55 to 60°C for 72 h.

Disinfectants. The commercial sterilants used in this test were freshly buffered alkaline 2.5% glutaraldehyde (Cidex; Johnson and Johnson Medical, Inc.), freshly mixed Exspor 4:1:1 (ClO_2; Alcide Corporation), and a mixture of 0.80% H_2O_2 and 0.06% peroxyacetic acid (Spor-Klenz; Calgon Vestal Laboratories, Inc.).

Preparation of spore-labeled cylinders. Dry, sterile cylinders prepared as described above were immersed in the 96-h culture of *B. subtilis* (1.6×10^8 CFU/ml) or *C. sporogenes* (4.0×10^8 CFU/ml) in a glass beaker and gently swirled for about 30 s. The titer of *B. subtilis* or *C. sporogenes* in 96-h cultures can range from 0.5 to 5×10^8 CFU/ml, and the critical measurement is the number of spores recovered from a dry cylinder. The spore-labeled cylinders were removed, placed upright in 15- by 100-mm petri plates matted with two layers of sterile filter paper (P5; Fisherbrand), and dried in a desiccator chamber over $CaSO_4$ for 72 h at an ambient (18 to 24°C) temperature. After being dried for 72 h, spore-labeled cylinders could be used for up to 7 days.

Test procedure. For each exposure time that was tested, eight 38- by 200-mm test tubes were each filled with 20.0 ml of test disinfectant. For each test disinfectant, five exposure time periods were selected to include a range of time points, from those in which few or none of a group of spore-labeled cylinders would be sterilized to exposure periods in which 100% of the cylinders would be expected to be sterilized. The tubes of disinfectant were brought to a temperature of 20°C in a water bath. By using sterile flamed stainless steel forceps, five spore-labeled cylinders were placed into the disinfectant without touching the sides of the test tube. The tubes were gently shaken to dislodge air pockets and ensure contact between the cylinders and the test disinfectant. Each tube of disinfectant was loaded with five cylinders, with 2.0-min intervals between test tubes. After the appropriate exposure time period, the cylinders were transferred with a nichrome wire hook, at 2.0-min intervals per tube, to individual 18- by 150-mm test tubes of neutralizing spore recovery medium. Neutralizing spore recovery medium is any medium that can be proven to neutralize the test disinfectant and support the growth of low numbers (1 to 10) of *B. subtilis* and *C. sporogenes* cells. If necessary to neutralize the disinfectant, the cylinder may be subtransferred to a second tube of spore recovery medium. In the tests reported on here, the neutralizing spore recovery medium was Dey-Engley medium for Spor-Klenz and Exspor and FTM for Cidex, and the process involved subtransfer into FTM. Eight tubes with five spore-labeled cylinders per tube, for a total of 40 spore-labeled cylinders, were tested at each exposure time. The tubes of recovery medium with cylinders were incubated for 7 days at 35°C and then scored for growth or no growth of *B. subtilis* or *C. sporogenes*. This test was repeated three times, with a different culture of spores and a different group of cylinders each time. The average percentage, from three different tests, of a group of 40 spore-labeled cylinders that were sterilized was plotted on a graph, with error bars indicating the highest and lowest numbers, versus the exposure time at 20°C.

Controls. The average number of *B. subtilis* spores on dry spore-labeled cylinders was measured by placing a cylinder into 10.0 ml of FTM in a plastic, screw-capped 18- by 150-mm test tube. The cylinder was agitated vigorously on a vortex mixer for 60 s to release the spores. The FTM was then diluted serially (1.0 ml into 9.0 ml of fresh FTM) through eight 10-fold dilutions. A 0.5-ml aliquot of each dilution was transferred to and spread on the surface of a nutrient agar plate, which was then incubated for 48 h at 35°C, at which time colonies of *B. subtilis* were counted. At least three cylinders per group of 200 were measured to determine the average number of spores per cylinder. The series of dilution tubes was also incubated for 48 h at 35°C.

Cylinders labeled with *C. sporogenes* were treated similarly; cylinders were placed in FTM and agitated to release spores, the suspension was diluted serially 10-fold in fresh FTM, aliquots were placed and spread on nutrient agar plates that were then incubated in a GasPak anaerobic jar (BBL) for 72 h at 35°C, and colonies of *C. sporogenes* were counted. The dilution tubes were incubated for 72 h at 35°C. Either the last dilution tube with growth or the colony counts could be used to determine the number of *B. subtilis* or *C. sporogenes* spores per cylinder, whichever gave the greater number. The standard for this test is that cylinders must carry an average of $\geq 2 \times 10^5$ *B. subtilis* or *C. sporogenes* spores per cylinder, measured as the average of the counts from 3 of 200 cylinders.

A comparison of growth versus no growth in the dilution tubes of neutralizing recovery medium with colony counts on petri plates verified that the neutralizing recovery medium could support the growth of 1 to 10 colonies of *B. subtilis* or *C. sporogenes*. This is a requirement for this test.

The resistance of the spore-labeled cylinders to 2.5 N HCl was measured by placing eight *B. subtilis*-labeled cylinders or eight *C. sporogenes*-labeled cylinders in 20.0 ml of 2.5 N HCl at

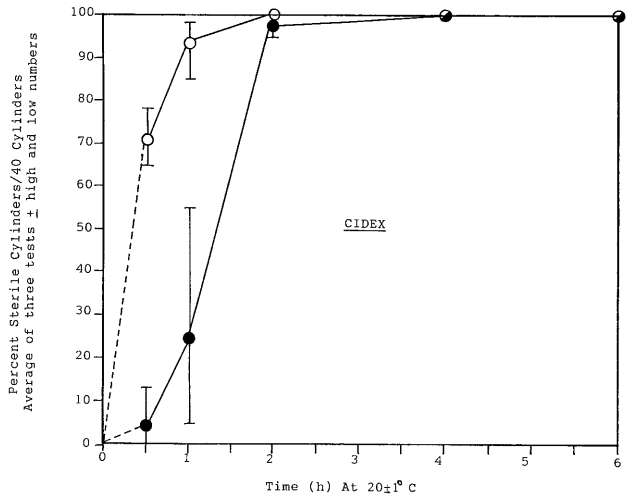

FIG. 1. The average percentage (± the high and low numbers [error bars]), for three tests, of groups of 40 *B. subtilis*-labeled cylinders (closed circles) or *C. sporogenes*-labeled cylinders (open circles) that were sterilized by exposure to Cidex for various time periods at 20°C. The three tests resulted in averages of 6.1×10^5, 10^6, and 4.3×10^5 CFU of *B. subtilis*/cylinder and of 2.8×10^5, 5.0×10^5, and 2.5×10^7 CFU of *C. sporogenes*/cylinder. All cylinders resisted 2.5 N HCl at 20°C for ≥2.0 min.

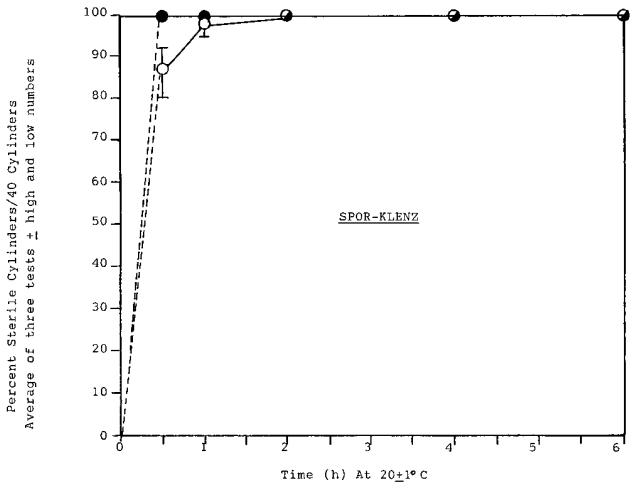

FIG. 2. The average percentage (± the high and low numbers [error bars]), for three tests, of groups of 40 *B. subtilis*-labeled cylinders (closed circles) or *C. sporogenes*-labeled cylinders (open circles) sterilized by exposure to Spor-Klenz for various time periods at 20°C. The three tests resulted in averages of 6.3×10^5, 1.1×10^6, and 1.6×10^6 CFU of *B. subtilis*/cylinder and of 5.0×10^4, 5.0×10^5, and 2.0×10^7 CFU of *C. sporogenes*/cylinder. All cylinders resisted 2.5 N HCl at 20°C for ≥5.0 min.

20°C. At time intervals of 2, 5, 10, and 20 min, two cylinders were transferred to modified FTM (2), subtransferred into FTM, incubated for 7 days at 35°C, and scored for growth or no growth. The spore-labeled cylinders must resist 2.5 N HCl at 20°C for at least 2.0 min.

To verify that the disinfectant was neutralized by the neutralizing spore recovery medium and transfer-subtransfer process, a sterile cylinder was placed in the test disinfectant, then transferred (with a residue of disinfectant), along with 0.1 ml of disinfectant, into 10.0 ml of the spore recovery medium, and then subtransferred to a second tube containing 10.0 ml of spore recovery medium. Approximately 100 CFU of *B. subtilis* or *C. sporogenes* was added to each tube. After incubation for 7 days at 35°C, growth of the test microbes verified that the disinfectant was neutralized by the recovery medium and/or transfer-subtransfer process. The standard for this test is that neutralization of the disinfectant must be validated regardless of the spore recovery medium and/or transfer-subtransfer process used to recover the surviving spores.

Results. Figure 1 shows the average percentage (± the highest and lowest numbers), for three different tests, of groups of 40 *B. subtilis* or *C. sporogenes* spore-labeled cylinders that were sterilized with Cidex, plotted as a function of exposure time at 20°C. *B. subtilis*-labeled cylinders were more resistant to Cidex than *C. sporogenes*-labeled cylinders. After exposure to Cidex for 4 h at 20°C, 100% of a group of 40 cylinders labeled with *C. sporogenes* or *B. subtilis* were sterilized. This result was repeated for three different batches of spore-labeled cylinders, for a total of 120 *B. subtilis*- and 120 *C. sporogenes*-labeled cylinders. Four hours at 20°C may be considered as the half-cycle exposure time required for Cidex (2.5% alkaline glutaraldehyde) to repeatedly kill $\geq 10^6$ dry spores, and a full-cycle exposure time would be 8 h at 20°C, for a SAL of 10^{-6}.

Figure 2 shows the average percentage (± the highest and lowest numbers), for three different tests, of groups of 40 *B. subtilis* or *C. sporogenes* spore-labeled cylinders that were sterilized by Spor-Klenz, plotted as a function of exposure time at 20°C. In contrast to the results for Cidex, *C. sporogenes*-labeled cylinders were more resistant to Spor-Klenz than *B. subtilis*-labeled cylinders. After exposure to Spor-Klenz for 2 h at 20°C, 100% of the cylinders labeled with *C. sporogenes* or *B. subtilis* were sterilized. The half-cycle exposure time for Spor-Klenz was 2 h, and its full-cycle exposure time was 4 h at 20°C, for a SAL of 10^{-6}.

Figure 3 shows the average percentage (± the highest and lowest numbers), for three different tests, of groups of 40 *B. subtilis* or *C. sporogenes* spore-labeled cylinders that were sterilized with Exspor, plotted as a function of exposure time at 20°C. *C. sporogenes*-labeled cylinders were more resistant to Exspor than *B. subtilis*-labeled cylinders. After exposure to

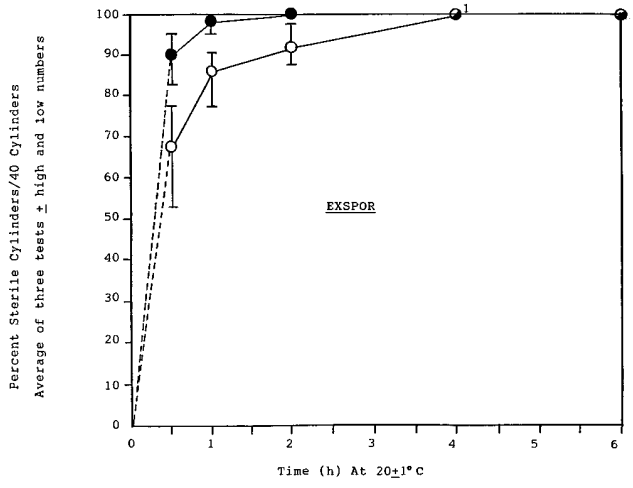

FIG. 3. The average percentage (± the high and low numbers [error bars]), for three tests, of groups of 40 *B. subtilis*-labeled cylinders (closed circles) or *C. sporogenes*-labeled cylinders (open circles) sterilized by exposure to Exspor for various time periods at 20°C. The three tests resulted in averages of 1.4×10^6, 1.1×10^6, and 1.5×10^6 CFU of *B. subtilis*/cylinder and of 5.0×10^5, 2.0×10^7, and 2.0×10^6 CFU of *C. sporogenes*/cylinder. All cylinders resisted 2.5 N HCl for ≥2.0 min at 20°C. The numeral 1 at the top indicates that there was one positive *B. subtilis*-labeled cylinder in one group of 40 cylinders.

Exspor for 4 h at 20°C, 100% of a group of 40 cylinders labeled with *C. sporogenes* or *B. subtilis* were sterilized. After the exposure time by which 100% of the 40 cylinders were sterilized was reached, this result was repeated for six different groups of *C. sporogenes*-labeled cylinders and, with the exception of one positive cylinder in 40, for eight different groups of *B. subtilis*-labeled cylinders. A statistical analysis of the group of 40 cylinders, contained as five cylinders per test tube in eight test tubes, indicates no difference, at the 95% confidence level, between 39 and 40 sterile cylinders per group of 40 cylinders.

The half-cycle exposure time for Exspor was 4 h at 20°C, and the full-cycle time, for a SAL of 10^{-6}, was 8 h.

Discussion. The modified, quantitative AOAC sporicidal test described in this paper retains the fundamental concept of testing two species of spore-forming bacteria thoroughly dried in their growth media onto a carrier surface. The modifications of test 966.04 include substitution of $MnSO_4$ for garden soil extract and filtering through manufactured paper filters instead of cotton. An average of $\geq 2.0 \times 10^5$ spores per cylinder is the required standard, along with resistance to 2.5 N HCl for at least 2.0 min at 20°C ± 1°C. In this test, each test tube holds five cylinders, with an average of $\geq 2 \times 10^5$ spores/cylinder, for a total challenge of $\geq 10^6$ spores per 20.0 ml of germicide. Previously, test 966.04 was scored as a failure if there was one positive carrier per 720 carriers tested. This method ignored the vast preponderance of data and lacked a mechanism by which to determine if the one positive carrier might be a false positive, in fact indicative of only a technical accident, a flawed cylinder, or occluded spores. The modified test can be statistically analyzed as eight groups (test tubes) with five cylinders per group. Under those conditions, one positive carrier in one of the eight test tubes is statistically insignificant at the 95% confidence level. The range of exposure times also provides data with which to determine if a positive carrier is a true positive or a false result.

This modified test is repeated with three different groups of 40 spore-labeled cylinders per exposure time period to demonstrate repeatability, and the exposure time required to kill $\geq 10^6$ spores in 24 separate test tubes (eight tubes of five cylinders per tube times three repeated tests) is considered the half-cycle exposure time. Considering that liquid chemical germicides are always directed for use on clean devices that in fact carry either no spores, dry or otherwise, or less than 100 spores at worst (11), a full-cycle exposure sterilization time (twice the half-cycle time) provides a wide margin of safety with a SAL of 10^{-6}.

Liquid chemical sterilants will always have limitations when compared with the penetrating ability of steam at 121°C. Any material that can withstand the heat of steam should be sterilized in that manner. However, as heat-sensitive, elastomeric, lensed, and electronic medical devices rapidly become more valuable minimally invasive devices, there is a need now for a repeatable, modified, quantitative AOAC sporicidal test with a large and quantifiable 10^{-6} SAL such as described here.

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