PUBLICATION

RESISTANCE OF VARIOUS MICROORGANISMS TO VAPORIZED HYDROGEN PEROXIDE IN A TABLE TOP STERILIZER

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ABSTRACT

A great deal of information is available in the literature on the relative resistance of microorganisms to aqueous hydrogen peroxide (hydrogen peroxide); however, the same cannot be said for hydrogen peroxide in the gaseous state. Stainless steel coupons, inoculated with approximately 10' of various bacteria, fungi, or bacterial spores, were allowed to air dry and then placed in the sterilizer chamber. A 30 wt.% hydrogen peroxide solution was vaporized into a flowing air stream at a rate sufficient to achieve a vaporized hydrogen peroxide gas concentration of 3.3 mg hydrogen peroxide/liter of air. The sterilant was continuously passed through the chamber at a vacuum level of 700 mmHg absolute and at a temperature of approximately 35°C for various exposure times. Each exposure was followed by a 2-minute chamber exhaust period. *Bacillus stearothermophilus* spores were found to be the most resistant to hydrogen peroxide gas. The presence of organic soil did not significantly affect the ability of hydrogen peroxide gas to sterilize stainless surfaces. The data suggest that hydrogen peroxide gas could offer a rapid alternative for sterilizing hard surface dental instruments and handpieces.

Introduction

Aqueous hydrogen peroxide has been recognized as a bactericidal and sporicidal agent for years and is used in several applications in the healthcare and food industries. Respective examples include contact lens disinfection using 3 wt. % hydrogen peroxide and aseptic packaging sterilization that requires 15 to 35 wt.% hydrogen peroxide at elevated temperatures. The use of vaporized hydrogen peroxide as a germicide is a relatively new concept although numerous applications are envisioned.

This study was undertaken to determine the most resistant microorganism to hydrogen peroxide gas. Representatives from several classes of vegetative bacteria, yeasts, and fungal and bacterial spores were exposed to hydrogen peroxide gas in a prototype Tabletop Sterilizer. The effect of organic material on sterilization efficacy was also determined by mixing the test microorganisms with 5% bovine serum.

MATERIALS AND METHODS

Test microorganisms. Most of the vegetative and spore-forming microorganisms selected for this study have previously shown some resistance to aqueous hydrogen peroxide and are listed in Table 1. Suspensions of *Pseudomonas aeruginosa, Serratia marcescens,* and *Streptococcus faecalis* were propagated in Trypticase Soy Broth (TSB; Becton Dickinson Microbiology Systems, Cockeysville, MD). Sabouraud dextrose broth (Difco Labs, Detroit, MI) was used for *Lactobacillus casei* and *Candida parapsilosis* while sabouraud dextrose agar (Difco Labs) was used to obtain the spores of *Aspergillus niger.* Middlebrook 7H9 broth (Difco Labs) containing 0.05% Tween 80 was used to grow *Mycobacterium smegmatis.* The spores of *Clostridium sporogenes* were prepared from soil-extract egg-meat medium (3). Spore suspensions of *Bacillus subtilis (globigii), B. pumilus,* and *B. stearothermophilus* were obtained from AMSCO Medical Products Division (Apex, NC). Each microorganism was incubated at its optimum growth temperature.

Preparation of bacterial and spore suspensions for testing. Following incubation, the suspensions were centrifuged at 4340 x *g* for 10 minutes. Each pellet was re-suspended in an equal volume of sterile, distilled water and re-centrifuged. The pellets were then suspended in an equal volume of either sterile, distilled water or 5% bovine serum (Sigma Chemical Co., St. Louis, MO). *C. Parapsilosis* and *L. casei* were re-suspended in one-tenth of their volumes because of lower populations.

Each suspension (10 µl) was inoculated onto sterile, stainless steel coupons and air-dried at ambient temperature. The viable population for each test microorganism was determined from 3 coupons. The remaining coupons were exposed to hydrogen peroxide gas within 5 minutes after the inoculum had

completely dried.

Exposure of inoculated coupons. Six inoculated coupons of a particular microorganism were placed in the sterilizer chamber while sitting on a glass petri dish. A 30 wt. % hydrogen peroxide solution was continuously vaporized into a flowing air stream at a pressure of 700 mmHg absolute, using an injection rate sufficient enough to achieve a hydrogen peroxide gas concentration of 3.3 mg/l at an air temperature of approximately 35°C in the chamber. Each exposure was followed by a 2-minute chamber exhaust period.

Recovery and determination of survivors. Immediately following the chamber exhaust, the coupons were aseptically transferred to 9 ml of phosphate buffer (USP XXI) containing 0.05% Tween 80. The tubes were vortexed vigorously to remove the inoculum from the coupon surfaces. The bacterial spore suspensions were sonicated for 15 minutes to accomplish their complete removal from the coupons. Serial dilutions (10^0 - 10^4) were prepared in phosphate buffer. With the exception of *A. niger* spores, dilutions of each microorganism were pour plated in duplicate into their appropriate agar medias. The tube dilution method was used to determine the number of *A. niger* spore survivors. The population was estimated to be a log less than the dilution exhibiting growth. The plates were incubated at the microorganism's optimum growth temperature for 48 to 72 hours while the fungal tubes remained at ambient temperature for 10 days prior to recording the results.

Results and Discussion .

hydrogen peroxide gas was found to have excellent bactericidal, fungicidal, and sporicidal properties against all of the test microorganisms when exposed to a sterilant concentration of 3.3 mg hydrogen peroxide/liter of air at approximately 35°C (Table 2). *B. stearothermophilus* spores were determined to be the most resistant to hydrogen peroxide gas requiring approximately 2 minutes to achieve a 6-log reduction in the viable population. All of the other microorganisms were completely inactivated within a 1-minute exposure except for *P. aeruginosa,* although its initial population was nearly 1-log higher upon drying than the others.

The majority of the microorganisms in Table 2 were chosen for evaluation because of their reported resistance to aqueous hydrogen peroxide (1, 4, 6, 8). This study did not show similar resistances to hydrogen peroxide in the gaseous state even at significantly lower concentrations. For example, Toledo et al. [reference 8] reported that *B. subtilis (globigii)* spores had a 1-minute D-value when exposed to 25.8 wt. % hydrogen peroxide (286,000 ppm in solution) at 35°C. Stainless steel coupons containing 10^6 spores of the same microorganism were sterile within 1 minute (D-value of ~10 seconds) when exposed to 3.3 mg/l (~2300 ppm) of hydrogen peroxide gas at the same temperature.

Repeating the experiments in the presence of 5% bovine serum did not significantly affect the results (Table 3). Curran et al. [2] have likewise shown that organic material has little effect upon the germicidal action of hydrogen peroxide.

Only *B. stearothermophilus* spores and the yeast *C. parapsilosis* had survivors after a 1-minute hydrogen peroxide gas exposure. Wagner et al. [9] in their research on the antifungal properties of hydrogen peroxide in neutral buffer showed that *C. parapsilosis* was the most resistant of the yeast and that it was also more resistant than fungal spores and bacteria.

Most of the literature on the sporicidal proper ties of aqueous hydrogen peroxide has agreed upon the fact that *B. subtilis* spores are among the most resistant to the germicide (4, 5, 7, 8, 10). However, there are a few studies available that indicate that *B. stearothermophilus* spores also exhibit similar resistances to low and high concentrations of hydrogen peroxide. Curran et al. [2] showed that the thermophile could not be completely inactivated in 7 days upon exposure to 1 wt. % hydrogen peroxide at 40°C while *B. cohaerens* and *B. albolactis* were inactivated in 10 and 12 hours, respectively.

In conclusion, several bacteria, yeast, and fungal and bacterial spores were rapidly inactivated by hydrogen peroxide gas at a concentration of 3.3 mg/l at 35°C when dried onto stainless steel coupons. *B. stearothermophilus* spores were found to be the most resistant to the sterilant. The presence of 5 % bovine serum did not significantly affect the ability of hydrogen peroxide gas to sterilize stainless surfaces. The data suggest that the rapid sterilization of hard surfaces, such as dental instruments and handpieces, may be attainable.

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TABLE 1

Microorganisms Evaluated For Their Relative Resistance to Vaporized Hydrogen Peroxide

Test Microorganisms	General Classification
Bacteria	
Lactobacillus casei (ATCC 4646)	Gram (+) rod
Mycobacterium smegmatis (PRD #1)	Gram (+) acid fast rod
Pseudonomas aeruginosa (ATCC 15442)	Gram (-) non-ferm. rod
Serratia marcescens (ATCC 14041)	Gram (-) enteric rod
Streptococcus faecalis (ATCC 16559)	Gram (+) cocci
Fungal Spore / Yeast	
Aspergillus niger (ATCC 16404)	Fungal spore
Candida parapsilosis (ATCC 7336)	Gram (+) yeast
Bacterial Spores	
Bacillus pumilus (ATCC 27142)	Gram (+) rod
Bacillus stearothermophilus (ATCC 12980)	Gram (+) thermo. Rod
Bacillus subtilis (ATCC 9372)	Gram (+) rod
Clostridium sporogenes (ATCC 3584)	Gram (+) anaerobic rod

TABLE 2

Exposure of Various Microorganisms Dried on Stainless Steel Coupons to 3.3 mg/l Vaporized Hydrogen Peroxide at 35°C

Test Microorganisms	Exposure Time, minutes			
	0	1	1.5	2
Bacteria				
Lactobacillus casei	7.1×10^5 [a]	0	0	0
Mycobacterium smegmatis	3.2×10^5	0	0	0
Pseudonomas aeruginosa	2.7×10^7	6	0	NT[b]
Serratia marcescens	7.8×10^5	0	0	0
Streptococcus faecalis	3.9×10^6	0	0	0
Fungal Spore / Yeast				
Aspergillus niger	8.0×10^5	0	0	0
Candida parapsilosis	3.1×10^5	0	0	0
Bacterial Spores				
Bacillus pumilus	2.9×10^6	0	0	0
Bacillus stearothermophilus	1.4×10^6	4.3×10^4	5.0×10^3	9.0×10^1
Bacillus subtilis	1.7×10^6	0	0	0
Clostridium sporogenes	4.6×10^6	0	0	0

[a]Surviving population (Colony Forming Units)

[b]NT = Not Tested

TABLE 3

Exposure of Various Microorganisms in 5% Bovine Serum Dried on Stainless Steel Coupons to 3.3 mg/l Vaporized Hydrogen Peroxide at 35°C

Microorganisms	Exposure Time, minutes			
	0	1	1.5	2
Bacteria				
Lactobacillus casei	6.0×10^5 [a]	0	0	0
Mycobacterium smegmatis	1.4×10^5	0	0	0
Pseudonomas aeruginosa	1.0×10^7	0	0	0
Serratia marcescens	1.1×10^6	0	0	0
Streptococcus faecalis	3.0×10^6	0	0	0
Fungal Spore / Yeast				
Aspergillus niger	8.0×10^5	0	0	0
Candida parapsilosis	5.2×10^5	6.6×10^1	0	0
Bacterial Spores				
Bacillus pumilus	5.6×10^6	0	0	0
Bacillus stearothermophilus	2.0×10^6	5.8×10^4	8.7×10^2	1
Bacillus subtilis	2.2×10^6	0	0	0
Clostridium sporogenes	2.5×10^6	0	0	0

[a]Surviving population (Colony Forming Units)