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Vapor-Phase Hydrogen Peroxide as a Surface Decontaminant and Sterilant

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The feasibility of utilizing vapor-phase hydrogen peroxide (VPHP) as a surface decontaminant and sterilant was evaluated in a centrifuge application. The prototype VPHP decontamination system, retrofitted into a Beckman L8-M ultracentrifuge, was designed to vaporize a 30% (wt/wt) solution of aqueous hydrogen peroxide continuously injecting and withdrawing VPHP in a deep-vacuum flow-through system. VPHP cycles of 4, 8, 16, and 32 min were examined for cidal activity against spores of *Bacillus subtilis* subsp. *globigii* and *Bacillus stearothermophilus*. Spore inocula (~10^6/coupon) were dried onto 0.5-in. (1.27-cm)-square stainless-steel coupons, and coupons were suspended in the centrifuge chamber, the space between the refrigeration can and the barrier ring (inner gap), and the space between the barrier ring and the vacuum ring (outer gap). At a chamber temperature of 4°C, *B. subtilis* subsp. *globigii* spores were inactivated within 8 min, while inactivation of spores located in the outer gap at 27°C required 32 min. The elevated temperature and high surface area/volume ratios in the outer gap may serve to decompose the gas more rapidly, thus reducing cidal efficacy. Of the two test spores, *B. stearothermophilus* was more resistant to VPHP. Nonetheless, VPHP was shown to possess significant sporicidal capability. For practical decontamination applications of the type described, VPHP shows promise as an effective and safer alternative to currently used ethylene oxide or formaldehyde vapors.

The germicidal properties of aqueous hydrogen peroxide (H_2O_2) have been recognized for more than a century. The literature contains numerous accounts of the satisfactory application of H_2O_2 as a disinfectant for inanimate materials and inert surfaces free of inactivating levels of catalase. In addition to bactericidal and virucidal properties, H_2O_2 in relatively high concentrations (10 to 30%) appears to be a good sporicide (11, 15). Hydrogen peroxide solutions have been recommended for the disinfection of surgical implant components (6), temperature-sensitive plastic equipment (17), spacecraft hardware (20), hydrophilic soft contact lenses (7, 16), commercial packing materials (10, 12), water (9, 21), and milk (8). Toledo (13) suggests that H_2O_2 shows great promise as an in-line sterilant for aseptic packaging of fluid foods. Recent studies have also concentrated on enhancing the germicidal properties of H_2O_2 by synergistic application with ultrasonic waves (1) or UV light (2, 18). A combined application of H_2O_2 with UV irradiation may be especially well suited for the surface sterilization of packaging materials prior to filling them with ultra-high-temperature-processed products (18).

While aqueous hydrogen peroxide has a long history of use as a sterilant, the concept of vapor-phase H_2O_2 (VPHP) sterilization has been developed within the past decade. VPHP represents a class of nontoxic cold gas sterilant which provides an opportunity to discontinue the use of such toxic or carcinogenic gaseous sterilants as ethylene oxide and formaldehyde. VPHP technology could have immediate applications for the sterilization of specialty medical products, especially those which would be destroyed by steam sterilization or require lengthy aeration to reduce toxic ethylene oxide residuals. In addition to the sterilization of health care products, potential applications of VPHP technology include pharmaceuticals, foodstuffs, processing equipment, packaging materials, fermentors, and dialyzers. The successful application of VPHP technology for the surface sterilization of contaminated equipment (e.g., incubators, lyophilizers, biological safety cabinets, glove boxes, and centrifuges) would be of particular significance to the medical and laboratory science community. This study addresses the feasibility of utilizing VPHP for in situ surface decontamination and sterilization of an ultracentrifuge.

MATERIALS AND METHODS

Test organisms. Spordex spore suspensions of *Bacillus subtilis* subsp. *globigii* ATCC 9372 (lot L072787GL-S; expiration date, January 1989) and *Bacillus stearothermophilus* ATCC 12980 (lot L111987ST-S; expiration date, May 1989) were provided by the American Sterilizer Co., Erie, Pa.

Description of prototype centrifuge unit. An L8-M ultracentrifuge (Beckman Instruments, Palo Alto, Calif.) was retrofitted with a VPHP system (American Sterilizer). The VPHP system consisted of a 30% (wt/wt) solution of aqueous H_2O_2 (Fisher Scientific Co., Pittsburgh, Pa.) which was vaporized and injected directly into the centrifuge through two ports (side and bottom) (Fig. 1). After the chamber had been charged with sterilant for 1 min, injections alternated between the ports at 1-min intervals. This is a deep-vacuum flow-through system utilizing simultaneous injection and withdrawal of VPHP sterilant. Injection cycles of 4 (~3 g of liquid H_2O_2), 8 (~5 g of liquid H_2O_2), 16 (~10 g of liquid H_2O_2), and 32 (~19 g of liquid H_2O_2) min were available. Sterilant injection was carried out at a rotor setting of 4°C. Each injection cycle was followed by a 16-min exhaust of the remaining sterilant. To facilitate VPHP dispersal, the centrifuge rotor was allowed to rotate at 2,000 rpm throughout both the injection and the exhaust cycles.

Although the weight-volume of aqueous H_2O_2 consumed

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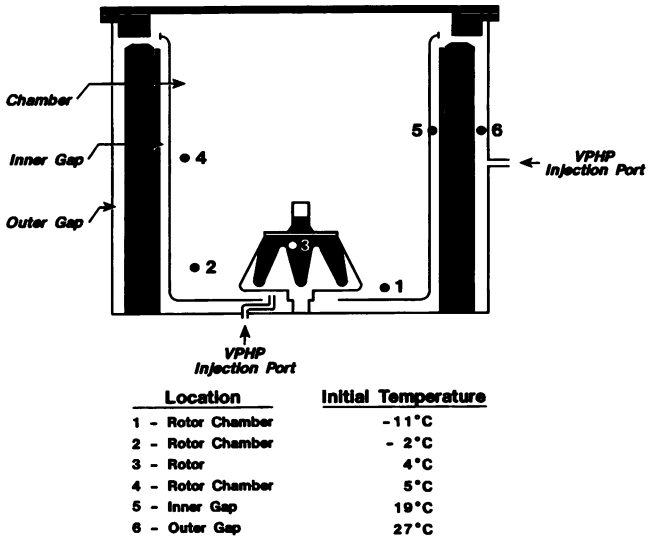

Location	Initial Temperature
1 - Rotor Chamber	-11°C
2 - Rotor Chamber	- 2°C
3 - Rotor	4°C
4 - Rotor Chamber	5°C
5 - Inner Gap	19°C
6 - Outer Gap	27°C

FIG. 1. Cross-sectional view of the prototype centrifuge unit.

during the injection cycle was measured for each trial, the prototype unit is not equipped with a mechanism for detection of VPHP concentration present at any location within the centrifuge. Therefore, the efficiency of the VPHP generation or distribution system or both could not be determined, and the run-to-run variation in the performance of this prototype unit could not be assessed.

Testing protocol. Four stainless-steel coupons (0.5 by 0.5 in. [1.27 by 1.27 cm]) were tied, at 2-in. (5.08-cm) intervals, to a length of nylon line. All coupon-lines were sterilized in a steam autoclave at 121°C for 30 min with a 15-min drying cycle. A 10-μl inoculum (~10^6 spores) of the appropriate test organism spore suspension was delivered to each coupon and allowed to air dry in an HEPA filter-equipped laminar-flow hood located in a restricted-access laboratory room.

For each trial, eight coupon-lines were placed in each of three locations within the centrifuge unit: (i) the centrifuge chamber, (ii) the space between the refrigeration can and the barrier ring (inner gap), and (iii) the space between the barrier ring and the vacuum ring (outer gap) (Fig. 1). Four access notches cut in the top of the chamber wall facilitated placement of the coupon-lines within the inner and the outer gaps. Since the chamber drum is approximately 10 in. (25.4 cm) deep, coupons were tied to each line at 2-in. intervals so that coupons 1, 2, 3, and 4 would be situated at depths of 2 (5.08 cm), 4 (10.16 cm), 6 (15.24 cm), and 8 (20.32 cm) in., respectively, when placed in the appropriate location.

After all sample coupon-lines had been taped in place and duct tape had been affixed over each of the four access notches, the chamber door was closed and the centrifuge cycle was initiated. At the completion of the VPHP cycle (injection and exhaust periods), each coupon-line was removed from the centrifuge. Dissecting scissors, which had been dipped in 95% ethanol and flamed, were used to detach each coupon aseptically from the nylon line. Each coupon was inoculated into a bottle (30 by 85 mm; 70-ml capacity) containing 27 ml of sterile tryptic soy broth (TSB; Difco Laboratories, Ann Arbor, Mich.). All TSB bottles were incubated for 7 days at 37°C (for *B. subtilis* subsp. *globigii*) or 55°C (for *B. stearothermophilus*). Results were recorded for each bottle as growth (contaminated) or no growth (sterile). Contaminated sample bottles were confirmed by Gram stain and colony morphology on Trypticase soy agar (BBL Microbiology Systems, Cockeysville, Md.) incubated at the appropriate temperature for the specific test organism.

The volume of aqueous H_2O_2 used, the injection and exhaust timing, and the rotor temperature profile and speed were monitored during each trial. The presence of H_2O_2 in the chamber, inner gap, and outer gap was verified qualitatively via a colorimetric dipstick indicator. Stability of the 30% (wt/wt) H_2O_2 solution from which vapor was generated was validated weekly, using the spectrophotometric method of Danner et al. (4).

Description of experimental controls. The following controls were run concurrently with each trial.

(i) Medium controls. Unopened bottles of sterile TSB were incubated at 37°C to verify that the growth medium did not contribute to a false-positive result.

(ii) Contaminated controls. Coupons sterilized by steam autoclave were inoculated with a known quantity of test spores (ranging from ~10^0 to ~10^6). Each coupon was inoculated into a TSB bottle and incubated along with trial samples to verify that a true-positive result could be detected by the TSB recovery system used.

(iii) Procedure controls. Uninoculated coupons tied to nylon line and sterilized by steam autoclave were aseptically removed, inoculated into TSB, and incubated at 37°C. Procedure controls were performed to demonstrate that the above-described coupon recovery and inoculation procedure did not contribute to a false-positive result.

(iv) Aseptic-handling controls. Coupons randomly selected from among the sterilized coupon-lines prepared for each trial were left uninoculated with test spores, subjected to the VPHP cycle, and then recovered as described above. These coupons provided validation that the procedure used to remove coupon-lines from the centrifuge did not contribute to a false-positive result.

Neutralizer experiments. Residual H_2O_2 present on any given coupon at the completion of the VPHP cycle could inhibit the recovery of test spores which survived the VPHP exposure. The following experiments were performed to assess this potential effect and determine the necessity of incorporating an H_2O_2 neutralizer (e.g., catalase) into the TSB recovery medium. Eight coupon-lines, which were sterilized by steam autoclaving but not inoculated with test spores, were placed in each of the three centrifuge locations and subjected to a specific VPHP cycle. After completion of the cycle, each coupon was aseptically inoculated into a TSB bottle. A second sterile coupon which had been inoculated with either *B. subtilis* subsp. *globigii* or *B. stearothermophilus* spores at approximately 10^0, 10^1, 10^2, or 10^6 was then aseptically added to the above-described TSB bottle. This protocol produced four TSB bottles for each combination of VPHP cycle-centrifuge location-test organism-spore concentration. All TSB bottles were incubated, and results were recorded as described above.

TABLE 1. Effects of various VPHP cycle times on *B. subtilis* subsp. *globigii* ATCC 9372 spores[a]

VPHP cycle (min)	No. of sterile coupons/total no. of coupons tested (sterilization probability)		
	Chamber	Inner gap	Outer gap
4	118/124 (0.95)	107/124 (0.86)	112/124 (0.90)
8	120/120 (1.0)	118/120 (0.98)	116/120 (0.97)
16	128/128 (1.0)	192/192 (1.0)	186/192 (0.97)

[a] Mean inoculum per coupon, 1.5 × 10^6 spores (range, 1.0 × 10^6 to 2.1 × 10^6 spores).

TABLE 2. Effects of various VPHP cycle times on *B. stearothermophilus* ATCC 12980 spores[a]

VPHP cycle (min)	No. of sterile coupons/total no. of coupons tested (sterilization probability)		
	Chamber	Inner gap	Outer gap
8	64/64 (1.0)	7/64 (0.11)	17/64 (0.27)
16	125/128 (0.98)	45/128 (0.35)	14/128 (0.11)
32	32/32 (1.0)	25/64 (0.39)	19/64 (0.30)

[a] Mean inoculum per coupon, 3.0×10^6 spores (range, 2.4×10^6 to 4.0×10^6 spores).

RESULTS

Results of the neutralizer experiments indicate that recovery of a minimal inoculum ($\sim 10^0$ spores) of either test organism was not inhibited by residual H_2O_2 levels present for any of the four cycle times. Therefore, incorporation of an H_2O_2 neutralizer into the TSB recovery medium does not appear necessary to ensure against a false-negative result. Composite results for the various controls which were run concurrently with each experiment are as follows. All of the 30 medium controls, 129 procedure controls, and 72 aseptic-handling controls were determined to be sterile after appropriate incubation. Conversely, all of the 47 *B. subtilis* subsp. *globigii*- and 45 *B. stearothermophilus*-contaminated controls showed abundant growth within 24 h of incubation at the appropriate temperature.

The composite results of trials in which coupons inoculated with *B. subtilis* subsp. *globigii* spores were subjected to various VPHP cycles are presented in Table 1. These data indicate that VPHP cycles of 8 min or more appear capable of sterilizing the centrifuge chamber. Sterilization of the centrifuge inner and outer gaps appears to be more difficult to achieve. A great many of the inner and outer gap coupons which remained contaminated were located at the positions most distant from the outer gap injection port. The observed pattern may be related to VPHP dispersion inefficiency or VPHP decomposition as VPHP diffuses to distant locations in the centrifuge gaps or to both. Nonetheless, the data show a trend toward increased decontamination in both gaps with longer VPHP cycles. Table 2 contains the composite results of similar experiments with coupons inoculated with *B. stearothermophilus* spores. The data suggest that *B. stearothermophilus* spores are more resistant than *B. subtilis* subsp. *globigii* spores to VPHP. However, direct comparison of the Table 1 and 2 data is not advisable due to run-to-run variation in the performance of this prototype machine. To obtain a direct comparison of *B. subtilis* subsp. *globigii* and *B. stearothermophilus* resistance to VPHP, each spore suspension ($\sim 10^6$ spores per coupon) was inoculated onto half of the coupons in an alternating fashion and the coupon-lines were then placed in each of the three centrifuge locations. Composite results of these trials, designed for simultaneous VPHP exposure of the two test spores, are shown in Table 3. The data clearly show that, of the two test spores, *B. stearothermophilus* is significantly more resistant to VPHP under the exposure conditions contained within the prototype centrifuge unit.

DISCUSSION

In laboratory practice, the decontamination of centrifuges has historically presented a difficult challenge. Spill problems encountered can range from leakage due to minor chips or cracks in tubes or overfilling of tubes to catastrophic rotor failure. Since spills often involve high concentrations of pathogenic organisms, the procedure used must adequately decontaminate the readily accessible rotor chamber area of the centrifuge while protecting the personnel performing the spill cleanup. In addition, for a spill occurring in a centrifuge operating under a vacuum, the potential contamination of the less accessible gaps and pumps must be addressed. While liquid disinfectants are of limited value for reaching inaccessible areas, vapor-phase germicides offer obvious penetration advantages. Formaldehyde and ethylene oxide, the vapors commonly used for centrifuge decontamination, require extended exposure times to achieve sterilization and are toxic or carcinogenic. Conversely, VPHP provides rapid inactivation rates and decomposes to water vapor and oxygen.

The results of this study indicate that VPHP has considerable potential for use in a centrifuge decontamination application. In the centrifuge chamber, spore inocula dried onto stainless-steel coupons ($\sim 10^6$ spores per coupon) were consistently killed within 8 min. This implies that VPHP cycle times of this magnitude, used for routine decontamination at the conclusion of an experiment or the end of a workday, could be depended upon to protect laboratory personnel. A longer cycle time ($\geq$32 min) appears desirable to achieve an adequate margin of safety following an obvious spill (tube breakage) or to assure decontamination of less accessible areas prior to servicing the centrifuge unit. In addition, VPHP was shown to be an effective decontaminant when applied at low temperatures. A temperature profile of the centrifuge (Fig. 1) indicates that the inner and outer gaps are near room temperature (19°C for the inner gap and 27°C for the outer gap), while the rotor is at 4°C. For both test spores, consistent and significantly greater kill was achieved in the chamber as opposed to the gap areas. This suggests that the low temperature served to keep the VPHP from decomposing before it had time to diffuse throughout the area and inactivate the spores, whereas the higher tempera-

TABLE 3. Comparative resistance of spores of *B. subtilis* subsp. *globigii* ATCC 9372 and *B. stearothermophilus* ATCC 12980 simultaneously exposed to VPHP cycles

VPHP cycle (min)	No. of sterile coupons/total no. coupons tested (sterilization probability)					
	B. subtilis subsp. *globigii*[a]			*B. stearothermophilus*[b]		
	Chamber	Inner gap	Outer gap	Chamber	Inner gap	Outer gap
16[c]	96/96 (1.00)	93/96 (0.97)	49/96 (0.51)	80/96 (0.83)	6/95 (0.06)	3/96 (0.03)
32[d]	32/32 (1.00)	48/48 (1.00)	43/48 (0.90)	30/32 (0.94)	15/48 (0.31)	4/48 (0.08)

[a] Mean inoculum per coupon, 1.4×10^6 spores (range, 1.1×10^6 to 1.8×10^6 spores).
[b] Mean inoculum per coupon, 3.9×10^6 spores (range, 3.2×10^6 to 4.7×10^6 spores).
[c] Composite results of six trials.
[d] Composite results of three trials.

tures promoted more rapid decomposition of the gas. Also, the comparatively higher surface area/volume ratio in the gaps may contribute to more rapid vapor decomposition in these locations. Engineering modifications of the prototype unit might resolve these apparent distribution differences and enhance the sporicidal activity of VPHP in the inner and outer gaps of the centrifuge.

The traditional biological indicator of choice for gaseous sterilants is *B. subtilis* subsp. *globigii* (3). In their pioneering evaluation of VPHP sterilization of polyethylene flexible packaging materials used in aseptic systems, Wang and Toledo (19) used *B. subtilis* A and *B. subtilis* subsp. *globigii* as indicator organisms. These spores are also the indicator organisms of choice for hot aqueous H_2O_2 systems used to sterilize plastic containers and laminated foil used in aseptic filling operations (5). Toledo et al. (14) have reported that *B. subtilis* SA 22 and *B. subtilis* subsp. *globigii* show a greater resistance to aqueous H_2O_2 than do spores of *B. stearothermophilus*, *B. coagulans*, or *Clostridium* sp. However, under the VPHP exposure conditions present in the ultracentrifuge, *B. stearothermophilus* exhibited considerably greater VPHP resistance than to *B. subtilis* subsp. *globigii*. An explanation for the observed resistance phenomenon is currently unavailable. The application of VPHP as a potential sterilant is clearly still in its infancy; definitive knowledge of the mechanism(s) of cidal action, and the factors which influence it, is lacking.

The bacterial spore is generally considered to be the most chemical germicide-resistant form of microbial life. As a surface decontaminant used in a centrifuge application, VPHP has been shown to possess significant sporicidal capability. Thus, for laboratory activities involving centrifugation of microorganisms, it is reasonable to presume that 8- to 32-minute decontamination cycles using VPHP will render the centrifuge safe for human exposure. Furthermore, VPHP, with its major advantage of innocuous breakdown products, has significant potential as a high-level disinfectant and chemosterilant for numerous decontamination applications, and further development of such applications should be encouraged.

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