Comparison of an In Vitro Method and an In Vivo Method of Giardia Excystation

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Giardiasis is the most frequently identified cause of waterborne outbreaks of gastroenteritis in the United States (9). It is also the enteric parasitic infection most commonly diagnosed in most clinical laboratories (18). One of the reasons for its ubiquity is the effective simplicity of the Giardia life cycle. Parasite trophozoites in the host small intestine are swept into the fecal stream and encyst into the dormant, infective cyst stage. Cysts that are ingested by a new host excyst in the upper gastrointestinal tract, producing a new infection.

Although the parasite life cycle is simple, encystation and excystation are not fully understood. In vivo excystation was first described in 1925 (11), but it was not until 1979 that Bingham and Meyer (6) reproduced the process under laboratory conditions. Despite the fact that the stimuli that trigger this critical stage have not been clearly delineated, excystation has been studied in the course of several areas of giardiasis. In vitro excystation (5, 13) and animal infectivity (12) have been used in studies of cyst viability. Excystation procedures have also been applied to questions of epidemiology. There is a need to retrieve and analyze (3, 19, 20) isolates from environmental, animal, and human sources to gain new knowledge in this area, but relatively few Giardia isolates are available for study (17). Both in vitro excystation and in vivo excystation have been used in the attempt to retrieve new parasite isolates (4, 14, 24). The purpose of this study was to compare in vitro and in vivo excystation in order to determine the most useful method for the retrieval of Giardia duodenalis (synonym, Giardia lamblia [17]) isolates.

MATERIALS AND METHODS

Parasite strains. Eleven different Giardia isolates were used in this study. Since variation in the biological behavior of isolates within the G. duodenalis morphological group has been described (16, 24), isolates obtained from both animal and human hosts were used. The isolates were representative of widely different geographical regions. The host source and geographical origins of the 11 isolates are noted in Table 1. Cysts were collected from Giardia-positive feces by a sucrose gradient method (21). Since cyst age is a potentially significant variable and since wide variation in excystation rates for the same strain of G. duodenalis (22, 23) has been reported, replicate experiments using cysts of the same age were deemed necessary. The original number of cysts retrieved from host feces was too small for carrying out replicate experiments, and the gerbil animal model was used to produce cysts. Cysts were quantified with a hemocytometer (Neubauer chamber) and stored at 4°C in distilled water for a maximum of 14 days prior to parallel excystations. No effort to allow for possible maturation requirements was made, although the age of the cysts used was recorded and analyzed. Data were included for analysis only if cyst suspensions demonstrated evidence of viability in either excystation procedure in at least one of the multiple in vitro or in vivo excystations carried out on that date or on a subsequent date. This resulted in the exclusion of data from two sets of excystations with one isolate (isolate 12 not included in analysis).

Excystation procedures. Excystations carried out on the same day by using same-age, same-strain cysts were considered one set for analysis purposes; 21 sets were analyzed. Two isolates were excysted in each of four sets, one isolate in each of three sets, two isolates in each of two sets, and six isolates in one set of excystations. Within each of the same-day sets, replicate individual excystation procedures were carried out, the number of replicates depending on the number of cysts available for that isolate. Duplicate in vitro excystations were carried out with eight sets, producing results from 29 individual in vitro excystations and 31 individual in vivo excystations.

Excystation procedures used were as follows. (i) In vitro excystation was done by the method described by Sauch (22); 5 ml of HCl-saline (0.7 ml of concentrated HCl, 100 ml of 0.85% NaCl, pH 1.4), 2.5 ml of Hanks balanced salt solution containing 32 mM glutathione with 57 mM L-cysteine, and 2.5 ml of 0.1 M NaHCO3 were added to a 0.5-ml
suspension of $1 \times 10^5$ to $5 \times 10^5$ cysts in a 15-ml conical centrifuge tube. The suspension was mixed by vortexing and incubated for 45 min at 37°C. After centrifugation at 650 × g for 2 min, the supernatant was discarded and the pellet was resuspended in 0.5 ml of Tyrode salt solution prewarmed to 37°C. A 0.1-ml aliquot of the suspension was removed from the tube and examined microscopically (Nikon, Labophot ×200), and the remainder of the suspension was inoculated into prewarmed TYI-S-33 broth medium (15) with the following modifications: 100 mg of L-cysteine · HCl (Sigma Chemical Company, St. Louis, Mo.) per ml and 100 mg of dried bovine bile (Sigma) per 100 ml. (ii) For in vivo excystation, six- to eight-week-old male Mongolian gerbils (Meriones unguiculatus) were obtained from Tumblebrook Farm (West Brookfield, Mass.). One animal, selected at random from each group, was sacrificed, and an aliquot of the small intestinal contents was examined for Giardia trophozoites. We have never found Giardia contamination of new animals. After the animals were allowed to rest for 1 week, each gerbil was gavage fed 20.0 mg of metronidazole daily on 3 consecutive days, as described by Belosevic et al. (2). Gerbils were allowed to rest for a further week, after which time a fecal specimen was concentrated and examined microscopically for Giardia cysts. Starting during this week and throughout the experiments, all animals were given dexamethasone (20 mg in 5 ml of phosphate-buffered saline [PBS]) by adding this concentrate to 250 ml of drinking water to produce a final concentration of 2 mg/100 ml. On the day of in vitro excystation, $1 \times 10^5$ to $5 \times 10^5$ cysts suspended in 0.5 ml of distilled water were inoculated into a gerbil by gavage. Five to seven days later, the inoculated gerbil was sacrificed, the small intestine was removed, and luminal contents were irrigated with 3 ml of prewarmed TYI-S-33. The bowel was then opened longitudinally, the mucosa was scraped, and material was pooled with the washings obtained previously. An aliquot of the pooled suspension was examined microscopically (Nikon, Labophot ×200). If trophozoites were not detected, a 3-mm section of bowel was removed approximately 1 cm from the pylorus of the stomach, suspended in PBS, and examined by light microscopy.

**Culture procedure and analysis.** Excysted material was inoculated into a test tube containing culture medium. Each disposable screw-cap borosilicate test tube (16 by 125 mm) contained 13 ml of filter-sterilized, prewarmed TYI-S-33 broth (15). Penicillin G (500 IU/ml), streptomycin (50 μg/ml), and amphotericin B (10 μg/ml) (GIBCO BRL, Life Technologies, Inc.) were added to each culture tube. An attempt was made to standardize the inocula obtained from the in vitro and in vivo excystation. We did not consistently observe excysted trophozoites by light microscopy immediately following in vitro excystation, and if trophozoites were not seen, a rate of 1% ($1 \times 10^5$ to $5 \times 10^5$ trophozoites) in vitro excystation was used to standardize culture inocula for the parallel in vivo excystation. After in vivo excystation, pooled intestinal washes and scrapings were examined microscopically and trophozoites were counted with a hemocytometer (Neubauer chamber). The trophozoite suspension diluted to produce the correct inoculum was added to TYI-S-33 prewarmed to 37°C, as described above. Large numbers of trophozoites were obtained from each gerbil, and these organisms were inoculated into extra cultures. When these extra in vivo cultures were inoculated, a series of dilutions of these extra trophozoites (inocula, $10^2$ to $10^5$) was used. All cultures were incubated at 37°C on a 45° incline and examined daily for 14 days with an inverted microscope (Nikon, Diaphot ×100). By using a semiquantitative scale, an estimate of trophozoite numbers was carried out during daily microscopic examination (a minimum of 10 microscopic fields) of each culture tube.

**RESULTS**

As noted in Fig. 1, motile trophozoites were produced from material excysted in vivo in all of the sets and from material excysted in vitro in 16 (76%) of the 21 sets. Of the five in vitro experiments that failed to produce motile trophozoites, two used cysts from the same isolate and the other three used cysts from three other isolates that successfully excysted in vitro in other experiments on different days. The cysts used in these five experiments were viable, since all parallel in vivo excystations produced motile trophozoites. Four of the five unsuccessful excystations occurred on days when in vitro excystations with other strains were successful. No pattern such as might be ascribed to aging or maturation of cysts could be observed with the five unsuccessful excystations.
Analysis which included replicate individual excystations within the sets showed that 24 (83%) of the 29 in vitro procedures and 31 of the 31 in vivo procedures produced trophozoites. There was no statistically significant difference \((P > 0.050)\), however, between the number of in vitro and in vivo excystations when either data from whole set or data including replicate individual excystations within the sets were included for analysis.

In numerous previous attempts to retrieve isolates by both methods, we observed that fewer isolates adapted to culture than were successfully excysted. The present study confirmed this observation. Bacterial contamination as a cause of culture failure was analyzed (Fig. 1). It was observed that only a few cultures inoculated with excysted material from either procedure were lost to contamination in this study. Four cultures (17%) obtained from the 24 successful in vitro excystations became contaminated. Seven (23%) of the 31 in vivo excystations became contaminated. There was no statistical difference \((P > 0.05)\) between the rates of contamination associated with in vitro and in vivo excystation.

Adaptation to culture was described as an increase in the number of trophozoites within the 7-day period following inoculation. By this definition of adaptation, there was success in the retrieval of isolated trophozoites in culture. Adaptation results were analyzed by using data that excluded contaminated cultures (Fig. 1). Adaptation to culture was observed more frequently following in vivo excystation. Of the 20 noncontaminated in vitro excystation cultures, 3 (15%) adapted. This compares with 7 (29%) of 24 cultures inoculated with in vivo excystation material. There was, however, no statistical difference in the adaptation rate between the two methods when either individual cultures or comparable sets were analyzed.

Analysis of isolate adaptation to culture showed that two \textit{Giardia} isolates adapted following in vitro excystation alone (18%) and four adapted after in vivo excystation alone (36%). None of the 11 isolates adapted following both types of excystation in these experiments. Five more isolates adapted from the extra, nonstandardized cultures inoculated from gerbils, so that in total 82% of isolates were retrieved following in vivo excystation. As noted above, extra cultures following in vivo excystations were inoculated by using the large numbers of trophozoites obtained from any one infected animal. The number of trophozoites inoculated did not appear to correlate with adaptation. Some cultures inoculated with 103 trophozoites increased in numbers and survived, while culture tubes with 104 and 105 trophozoites of the same strain inoculated at the same time following the same excystation did not adapt.

**DISCUSSION**

In vitro excystation has been used to assess \textit{Giardia} cyst viability (5, 23) and to retrieve isolates from various sources (4, 14, 16). Although retrieval of isolates for laboratory study is important for understanding the epidemiology of giardiasis, Meyer notes (17) that relatively few \textit{Giardia} strains have been axenized in culture to date. In 1986, Wallis and Wallis (24) described a method using the Mongolian gerbil (\textit{Meriones ungulatus}), referred to as in vivo excystation, for retrieving \textit{Giardia} isolates. Having used both in vitro and in vivo excystation, it was our impression that the animal model was more often successful in isolate retrieval. The aim of this study was to compare an in vitro method and an in vivo method of excystation, but the overall results also demonstrate the difficulty in retrieving new isolates.

In a recent study of human source cysts, Hautus et al. (10) reported that isolates adapted to culture 35% of the time following in vitro excystation. These results were similar to in vitro results of the present study. No reports describe isolate retrieval rates by using in vivo excystation. In vivo excystation in the present study showed that 36% of isolates adapted to culture and, if all attempted cultures are included, this adaptation rate increases to 82%.

Aggarwal and Nash (1) reported that trophozoites with different surface antigens can be selected from populations derived from a single isolate. Heterogeneity in \textit{Giardia} trophozoite populations has also been suggested in other reports (7, 8), and while it did not appear in the present study that isolate retrieval improved with inoculum size, it was observed that the more excystation procedures that were carried out, the more likely the procedure was to produce trophozoites. We have also repeatedly observed that only one of many culture tubes inoculated in an identical manner will adapt to in vitro propagation. Our observation that in vivo retrieval rates increased to a significantly high level when all cultures inoculated were analyzed could be explained on the basis of the selection of culture-adaptable subpopulations of trophozoites. The lack of observed correlation with inoculum size could be explained on the basis of the infrequency of culture-adaptable trophozoites in some populations. The advantage of the in vivo technique is largely due to the large number of trophozoites produced in every excystation. Another advantage of in vivo excystation is that the minimal infective dose of \textit{Giardia} cysts is small, particularly when immunosuppressed animals are used. This is important in the retrieval of environmental or animal source isolates when the number of cysts available is small. We have recently adapted water and beaver source isolates to culture by using this in vivo method.

Cysts for these experiments were obtained by gerbil inoculation in order to provide adequate numbers of cysts of the same strain and exactly the same age. We recognize, therefore, that a bias may have been introduced that favored in vivo excystation. In our experience with the gerbil model in viability testing of drinking water samples, however, it was found that over half of 200 inoculations, largely from environmental samples containing small numbers of cysts, resulted in infection. Use of this relatively nonselective animal model was deemed useful despite reservations regarding bias. The same sample can be inoculated into the gerbil to test for viability and to retrieve isolates.

While this present study, which compared a relatively small number of experiments, did not demonstrate the statistical superiority of one method, comparison of the overall retrieval of isolates does support the impression that the in vivo method is superior to in vitro excystation. The fact that large numbers of cultures can be inoculated following a single in vitro excystation is advantageous to isolate recovery. We now routinely use the in vivo method to retrieve new isolates, particularly in cases where numbers of cysts are small, such as those from environmental sources. It is also clear, however, as pointed out by Schaefer (23), that the use of such a biological system is expensive and time-consuming. Further application of in vitro excystation in isolate retrieval will develop as the factors that trigger this process are identified, although it is equally plausible that this approach will also be subject to a selection bias.

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REFERENCES