Ethanol and Isopropanol in Concentrations Present in Hand Sanitizers Sharply Reduce Excystation of *Giardia* and *Entamoeba* and Eliminate Oral Infectivity of *Giardia* Cysts in Gerbils

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Enteric protozoan parasites, which are spread by the fecal-oral route, are important causes of diarrhea (*Giardia duodenalis*) and amebic dysentery (*Entamoeba histolytica*). Cyst walls of *Giardia* and *Entamoeba* have a single layer composed of fibrils of β-1,3-linked GalNAC and β-1,4-linked GlcNAC (chitin), respectively. The goal here was to determine whether hand sanitizers that contain ethanol or isopropanol as the active microbicide might reduce transmission of these parasites. We found that treatment with these alcohols with or without drying in a rotary evaporator (to model rapid evaporation of sanitizers on hands) kills 85 to 100% of cysts of *G. duodenalis* and 90 to 100% of cysts of *Entamoeba invadens* (a nonpathogenic model for *E. histolytica*), as shown by nuclear labeling with propidium iodide and failure to excyst in vitro. Alcohols with or without drying collapsed the cyst walls of *Giardia* but did not collapse the cyst walls of *Entamoeba*. To validate the in vitro results, we showed that treatment with alcohols eliminated oral infection of gerbils by 1,000 *G. duodenalis* cysts, while a commercial hand sanitizer (Purell) killed *E. invadens* cysts that were directly applied to the hands. These results suggest that expanded use of alcohol-based hand sanitizers might reduce the transmission of *Giardia* and *Entamoeba*.

*Giardia duodenalis* (also known as *Giardia lamblia*) is an important cause of human diarrhea in both the developing and developed world and is an important zoonosis of companion and production animals (1–8). *Entamoeba histolytica* may cause amebic dysentery and liver abscesses, primarily in the developing world (9–11). Because these parasites may cause severe infections and are readily transmitted by asymptomatic carriers, the National Institute of Allergy and Infectious Diseases (NIAID) has designated *Giardia* and *Entamoeba* category B priority pathogens.

Our laboratory has extensively characterized the cyst walls of *Giardia* and *Entamoeba*, each of which contains a prominent sugar polymer and a small set of proteins (12, 13). In contrast, the *Saccharomyces cerevisiae* cell wall contains two sugar polymers (chitin and β-1,3-linked glucan) and ~100 glycoproteins (14). The *Giardia* cyst wall consists of fibrils of β-1,3-linked N-acetylgalactosamine (GalNAC) and cyst wall proteins (CWPs) that bind the GalNAC homopolymer (15, 16). Cyst wall proteins have very short Asn-linked glycans composed of two GlcNAC residues (17). While cytosolic proteins of *Giardia* may contain GlcNAC linked to Ser or Thr residues, no O-linked glycans have been identified in its secreted or cyst wall proteins (18).

The *Entamoeba* cyst wall is modeled by *Entamoeba invadens*, which infects reptiles but not humans, because *E. histolytica* does not encyst in axenic culture or in the mouse model. The *Entamoeba* cyst wall is composed of fibrils of β-1,4-linked N-acetylglucosamine (GlcNAC) (chitin), which are labeled by wheat germ agglutinin (WGA) (19, 20). *Entamoeba* cyst walls contain three abundant chitin-binding glycoproteins (lectins) that cross-link chitin, degrade chitin, or self-aggregate (21). These glycoproteins contain N-glycans that are longer than those of *Giardia* but shorter than those of the host. Cyst wall glycoproteins also contain unique O-phosphodiester-linked glycans (22). When cysts of *Giardia* or *Entamoeba* are placed in excystation media that mirror conditions in the small intestines, trophozoites (the motile, colonizing form) are released (23, 24).

We are interested in alcohol-based hand sanitation methods to reduce transmission of *Giardia* and *Entamoeba*, because these sanitizers, which were identified >100 years ago, are effective against many viral, bacterial, and fungal infections; alcohol-based sanitizers are relatively inexpensive and do not depend upon scarce supplies of clean water; and there are no human vaccines for *Giardia* and *Entamoeba* (25–32). Hand sanitizers contain 63% to 80% ethanol or isopropanol. They kill microorganisms by penetrating walls, disrupting membranes, and denaturing proteins. Here, we show that alcohol treatment blocks excystation of *G. duodenalis* and *E. invadens* in vitro and blocks oral infection of gerbils by *G. duodenalis* cysts.

**MATERIALS AND METHODS**

*Giardia duodenalis*. Cysts of the H3 strain of *G. duodenalis* (assemblage B), which are produced in gerbils, were purchased from Waterborne, Inc. (New Orleans, LA). The cysts (1,000 cysts/200 μl) were incubated with occasional shaking in water, 63% ethanol, 80% ethanol, 63% isopropanol, or 80% isopropanol for 5 min at room temperature (−21°C) with or without drying in a rotary evaporator (to model rapid evaporation of sanitizers on hands). To validate the in vitro results, we showed that treatment with alcohols eliminated oral infection of gerbils by 1,000 *G. duodenalis* cysts, while a commercial hand sanitizer (Purell) killed *E. invadens* cysts that were directly applied to the hands. These results suggest that expanded use of alcohol-based hand sanitizers might reduce the transmission of *Giardia* and *Entamoeba*. 
without drying in a rotary evaporator (Savant SpeedVac SC110A Plus concentrator; Thermo Fisher Scientific, Waltham, MA) for 5 min. These times, which are longer than those used to wash hands with alcohol-based hand sanitizers, allowed us to process multiple samples as a group and so avoid the variability that might occur when samples were processed sequentially. WHO formulations for hand sanitizers are available online (http://www.who.int/gpsc/5may/Guide_to_Local_Production.pdf). Cyst walls were labeled for 1 h at room temperature with an anti-CWP1 monoclonal antibody (MAb) (Waterborne Inc., New Orleans, LA), which was diluted 1:500 in phosphate-buffered saline (PBS) (16). After washing three times in PBS, the cysts were incubated with 4′,6-diamidino-2-phenylindole (DAPI) (0.2 μg/ml) and/or propidium iodide (PI) (0.2 μg/ml) for 5 min at room temperature and then washed twice in PBS. DAPI is a vital stain that penetrates membranes and binds nuclei, although it may be excluded by cyst walls. PI, which is often included in LIVE/DEAD kits, cannot penetrate intact membranes and so labels only nuclei of dead cells. Cysts and trophozoites were visualized with a DeltaVision deconvoluting microscope (Applied Precision, Issaquah, WA). Images were taken with a 100x objective and deconvolved using Applied Precision’s softWoRx software (16).

G. duodenalis excystation was induced by incubating cysts in excystation medium containing 1 mg/ml chymotrypsin in Tyrode salt solution for 30 min at 37°C (23). Excystation rates were determined by counting intact versus excysted walls with a Labophot phase-contrast microscope (Nikon Instruments, Inc., Melville, NY) using a 20x objective. In each experiment, 100-μl aliquots that contained ~100 cysts were counted for each sample, and the experiments were performed at least 3 times each. The rate of excystation for each alcohol treatment was compared with that of cysts incubated in water using an unpaired t test (GraphPad Prism software). Similarly, the rate of excystation for each alcohol treatment plus drying with the rotary evaporator was compared with that of cysts incubated in water and then dried. No statistical comparisons were made between groups treated with different alcohols. Excysted Giardia trophozoites were cultured in TYI-S-33 medium at 37°C to confirm their viability (33).

Four- to 6-week-old female gerbils (Charles River Laboratories, Wilmington, MA), none of which were infected with Giardia, were experimentally infected by oral gavage with 1,000 G. duodenalis strain H3 cysts in 100 μl water (34). The cysts were untreated, incubated in 65% or 80% ethanol and dried, or incubated in 80% isopropanol and dried. After 7 days of infection, the gerbils were euthanized by CO2 asphyxiation, and the duodenum was removed, minced, and incubated in 1 ml TYI-S-33 medium for 60 min to release Giardia trophozoites, which were counted with phase microscopy and a hemocytometer. Three aliquots from each animal were counted with a hemocytometer. Four animals were in each experimental group, and the experiments were performed two times each. The averages and standard deviations for 8 animals in each group are shown in Fig. 11. No statistical test was used, as we were unable to identify any Giardia organisms in the lumen of the duodenum in gerbils infected with cysts treated with ethanol or isopropanol.

The manipulation of G. duodenalis cysts was approved by the Boston University Institutional Biosafety Committee. Infecting gerbils with G. duodenalis cysts was approved by the Boston University Institutional Animal Care and Use Committee.

Entamoeba invadens. The IP-2 strain of E. invadens was cultured in TYI-SS medium at room temperature, and trophozoites (107/ml) were induced to encyst by transfer to medium with reduced glucose, osmolarity, and serum for 72 h (20, 33, 35). After encystation, the cysts were suspended in sterile water and left at 4°C for 6 h to lyse trophozoites. E. invadens cysts were treated with alcohols, and then DAPI and PI labeling of the cysts were performed as described for Giardia; except cyst walls were labeled with 10 μg/ml WGA, which binds to chitin fibrils (21). E. invadens excystation rates were determined by incubating 106 cysts in 10 ml of excystation medium containing 40 mM sodium bicarbonate and 1.25 mM taurodeoxycholate for 24 h at room temperature and counting intact versus excysted walls with the phase microscope (24). Alternatively, ~107 E. invadens cysts in 1 ml of water were applied with a pipette to the index and pointer fingers of one hand, and hands were washed to dryness (~2 min) with 1 to 2 ml of a commercial hand sanitizer (Purell; Gojo Industries, Akron, OH). Purell contains 70% ethanol. Residual cysts were eluted with 300 ml of water; concentrated to 1 ml by centrifugation; and labeled with PI, DAPI, and WGA, as described above. The numbers of live cysts (with DAPI staining but no PI staining) and dead cysts (with PI-stained nuclei or walls torn apart) recovered from the hands were compared to those of untreated E. invadens cysts. Hands were extensively washed with soap and water, and all wash solutions were decontaminated with Wesco-dyne germicidal detergent (Steris, Mentor, OH).

RESULTS

Ethanol and isopropanol permeabilize membranes of G. duodenalis trophozoites within cysts, collapse cyst walls, dramatically reduce excystation in vitro, and block oral infection of gerbils. The protocol for testing the effects of alcohols on the infectivity of Giardia cysts is shown in Fig. 1A. All G. duodenalis cysts, regardless of experimental treatment, labeled with a MAb to the most abundant cyst wall protein (CWP1) (Fig. 1B). In contrast, only ~20% of G. duodenalis cysts, which were washed in PBS and incubated in water, labeled with DAPI, a nuclear stain that penetrates membranes. This result shows that the intact cyst wall is relatively impermeable to small molecules, as the formula weight of DAPI is ~227 and DAPI readily penetrates intact plasma membranes of live trophozoites (data not shown). Zero to 5% of these cysts labeled with PI (the “DEAD” stain in LIVE/DEAD kits), depending upon the batch of commercially obtained cysts (Fig. 1B). Cysts that had been incubated in water remained viable (they might label with DAPI but not with PI) after being quickly dried with the rotary evaporator (Fig. 1C). In contrast, drying overnight in the cold decreased the viability of G. duodenum cysts (36).

After treatment for 5 min with ethanol or isopropanol, nuclei of >95% of G. duodenalis trophozoites within cysts label with PI, indicating that the trophozoites within are dead (Fig. 1D to G). Staining with the anti-CWP1 MAb shows that cyst walls are often collapsed by incubation with ethanol. Drying in the rotary evaporator modestly improved killing. Supporting the idea that alcohols kill G. duodenalis cysts, ethanol or isopropanol treatment with or without drying dramatically reduces excystation in vitro to 0 to 15% of parasites examined (Fig. 1H). The effect of each alcohol treatment on excystation was highly significant (P < 0.001) compared with treatment in water. No statistical comparison was made between the alcohol treatments, as they all worked well to block excystation. Excysted parasites are viable, as shown by their motility and by their growth in culture. Consistent with the absence of PI labeling of their nuclei (Fig. 1C), drying of water-treated cysts in the rotary evaporator did not prevent excystation.

Treatment of 1,000 Giardia cysts with 63% and 80% ethanol or 80% isopropanol, all with drying in the rotary evaporator, completely prevents gerbil infection by oral gavage, which was determined by counting trophozoites in the duodenal lumen after 1 week of infection (Fig. 1I) (34). Eight of 24 gerbils were infected with untreated cysts, and we recovered >107 G. duodenalis trophozoites (average) from their duodenums. In contrast, we were unable to recover any trophozoites from 24 of 24 gerbils infected with alcohol-treated cysts (the lowest detection limit with the hemocytometer was 103 parasites/ml).
Alcohols in the test tube and on the hands kill *E. invadens* cysts, as shown by PI staining, disruption of cyst walls, and/or reduced rates of excystation. The protocols for testing the effects of alcohols on cysts of *G. duodenalis* are shown in Fig. 2A and G. All the control *E. invadens* cysts in water labeled with WGA (chitin in the wall) and DAPI but did not label with PI (Fig. 2B). Treatment of *E. invadens* cysts with ethanol or isopropanol makes the cysts permeable to PI (Fig. 2C to E). While dehydration with alcohols collapses *G. duodenalis* cyst walls (Fig. 1D to G), alcohol treatment and drying do not collapse the cyst wall of *E. invadens*. Alcohol treatment with or without drying with the rotary evaporator blocks excystation in vitro of *E. invadens* cysts (Fig. 2F). Again, the difference between the excystation rates of alcohol-treated organisms and that of cysts incubated in water was highly significant (*P* < 0.001). As described for *G. lamblia* cysts, drying of water-treated *E. invadens* cysts in the rotary evaporator did not prevent excystation.

When *E. invadens* cysts, which are not infectious to people, are applied to the index and pointer fingers and the hands are cleaned with a commercial hand sanitizer (Purell), the cysts are killed, as shown by PI staining of nuclei (Fig. 2H). In addition, cyst walls, which are heavily contaminated with squamous epithelial cells and bacteria, are frequently disrupted (Fig. 2I). Because of this heavy contamination, we did not perform excystation experiments but instead counted 500 Purell-treated cysts (total) in three separate experiments. All of these cysts appeared dead (they were labeled with PI).
and/or had disrupted walls), and none appeared alive (i.e., labeled with DAPI but not PI and had intact walls).

**DISCUSSION**

Arguments for using alcohol-based hand sanitizers to reduce the spread of *Giardia* and *Entamoeba*. As shown by the labeling of the nuclei of alcohol-treated cysts with PI, alcohols penetrate cyst walls and disrupt plasma membranes of trophozoites of *G. duodenalis* and *E. invadens* that are inside (Fig. 1 and 2). Alcohols dramatically reduce excystation of both parasites and block gerbil infections with 1,000 *G. duodenalis* cysts, which is well above the minimal infectious dose (34). As the vast majority of *Giardia* and *Entamoeba* organisms were killed by alcohols without drying, the effect of the rotary evaporator was minimal. The effect of alcohol treatment on *G. duodenalis* infections is much greater than that of oral consumption of whole wheat and/or wheat germ, which contain WGA and are common components of the human diet. In a rodent model and in human infections, WGA, which binds to short N-glycans of *G. duodenalis* and reduces excystation *in vitro*, showed modest reduction in cysts shed and in symptoms (humans) (37–39). A commercial alcohol-based hand sanitizer killed *E. invadens* cysts directly applied to the hands, validating the findings when *E. invadens* cysts were treated with alcohols in a microcentrifuge tube and dried in the rotary evaporator.

The alcohol-based hand sanitizers are safe, relatively inexpensive, and well tolerated; they do not need water; they do not select for antibiotic-resistant organisms; they also reduce infections with viruses, bacteria, and fungi; and they are packaged in small volumes that are stable at room temperature and so can be used by travelers (25–32, 40). While hand washing with soap and water will also reduce diarrhea caused by *Giardia* and *Entamoeba*, as well as other parasites, bacteria, and viruses, this solution is unavailable to hundreds of millions of people who lack access to abundant clean water (41–43).
There is presently no human vaccine for Giardia or Entamoeba. While drugs to treat Giardia (albendazole and metronidazole) and Entamoeba (metronidazole) are inexpensive and efficacious, the broader public health goal is to prevent infections by whatever reasonable means are available (44). This is not to diminish the appropriate excitement about new anti-Giardia drugs that (i) overcome antibiotic resistance (e.g., derivatives of nitroimidazoles and benzimidazoles), (ii) have already been approved for other indications (e.g., auranofin), or (iii) target enzymes essential to parasite viability that are absent from the host (e.g., arginine deaminase) (reviewed in reference 45).

Finding drugs that inhibit cyst formation, which might break the life cycle of each parasite, faces three difficulties (46). First, trophozoites are the dividing forms that cause diarrhea (Giardia) or dysentery (Entamoeba), so inhibition of wall formation would temporarily stop shedding of infectious cysts but would not clear the primary infections. Second, the search for chitin synthase inhibitors to treat fungal infections has produced candidate compounds (e.g., nikkomycin Z), none of which is in clinical use (47). It is therefore unlikely that an inhibitor for the Entamoeba chitin synthase will be easily found, and the synthase that makes the GalNAC polymer in Giardia cyst walls has not been molecularly characterized (15). Third, because Giardia is a zoonotic infection, inhibitors of cyst wall formation would also need to treat animal reservoirs, which are most likely not household pets but are likely infected production animals (6–8).

**Caveats.** Only a few alcohol-based hand sanitation solutions were modeled here, so there are likely hand sanitizers with additional ingredients (e.g., detergents, organic acids, or quaternary ammonium compounds) that might be even more effective or last longer after washing. Because we were handling sets of parasites, organisms were treated with alcohol for 5 min and dried for 5 min, which is longer than it takes to use hand sanitizers. G. duodenalis cysts were not directly applied to the hands and washed with alcohol-based sanitizers. E. invadens was used to model E. histolytica cysts, and we did not validate alcohol treatments in an animal model (infecting reptiles with E. invadens cysts is beyond our capabilities).

While these hand-sanitizing solutions might reduce food contamination by infected handlers, they would not be expected to reduce waterborne infections with cysts of Giardia or Entamoeba (1, 30). Hand washing or use of hand sanitizers is uneven at best in day care centers and homes in developed countries and is likely sporadic in developing countries (2–5, 26, 28–32). The role of alcohol-based hand sanitizers in schools, day care centers, and homes in developed countries, where water is plentiful for washing hands with antimicrobial soaps, is unclear (30, 32). The dermatologic effects of alcohols (e.g., dryness) may reduce their routine use to prevent infections with Giardia and Entamoeba (48). Finally, because hand sanitizers cost more than soap and water, their use would likely need to be subsidized and targeted (e.g., to mothers or caregivers of infants).

Despite these caveats, it appears that expanded use of alcohol-based hand sanitizers, which rapidly kill cysts, has a reasonable chance of reducing transmission of Giardia and Entamoeba.

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**REFERENCES**


