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Bacterial Spores Survive Treatment with Commercial Sterilants and Disinfectants

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This study compared the activity of commercial liquid sterilants and disinfectants on *Bacillus subtilis* spores deposited on three types of devices made of noncorrodible, corrodible, or polymeric material. Products like Renalin, Exspor, Wavicide-01, Cidexplus, and cupric ascorbate were tested under conditions specified for liquid sterilization. These products, at the shorter times indicated for disinfection, and popular disinfectants, like Clorox, Cavicide, and Lysol were also studied. Data obtained with a sensitive and quantitative test suggest that commercial liquid sterilants and disinfectants are less effective on contaminated surfaces than generally acknowledged.

Different reports agree that 5 to 10% (1.75 to 3.5 million) of the 35 million patients annually admitted to hospitals in the United States acquire an infection during hospitalization (5, 6, 22). More than 850,000 of these have been estimated to be implant- and device-related infections (2). Abundant data linking the transmission of various diseases (including AIDS, tuberculosis, and Creutzfeldt-Jakob disease, as well as hospital epidemics of infections with *Pseudomonas*, *Serratia*, and *Bacillus* species) to medical devices suggest that the effectiveness of disinfection and sterilization practices has been overestimated (21).

The capacity to kill bacterial spores determines how a commercial product will be marketed. Disinfectants are not expected to kill all bacterial spores and are used to decontaminate devices that ordinarily do not penetrate tissues or that touch only intact skin (3, 16, 25). Sterilants are expected to kill all microorganisms, including bacterial spores, and are used to treat devices that penetrate tissue or present a high risk if unsterile. Viable spores still attached to various materials could remain undetected by current sporicidal tests (1), resulting in overestimation of the sporicidal activity of sterilizing agents (4, 7, 11, 12, 14, 15). The goal of this study was to compare the sporicidal activities of commercial liquid sterilants under manufacturer-specified conditions by using a sensitive method able to quantitatively account for the survival of all spores on contaminated carrier devices.

Selection of carrier devices. The device to which spores are attached might alter the sporicidal activity of some germicidal agents (19). Therefore, the criteria used to select the carrier devices that we tested were based on the following practical considerations: (i) diverse material composition, (ii) geometry representative of medical devices, (iii) similar spore load capacities, (iv) size amenable to microtesting, and (v) cost. Miniature stainless steel machine screws (no. 0/80, pan head, 1.5 mm in diameter, and 12.5 mm long) were purchased at a local hardware store (Home Depot, Rockville, Md.) or from Thompson & Cooke (Bladensburg, Md.). Dental burs (FG 557) made of carbon steel were manufactured by Midwest Dental Products Corporation (Des Plaines, Ill.). Medical-grade silicone rubber tubing, 3.1-mm outer diameter and 1.5-mm inner diameter (Silastic; catalog no. 602-285), was manufactured by Dow Corning Corporation Medical Products (Midland, Mich.) and used in 12.5-mm-long sections. All devices were cleaned prior to use by washing with detergent, rinsing three times with distilled water, washing once in acetone, and rinsing again in distilled water before sterilization by autoclaving. The devices were immersed 5 mm deep in spore-loading suspensions. This procedure contaminated areas of 20, 40, and 78 mm^2 on dental burs, screws, and tubing, respectively. Likely due to differences in geometry and materials, the test described below loaded similar numbers of spores onto the three devices in spite of the different immersed areas. The miniature stainless steel screws and small sections of medical-grade silicone rubber tubing were small enough to fit our microtest format and inexpensive (costing 6 and 3 cents each, respectively). Easy availability of tubing, burs, and screws made custom manufacturing of carriers unnecessary. Their low cost allowed these carriers to be used only once and then discarded, thus preventing spore carryover and the need to wash and sterilize the carriers between tests.

Direct measurement of spores loaded onto carriers. Spores of *Bacillus subtilis* subsp. *globigii* (Spordex) were purchased from AMSCO American Sterilizer Co. (Erie, Pa.) with a reported *D* value for dry-heat killing at 160°C of 2.2 min and a *D* value for ethylene oxide killing (600 mg/liter at 54°C) of 3.5 min, respectively. The number of spores loaded onto carriers was determined by using radioactively labeled spores. A method that produces dry-heat-resistant spores in synthetic medium (8, 13, 23) was modified in our laboratory so that it would result in maximum incorporation of radiolabeled precursor as previously described (19). A rapidly growing culture (10^6 bacteria in 5 ml) was inoculated into 300 ml of synthetic sporulating medium in which methionine was replaced with radioactive L-[*methyl*-^{14}C]methionine (0.33 Ci/ml; NEC165H; 50 mCi/mmol; New England Nuclear, Boston, Mass.). After 5 days of incubation at 32°C in a shaker operating at 140 rpm, cultures were chilled in ice and spores were pelleted by centrifugation for 30 min at 900 × *g* in a Beckman TJ-R refrigerated centrifuge. After five cycles of centrifugation and resuspension in new Luria-Bertani (LB) broth, the radioactivity in the supernatant was reduced to less than 2% of the radioac-

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tivity in the pellet containing the spores. Samples from each batch of spores radioactively labeled and concentrated in our laboratory or nonradiolabeled spores obtained commercially (Spordex) were microscopically examined and exposed to acid for confirmation of spore morphology and chemical resistance as previously described (18). No vegetative cells (rods) were observed during the counting of 1,000 radioactively labeled or nonlabeled spores. Spores were exposed for various time periods to either deionized, glass-distilled, autoclave-sterile water (controls) or hydrochloric acid (2.5 N). After exposure they were neutralized with ice-cold LB broth (Advanced Biotechnology IC, Columbia, Md.) and titrated onto broth-agar (LB broth [Miller-Difco, Detroit, Mich.], 1.5% Agar Select [Gibco-BRL, Paisley, Scotland]) plates 100 mm in diameter. Typical spore survival in hydrochloric acid for 5 and 10 min was 100 and 88%, respectively.

Spores labeled with [^{14}C]methionine were diluted in LB broth, and identical aliquots were either titrated for viability or counted for radioactivity. The specific activity of each spore preparation was obtained from the slope of the regression line of spore number (as determined by titration) versus incorporated ^{14}C label (measured by scintillation counting). We transferred various devices to Eppendorf polypropylene tubes (1.5 ml) containing 50 μl of ^{14}C-labeled spores at different concentrations. Each device was immersed in a separate spore-loading suspension for 30 min. The devices were then removed from the loading suspension with forceps and dried for 10 min under vacuum (Speed Vac; Savant, Farmingdale, N.Y.). Each 50-μl suspension was used once and then discharged.

The spore load on each device was estimated by immersing the loaded devices in scintillation liquid, measuring radioactivity, and multiplying this value by the specific activity of the preparation. One large batch with a specific activity of $1.7 \times 10^3 \pm 0.3 \times 10^3$ spores per cpm was used for final calibration of all devices. The number of spores attached to no. 0/80 stainless steel screws (ranging from 6.0×10^6 to 6.5×10^6) was comparable to that loaded into medical-grade silicone rubber tubing (3.8×10^6) immersed in a spore suspension with a similar spore concentration (1.7×10^9/ml). The increase in the number of spores loaded onto the stainless steel screws or silicone rubber tubing was approximately linear with increasing concentrations of the loading suspension in the range of 10^7 to 10^{10} spores/ml. This contaminating procedure loaded, on average, 3 spores per 1,000 spores/ml of the loading suspension.

Sterilants and disinfectants. Cidexplus (3.4% glutaraldehyde, pH 8.0; Johnson and Johnson Medical Inc., Arlington, Tex.) was activated as specified and used full strength at 21°C over a period of either 10 h, for sterilization, or 20 min, as indicated for high-level disinfection. Exspor (Alcide Corp., Redmond, Wash.), containing 1.52% sodium chlorite, was activated daily before experiments by mixing 1 part base concentrate, 4 parts water, and 1 part activator (yielding a pH between 2.3 and 2.7). The label prescribes the treatment of medical items with an Exspor-activated solution for 10 h to achieve sterilization and for 1 to 3 min for killing of *Mycobacterium* sp. and other bacteria, pathogenic fungi, and viruses on hard surfaces. Renalin (Renal Systems Division of Minntech Corp., Minneapolis, Minn.), a mixture of 20.0% hydrogen peroxide and 4.0% peroxyacetic acid, was used as recommended for sterilization at a dilution of 1:5 (final dilution; pH 1.8) in sterile, deionized, and glass-distilled water for an 11-h exposure. Wavicide-01 (2% glutaraldehyde; Wave Energy Systems, Wayne, N.J.) was used full strength for 10 h at 21°C as a sterilant or at a 1:4 dilution for 10 min (at room temperature [21°C]), as specified for killing of vegetative bacteria and viruses. Clorox (5.25% sodium hypochlorite, manufactured by The Clorox Company, Oakland, Calif.) was used at a 1:21 dilution, as recommended for disinfection. Lysol I.C. (7.24% *o*-benzyl-*p*-chlorophenol and 2.23% *o*-phenylphenol; National Laboratories, Montvale, N.J.) was used at the 1:128 dilution specified for use in hospitals, nursing homes, dental offices, and other institutional facilities as a germicidal, tuberculocidal, pseudomonacidal, staphylococcidal, fungicidal, and virucidal compound. Cavicide (15.30% isopropanol and 0.25% diisobutyl phenoxyethoxyethyl dimethyl benzyl ammonium chloride; Micro Aseptic Products, Inc., Palatine, Ill.) was used full strength, as specified for disinfection of noncritical medical instruments. Cupric chloride ($CuCl_2 \cdot 2H_2O$; Mallinckrodt Specialty Chemicals, Paris, Ky.), L-ascorbic acid, and (30% wt/vol) hydrogen peroxide (both from Aldrich Chemical, Milwaukee, Wis.) were used in a mixture (0.5% cupric ions [as cupric chloride]–0.1% ascorbic acid–0.003% hydrogen peroxide, pH 2.9).

Sporicidal test on contaminated medical devices. Each carrier device was independently immersed in a tube with 50 μl of a suspension of radiolabeled spores (1.7×10^9 spores/ml). After drying, the devices were divided into two identical groups. In one group, the number of spores loaded into each device was measured radioactively. Devices in the second group were incubated at 20°C in 400 μl of disinfectant (three devices per disinfectant in separate tubes) for the time period specified on the respective product label or in an equal volume of sterile distilled water for 30 min, as a control for spore survival. After incubation, each device was removed from the test tube, the remaining disinfectant was diluted with 600 μl of ice-chilled LB broth, and the tube was centrifuged (5 min at 15,000 rpm in a model 5414 Microfuge [Brinkman Instruments Inc., Westbury, N.Y.]). The supernatant with diluted disinfectant was discarded; the spores in the pellet were resuspended by vortexing in fresh, ice-chilled LB broth (1 ml); and this sample containing loosely adherent spores was named fraction a. The device removed in the step described above was transferred to 400 μl of distilled water and sonicated for 5 min (Ultrasonic Cleaner; Cole Parmer, Chicago, Ill.). After sonication, the device was removed and 600 μl of ice-chilled LB broth was added to the 400 μl of water. This sample with spores removed by sonication was named fraction b. To recover viable spores still remaining on the carriers after fractions a and b had been obtained, the devices were incubated in 400 μl of fresh LB broth for 30 min at 32°C in a shaker operating at 140 rpm. The device was removed and counted in scintillation liquid, and lack of radioactivity confirmed the absence of detectable spores. Six hundred microliters of ice-chilled LB broth was added to the broth left after device removal, and this sample with spores dislodged after 30 min of shaking in medium was named fraction c. The incubation time of fraction c (30 min) was shorter than the period required for spores of *B. subtilis* to germinate and replicate, thus preventing overestimation of surviving organisms (data not shown). Fractions a, b, and c were serially diluted in LB broth, and the surviving spores in each fraction were titrated by serial dilution on LB broth agar plates.

The overall recovery ratio of the method was calculated as the sum of spores titrated in fractions a, b, and c (ranging from 2.9×10^6 to 10.9×10^6 spores) after treatment with water divided by the average number of spores loaded (estimated radioactively). The spore recovery of the three-step method was 1.02 ± 0.22 (average fraction of the starting spore number $\pm$ the standard error (SE) in six independent experiments) for 0/80 stainless steel screws, a value nearly identical to the recovery previously obtained for silicone catheter tubing (1.02 ± 0.59). The recoveries of nonradiolabeled or radiola-

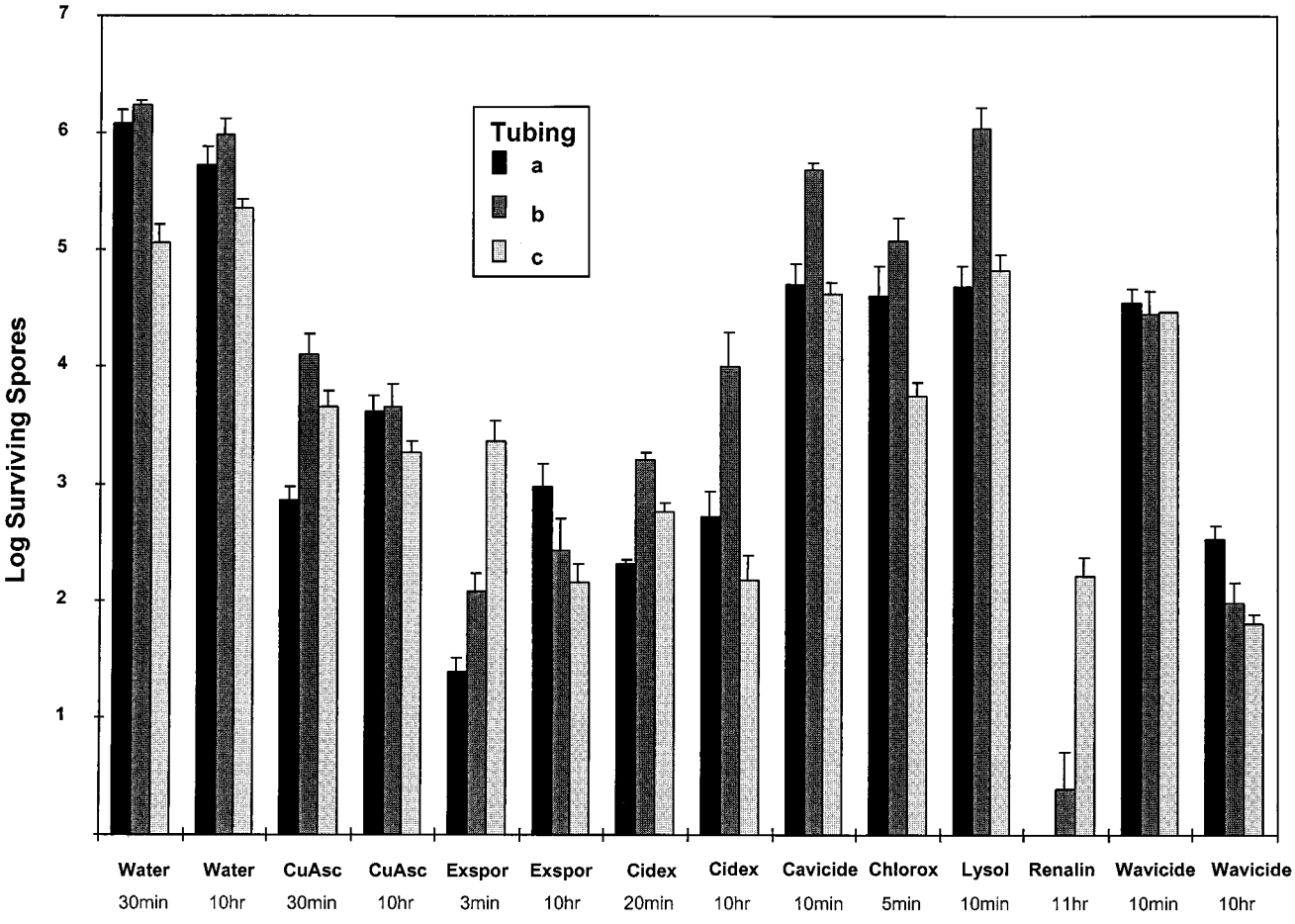

FIG. 1. Effects of germicides on spores deposited on silicone rubber medical tubing. Spores were loaded onto tubing, dried, and exposed for the times prescribed on the products' labels either for sterilization or for disinfection (indicated on the *X* axis). Viable spores were measured in fractions a, b, and c as described in the text. Bar height represents mean survival, and the bracket over the bars indicates the SE of triplicate determinations in three to eight independent experiments.

beled spores in fractions a, b, and c were similar with all of the devices studied. Therefore, nonradioactive spores were used after the number of spores loaded onto each device was calibrated and it was established that the three-step method accounted for all of the challenge spores. By using the same devices and procedure, other laboratories could reproduce this test without further calibration or need for radioactive spores.

We included positive and negative controls for sporicidal activity in each experiment to allow monitoring of intertest performance. Water was chosen as the negative control because of its lack of sporicidal activity and common availability (no killing or 100% spore survival). Stability in dry chemical form and relatively low cost made cupric ascorbate a convenient positive control for sporicidal activity that produced a significant, consistent, and relatively time-independent (between 30 min and 10 h) reduction in spore survival (see Table 1).

The sporicidal test that we developed has several valuable characteristics. (i) It is quantitative. The number of spores attached to the devices before disinfection was directly measured with radiolabeled spores. Absence of spore attachment to the carrier at the end of the testing process is easily confirmed by determining lack of remaining radioactivity. A recovery value of nearly 1 in the negative controls demonstrates that all of the loaded spores are accountable for by the test. This is a clear advantage over methods that estimate carrier load indirectly by measuring the spores dislodged from the device to an unknown extent. Furthermore, determining the surviving fraction at each step of the test by counting colonies from surviving spores is more precise and informative than scoring growth or nongrowth as in other sporicidal tests. (ii) It is rapid. Our procedure was completed within 4 h, not counting overnight colony development. (iii) It is economical and environmentally friendly. The technique uses only 400 μl of disinfectant, resulting, for all practical purposes, in a nondestructive test that saves reagents and reduces the amount of toxic and infectious waste produced.

Effect of germicides on contaminated devices. Devices carrying 3.8×10^6 to 6.2×10^6 spores of *B. subtilis* were exposed once to various sterilizing agents or to water, and the spores titrated in fractions a, b, and c are shown in Fig. 1 (tubing) and 2 (screws). It was unclear how much the sporicidal activity of products labeled as liquid sterilants differed from that of common disinfectants. To answer this question, we also measured the relative sporicidal activities of products not intended for liquid sterilization but recommended for disinfection of medical devices used in patients with AIDS or decontamination of surfaces during epidemics or bacteriological warfare or widely used as household disinfectants (9, 10, 20, 24). The spore survival results shown in Fig. 1 and 2 and Table 1 confirm that

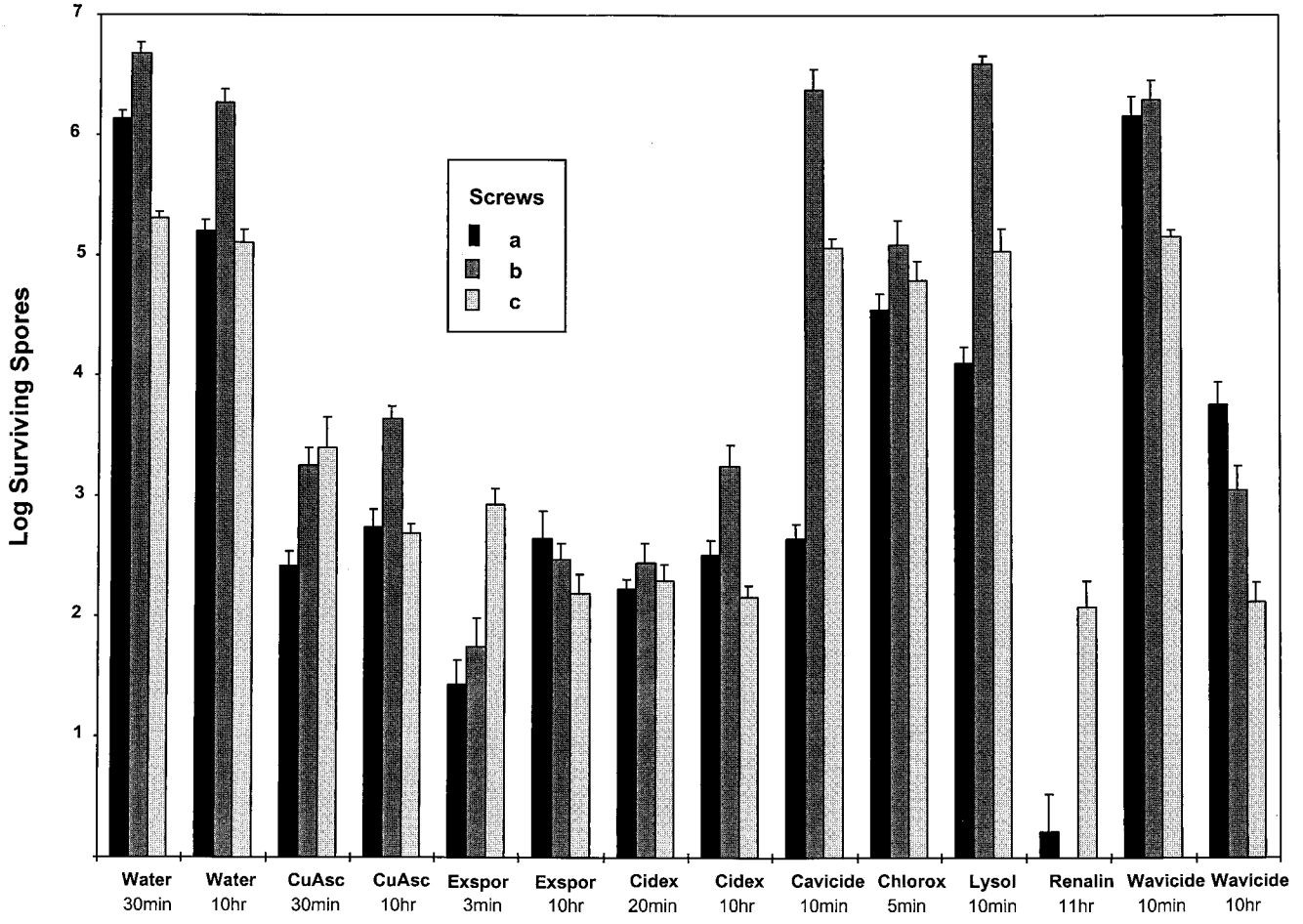

FIG. 2. Spore survival after treatment of stainless steel screws. Reagents and conditions were as described in the text and in the legend to Fig. 1. Bar height represents the mean ± the SE of the number of viable spores obtained in fractions a, b, and c determined in triplicate by titration in four to eight independent experiments.

general disinfectants (not specifically labeled for liquid sterilization, like Cavicide, Clorox, and Lysol) do not kill spores on contaminated devices and, thus, should never be employed in this capacity.

Figures 1 and 2 show that the proportion of viable spores recovered in each fraction varies for different products and treatment times. For all products, a one-step procedure (fraction a with loosely adherent spores) failed to detect all of the spores remaining viable after treatment (Fig. 1 and 2). Spore recovery in fraction a was often lower than after sonication and 30 min of shaking in culture medium, respectively (fractions b and c in Fig. 1 and 2). This could be a consequence of fixing or trapping of viable spores on the device surface by chemical cross-linking with the germicide. No viable spore could be detected in fraction a after treatment of tubing with the most active disinfectant (Renalin incubated for 11 h; Fig. 1). However, by using a procedure that completely recovers attached spores, a few spores were detected after sonication in fraction b and several hundred spores were easily detected after 30 min of shaking in medium (fraction c). Often, the number of surviving spores detected in fraction a differed by more than 1 log from the total number of viable spores (fractions a, b, and c in Fig. 1 and 2). Products whose effectiveness would be overestimated by more than 10-fold by a one-step recovery method included Cavicide, Cidexplus, Exspor, Lysol, and Renalin. Thus, these findings indicate that the sporicidal activity of disinfectants and sterilants will likely be overestimated by methods that dislodge spores in only one step (obtaining results equivalent to those obtained with fraction a) or by tests in which the recovery of loaded spores is unknown.

Comparative sporicidal activity. The total log of spore killing was obtained by subtracting the log of the total number of viable spores after exposure of devices to disinfectants (titrated in fractions a, b, and c) from the log of the number of spores surviving treatment with water. The values obtained for each device-disinfectant combination are displayed in Table 1. The survival of spores on contaminated dental burs was higher than on the other two devices. Disinfection of carbon steel dental burs produced corrosion stains on the devices and a fine precipitate at the bottoms of the test tubes. The higher spore survival correlated with obvious corrosion, and therefore, data on burs were not considered for comparison or ranking of products. The severe corrosion observed after treatment with commercial disinfectants made carbon steel dental burs inadequate as carriers for sporicidal testing. Deterioration after a single test and increased spore survival demonstrate that dental burs (and likely other devices containing carbon steel) must not be decontaminated with liquid disinfectants in spite of instructions to the contrary on the labels of some carbon steel devices. In contrast, the other two materials in this study were

TABLE 1. Log of spore killing under conditions specified for sterilization and disinfection[a]

Conditions and treatment	Time[b]	Mean log killing ± SE (no. of expts)			
		Tubing	Screws	Burs[c]	Avg[d]
Sterilization					
Water	10 h	0.25 ± 0.09 (8)	0.50 ± 0.09 (8)		0.38 ± 0.06 (16)
Cupric ascorbate	10 h	2.62 ± 0.20 (6)	3.24 ± 0.17 (8)[e]		2.93 ± 0.14 (14)
Exspor	10 h	3.47 ± 0.30 (4)	3.95 ± 0.30 (4)		3.71 ± 0.16 (8)
Cidexplus	10 h	3.28 ± 0.25 (4)	3.50 ± 0.25 (4)		3.39 ± 0.27 (8)
Renalin	11 h	4.34 ± 0.37 (4)	5.16 ± 0.30 (6)		4.75 ± 0.24 (10)
Wavicide-01	10 h	3.81 ± 0.34 (4)	3.44 ± 0.34 (4)		3.62 ± 0.24 (8)[f]
Disinfection					
Water	30 min	0 (5)	0 (6)	0 (5)	
Cupric ascorbate	30 min	2.36 ± 0.22 (5)	3.29 ± 0.20 (6)[e]	1.51 (5)	2.83 ± 0.13 (11)
Expor	3 min	3.36 ± 0.27 (5)	4.15 ± 0.30 (6)[e]	0.49 (5)	3.76 ± 0.20 (11)
Cidexplus	20 min	3.07 ± 0.22 (5)	4.00 ± 0.21 (6)[e]	2.43 (5)	3.54 ± 0.06 (11)
Cavicide	10 min	0.73 ± 0.29 (5)	0.80 ± 0.27 (6)	−0.22 (5)	0.76 ± 0.20 (11)
Clorox	5 min	1.95 ± 0.39 (5)	1.66 ± 0.35 (6)	−0.40 (5)	1.81 ± 0.26 (11)
Lysol	10 min	0.62 ± 0.18 (5)	0.18 ± 0.17 (6)	−0.33 (5)	0.40 ± 0.12 (11)
Wavicide-01	10 min	1.63 ± 0.40 (3)	0.63 ± 0.28 (6)	−0.11 (3)	1.13 ± 0.24 (9)[f]

[a] Spore survival was calculated by adding the numbers of spores recovered in fractions a, b, and c of the test described in the text. Log of spore killing was calculated as the log of the number of spores surviving treatment with water for 30 min minus the log of the number of spores surviving after each treatment.
[b] Incubation was done for the times specified by the manufacturer's label instructions, generally consisting of a short time (on the order of minutes) for disinfection and an extended time (10 to 11 h) for sterilization.
[c] Metal flaking and corrosion products made spore killing on burs imprecise, and the data were omitted from further analysis.
[d] Average of spore killing by each disinfectant in tubing and screws.
[e] Significant difference ($P > 0.95$) between tubing and screws for the same treatment.
[f] Significant difference ($P > 0.05$) between conditions for sterilization and disinfection for the same disinfectant.

impervious to all disinfecting treatments. Stainless steel screws and silicone rubber catheter tubing did not show signs of deterioration after visual and microscopic examination (×160 magnification). These findings agree with the relative resistance of stainless steel and medical-grade silicone rubber to corrosion (17). Similar spore recovery and killing (within 1 log) by the same disinfectant on both devices (Table 1) suggest that testing on stainless steel screws and medical silicone rubber tubing should provide an adequate estimation of sporicidal activity on medical devices.

Cidexplus is specified to be used for up to 28 days after activation. The label of Renalin indicates that the diluted solution must be used within a 7-day period as a sterilant for dialyzer reprocessing. These sterilants were tested at various times after activation or dilution. No significant change in the sporicidal activity of Cidexplus or Renalin was detected on contaminated silicone tubing, dental burs, and stainless steel screws during a 28- or 7-day test period, respectively (data not shown).

The incubation time specified in the labeling of products intended for sterilization is 10 or 11 h. Much shorter incubation times (a few minutes) are specified for use of the same products as sterilants. Changes in incubation time had a distinct effect on spore killing produced by different formulations specified as sterilants (Table 1). Extending treatment with Wavicide-01 from 10 min to 10 h caused a relatively large increase (more than 100 times) in sporicidal activity. In contrast, extending treatment with Exspor or cupric ascorbate from a few minutes to 10 h did not produce a substantial increase in sporicidal activity (less than a 10-fold difference between short and long exposures). Unexpectedly, spore killing on screws was slightly higher after 20 min than after 10 h of incubation with Cidexplus in four independent comparative experiments (Table 1). These findings suggest that the sporicidal activity of some products may be exhausted after a relatively short incubation period and highlight the importance of precise adherence to the times specified by the particular product's label.

Glutaraldehyde and peroxi compounds are common active ingredients used in liquid sterilization and high-level disinfection (3, 9, 10, 16, 21, 25). However, commercial products with these active ingredients had quite different sporicidal potencies after incubation for the similar periods (10 and 11 h of treatment) recommended for sterilization. The reduction of spore numbers ranged from 2,500- to 56,000-fold for Cidexplus and Renalin, respectively (Table 1).

The substantial spore survival detected in this study after treatment of devices with commercial sterilants (Table 1) conflicts with the concept of sterilization, defined as the destruction of all life, including bacterial spores. The data that we obtained with a sensitive and quantitative test suggest that commercial liquid sterilants and disinfectants are less active on contaminated surfaces than generally acknowledged.

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