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Preliminary experiments on use of zebrafish as a laboratory model for *Giardia duodenalis* infection

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Abstract

Although *Giardia duodenalis* is considered a parasite of mammals, different genotypes have been identified as infecting several species of freshwater and marine fish in Australia. Establishment of *G. duodenalis* infection in common laboratory zebrafish (*Danio rerio*), could provide an excellent tool for a range of studies on *Giardia*. We conducted preliminary experiments to investigate this possibility. Zebrafish were inoculated with viable *G. duodenalis* cysts from two different Assemblages (A and D) using a modified oro-gastric tube. Direct microscopy and immunofluorescent antibody test were used to check for *Giardia* cysts/trophozoites in the intestine, and histology was performed on intestinal mucosa to evaluate possible pathological changes. *Giardia* cysts were successfully deposited in the zebrafish alimentary tract using a modified oro-gastric tube, and were maintained in the fish gut for at least 8 days. Although a single trophozoite was observed in one fish three days post-exposure, we were unable to demonstrate established, propagative infection under the conditions tested.

Keywords

Animal model, cyst, *Giardia*, infection, trophozoite, zebrafish

Introduction

Giardia duodenalis (syn: *G. intestinalis* and *G. lamblia*) is a well-known intestinal protozoan parasite of mammals. *Giardia* infection is often associated with diarrhoea, but can be asymptomatic or associated with a wide range of symptoms, ranging from chronic to acute (Robertson *et al*. 2010).

Based on molecular, but not morphological, differences, various *G. duodenalis* Assemblages and genotypes have been identified which are characterised by particular host-specificities. *Giardia* in Assemblage A1 are the most important zoonotic genotype, A2 predominantly infect humans, but may also be zoonotic, while A3 are common among wild ungulates (Sprong *et al*. 2009, Beck *et al*. 2011). *Giardia* in Assemblage B appear to be more heterogenic, but are predominantly found in humans and can also be zoonotic. *Giardia* in Assemblages C and D appear to infect canids exclusively, *Giardia* in Assemblage E infect ruminants, in Assemblage F infect felids, in Assemblage G infect rodents, and in Assemblage H infect pinnipeds (Lasek-Nesselquist *et al*. 2010). In addition, members of the different Assemblages have different phenotypic traits related to *in vitro* and *in vivo* cultivation. Whilst isolates from Assemblage A appear to be relatively easy to cultivate *in vitro*, and can also be readily established in common laboratory animals such as gerbils (Schupp *et al*. 1988), other Assemblages, particularly those associated with animal infections (especially C, D, and F), appear refractory to laboratory cultivation and do not readily establish in laboratory animals (Bénéré *et al*. 2010). Taken together, host specificity and genetic differences have led to the suggestion that *Giardia duodenalis* is a species complex and should be redescribed as a number of different species (Monis *et al*. 2009).

The establishment of *Giardia* isolates from different Assemblages in a laboratory setting has been identified as a clear need for investigating different aspects of biology, pathogenicity, immunopathology, and host-parasite interactions (Thompson 2009), particularly for those isolates that are refractory to *in vitro* cultivation. Although generally considered a mammalian parasite, Yang *et al*. (2010) reported that *G. duodenalis* could also establish infections in some species of fish.

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In their Australian study, *G. duodenalis* was detected by PCR at various genes in 3.8 % of the 709 fish studied, including 8 species of cultured fingerlings (n = 227), 5 species of wild marine fish (n = 255), and 8 species of wild freshwater fish (n = 227). The highest prevalence (8.4 %) was in cultured fingerlings of various species. Examination of tissue sections demonstrated trophozoites in 10 samples, and it was reported that several isolates had large numbers of parasites (> 100 *Giardia* per field of view) (Yang *et al*. 2010). Therefore, this finding was apparently not simple passive passage of ingested cysts, but suggested true establishment of infection. Interestingly, the *G. duodenalis* found in these fish were not only Assemblage A, which has been shown to be the most promiscuous genotype, but also Assemblage B and additionally some isolates previously only associated with host-specific infections.

The findings by Yang *et al*. (2010) suggested a possible novel solution for resolving the problems with laboratory culture of *Giardia*. The observation of *G. duodenalis* infections established in wild or cultured fish, with presumably relatively low exposure to infective *Giardia* cysts, suggested that it might be possible to establish experimental *G. duodenalis* infections in some piscine species. Zebrafish (*Danio rerio*) have a range of advantageous features that have resulted in them being an important, widely used model organism. As they are tropical fish, thriving best at 26–28°C, they can be kept at the above ambient temperatures that are considered necessary for establishing and maintaining *Giardia* infections. Additionally, the use of zebrafish as an infection model for *Giardia* would circumvent some of the ethical issues associated with use of small mammals in the laboratory.

Thus, the main aim of this study was to make a preliminary investigation of whether *Giardia* infections could be successfully established in zebrafish using gavage methods to deliver defined doses of the infectious cysts to the fish. A secondary aim was to compare different methods for detection of *Giardia* cysts and trophozoites in intestinal samples collected from zebrafish.

Materials and methods

Sources of Giardia *cysts*

Assemblage D

Giardia cysts were purified from stool samples from a naturally-infected puppy, by a series of filtration and flotation on zinc sulphate steps. Sequencing of PCR products from amplification of a partial sequence of the glutamate dehydrogenase (GDH) gene was used to determine this isolate as Assemblage D. The cysts were stored refrigerated at concentrations of approximately 1.9×10^4 cysts/ml in distilled water with antibiotics (penicillin/streptomycin 100 U/ml and amphotericin B 0.1 µg/ml). The cysts were used for experimental infections within 24 h of isolation, and viability was established by *in vitro* excystation subsequent to the inoculation of the zebrafish.

Assemblage A

Viable *Giardia* cysts of Assemblage A were purchased (in PBS) from a commercial distributor (Waterborne Inc., New Orleans, LA, USA). Sequencing of PCR products from amplification of a partial sequence of the GDH gene was used to confirm the identity of this isolate. After arrival at the laboratory, the cysts were stored refrigerated for 24 h before the experimental infections were initiated. Viability of the cyst suspension was established by *in vitro* excystation and culture subsequent to the inoculation of the zebrafish.

Maintenance of zebrafish

Zebrafish used in this study were maintained within a dedicated zebrafish laboratory (Aleström-laboratory, www.zebrafish.no), at the Norwegian School of Veterinary Science, under the standard operating procedures (SOPs) of the laboratory. For the duration of the study, the zebrafish were kept in two small glass aquaria, with water temperature at 30°C (± 1°C). The fish were acclimatised to these conditions for 2 days before exposure. The fish were starved for 12 h prior to infection, and fed according to appetite twice daily post-infection. Throughout the study period, fish were monitored by visual observation at least three times per day. Fish with abnormal behaviour were withdrawn from the study and euthanized.

Study set-up

This study was conducted in two consecutive rounds. Round 1 was performed during December 2010 using the Assemblage D isolate, and in the second round (February 2011) the Assemblage A isolate was used. For each round, 20 fish were inoculated with *Giardia* cysts and 3 control fish were inoculated with PBS using the gavage method described below. The fish were sampled at different time points post exposure, and examined for *Giardia* infection using a variety of different techniques (e.g. direct microscopy, immmunofluorescent antibody test (IFAT), histology). Table I provides an overview of infection and sampling for each round and the techniques used are detailed in the sections below.

Inoculation of zebrafish by gavage

Gavage of zebrafish is not a standard procedure. Thus, the feasibility of the gavage technique was initially tested on euthanized zebrafish. For these preliminary studies, transparent ("casper" mutant) zebrafish were used and gavage was attempted using a blue dye (methylene blue) solution, in order for the success of oro-gastric deposition to be ascertained. Following experimentation with different methods for de-

Table I. Overview of zebrafish inoculation and sampling

Day	No. fish inoculated	No. controls	Sampling	Infection assessment method
Round 1: Assemblage D				
0	20	3	3 controls 2 experimental	5 – IFAT
1			5 experimental	5 – direct microscopy 5 – IFAT
4			5 experimental	5 – direct microscopy 5 – IFAT
6			4 experimental	4 – IFAT 1 – direct microscopy 2 – histology 2 – culture
8			4 experimental	4 experimental 1 – direct microscopy 2 – histology 2 – culture
Round 2: Assemblage A				
0	25	3	3 controls 10 experimental	13 – IFAT
1			5 experimental	2 – histology 5 – IFAT
3			4 experimental	5 – direct microscopy 5 – IFAT
6			6 experimental 4 – direct microscopy 4 – histology 4 – histology	4 – IFAT

livering the dose (e.g. pipette tip, syringe), it was apparent that use of a 24 gauge catheter sheath of flexible plastic (BD Neoflom™) connected to a standard 2–20 µl volume pipette was the most appropriate. This set up provided sufficient sturdiness and flexibility, as well as being relatively easy to use for a 2-person team, and enabling delivery of relatively precise volumes.

For the *in vivo* experiment, each fish was anaesthetized by exposure to tricaine mesylate solution (TMS; 0.17 mg/ml), until loss of equilibrium and touch reflexes while gill movements were maintained. As per the SOPs for anaesthesia of zebrafish, the fish was then placed in a position of dorsal recumbency on a moist sheet of absorbent tissue.

As the fish was being anaesthetized, 5 µl of suspension containing approximately 95 (Assemblage D) or 6,250 (Assemblage A) cysts were drawn up into the catheter, then detached from the pipette and carefully inserted, by one operator, into the fish's mouth so that approximately 10 mm of the catheter was inside the fish. While the operator held the fish and gavage in position, the pipette was reattached to the catheter by another operator, and the dose delivered. The catheter was then removed from the fish, and the fish immediately placed in a separate jar of fresh water. From sedation onwards, the entire gavage procedure was completed in less than 3 minutes.

Assessment of establishment of infection in zebrafish

Sampling of zebrafish

Zebrafish were sampled according to the schedule in Table I. Fish that were withdrawn from the study due to behavioural irregularities were selected first. Aside from these, fish were selected at random from the tanks and euthanized by subjecting them to an overdose of TMS (1.2 mg/ml).

Dissection of zebrafish

Freshly killed fish were dissected under a dissection microscope. The intestines were removed and opened longitudinally. The longitudinally opened intestines were used for direct microscopy, IFAT, histology, and direct *in vitro* cultivation according to the schedule in Table I.

Direct microscopy

Sections of longitudinally-opened intestines were placed into 2 ml vials with phosphate-buffered saline (PBS) and vortexed briefly. 15 µl aliquots of the suspension (three from each suspension) were placed on a microscope slide, a cover slip added, and the whole area examined under a light microscope at × 200 and × 400 for *Giardia*, with particular attention to detection of motile *Giardia* trophozoites.

IFAT using dual-label monoclonal cocktail against Giardia *cysts and* Giardia *trophozoites*

Sections of longitudinally-opened intestines were spread out thinly onto microscope slides, air-dried and methanol-fixed. The surfaces were then covered with a cocktail of monoclonal antibodies against *Giardia* cysts and *Giardia* trophozoites, the former labelled with FITC and the latter labelled with Cy-3 (TOG-COG, Waterborne Inc. New Orleans, USA), and incubated at 37°C for 30 min. The samples were then rinsed gently in PBS, and a drop of mounting medium (Waterborne Inc.) and a cover-slip applied. The preparations were viewed at × 200 and × 400 using a fluorescence microscope with appropriate filters for viewing Cy3 and FITC. As TOG-COG is a research product, its efficacy was first confirmed on dried suspensions of *Giardia* cysts and *Giardia* trophozoites from regular *in vitro* culture.

Direct cultivation from zebrafish intestines

Sections of longitudinally-opened intestines were put in 2 ml vials and briefly vortexed with PBS, then transferred to 10 ml flat-sided culture tubes containing modified TYI culture medium (Keister 1983) and antibiotics (200 µg/ml of gentamycin and ampicillin). These tubes were incubated at 37°C and examined approximately every 30 min for the next 3 h. At 5 h, 1 ml was removed from the culture media into 2 new tubes of culture media, and all three tubes examined after 12 h and 24 h.

Histology

Pieces of longitudinally-opened intestines were fixed in 4% formaldehyde, embedded in paraffin and sectioned. Sections of 5 µm thickness were stained with haematoxylin and eosin (H & E) by routine methods (Bancroft and Gamble 2002) and examined by light microscopy at 100–1000 times magnification.

Examination of aquarium water for Giardia *cysts*

Water from the fish tanks was collected and investigated for cysts presence by a modified US EPA method 1623 (US EPA, 2005). Briefly, the water (approx. 5 l per tank) was filtered through a 47 mm track-etched Isopore membrane (2.0 µm TTTP) filter under negative pressure, the filter washed in a membrane elution solution, and the eluate concentrated by centrifugation. *Giardia* cysts were isolated by immunomagnetic separation (Dynal, Invitrogen Life Sciences, Oslo), and the final concentrate air-dried to a welled microscope slide, stained with FITC-monoclonal antibody (Aqua-Glo, Waterborne Inc. New Orleans, USA), and 4'2 diamino-2-phenyl indole (DAPI) and examined by fluorescence microscopy at × 200 using appropriate filters. The recovery efficiency of this method in this laboratory is between 50 and 80% depending on matrix variables.

Ethical approval for study

This study was approved in advance by the laboratory's Animal Research Officer, acting under authorisation from The Norwegian Animal Research Authority (NARA).

Results

Inoculation of zebrafish by gavage

In the first round of inoculation of zebrafish, the gavage method was considered to be successful. Only 2 out of 23 fish did not recover from the procedure; 1 inoculated and 1 control – these were included as the sample fish for day 0. All other fish appeared healthy in the subsequent days of the trial.

In the second round of inoculation, 11 out of 28 fish did not fully recover from the procedure. Seven fish did not recover from the anaesthesia, and 4 died within two hours after apparent recovery, with bleeding from the gills indicating mechanical damage caused by the gavage instruments. All these fish were included as sample fish for day 0. Two fish demonstrated abnormal behaviour at day one post infection and were therefore euthanized first.

In all fish that survived the inoculation procedure, IFAT was able to reveal that *Giardia* cysts had been deposited in the intestinal mucosa (see Fig. 1).

Establishment of infection in zebrafish as determined by microscopy, IFAT, direct cultivation and histology

No indication of *Giardia* infection was detected in any of the control fish, either by direct microscopy, IFAT, or histology.

For the inoculated fish, neither *Giardia* cysts nor motile trophozoites were detected by direct microscopy of PBS in which sections of intestine had been vortexed. IFAT demonstrated successful deposition of *Giardia* cysts in the intes-

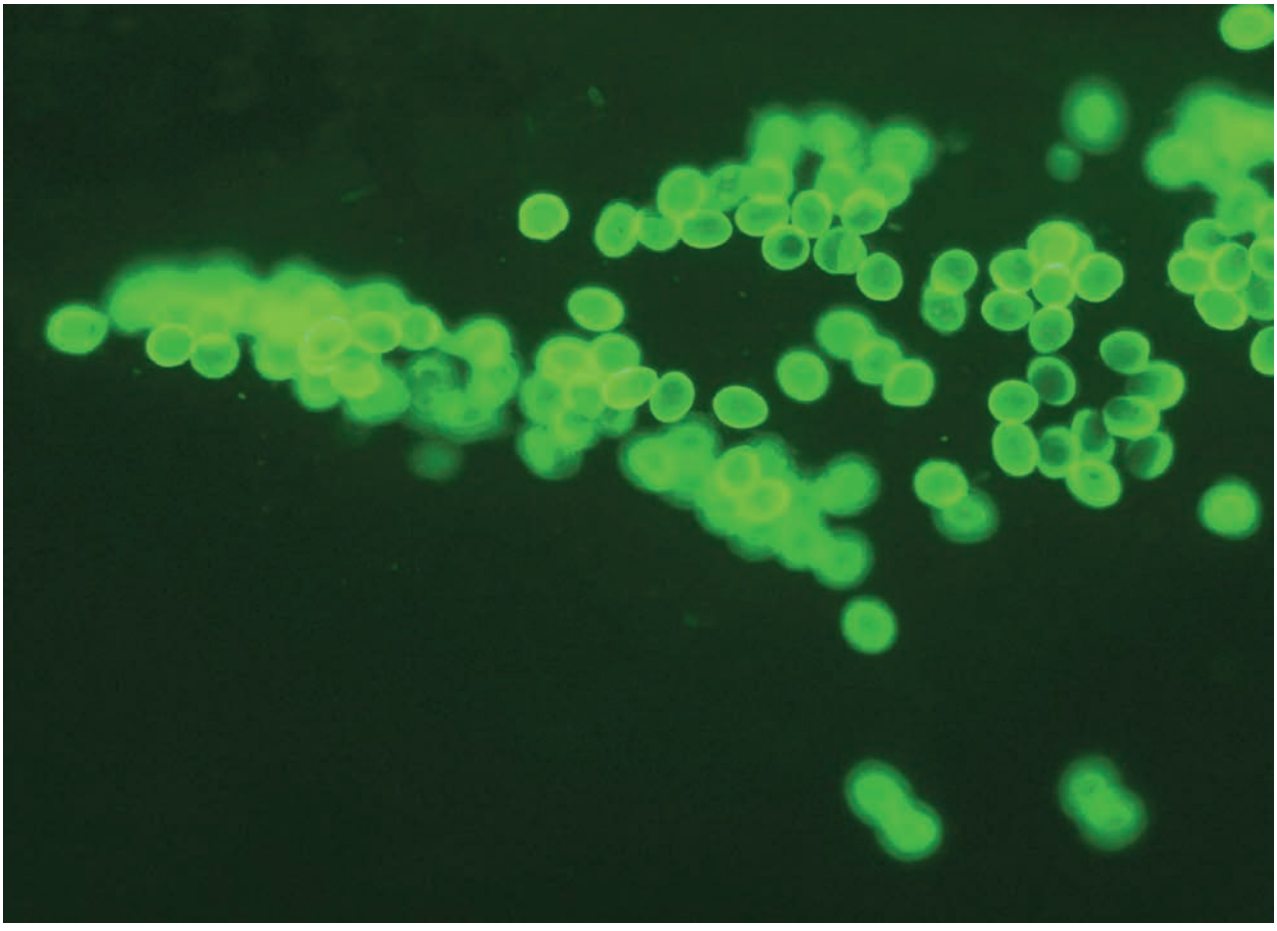

Fig. 1. *Giardia* cysts deposited by gavage in the intestine of a zebrafish

tine, and also that the cysts were retained in the intestine for several days. However, the numbers of cysts in the intestine were found to decline over time, indicating excretion of some cysts, although it was not possible to quantify this as the sections of intestine examined at each sampling were not directly comparable. A single trophozoite was identified in a sample from day 3 after inoculation with *Giardia* from Assemblage A. Direct cultivation from zebrafish intestines did not result in the production of viable trophozoites from any of the samples.

During histological examination, it was noted that several samples had suffered mechanical damage, which affected the assessment. These changes were considered to have occurred during dissection when splitting the intestine longitudinally. For those fish that provided specimens that were satisfactory for histological examination, *Giardia* trophozoites were not identified, nor were any intestinal pathological changes seen that could be conclusively associated with *Giardia* infection. Some mechanical damage was observed, but considered to have been caused by dividing the intestines during autopsy; control fish with intact intestines did not demonstrate similar changes by histology.

Giardia *cysts in aquarium water*

Examination of the aquarium water concentrates demonstrated the presence of *Giardia* cysts, indicating excretion of *Giardia* cysts by the zebrafish. Total numbers of cysts detected per aquarium ranged from 73 to 103, indicating that as each tank held at least 10 fish, each inoculated with 95 or 6250 *Giardia* cysts, a large proportion of the cysts were retained inside the fish.

Discussion

The results of this pilot project suggest that experimental oral exposure of adult zebrafish by gavage is a useful, relatively simple method of delivering volumes up to 5 µl to the zebrafish intestine, and adds a further tool to the armoury of techniques available for use in investigating host-pathogen interactions with this model organism (Meijer and Spaink, 2011). However, we were unable to confirm the findings of Yang et al. (2010), that *Giardia duodenalis* infections are able to establish in fish, and we were unable to demonstrate that

live zebrafish could be used as an appropriate laboratory model for cultivation of *Giardia*. The diversity among piscine species may mean that different, phylogenetically-distant, warm-water species may be more suitable hosts for *Giardia*.

The higher mortality experienced in the second round of inoculation was retrospectively attributed to an over-representation of so-called "egg-bound" females in this group of fish (as determined at dissection), as the large, over-ripe ovaries tended to displace the fish gut. Thus, the catheter may have become incorrectly placed or may have perforated the oesophagus or ventricle. Therefore, we would recommend for any similar experiments in which doses are delivered by gavage, male fish or freshly-spawned female fish are used preferentially.

The use of a dual-label *Giardia* trophozoite/*Giardia* cyst monoclonal cocktail was considered to be successful, and provided a rapid method for determining the presence of cysts or trophozoites in air-dried preparations, despite the presumption that the relatively fragile trophozoites might rupture and become indistinguishable on drying.

Although we were unable to establish prolonged identifiable infections in the zebrafish, the observation of one trophozoite suggest that a short-lived infection may have established in some fish. It would be interesting to determine whether this was indeed the case, and why *Giardia* infections are short-lived, or do not establish at all, in zebrafish, but apparently do establish in other fish species (Yang *et al*. 2010). As piscine host immune responses are temperature-dependent, then this may be one factor worth considering, and comparative studies between successful and unsuccessful conditions for *Giardia* infection establishment in fish may provide insights into immunological reactions that may also be of relevance for mammalian *Giardia* infections. It has long been recognised that whilst some individuals are relatively unaffected by *Giardia* infections, others may have an acute diarrhoea that resolves rapidly and the infection is expelled, whereas others again may have a prolonged symptomatic picture, and refractory infections (Robertson *et al*. 2010).

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