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Research paper

Propagation of *Giardia duodenalis* cysts in immunosuppressed CF-1 mice

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ABSTRACT

This study developed and evaluated *Giardia duodenalis* cyst propagation using a dexamethasone immunosuppressed CF-1 mouse model as an alternative to a previously described Mongolian gerbil model. The CF-1 mouse model shed significantly more cysts per animal during a 16–18 h collection period compared to the gerbil (averages: 7.8×10^6 cysts/CF-1 mouse and 2.5×10^6 cysts/gerbil). In addition, the patency period for this model differed from both *G. muris* in mice and *G. duodenalis* in gerbils in that cysts were shed continuously for over 20 days. Results further showed that the *β*-giardin gene sequences from gerbil derived and mouse derived *G. duodenalis* were identical, after 34 serial passages through the CF-1 mouse model. Overall, the CF-1 mouse model produced higher concentrations of cysts per animal, and were genetically and phenotypically stable based on *β*-giardin gene sequences.

1. Introduction

Giardia duodenalis is a protozoan parasite that commonly causes waterborne gastrointestinal illness in humans, with outbreaks across the world (Efstratiou et al., 2017). *Giardia* cysts are often found in wastewater and biosolid samples at high concentrations, and remains an important waterborne pathogen (King et al., 2016; Ramo et al., 2017a, b; Rhodes et al., 2015). Since the cyst is the only stable life stage in the environment, viable *G. duodenalis* cysts are required to study this pathogen in environmental matrices. *In vitro* culture of *G. duodenalis* cysts has been described and used for gene expression studies; however, axenic culture is challenging, cyst yields are limited, and these cysts may not be as robust or viable as those produced in animals (Einarsson et al., 2016; Lujan et al., 1996). The Mongolian gerbil (*Meriones unguiculatus*) has been used to propagate large amounts of cysts ($> 2 \times 10^7$ cysts with 10 gerbils); however, gerbils can be cost prohibitive and challenging to maintain (Belosevic et al., 1983). In addition, although infected gerbils may shed *G. duodenalis* cysts for over 30 days post-infection, the patency period is irregular and includes days with minimal or no cysts detected in the feces (Faubert et al., 1983; Visvesvara et al., 1988). Cysts have also been propagated using neonatal mice and rats (< 14 days old); however, obtaining and using neonatal animals is challenging and will likely yield only a limited number of cysts (Craft, 1982; Reynoldson et al., 1991; Vinayak, 1979). An alternative to these gerbil or neonatal models for cyst propagation is needed. In a previous study, we described an immunosuppressed CF-1 mouse model to propagate *Cryptosporidium parvum* oocysts (Ware and Villegas, 2010). Like *C. parvum*, *G. duodenalis* does not infect adult mice so we investigated whether the CF-1 immunosuppressed mouse model will also infect and shed *G. duodenalis* cysts. We compared the number of cysts produced by the mouse model and the cyst shed pattern to historical results obtained with Mongolian gerbils. These *G. duodenalis* cysts were then confirmed by genotype sequencing.

2. Material and methods

2.1. G. duodenalis and animal models

G. duodenalis cysts (H3 strain, Assemblage B) were originally obtained from Waterborne, Inc. (New Orleans, LA) and serially passed in Mongolian gerbils (gerbils) and in chemically immunosuppressed CF-1 mice. Mixed sex CF-1 (3–4 week old) mice or gerbils were originally acquired from Charles Rivers Laboratories (Wilmington, MA) and bred in-house. Animals used for the study were between 3–52 weeks of age. After breeding, mice and gerbils were separated by sex in groups of 2–5 animals per cage, and housed in sterile, individually-vented cages. Both received Pico Lab mouse diet (Brentwood, MO) and sterile water (gerbils) or sterile amended water (mice, as described below) *ad libitum*. All animal studies had an approved IACUC protocol and were overseen and monitored by the Cincinnati EPA IACUC.

2.2. Propagation and purification of G. duodenalis cysts

Gerbils were exposed to at least 5000 cysts by oral gavage using a

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22-gauge feeding needle. On day 6 and 13 post-exposure, gerbils were placed on raised wire mesh above a layer of sterile water, and the feces collected for 16–18 h. These collection days were based on published cyst yields described in the literature (Faubert et al., 1983; Visvesvara et al., 1988).

CF-1 mice were chemically immunosuppressed as previously described (Ware and Villegas, 2010). Briefly, sterile drinking water was amended with 288 mg/l dexamethasone phosphate (Gold Biotechnology, St. Louis, MO) or 50 µg/ml tetracycline (Bimeda, Oakbrook Terrace, IL) on alternate days beginning 7 days prior to exposure and continuing through the end of each experiment. Mouse exposures and collections were the same as the gerbils except that only day 6 post-exposure samples were collected. All cysts were purified by sieving followed by sucrose and Percoll flotations as previously described (Belosevic et al., 1983; Sauch, 1984). Purified cysts were enumerated by hemocytometer (Bennett et al., 1999).

2.3. Patency pattern in CF-1 mouse model

The patency pattern of the mouse model was determined by collecting 3–10 fresh fecal pellets daily at approximately the same time and placing them into tared 2 ml screw cap tubes preloaded with 100 µl water (t_L) and weighed (W_i). The cysts were purified by adding 1 ml $ZnSO_4$ solution (specific gravity 1.18), and the sample was then macerated, mixed, centrifuged at 1000 x g for 5 min, and decanted into an empty tared 2 ml tube (t_E) (Ash and Orihel, 1987). The purified cysts in the second tube were washed with 1 ml reagent water, centrifuged at 2000 ×g for 2 min, most of the supernatant was removed by aspiration, and weighed (W_f) which was converted into volume assuming that it was water. Cysts per ml was determined by either number of cysts per 1 mm^2 hemocytometer counting square (C_s;100 nl volume) (if $\geq 1 \times 10^4$cysts/ml) or 1 hemocytometer chamber (C_c; 10 µl). The total number of cysts per gram of feces was calculated by following formula.

$$\text{Cysts/g} = [(W_f\text{-}t_E)\ \text{x1 ml/g x}\ (C_s \times 10{,}000 + C_C \times 100)]/(W_i\text{-}t_L)$$

2.4. DNA extraction, PCR, and sequencing

DNA was obtained from 1×10^6 mouse derived *G. duodenalis* cysts or 1×10^6 archived frozen trophozoites originally isolated from gerbil derived *G. duodenalis* cysts. Cyst and trophozoites were lysed by 8 freeze-thaw cycles (1 min liquid N_2; 1 min 95 °C) and the DNA extracted using the DNA mini-kit (Qiagen, Valencia, CA) following the tissue protocol. A 583 bp *β*-giardin PCR was designed using the following primers: forward 5′-AGC GCC AGG CCT CGT T-3′and reverse 5′-GCT TAG TGC TTT GTG ACC ATC G-3′primers. Each 50 µl *β*-giardin reaction was performed in triplicate with 1.5 mM $MgCl_2$, 1 X PCR II buffer, 200 nM each primer, 5 U AmpliTaq Gold (Applied Biosystems), 200 µM dNTPs (Promega, Madison, WI), and 5 µl DNA. Thermocycling conditions were 5 min at 95 °C, followed by 40 cycles at: 95 °C 30 s, 63 °C 30 s, and 72 °C 1 min; and then 10 min at 72 °C using the GeneAmp 9700 (Applied Biosystems). Amplicon sizes were verified using E-gels following manufacturer's protocol (Life Technologies). All sequences were bi-directionally sequenced using the same primers listed above using the ABI PRISM Big Dye Terminator cycle sequencing kit with the ABI PRISM 3730xl DNA Analyzer (Applied Biosystems). Sequences analyzed and aligned using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/) and Lasergene software (DNASTAR Inc., Madison, WI).

Nucleotide sequence data reported in this paper are available in GenBank™ under the following accession numbers: MH669074 and MH669075.

2.5. Statistical analyses

A student *t*-test was used to determine statistical significance. $P \leq 0.05$ were considered significant. Microsoft Excel was used to perform all statistical analyses reported.

Table 1
Giardia duodenalis cyst yields in overnight fecal collections by two animal models.

Model	Number of collections	Total cysts/animal* (mean ± SD)
Immunosuppressed CF-1 Mouse	35	$7.8 \times 10^6 \pm 4.4 \times 10^6$
Mongolian Gerbil	35	$2.5 \times 10^6 \pm 1.8 \times 10^6$

*Significantly different ($P < 0.005$).

3. Results

3.1. G. duodenalis cyst propagation in two animal models

The average number of cysts per animal collected in 16–18 h collection periods was determined for 34 propagation studies of each model. The results in Table 1 show that the average number of cysts per CF-1 mouse was significantly higher than the gerbil ($7.8 \times 10^6 \pm 4.4 \times 10^6$ [SD] cysts/mouse and $2.5 \times 10^7 \pm 1.8 \times 10^6$ [SD] cysts/gerbil; $p < 0.001$).

3.2. Pattern of infection in the mouse model

Fig. 1 shows the number of *G. duodenalis* cysts/g shed in the feces starting on day 2. No cysts were detected on day 2 post-exposure, only 50% of the mice shed cysts on day 3, and on the remaining days all mice shed cysts, except for one mouse on day nine. It should be noted that some mice died during the shed period and thus the mouse number was not constant. The mean number of cysts/g shed by day was 1.1×10^6 cysts/g and ranged from 4.4×10^4 (day 3) to 2.6×10^6 cyst/g (day 5). The number of cysts/g shed by mouse per day ranged from 1.5×10^2 to 1.1×10^7 cysts/g. There was no apparent shed cycle or pattern observed either by daily mean or by individual mouse (data not shown). To verify that immunosuppression is required for *G. duodenalis* cyst propagation in mice, immunosuppression was stopped on day 24. Fecal monitoring continued beyond day 24 and both the cysts/g and percent of mice shedding decreased incrementally to none by day 30. Monitoring continued seven more days after the last positive sample to verify the clearance of infection (Fig. 2).

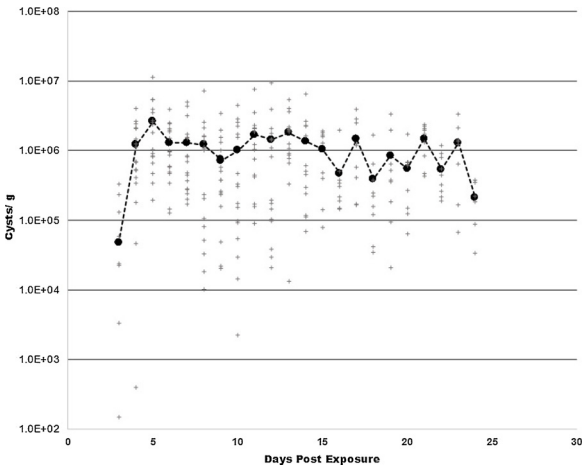

Fig. 1. *Giardia duodenalis* cyst release pattern by the mouse model. The number of cysts/g feces per mouse is represented by (+) and the mean cysts/g propagation is represented by (●). Only animals that shed cysts are reported.

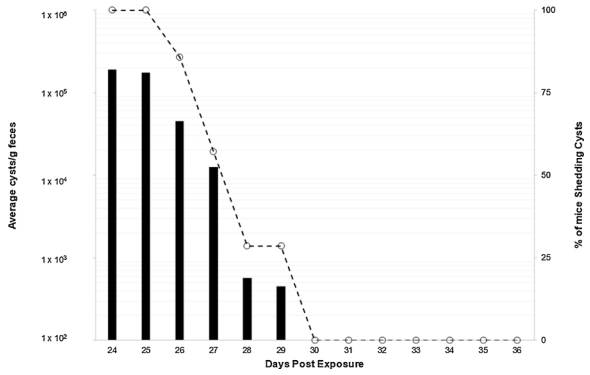

Fig. 2. *Giardia duodenalis* cyst shed pattern after stopping dexamethasone treatment on day 24 post-exposure. The mean cysts/g of feces from all mice is represented by the columns and plotted on the left-side y-axis. The percent of all mice shedding cysts are open circles and plotted on the right-side y-axis. Note: day 24 results are also shown in Fig. 1.

3.3. Sequence analysis

β-giardin PCR amplicons of DNA isolated from gerbil and mouse derived *G. duodenalis* cysts were sequenced and aligned. The PCR sensitivity with this amplicon is < 10 cysts (data not shown). It is important to note that the gerbil derived *G. duodenalis* H3 (Assemblage B) cysts were used to initially infect the CF-1 mice and then sequenced after 34 serial passages through the mouse model. The *β*-giardin sequence results showed that the gerbil and mouse sequences are identical to each other in the forward and reverse direction (data not shown). In addition, the *β*-giardin sequence results were also compared to two *G. duodenalis* reference strains M36728.1 (Portland strain; Assemblage A) and DQ116605.1 (H3 strain; Assemblage B) (Fig. 3). The mouse, gerbil, and DQ116605.1 sequences were identical to each other, in contrast the reference Portland strain M36728.1 which shared only 95% identity to the H3 sequences.

4. Discussion

This study showed that the immunosuppressed CF-1 mouse model can produce the same number of *G. duodenalis* cysts using significantly fewer animals compared to the gerbil model. In addition, the mouse model had a prolonged patency period averaging 1.1×10^6 cysts/g being shed for at least 24 days with a pre-patent period of approximately 3 days (Fig. 1). There was substantial variation in the number of cysts per gram of feces by both individual mouse and daily mean which was likely due to the limitations of the enumeration method and/or natural variation. It is also possible that the increase in fecal cyst burden in mice could be due to their immunosuppressed condition. This extended patency period was in contrast with the short *G. muris* cyst patency period reported in mice or the intermittent patency period pattern in reported in *G. duodenalis* infected gerbils (Faubert et al., 1983; Visvesvara et al., 1988). A recent study reported that sex of the gerbil may influence shedding pattern; however, the overall cyst shed pattern remained intermittent (Pecková et al., 2018). Other than overall patency pattern through time, it is difficult to directly compare these studies because they used different sampling methods, processing methods, and *G. duodenalis* isolates. After stopping the immunosuppression protocol, the cyst output rapidly decreased to zero within a few days and remained cyst free for one week indicating that the mice cleared infection and immunosuppression is required to maintain infection (Fig. 2).

Because the models use different hosts, it is possible that some unknown host factor could affect the cysts which could affect cyst behavior or serial passage in an alternative host could allow for genetic drift. To test for genetic drift, the *β*-giardin gene was sequenced before and after 34 serial passages in the mouse model and compared to the original DNA from cysts acquired from the gerbil model. The *β*-giardin sequences were identical to each other as well as to a published *G. duodenalis* Assemblage B H3 isolate strain (DQ116605.1) demonstrating no genetic drift was detected at this locus, and that it differed from an Assemblage A isolate sequence (Portland strain, M36728.1).

In conclusion, while both the mouse and the gerbil models produce high concentrations of *G. duodenalis* cysts, the mouse model shed significantly more cysts per animal with a much longer patency period. The mouse derived cysts can be a suitable source of cysts for studying the basic biology of *G. duodenalis*. Furthermore, no significant genetic differences were detected in *β*-giardin sequences between *G. duodenalis* cysts in gerbils and mouse after prolonged serial passages through the

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                Forward primer
                AGCGCCAGGC CTCGTT
M36728.1        AGCGCCAGGC CTCGTTCGAG GACATCCGCG AGGAGGTCAA GAAGTCCGCC GACAACATGT ACCTAACGAT CAAGGAGGAG ATCGACACCA TGGCTGCAAA   338
DQ116605.1      .......... .......... .......... ....A..... ......T... .......... ....G..... .......... .......... ....C.....
Gd (gerbil)     ---------. .......... .......... ....A..... ......T... .......... ....G..... .......... .......... ....C.....
Gd (CF-1)       ---------. .......... .......... ....A..... ......T... .......... ....G..... .......... .......... ....C.....

M36728.1        CTTCCGCAAG TCCCTTGCGG AGATGGGCGA CACACTCAAC AACGTTGAGA CAAATCTCCA GAACCAGATC GCCATCCATA ACGACGCCAT CGCGGCTCTC   438
DQ116605.1      .......... ..T.....T. .......... T..G...... .....C.... .G..C..... .......... ........C. .......... ...G..C...
Gd (gerbil)     .......... ..T.....T. .......... T..G...... .....C.... .G..C..... .......... ........C. .......... ...G..C...
Gd (CF-1)       .......... ..T.....T. .......... T..G...... .....C.... .G..C..... .......... ........C. .......... ...G..C...

M36728.1        AGGAAGGAGG CCCTCAAGAG CCTGAACGAT CTCGAGACGG GCATTGCCAC GGAGAACGCA GAAAGGAAGA AGATGTACGA CCAGCTCAAC GAGAAGGTCG   538
DQ116605.1      .......... .......... .........C ........A. ....C..... .........C ..G....... .......T.. .......... .....A....
Gd (gerbil)     .......... .......... .........C ........A. ....C..... .........C ..G....... .......T.. .......... .....A....
Gd (CF-1)       .......... .......... .........C ........A. ....C..... .........C ..G....... .......T.. .......... .....A....

M36728.1        CAGAGGGCTT CGCCCGCATC TCCGCCGCGA TCGAGAAGGA GACGATCGCC CGCGAGAGGG CCGTTAGCGC TGCCACGACA GAAGCGCTCA CAAACACGAA   638
DQ116605.1      .......... .......... .....T..C. .......... .......... .......... ....C..... C......... ..G..C.... ..........
Gd (gerbil)     .......... .......... .....T..C. .......... .......... .......... ....C..... C......... ..G..C.... ..........
Gd (CF-1)       .......... .......... .....T..C. .......... .......... .......... ....C..... C......... ..G..C.... ..........

M36728.1        GCTCGTCGAG AAGTGCGTCA ACGAGCAGCT CGAGAACGTC GCCTCGGAGA TCCGCGCTAT CCAGGAGGAG ATCGACCGCG AGAAGGCCGA ACGCAAGGAG   738
DQ116605.1      .......... .......... .......... .......... .......... .......C.. .......... .......... .......A.. G.........
Gd (gerbil)     .......... .......... .......... .......... .......... .......C.. .......... .......... .......A.. G.........
Gd (CF-1)       .......... .......... .......... .......... .......... .......C.. .......... .......... .......A.. G.........

M36728.1        GCAGAGGACA AGATCGTCAA CACTCTCGAG GACGTCGTCT CGAAGATCCA GGGCGGCCTC TCGATGGTCA CAAAGCACTA AGC 811
DQ116605.1      .......... .......... ...A...... .......... .--------- ---------- ---------- ---------- ---
Gd (gerbil)     .......... .......... ...A...... .......... .......... A.....T..T .......... .......... ...
Gd (CF-1)       .......... .......... ...A...... .......... .......... A.....T..T .......... .......... ...
                                                                                   GATGGTCA CAAAGCACTA AGC
                                                                                   Reverse primer
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Fig. 3. Alignment of *β*-giardin sequences Row 1: *Giardia duodenalis* primary reference sequence (M36278.1 Portland strain, Assemblage A); Row 2: *G. duodenalis* reference sequence (DQ116605.1 H3 strain, Assemblage B); Row 3: gerbil derived *G. duodenalis* (Gd (gerbil)); and Row 4: mouse derived *G. duodenalis* (Gd (CF-1)). Dots represent identical nucleotide to primary reference sequence, letters represent nucleic acid substitution (A,T,C, and G), and dashes represent either area of no overlap or unresolvable sequences.

immunosuppressed mouse model. Therefore, the mouse model is a viable and more cost-effective alternative for the propagation of *G. duodenalis* cysts.

Ethics: All animal studies were overseen and monitored by the Cincinnati EPA IACUC.

Conflicts of interest

None.

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