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UNIVERSITY MEDICAL FACULTY GROUP

DEPARTMENT OF MICROBIOLOGY
AND IMMUNOLOGY

Study Title: Effect of Ozone on *Acanthamoeba castellani*

Study Sponsor: Alab, LLC
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Purpose

The purpose of this experiment was to validate QuickPure™ Contact Lens Sanitizer's ability to eradicate *Acanthamoeba castellani* cysts and trophozoites.

Materials

Organism. The test organism was *Acanthamoeba castellani* ATCC 30011 (American Type Culture Collection, Manassas, VA).

Culture Medium. Ameba were cultured on Middlebrook 7H11 agar (Remel, Lenexa, KS) with *Escherichia coli* ATCC 25922.

Saline. Buffered isotonic saline, pH 7.0 was used; the formula is proprietary to Alab, LLC. (Note: The formula is available but not included in this report at the request of Alab, LLC.)

Inoculum Preparation. *Acanthamoeba castellani* ATCC 30011 was cultured with *Escherichia coli* ATCC 25922 on Middlebrook agar for 7 days at 35° C in ambient air.

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Ameba from several agar plates were harvested by washing each agar surface with approximately 3 to 4 mls of sterile saline followed by centrifuging at 3,400 x g for 10 minutes in sterile plastic disposable screw cap centrifuge tubes. After centrifugation, supernatants were discarded and pellets combined and re-suspended in fresh saline; this process was twice repeated to wash the ameba and remove organics. After the final wash, ameba were microscopically enumerated using a disposable hemocytometer (C-Chip, SKC Company, Seoul, Republic of Korea) and diluted to approximate a desired inoculum of 1×10^6 trophozoites and 10^2 cysts per ml.

Exposure to Ozone. The ozone generating instrument was operated by and its calibration and adjustments were set by Alab, LLC.

Methods and Results

On the day of test, room temperature was 22° C and temperature of saline was 22° C.

The harvested ameba prepared as described above were diluted to yield 1×10^6 trophozoites and 7.5×10^4 cysts per ml.

The washed suspension of trophozoites and cysts in proprietary isotonic saline was used to calibrate the ozone-generating instrument (performed by Alab, LLC) to ensure that possible interfering organics had been removed and discarded by washing. In brief, 10 mls of ameba suspension were pipeted into an aeration cup attached to a support stand placed inside a Biological Safety Cabinet. After the appropriate time of ozonation was reached, the ameba suspension was poured into a sterile cup and mixed with 10 ml to 30 ml of proprietary saline solution. A 10 ml sample was extracted with Hach™ Ozone Reagent AccuVac™ to measure and record O_3 using a Hach DR/820 Colorimeter.

After the ozone-generating instrument was calibrated, 10 ml aliquots of the organism suspension were pipeted into the aeration cup and exposed to aeration with concentrations of ozone ranging from 1.24 to 2.73 mg/L for 1 minute, 2 minutes, 3 minutes, 4 minutes, 5 minutes and 6 minutes, respectively; duplicate samples for each exposure time were prepared. After each exposure time had elapsed, the ameba suspension was poured into a sterile cup containing 2 drops of 1N sodium thiosulfate with immediate thorough mixing, centrifuged at 3400 x G for 10 minutes, the supernatant discarded and the pellet re-suspended in 100 microliters saline. One set of samples was counted microscopically in a hemocytometer to determine the number of organisms following exposure to ozone. A second set of samples was inoculated onto agar plates (inoculated with *Escherichia coli* ATCC 25922 the previous day) and incubated at 35° C in ambient air for a total of 10 days to determine viability of organisms following exposure to ozone.

After exposure to ozone, microscopic counts were performed in a hemocytometer.

After 2, 3, 6, 7, 8, 9 and 10 days incubation, agar plate cultures were examined by light microscopy at 100X magnification for growth. In brief, control cultures of ameba with or without sodium thiosulfate and not exposed to ozone had too numerous to count (TNTC) trophozoites and cysts in the area of inoculum deposition and had typical morphology on each day of examination; microscopic fields with TNTC organisms had approximately 500-1000 organisms per field, i.e., the entire field of vision was filled with organisms. Ozone-exposed cultures, however, had significantly fewer organisms in the area of inoculum deposition and had atypical morphology, i.e., organisms appeared to be intact but smaller in size and did not have typical appearance of non-ozone-exposed trophozoites or cysts. Although culture of organisms exposed to ozone for 1 minute showed no evidence of growth after three days incubation, growth was evident after 6 and 7 days incubation and the number of trophozoites and cysts was TNTC by day 8, and had typical size and appearance. Cultures of organisms exposed to ozone for ≥2 minutes showed no evidence of growth over 10 days incubation.

Conclusions

Exposure to ozone concentrations ranging from 1.24-2.73 mg/L for 1 to 6 minutes significantly reduced the number of *Acanthamoeba castellani* ATCC 30011 trophozoites and cysts. As evidenced by microscopic enumeration of organisms, the number of trophozoites/ml was decreased by approximately 2 logs within 1 minute of exposure, while the number of cysts/ml was decreased by approximately 1 log and 4 logs within 1 minute and ≥2 minutes, respectively, compared to non-ozone exposed organisms.

Exposure of *Acanthamoeba castellani* ATCC 30011 to ozone for ≥2 minutes was shown to have been amebicidal. No evidence of growth following 2, 3, 4, 5 or 6 minutes of exposure to ozone was noted for at least 10 days. Although exposure to ozone for 1 minute was shown to inhibit organism growth for at least 3 days, growth was observed after 6 or 7 days of incubation.

In brief, exposure to ozone for ≥2 minutes was shown to kill 6 logs of *Acanthamoeba castellani* ATCC 30011 trophozoites and 4 logs of cysts per ml.

D.J.Hardy, Ph.D.
URMC
April 6, 2010

Summary of Results of Exposure of *Acanthamoeba* to Ozone				
Test Organism: *A. castellanii* ATCC #30011			Ozone Exposure Date February 9,2010	
Testing performed by Dwight Hardy, PhD. At University of Rochester Medical Center Lab 2-5336				
Ozone Exposure Time (Minutes)	PPM Ozone Concentration		*Acanthamoeba* Trophozoite Inoculation with 10 mls @ 1.1×10^6/ml	*Acanthamoeba* Cyst Inoculation with 10 mls @ 2.0×10^4/ml
	Saline Only	With Inoculation	Deactivation (Log Reduction)	Deactivation (Log Reduction)
0	X	X	0	0
1	3.30	1.52	0	0
2	3.12	1.24	7 logs	5 logs
3	3.30+	2.04	7 logs	5 logs
4	4.05	2.73	7 logs	5 logs
5		2.31	7 logs	5 logs
6		1.80	7 logs	5 logs

Organism suspended in Alab Saline Solution
Incubation Period 10 days
Testing particulars can be found in study titled Effect of Ozone on *Acanthamoeba castellani*