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Excystation and Culturing of Human and Animal *Giardia* spp. by Using Gerbils and TYI-S-33 Medium

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Mongolian gerbils were used as an animal model to excyst and host *Giardia* spp. isolated from meadow voles, dogs, beavers, and humans. Both cysts and trophozoites were used to establish infections. Gerbils were infected with *Giardia duodenalis* from beaver, dog, and human sources, and the trophozoites were extracted and cultured in Diamond TYI-S-33 medium. The use of gentamicin and ampicillin in the medium, coupled with treatment of gerbils with gentamicin before they were sacrificed, permitted the elimination of trophozoite purification techniques before culturing. An extract of whole bovine calf blood, CLEX, was substituted for fetal bovine serum in TYI-S-33 medium and was found to be both adequate and less expensive.

Formerly, the study of various species of the parasite genus *Giardia* was limited owing to difficulty in obtaining cultured stocks for study. This situation has changed over the past 20 years, and a number of methods for the excystation and culturing of *Giardia* spp. have been described. In this paper we describe a refinement of some of these methods for use in the isolation and propagation of *Giardia* trophozoites for experimental purposes.

Giardia muris has been extensively studied by using the mouse model, but an equivalent model for the study of *Giardia duodenalis* has not yet been generally accepted. For this reason, we tested gerbils (*Meriones unguiculatus*) as an animal model for the excystment of *Giardia* from humans, wild meadow voles (*Microtus pennsylvanicus*), domestic dogs (*Canis familiaris*), and beavers (*Castor canadensis*) by using the methods of Belosevic et al. (4). We were able to culture trophozoites obtained from gerbils infected with human, dog, and beaver *Giardia* isolates, but not vole *Giardia* isolates, by using Diamond TYI-S-33 medium. The *Giardia* isolates extracted from voles were assumed to be *G. muris*, and the *Giardia* isolates extracted from humans, dogs, and beavers were assumed to be *G. duodenalis*, using the terminology of Filice (9).

MATERIALS AND METHODS

Sources of *Giardia* spp. Cysts were obtained from beaver, dog, and human feces. The beaver feces were obtained from a small pond 3 km north of the Kananaskis Field Station in southwestern Alberta, Canada. Infested dog feces were obtained from the Calgary Animal Shelter, Calgary, Alberta, and human stools containing *Giardia* cysts were obtained from the Alberta Provincial Health Laboratory and Foothills General Hospital in Calgary, Alberta, or from the University of Alberta Hospital, Edmonton. All fecal specimens obtained in Calgary were shipped directly to our laboratory over ice within 6 h of excretion and were processed immediately. *G. muris* trophozoites were obtained by stripping the entire small intestines of naturally infected meadow voles that were trapped live in the vicinity of the Kananaskis Field Station.

Cyst concentration. Beaver feces consisted mostly of large particulate matter that was easily separated from the finer sediment and cysts by straining and rinsing with phosphate-buffered saline through four layers of cheesecloth. The centrifuge-concentrated pellet was usually pure enough to be used without further treatment. If the concentrate required clarification, the sucrose centrifugation method was used, as described below.

Good cyst recovery rates (about 17% of the cysts were recovered during seeding trials) were obtained by using the sucrose centrifugation method of Roberts-Thomson et al. (15). In order to obtain approximately 100,000 cysts, 25 g (wet weight) of feces was placed in 50 ml of phosphate-buffered saline and thoroughly mixed with a spatula. The fecal suspension was then strained through four layers of cheesecloth, and about 5 ml of the mixture was carefully layered over 5 ml of 1.0 M sucrose in each of 12 centrifuge tubes (capacity, 15 ml). The tubes were centrifuged at 500 × *g* for 5 min, and the clarified upper layer was removed with a pipette. The samples were then pooled and centrifuge concentrated to a volume of 2 ml. In the case of human feces, fat was removed by mixing 8 ml of a fecal suspension with 1 ml of diethyl ether and centrifuging the preparation at 500 × *g* for 5 min. The emulsified fat and liquid were poured off, and the pellet was suspended in phosphate-buffered saline for further clarification over sucrose.

Gerbil infection. Mongolian gerbils that were 5 weeks old were obtained from Tumblebrook Farm, West Brookfield, Mass., transferred to separate cages, and cleared of intestinal protozoa with metronidazole as described by Belosevic et al. (4). Before the animals were infected, stool specimens were collected from each gerbil on 3 consecutive days and examined for *Giardia* cysts. After at least 1 week of rest, the gerbils were inoculated by gastric intubation with cysts concentrated from feces or with trophozoites obtained from intestinal scrapings. After 72 h, stool samples were collected on a daily basis for examination. This practice was eventually discontinued because of poor correlation between the presence of cysts in the feces and the presence of trophozoites in the gut. In all, 9 gerbils were infected with trophozoites from meadow voles, 13 gerbils were infected with human *Giardia* cysts, 12 gerbils were infected with cysts from dogs, and 11 gerbils were infected with cysts from beavers. Gerbils were sacrificed 4 to 20 days after infection.

In some cases, infected gerbils were given two 25-mg doses of gentamicin (GIBCO Laboratories, Grand Island, N.Y.) by gastric intubation 48 and 24 h before they were

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sacrificed in order to reduce the number of intestinal bacteria. The animals were anesthetized with diethyl ether and killed by cervical dislocation, and the peritoneal cavity was opened. A small portion of the upper duodenum 2 cm from the pylorus of the stomach was removed, mounted in phosphate-buffered saline on a slide, and examined at ×400 by using dark phase-contrast microscopy. If no trophozoites were detected, a second sample was taken approximately 10 cm from the pylorus. If trophozoites were detected in either sample, the small intestine was removed and placed in RPMI 1640 medium. If trophozoites were not detected in either sample, the gerbil was judged to be uninfected.

Concentration of trophozoites. The entire small intestine was opened longitudinally by using scissors, and the gut contents were scraped with a scalpel into a disposable petri dish containing RPMI 1640 medium. The entire contents of the petri dish were rinsed into six 15-ml centrifuge tubes after passage through a quadruple layer of cheesecloth. The petri dish was examined by using a Zeiss inverted microscope to make sure that most of the trophozoites had been rinsed off. If large numbers of trophozoites were still adhering to the bottom of the dish, the dish was placed in a refrigerator at 4°C for 5 min to detach the trophozoites and rinsed again. All of the trophozoites were then concentrated by centrifugation at 500 × *g* for 5 min. Attempts were made to separate the trophozoites from intestinal bacteria by using the nylon fiber column method of Andrews et al. (1) or by allowing the trophozoites to adhere to clean petri dishes, using the method of Feely and Erlandsen (8).

Culturing. Concentrated trophozoites were inoculated into Diamond TYI-S-33 medium (5). Glass tubes (15 ml) were used routinely, although 50-ml tissue culture flasks were useful for growing large numbers of trophozoites. Culture tubes were subcultured every 3 to 4 days. The medium was prepared as described by Diamond et al. (5). The vitamin solution of Diamond et al. was initially purchased from North American Biologicals, Miami, Fla., but was later made in our laboratory by using the formulation described by Diamond et al. (5) and Evans et al. (6). Both fetal bovine serum and CLEX (Dextran Products Ltd., Scarborough, Ontario, Canada) were used as medium supplements at concentrations of 10%. TYI-S-33 medium rapidly inactivated antibiotics, so the medium was filter sterilized and stored for no more than 7 days at 4°C. Antibiotics were added to each culture tube when the medium was changed (see below).

Cultures were stored by freezing. Mature culture tubes were placed in the refrigerator for 30 min, centrifuged at 500 × *g* for 5 min, and decanted. The resulting pellet was mixed in 1 ml of fresh TYI-S-33 medium in a fresh disposable culture tube and diluted with 1 ml of 10% dimethyl sulfoxide (final concentration, 5%). The culture tube was frozen at −25°C for 60 min and then transferred to a Revco Ultra Low freezer and held at −60°C. Cultures were recovered by thawing them quickly in a 37°C water bath and filling the tubes with fresh medium. When the majority of trophozoites had become attached to the sides of a culture tube (about 60 min), the medium was changed in order to avoid toxicity problems from the dimethyl sulfoxide (14). All strains of *Giardia* isolated during this study have been maintained in the Kananaskis Centre for Environmental Research laboratory and are available for study.

Control of bacterial overgrowth. Initially, we used streptomycin (50 μg/ml) and penicillin G (50 μg/ml) to suppress bacterial overgrowth in the medium. These antibiotics were not very effective, even at concentrations of 200 μg/ml. In order to find more efficient antibiotics, complete TYI-S-33 medium containing serum was solidified with 1.5% agar, and antibiotic susceptibility disks (BBL Microbiology Systems, Cockeysville, Md.) were added. The resulting plates were inoculated with 0.1 ml of medium that had been overgrown with bacteria the night before. The following antibiotics were tested: penicillin G (2 U), kanamycin (30 μg), streptomycin (2 μg), trimethoprim (23.75 μg)-sulfamethoxazole (1.25 μg), erythromycin (15 μg), gentamicin (10 μg), ampicillin (10 μg), tetracycline (5 μg), and nalidixic acid (30 μg). The results of this test showed that overgrown bacteria were susceptible to nalidixic acid, gentamicin, and ampicillin. Nalidixic acid inhibited the growth of human *Giardia* trophozoites so it was not tested further. A similar trial with gentamicin and ampicillin showed that the growth of human *Giardia* isolates was not inhibited by these antibiotics at concentrations up to 200 μg/ml. In order to determine the best concentration range for routine culturing, we prepared replicate tubes of TYI-S-33 medium containing concentrations of gentamicin and ampicillin ranging from 50 to 200 μg/ml each in 25-μg/ml increments. In addition, one set of tubes contained 400 μg of gentamicin per ml and 400 μg of ampicillin per ml. Three replicate tubes were prepared for each concentration. Each tube was inoculated with approximately 10^6 human *Giardia* trophozoites and was incubated for 4 days without changing the medium.

RESULTS

Gerbil infection. Gerbils were successfully infected with *Giardia* isolates from meadow voles, humans, dogs, and beavers. Both cysts and trophozoites were capable of infecting gerbils.

The results of the infection experiments with meadow vole *Giardia* isolates are shown in Table 1. All of the gerbils, with the possible exception of gerbil 6, became infected, as demonstrated by either gut or fecal examination. Negative results in gut analyses seemed to be correlated with longer incubation times. The overall infection rate was at least 89% when both fecal and gut analyses were considered.

Table 2 shows the results of infection of gerbils with human *Giardia* isolates. The overall rate of infection with human parasites (46%) was lower than the rate with vole parasites, and there was only one case in which both fecal and gut specimens were positive. Trophozoites were found in the small intestines of animals after incubation times ranging from 4 to 18 days.

TABLE 1. Infection of gerbils with *Giardia* isolates from meadow voles

Animal no.	Inoculum	Incubation time (days)	Results of[a]:	
			Fecal analysis	Gut examination
1	16,000 trophozoites	15	+	+
2	5,000 trophozoites	16	+	−
3	10,000 trophozoites	7	+	+
4	10,000 trophozoites	11	+	+
5	10,000 trophozoites	11	+	+
6	10,000 trophozoites	18	ND[b]	−
7	10,000 trophozoites	18	ND	+
8	10,000 cysts	15	+	−
9	10,000 cysts	17	+	−

[a] An analysis was considered positive if cysts were found in at least one specimen.
[b] ND, No data.

TABLE 2. Infection of gerbils with human *Giardia* isolates

Animal no.	Inoculum[a]	Incubation time (days)	Results of[b]:	
			Fecal analysis	Gut examination
1	200,000 cysts	21	−	−
2	200,000 cysts	14	+	−
3	200,000 cysts	21	−	−
4	40,000 cysts	13	−	−
5	40,000 cysts	13	−	−
6	4,000 cysts	17	−	−
7	8,000 cysts	16	+	−
8	15,000 cysts	5	ND[c]	−
9	15,000 cysts	5	ND	−
10	100,000 cysts	10	−	+
11	100,000 cysts	4	+	+
12	100,000 cysts	18	−	+
13	500,000 trophozoites (from culture)	20	+	−

[a] The fecal samples used to infect gerbils 1 through 5 were from 7 to 10 days old, and gerbils 6 through 10 were contaminated with *Trichomonas* sp.
[b] An analysis was considered positive if cysts were found in at least one specimen.
[c] ND, No data.

The results of infections with dog *Giardia* isolates are shown in Table 3. In this case, no fecal specimens were positive, although the overall infection rate was 50%. A higher incidence of trophozoites in the small intestines appeared to be correlated with shorter incubation times.

Table 4 shows the results from the beaver *Giardia* infection experiments. As with the dog parasites, no positive fecal samples were found, although the overall infection rate was 91%. No trend was apparent between positive gut specimens and incubation times.

A number of gerbils were found to be contaminated with *Trichomonas* sp. or *Hexamita* spp. (from voles) or both, which may have influenced the results of the experimental infections.

Culturing. Despite a few unsuccessful attempts, trophozoites from gerbils infected with dog, human, and beaver *Giardia* isolates were cultured in TYI-S-33 medium. Vole *Giardia* isolates could not be cultured. Trophozoites grew as well in medium prepared with CLEX (which is about one-half as expensive as fetal bovine serum) as in medium prepared with fetal bovine serum. Similarly, we found no difference between the vitamin supplement purchased from North American Biologicals and that prepared in our laboratory.

TABLE 3. Infection of gerbils with *Giardia* isolates from dogs

Animal no.	Inoculum (no. of cysts)	Incubation time (days)	Results of:	
			Fecal analysis	Gut examination
1	18,000	13	−	−
2	18,000	13	−	−
3	250,000	16	−	−
4	250,000	16	−	−
5	50,000	6	−	+
6	40,000	7	−	+
7	40,000	7	−	+
8	100,000	8	ND[a]	+
9	24,000	9	ND	+
10	25,000	10	ND	−
11	50,000	18	ND	−
12	50,000	7	ND	+

[a] ND, No data.

TABLE 4. Infection of gerbils with *Giardia* isolates from beavers

Animal no.	Inoculum	Incubation time (days)	Results of:	
			Fecal analysis	Gut examination
1	30,000 cysts	7	−	+
2	30,000 cysts	10	−	+
3	30,000 cysts	14	−	+
4	60,000 cysts	13	−	+
5	75,000 cysts	11	−	+
6	1,500,000 trophozoites	16	−	+
7	1,000,000 trophozoites	17	−	−
8	1,000,000 trophozoites	17	−	+
9	500,000 trophozoites	7	ND[a]	+
10	500,000 trophozoites	16	ND	+
11	500,000 trophozoites	11	ND	+

[a] ND, No data.

Attempts to purify inocula of trophozoites scraped from small intestines by using the nylon fiber method (1) or the attachment method (8) met with only limited success. In general, we found that separation of bacteria and trophozoites could be obtained only at the cost of losing most of the parasites. The resulting inoculum still contained enough bacteria to contaminate cultures overnight in the absence of antibiotics. Gerbils that had been treated with gentamicin before being sacrificed were found to be almost completely free of bacteria, and no deleterious effects on trophozoite recovery were observed.

We found that a combination of gentamicin and ampicillin in equal quantities was very effective in suppressing bacteria. When human *Giardia* isolates were cultured in TYI-S-33 medium containing a range of concentrations of these two antibiotics, we found that complete inhibition of bacteria occurred at concentrations of 175 μg of each antibiotic per ml with no inhibition of the growth of trophozoites. After 4 days, all of the tubes were overgrown with either *Giardia* trophozoites or bacteria. A combination of 200 μg of gentamicin per ml and 200 μg of ampicillin per ml was used thereafter for routine culturing.

Attempts to culture trophozoites from intestinal scrapings were not always successful initially. Although up to 500,000 trophozoites were inoculated into each tube, most of these died within 72 h. If trophozoites were still alive after this time, they began to replicate and slowly build up their numbers. In a typical successful run, a tube was not ready to be subcultured for about 2 weeks. Sometimes the initial subculture was unsuccessful, but if cultures survived to this point, usually very little trouble was encountered thereafter.

Repeated attempts to culture *Giardia* spp. from meadow voles were unsuccessful. The trophozoites did not stick well to the culture tubes and did not survive for more than 72 h. Boosting the concentrations of cysteine and ascorbic acid and adding glutathione had no effect. Cultures of dog, human, and beaver *Giardia* trophozoites frozen in dimethyl sulfoxide have been successfully recovered after up to 6 months of freezing.

DISCUSSION

The use of gerbils to excyst *Giardia* isolates obtained from different animal sources was generally effective, although problems were encountered. The first of these was the age of the cysts that were obtained for infection. The experiments were begun with human cysts obtained from the Provincial

Laboratory of Public Health in Calgary. Samples submitted for parasite analysis are invariably preserved, and it is possible to obtain nonpreserved material only if a sample for bacteriological testing is also submitted. By the time a positive diagnosis was made and the corresponding bacteriology sample was located, up to 7 days had passed before we received the sample. It is probable that this was the reason for the low success rate of infection in the first five attempts shown in Table 2. If these data had not been included, the success rate would have been much higher. Another problem may have resulted from the use of diethyl ether in the extraction of cysts. Young et al. (19) recommended substitution of ethyl acetate for diethyl ether, although they concluded that neither solvent caused any visible distortion or morphological change in cysts. In order to test for cyst inactivation by diethyl ether, five additional gerbils were infected with approximately 50,000 *Giardia* cysts that had been extracted from dog feces by using diethyl ether, and five gerbils were infected with the same number of cysts extracted without diethyl ether from the same fecal sample. In both cases four of the five gerbils were found to be infected after 7 days. It is possible that the use of diethyl ether was detrimental to cyst viability, but enough viable cysts survived to cause infection. However, we noticed that in the case of dog feces the yield of cysts was lower when diethyl ether was used.

Similar problems were experienced with dog and beaver feces. The dog feces were less than 6 h old when they were collected, but they had been deposited in an open, shaded runway and were subject to some desiccation. Even less control was possible with beaver scats because samples were taken from the bottom of a local pond and it was difficult to ensure that the samples were fresh. The high infection rate (91%) of these cysts was probably the result of the beneficial effects of storage in cold (6°C), alkaline water.

The infection of gerbils with trophozoites suggests that this parasite stage is less fragile than previously thought. Hewlett et al. (11) were able to infect dogs with cultured human *Giardia* trophozoites. Belosevic et al. (4) reported successful infection with cultured human trophozoites (Portland 1 strain) in gerbils, and Schleinitz et al. (16) obtained similar results with cultured trophozoites that were originally obtained from rabbit and human small intestine contents.

As discussed by Faubert et al. (7), the output of *G. duodenalis* type cysts from gerbils is irregular. For this reason, a very poor correlation was found between cyst output in the feces and the ultimate discovery of trophozoites in the guts of infected gerbils. Therefore, the use of stool examination to demonstrate infection in animals infected with *G. duodenalis* may result in false-negative findings.

We concluded that the gerbil model is efficient for excysting *G. duodenalis* from dogs, beavers, and humans if the cysts are viable. These results confirm the results of Faubert et al. (7), who infected gerbils with cysts from humans, beavers, and mice. In addition, Belosevic et al. (3) reported that they infected gerbils with cysts from cats (*Felis domesticus*).

During this study we encountered problems with contamination by *Trichomonas* spp. in gerbils. When *Trichomonas* was found in gerbils, its presence usually meant that *Giardia* cysts and trophozoites were either reduced or absent after more than 1 week of incubation. If cysts were detected in the feces but not in the gut, it may have been because *Giardia* failed to establish itself in the presence of *Trichomonas* spp. This may also be the reason why more positive gut samples were found after shorter incubation times. The source of *Trichomonas* was eventually tracked down to the Animal Sciences Vivarium at the University of Calgary, where the gerbils were held for from a few hours to 1 day before being shipped on to the Kananaskis Centre for Environmental Research laboratory. The problem was solved by sacrificing all of the animals in the laboratory, sterilizing the animal room, and arranging for all shipments to come directly to our laboratory.

When gerbils were infected with intestinal contents removed from meadow voles, *Hexamita* spp. were also often present, but these organisms did not appear to influence the population of *Giardia*, suggesting that the two parasites can coexist. We noted that meadow voles were often naturally infected with *Giardia*, *Hexamita*, and *Trichomonas* isolates without any deleterious effects on either the parasites (commensals?) or the hosts. It is possible that these organisms have evolved to coexist with *G. muris* but outcompete *G. duodenalis* in unnatural hosts.

In this study we demonstrated that *G. duodenalis* isolates from several hosts can be cultured in Diamond TYI-S-33 medium. *Giardia* isolates from beavers and humans have been cultured previously (2, 7, 13, 17), but we have found no previous reference to the culturing of dog *Giardia* isolates in the literature. Once established, these strains were amenable to culturing even when CLEX was substituted for fetal bovine serum. The trophozoites grow rapidly and can withstand being held at room temperature for several days and even refrigeration (4°C) for up to 48 h.

A major problem encountered in culturing *Giardia* trophozoites was overgrowth by bacteria. Early in the study we found that TYI-S-33 medium can inactivate antibiotics within 24 to 48 h. A great many overgrowth problems were avoided by adding the antibiotics when fresh medium was added to the cultures and by occasionally supplementing the tubes. Attempts to remove bacteria from culture inocula by using nylon fiber columns or adherence methods were only partially successful and resulted in a large loss of trophozoites. Much better results were obtained when the gerbils were treated with gentamicin and cleared of intestinal flora before being sacrificed. This method, combined with the use of gentamicin and ampicillin in the medium, allowed the use of concentrated gut scrapings as culture inocula, thus avoiding the use of any further purification technique. A considerable saving in medium also resulted as the medium needed to be changed only twice per day at most for fresh inocula. After 2 to 3 days, the medium was changed only when the trophozoites were subcultured.

We have not yet succeeded in producing axenic cultures. All of our cultures are contaminated with *Pseudomonas cepacia*, and one (human strain WB) also contains *Alcaligenes faecalis*; both of these contaminants are resistant to gentamicin and ampicillin. These organisms are motile so we have not been able to separate them from trophozoites. Additional antibiotic susceptibility tests have been carried out by using Mueller-Hinton medium, but so far we have discovered only that these bacteria are resistant to the following antibiotic disks supplied by Difco Laboratories, Detroit, Mich.: colistin (10 μg), carbenicillin (100 μg), amikacin (30 μg), cefoxitin (30 μg), and tobramycin (10 μg). In the meantime, we have been able to control bacterial overgrowth in our cultures with gentamicin and ampicillin but have not yet succeeded in eliminating the bacteria.

The culturing methods described in this paper are similar to those used by Gordts et al. (10; B. Gordts et al., Abstr. Annu. Meet. Am. Soc. Microbiol. 1985, C13, p. 302), who

cultured *Giardia lamblia* isolated from 10 human patients in modified TPS-1 medium. These authors obtained their inocula from duodenal aspirates and used streptomycin (100 μg/ml), penicillin (100 IU/ml), vancomycin (20 μg/ml), and clindamycin (20 μg/ml) to control bacteria. All of these strains were subcultured in media that contained only streptomycin and penicillin. The use of these antibiotics avoided the need for purification of trophozoites.

The survival rate of frozen trophozoites after recovery was low, but enough viable trophozoites were present to recolonize tubes within 72 h, as found by Meyer and Chadd (14). The final concentration of dimethyl sulfoxide which we used (5%) was close to the concentration recommended (6.5%) by Lyman and Marchin (12), but we did not store our cultures in liquid nitrogen as recommended by these authors or by Warhurst and Wright (18). The cooling rate which we used was close to −1°C/min, as recommended by Lyman and Marchin (12) and Warhurst and Wright (18). If we had used the Dewar flask and ethanol cooling method described by Lyman and Marchin (12) and liquid nitrogen storage, our recovery method could have been improved.

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