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Gaseous Disinfection of *Cryptosporidium parvum* Oocysts

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Purified oocysts of *Cryptosporidium parvum* suspended in ~400 μl of phosphate-buffered saline or deionized water in microcentrifuge tubes were exposed at 21 to 23°C for 24 h to a saturated atmosphere of ammonia, carbon monoxide, ethylene oxide, formaldehyde, or methyl bromide gas. Controls were exposed to air. Oocysts in each tube were then rinsed and resuspended in fresh, deionized water, and 1 million oocysts exposed to each gas were orally administered to each of three to six neonatal BALB/c mice in replicate groups. Histologic sections of ileum, cecum, and colon tissues taken from each mouse 72 h after oral administration of oocysts were examined microscopically to determine if infection had been established. All 15 mice given oocysts exposed to carbon monoxide had numerous developmental stages of cryptosporidium in all three intestinal segments. Of 10 mice given oocysts exposed to formaldehyde, 6 had a few developmental stages of cryptosporidium in the ileum. No mice given oocysts exposed to ammonia, ethylene oxide, or methyl bromide were found to be infected. These findings indicate the efficacy of these low-molecular-weight gases (ammonia, ethylene oxide, and methyl bromide) as potential disinfectants for *C. parvum* oocysts where soil, rooms, buildings, tools, or instruments might be contaminated.

Environmental contamination with the oocyst stage of *Cryptosporidium parvum*, excreted in the feces of infected mammals, can result in infection of immunologically impaired or naive humans and animals. The tough, nearly impervious wall of the oocyst has made disinfection by chemical methods extremely difficult, with only a few of 59 reported agents showing significant efficacy in reducing oocyst viability or infectivity (7). Disinfectants that were most effective at temperatures below 30°C were compounds such as ammonia, chlorine, chlorine dioxide, monochloramine, formaldehyde, and hydrogen peroxide. However, studies varied markedly in the concentrations of the chemicals used, the length of exposure, the methods used to determine the viability or infectivity of oocysts, and the quantitation of efficacy. The most effective disinfectants were low-molecular-weight compounds in gaseous form. Some such compounds used as disinfectants in agricultural and medical environments had not been previously tested. The present study was undertaken to test gaseous forms of such disinfectants. The practical considerations leading to this study included the need to disinfect contaminated buildings where poultry, livestock, or other animals might acquire cryptosporidiosis and the need to disinfect contaminated instruments, tools, or utensils.

Source of *C. parvum* oocysts. Oocysts of *C. parvum* (AUCP-1 isolate) were collected in the feces of an experimentally infected neonatal calf and cleaned of fecal debris as previously described (6). Oocysts were stored in 2.5% aqueous potassium dichromate at 4°C and were less than 1 month old when used.

Bioassay for infectivity. Sixteen BALB/c mouse dams, each with 3 to 6 pups 2 to 3 days old, were purchased from the National Cancer Institute, Frederick, Md. Each litter was housed separately. Fresh water and food (Agway Prolab Diet 3000; Agway Inc., Syracuse, N.Y.) were available at all times. After 1 week of acclimation, pups were utilized for bioassay. Each pup received 10^6 oocysts in 50 μl of water or water alone by gastric intubation with a 24-gauge gavage needle and was killed 72 h later by overexposure to CO_2. As indicated in a previous study (5), 1,000 oocysts were sufficient to infect 100% of 16 neonatal mice, but the present objective was to detect as little as 0.1% survival, which might result in infection after exposure to disinfectants. If at least 10^3 of the 10^6 oocysts inoculated per mouse remained infectious, it was expected, on the basis of a previous study (5), that all of the mice in that treatment group would become infected. Control groups received oocysts that were exposed to air at the same temperature for the same length of time that other oocysts were exposed to the gaseous disinfectants.

Histology. Five- to 10-mm-long ileum, cecum, and colon segments from each mouse were fixed in neutral buffered formalin, stained with hematoxylin and eosin, and examined as previously described (6). In each segment, five or more ×400 microscopic fields were examined, each field consisting of 100 or more epithelial cells. Scores of 0, 1, 2, 3, and 4 (0%, ≤1%, 2 to 33%, 34 to 66%, and ≥67%, respectively) were used to designate percent infected epithelial cells. For each mouse, the scores for the three segments were added. For each group, the individual mouse scores were summed and a mean cumulative score was calculated for each treatment group (Table 1).

Disinfectants. Ammonia gas was obtained in a pressurized canister from Compass Foods (Montvale, N.J.). Carbon monoxide was obtained in a pressurized canister from Air Products, Inc. (Cockeysville, Md.). Ethylene oxide was obtained as Anprolene (84% ethylene oxide, 16% inert gases) from H. W. Andersen Products, Inc. (Health Science Park, Hall River, N.C.). Formaldehyde gas was generated by addition of 0.3 g of potassium permanganate to 0.6 ml of 10% formaldehyde (both reagents were from Sigma Chemical Co., St. Louis, Mo.). Methyl bromide (99.5% bromomethane) was obtained in a pressurized canister from Aldrich Chemical Co., Inc. (Milwaukee, Wis.). Ethylene oxide was supplied in a kit consisting of a crushable vial within a sealable plastic bag into which a rack supporting the microcentrifuge tubes was inserted. All other

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TABLE 1. Infectivity for neonatal BALB/c mice of *C. parvum* oocysts exposed to gases

Expt no.	Mouse group no.	Treatment	No. infected/ no. in group	Group histologic score
1	1	Carbon monoxide	6/6	11.8
1	2	Carbon monoxide	6/6	11.8
1	3	Ethylene oxide	0/6	0
1	4	Ethylene oxide	0/6	0
1	5	Methyl bromide	0/5	0
1	6	Methyl bromide	0/5	0
1	7	Air (control)	6/6	10.8
1	8	Air (control)	6/6	7.2
2	9	Carbon monoxide	3/3	9.0
2	10	Air (control)	3/3	9.3
3	11	Ammonia	0/5	0
3	12	Ammonia	0/5	0
3	13	Formaldehyde	5/5	1.4
3	14	Formaldehyde	1/5	0.2
3	15	Air (control)	4/4	9.3
3	16	Air (control)	5/5	10.0

gases were applied by displacing air in covered, rigid containers (e.g., desiccator jars) which held a rack of microcentrifuge tubes.

Experimental design. Three experiments were conducted with 16 litter groups of mice (Table 1). In each of 16 polypropylene microcentrifuge tubes (USA Scientific Plastics, Ocala, Fla.), 8×10^6 *C. parvum* oocysts were suspended (an excess of the number actually needed) in 400 μl of deionized water (groups 1 to 12) or phosphate-buffered saline (groups 13 to 16). Oocysts from each tube were used to inoculate one litter group of mice at a rate of 10^6 oocysts per mouse. After displacement of air by test gases, all containers were stored for 24 h in a chemical fume hood at room temperature (21 to 23°C). Control tubes remained exposed to air in the same location for the same time at the same temperature. All of the tubes were then filled with deionized water to displace the gas, centrifuged at $3,575 \times g$ for 5 min, rinsed, and centrifuged a second time, and the contents of each tube resuspended in 400 μl of deionized water. Oocysts were then administered to mice for bioassay as described above. All treatments were replicated in experiments 1 and 3. The gases used and the groups that received oocysts exposed to each gas are shown in Table 1.

Histologic findings. The lack of parasites at any stage of development in histologic sections from mice inoculated with oocysts exposed to ammonia, ethylene oxide, and methyl bromide suggested that all such oocysts had been rendered noninfectious or that so few remained infectious that the number of developmental stage parasites was below the ability of the bioassay to detect them (Table 1). Parasites were found in all intestinal segments from mice inoculated with oocysts exposed to air (controls) or carbon monoxide. They were also found in ileal segments from 6 of 10 mice inoculated with oocysts exposed to formaldehyde. The histologic scores for mice inoculated with oocysts exposed to carbon monoxide were actually higher than the scores for controls, suggesting that carbon monoxide had little or no effect on oocyst infectivity. The scores for mice inoculated with oocysts exposed to formaldehyde reflected the finding of relatively few parasites only in the ileum. The scores for formaldehyde-exposed oocysts were much lower than those for controls and suggested that formaldehyde had greatly reduced the number of infectious oocysts but had not killed all of them.

In the present study, oocysts were exposed to a higher concentration of ammonia for a longer period of time than previously reported. The present findings related to ammonia support the previous finding that oocysts exposed to 5% ammonia for 18 h were no longer infectious (4). From studies using excystation as an indicator of viability, ammonia was reported to have reduced viability 60 to 94% (1), 75 to 85% (9), and 97% (1, 10), suggesting significant anticryptosporidial activity. Ammonia, although irritating and potentially very toxic, might be used for disinfection when the gas can be contained and evacuated without exposing humans or animals.

In the present study, oocysts were exposed to a higher concentration of formaldehyde than in previous studies. Infectivity was reduced but not eliminated. In contrast, exposure to 10% formol saline for 18 h was reported to render oocysts noninfectious (4). Other studies indicated that after exposure to 5% formaldehyde for 24 h, oocysts were still infectious (8) and after exposure to 10% formalin for 1 and 7 days (3), excystation was reduced but not completely inhibited. On the basis of data from these four studies, it would be prudent to be cautious in handling feces or objects thought to be contaminated with *C. parvum* oocysts, even after several days of exposure to formaldehyde.

Ethylene oxide and methyl bromide, examined for the first time as potential disinfectants for *C. parvum* oocysts and found effective, have been used for many years as microbial disinfectants in different environments. Ethylene oxide has been used as a fumigant for textiles and foodstuffs, to sterilize surgical instruments, and as an agricultural fungicide (2). Methyl bromide has been used as an insect fumigant in mills, warehouses, vaults, ships, and freight cars and as a soil fumigant (2). Both are extremely toxic to humans and animals and must be used with caution. However, the variety of applications and diverse environments in which these gases have been used suggest that, with appropriate testing targeted to specific conditions, either or both might be useful for disinfection of oocysts in agricultural settings where soil, housing, or tools might be contaminated.

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