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REVISED

Title: *Giardia duodenalis* Mouse Model for the Development of Novel Antigiardial Agents

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Abstract:

This study describes a neonatal mouse model of *Giardia* infection for development of novel antigiardials. Mice were infected with the axenically cultured Assemblage A BAH2c2 strain, with 10^5 trophozoites per animal recovered. This model proved to be robust and consistent for use in preliminary drug efficacy trials and drug development.

Keywords: infection model, giardiasis, drug discovery

Giardia duodenalis (aka. *G. lamblia*) is one of the most common enteric pathogens worldwide and is especially prominent in children in developing nations, often resulting in chronic infections with long lasting side effects such as malnourishment, developmental delays and general failure to thrive (Berkman et al., 2002, Homan and Mank, 2001, Lengerich et al., 1994). In addition, a correlation between giardiasis and the development of other diseases such as irritable bowel syndrome has also been established (Hanevik et al., 2014). Although there are current treatments available for giardiasis, treatment failures occur often, due in part to poor adherence to dosing schedules, unpleasant side effects and the development of drug resistance in the parasite (Jokipii and Jokipii, 1979, Wright et al., 2003). An important step in the development of novel chemotherapeutics for the treatment of

giardiasis is preliminary evaluation of potential antigiardials in animal models, as *in vitro* efficacy does not always translate to *in vivo* efficacy (Sande and Zak, 1999).

Attempts to develop mouse models of giardiasis have been undertaken in both adult and neonatal mice. In immunocompetent adult mice only one characterised human derived isolate (GS(M)/H7 – assemblage B) was able to cause infection over several weeks. However, development of infection is highly dependent on mouse strain and intestinal microflora (Byrd et al., 1994, Singer and Nash, 2000). Other models using cysts of uncharacterised human derived strains (e.g. H3 – assemblage B) have been shown to cause longer infections. However, these models are not ideal for drug development as the strains cannot be characterised *in vitro,* they require the animals be maintained on an antibiotic cocktail (which could interfere with the activity of novel compounds) (Bartelt et al., 2013, Singer and Nash, 2000)In addition, not all isolates can cause successful infections in mice, identified by the shedding of cysts and presence of trophozoites in the intestine upon necropsy. The neonatal mouse model, on the other hand, is ideal for early antigiardial compound testing as pre-weaned pups have been shown to be susceptible to a wide variety of *Giardia* strains including those available in axenic culture allowing comparison between different strains and *in vitro* and *in vivo* results (Lemee et al., 2000, Reynoldson et al., 1991).

In this study we describe modifications of the suckling mouse model of *Giardia* infection first described in 1983 and used in preliminary testing of albendazole efficacy in the 1990's (Hill et al., 1983, Reynoldson, Thompson and Meloni, 1991). The *Giardia* strain used, BAH2c2, was isolated from an Australian human patient, maintained in axenic culture (TYI-S-33 media, passaged 2-3 times per week) and has not previously been reported to infect mice (Wielinga et al., 2011). The additional modifications made to this model include highly sensitive techniques for detection and monitoring of infection including fluorescent antibody staining and qPCR. These modifications make the identification of infection more accurate and reliable.

All work was performed with the approval of Murdoch University Animal Ethics Committee (Permit #R2855/16) Animals used were arc:arc (S) Swiss origin mice sourced from the Animal Resource Centre, Murdoch, Perth, Western Australia. Suckling mice were obtained when 6 days old (7-13 mice

per group, number of mice in initial trial determined using a power calculation with a confidence of 95%, and an estimated 30% of animals becoming infected) and were acclimatised for 2-3 days in the PC2 facility. Mice were housed in plastic cages with wire lids with food and water supplied ad lib. Housing and nesting material were also provided. All mice were inoculated when nine days old via oral gavage using a flexible gavage needle (Walker Scientific, Joondalup DC WA Australia 6919). In the initial establishment model mice were inoculated with either $1x10^4$ or $1x10^5$ *Giardia duodenalis* trophozoites of the P1c10 strain (a clone of the Portland 1 strain, previously shown to cause infection in neonatal mice) or the BAH2c2 strain (a clone isolated from a human from Woodanilling, south-western Australia (Meloni et al., 1990, Wielinga, Ryan, Andrew Thompson and Monis, 2011) in a volume of 100 μL of 0.9% saline. In all subsequent trials animals were inoculated with $1x10^5$ trophozoites of the BAH2c2 strain in a volume of 100 μL of 0.9% saline. Faecal samples were collected by removing groups to a separate cage without bedding. Due to the small volume of faeces produced by neonatal mice, faecal samples were pooled by group in order to obtain sufficient sample for testing. Faeces were examined for cysts via sucrose gradient centrifugation and observed via optical microscopy, immunofluorescent microscopy as described previously and qPCR (O'Handley et al., 2000). Briefly, cysts were concentrated by sucrose gradient centrifugation (prepared in-house, specific gravity 1.13, centrifugation: 900 X g, 5 minutes) and samples taken for each cyst detection method. For immunofluorescent detection 20 μl of concentrated cysts were placed onto a glass slide (Polysciences, Inc., Warrington, PA United States 18976) and air dried. An aliquot of *Giardia* specific fluorescein-labelled antibody (Giardi-a-Glo, Sapphire Bioscience, Redfern NSW Australia 2016, 20 μl) was placed over the top of the sample and incubated at 37°C in a humidified box for 30 minutes. Excess antibody was removed by gentle washing with phosphate buffered saline (PBS) and a coverslip was mounted onto a slide using aquapolymount (Thermo Fisher Scientific, Scoresby VIC Australia 3179) . Samples were viewed with an Olympus BX41 microscope and the number of *Giardia* cysts/mg faeces calculated. The qPCR for the detection of giardial glutamate dehydrogenase (*gdh*) DNA in faeces was based on the method of Yang et al (Yang et al., 2014). The protocol used the primers gdhF1 F1 5′ GGGCAAGTCCGACAACGA 3′, the reverse primer gdhR1 5′ GCACATCTCCTCCAGGAAGTAGAC 3′, developed by Yang *et al*., and the probe modified to

include FAM as the reporter dye 5′-(6FAM)-TCATGCGCTTCTGCCAG BHQ2 3' (Yang, Jacobson, Gardner, Carmichael, Campbell and Ryan, 2014). Concentrated cyst material from the sucrose clarification was freeze-thawed five times using liquid nitrogen, before total DNA was extracted using a Purelink Genomic DNA extraction kit (Invitrogen, Thermo Fisher Scientific, Scoresby VIC Australia 3179). Reactions were preformed using TaqPath qPCR mastermix (Thermo Fisher Scientific, Scoresby VIC Australia 3179) on a Quantstudio6 platform. At the end of the trial period, mice were euthanized via cervical dislocation and the entire small intestine was removed, incised longitudinally and placed in ice-cold PBS. Samples were chilled for at least 30 minutes before enumeration of trophozoites using a haemocytometer.

The BAH2c2 strain with an inoculum of 1 x 10^5 cells/mouse was found to be the most promising in establishing infection in pups with 100% of animals infected in the first experiment and observation of cysts 13 days post-inoculation. The smaller inoculum of 1 x 10^4 cells only caused infection in 22% (2/9) of animals and no cysts were observed in the faeces. The P1c10 strain infected 8% (1/13) of mice at the highest inoculum used but failed to infect any mice at the lower inoculum and no faecal cysts were observed in either group 15 d p.i. (Figure 1).
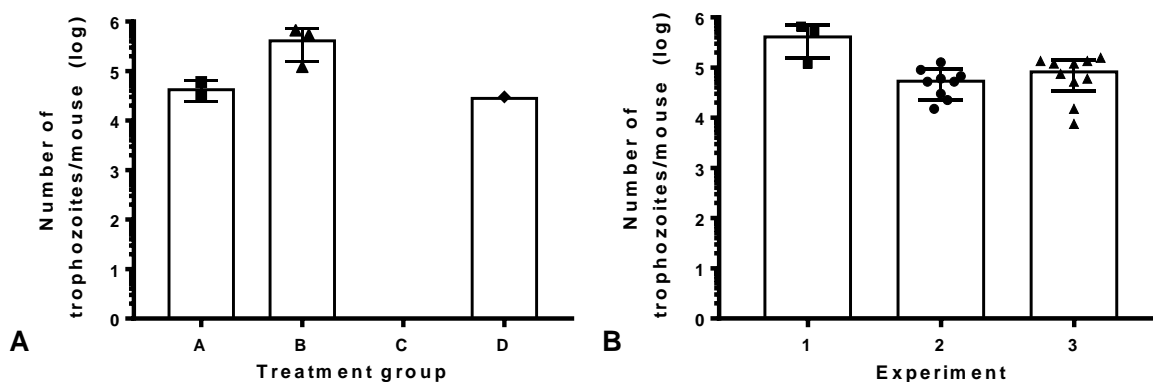

***Figure 1:** Experimental infection of neonatal mice with* Giardia duodenalis. ***1A: Establishment of infection** with various strains and inoculum densities of* G. duodenalis. *Neonatal mice were infected at 9 days old with either the P1c10 or BAH2c2 strain of* G. duodenalis. *15 days p.i. trophozoites were collected from the small intestine and enumerated. A – inoculum BAH2c2, 1 x 10^4 cells/mouse, 22% of*

mice infected; B – BAH2c2, 1 x 10^5 cells/mouse,100% of mice infected (only subset had trophozoites counted); C -P1c10, 1 x 10^4 cells/mouse, 0% of mice infected and D – P1c10, 1 x 10^5 cells/mouse, 8% of mice infected. **1B: Reproducibility of infection** *of neonatal mice with the BAH2c2 strain of* G. duodenalis. *Mice were inoculated at ~9 days old with 1 x 10^5 trophozoites/mouse of the BAH2c2 strain. Once cyst shedding had been observed, trophozoites were harvested from the small intestine and enumerated. Infection was established in 100% of animals based on the presence or absence of trophozoites in the small intestine. Error ± SD. Individual points represent single animals.*

Based on the initial experiment the BAH2c2 strain at the inoculum of 1×10^5 cells/mouse was chosen for future experiments. To ensure reproducibility between litters, infection with the BAH2c2 strain was repeated on two separate occasions. In both cases an infection was established in 100% of the exposed mice with faecal cysts observed 7-8 days post inoculum. Across all three replicates of the trial, a similar number of trophozoites was observed in intestinal samples (Figure 1).

Three cyst detection methods were evaluated with immunofluorescence microscopy being the most sensitive, detecting the presence of cyst material in the faeces a day earlier than qPCR and traditional microscopy, and is easily used to quantify the number of cysts in a sample when the initial faecal weight of the sample is known. qPCR was the second most effective detection method giving a positive or negative answer while traditional microscopy was least effective (Table 1). In addition, qPCR and immunofluorescence do not require the specialist training and experience that is required for light microscopy. However, qPCR should be used in conjunction with microscopy detection, rather than as a stand-alone detection method, to ensure infection has been established, as the presence of *gdh* DNA does not necessarily indicate the presence of viable cysts. Concentration of the cysts via sucrose gradient centrifugation was an important addition to the model in detection of faecal cysts.

Days post inoculation	Light microscopy (+/-)	qPCR (+/-)	Immunofluorescent microscopy (cysts/mg faeces)
7	-	+	Positive but not quantified
8	+	+	97
9	-	+	60
10	+	+	59
11	NT	+	139

Table 1: Comparison of three methods to detect Giardia *infection in mice via faeces. Faecal samples from individual animals were pooled and subjected to sucrose gradient centrifugation techniques to concentrate cysts before preparation for microscopy or qPCR. Representative data from pooled samples collected in one experiment with 7 mice per group are shown (the final trial). NT – not tested.*

In this study we demonstrate that the neonatal suckling model is useful in the pre-clinical development of antigiardial agents as it is reproducible, predictable and easy to perform. It has demonstrated an improvement on previous models by utilising immunofluorescence to increase specificity and ease of cyst identification and qPCR for rapid identification of infection and confirmation of immunofluorescence results. This model has been developed specifically for antigiardial drug screening and development.

Competing interests: The authors have no competing interests to declare

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Highlights

- A neonatal mouse model of giardiasis was established
- Fluorescent microscopy and qPCR were used to identify infection
- This model can be used for antigiardial efficacy screening