International Journal of Infectious Diseases 16 (2012) e350–e353

Contents lists available at SciVerse ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

ECC–RT-PCR: a new method to determine the viability and infectivity of Giardia cysts

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ARTICLE INFO

Article history:
Received 3 August 2011
Received in revised form 3 January 2012
Accepted 11 January 2012

Corresponding Editor: Karamchand Ramotar, Ottawa, Canada

Keywords:
Giardiasis
Molecular diagnostic method
Infectious Giardia cysts

SUMMARY

Background: *Giardia sp* is a major cause of diarrheal illness worldwide, and millions of people are infected each year. Rapid methods to determine the infectivity and virulence of isolates are critical for the development of intervention strategies to control the transmission of *Giardia sp* cysts, which occurs through contaminated surfaces, food, and water. However, determining the viability, infectivity, and virulence of *Giardia sp* cysts using molecular methods is a technical challenge because of the lack of a cell culture model.

Method: This study was designed to evaluate mRNA expression in trophozoites and to assess trophozoite attachment to cell monolayer and changes in transcellular resistance as an indicator of *Giardia sp* viability and infectivity. Heat shock mRNA in Giardia cysts and variant-specific protein (VSP) mRNA in trophozoites were quantified by reverse transcription polymerase chain reaction (RT-PCR). C2bb (Caco-2) cells were grown on transwell chambers to study the attachment of trophozoites, changes in transcellular resistance, and expression of VSP in trophozoites.

Results: The results of these molecular and cell culture studies indicate a direct linear correlation between the viability and infectivity of fresh stocks of *Giardia sp* cysts. The attachment of trophozoites to cell monolayer, expression of VSP, and change in the transcellular resistance was directly correlated with their infectivity in neonatal mice. PCR was successfully combined with the electrophysiological analysis of cell culture (ECC–RT-PCR) post-trophozoite attachment.

Conclusion: This study shows that the ECC–RT-PCR, a new integrated cell culture assay, can be used as a rapid and cost-effective tool for assessing the viability and infectivity of environmental isolates of *Giardia sp* cysts.

1. Introduction

Giardia sp is a common cause of gastrointestinal infections in humans and many non-human mammals. Giardiasis is reported in both developing and developed countries, however the incidence is generally higher in underdeveloped and low-income countries. In developed countries, approximately 5% of gastroenteritis cases are caused by *Giardia sp*, compared to 15–55% of cases in developing countries.[1–3] In the USA alone, an estimated 45 000 cases of Giardiasis occur annually.[1]

The *Giardia sp* parasite can proliferate extensively in the intestine of the infected subject, releasing millions of cysts in the feces. *Giardia sp* infections are acquired through the fecal–oral route, often through the ingestion of contaminated water or food. In economically disadvantaged communities, person-to-person transmission is also reported due to poor hand hygiene practices. Typically such transmission has been documented in children attending day care facilities in low-income communities.[4]

Infection with *Giardia sp* during early childhood has been reported to be responsible for impaired cognitive function and a failure to thrive.[5,6] Despite the health significance of its incidence, *Giardia sp* has largely been ignored as a public health threat during the last century; it was finally included in the 'Neglected Diseases Initiative' in 2004.[7] In 2001, the US Environmental Protection Agency (US EPA) established a method to detect *Giardia sp* cysts in environmental water samples.[8] This is a multi-step assay using *Giardia sp*-specific antibody and a fluorescence microscope. However, the method is not able to determine the viability/infectivity of *Giardia sp* cysts.

Information on the viability/infectivity of cysts is a critical factor in establishing the public health significance of environmentally prevalent *Giardia sp* cysts.[9] Several molecular-based methods such as RT-PCR, DNA intercalating dye, immunofluorescence, and flow cytometry have been reported for assessing the

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doi:10.1016/j.ijid.2012.01.004

viability of *Giardia sp* cysts.[10–14] Animal infectivity has been considered the gold standard to determine the infectivity of *Giardia sp* cysts.[15]

Heat shock protein (HSP) has been used widely to study the viability of parasites such as Cryptosporidium and Giardia;[16] however, its relationship with the infectivity status of *Giardia sp* has been questioned.[17] The Giardia trophozoites that are attached to the human intestinal tract during infection are covered with a layer of variant-specific surface protein (VSP).[18–20] Caco-2 is the most commonly used cell line to study host–parasite interactions,[21–25] because of the ability of this cell line to develop apical brush border membranes, which express brush border enzymes such as disaccharidases and alkaline phosphatase.[22] These features give this cell line functional and structural similarity to the digestive tract.

In this study we developed a method combining the measurement of electrical resistance in the trans-cell culture monolayer electrophysiologically post-trophozoite attachment and an RT-PCR assay (ECC–RT-PCR) to detect the viable/infective *Giardia muris* cysts in vitro, and compared this ECC–RT-PCR assay with the mouse infectivity assay.

2. Methods

2.1. Parasite stocks and excystation

In the present study *G. muris* cysts were used as a surrogate for *Giardia duodenalis* or *Giardia intestinalis*. The US EPA has accepted *G. muris* as an acceptable substitute for *Giardia sp* infecting humans.[26] The *G. muris* cysts were obtained from Dr Shivaji Ramalingam (Oregon Health Sciences University, Portland, OR, USA). Upon receipt, the stocks of cysts were stored at 4 °C until used in the assays. Before the experiment, cyst stocks were washed with and re-suspended in fresh phosphate buffered saline (PBS). In vitro excystation was performed in two phases, as previously described.[27]

2.2. RNA extraction

The Dynabeads® mRNA Direct Kit (Invitrogen, Grand Island, NY, USA) was used for the extraction of mRNA from Giardia cysts. Cyst stocks were diluted to the desired concentrations by 10-fold serial dilutions with PBS. To confirm the accuracy of the dilution process, an aliquot from each was counted using a hemocytometer (Hausser Scientific, Horsham, PA, USA). To induce *hsp*70 mRNA transcription, cysts were exposed to 42 °C for 20 min, immediately followed by six cycles of freeze–thaw. The freeze-thawed sample was processed using the Dynabeads mRNA Direct Kit (Invitrogen) in accordance with the manufacturer's instructions.

2.3. RT-PCR

The RT-PCR conditions involved a reverse transcription step followed by 40 cycles of 95 °C denaturation for 1 min, 50 °C annealing for 1 min, and 72 °C extension for 2 min, followed by a final extension at 72 °C for 7 min. The amplified product was analyzed by gel electrophoresis, followed by ethidium bromide staining (0.5 μg/ml), UV transillumination (Kodak, Rochester, NY, USA), and image capture using a Kodak camera (Biophotonics, Ann Arbor, MI, USA). The target products were quantified by image analyses of each band on the electrophoresis gel. The primers used for the RT-PCR are shown in the Table 1.

Table 1
Primers for the RT-PCR assays

Gene	Primer name	Primer sequences	Product (bp)
HSP	HSPGm 1F	5′-GTATCTGTGACCCGT CCGAG-3′	163
	HSPGm 1R	5′-AGGGCT CCGGCATAACTTTCC-3′	
VSP	VSPGm 1 F	T C A T G T G C A C T G A A G C A A C A	122
	VSPGm 1 R	C A G G G G G T T A C C A C A A G C T A	

RT-PCR, reverse transcription polymerase chain reaction; HSP, heat shock protein; VSP, variant-specific protein.

2.4. Cell culture and PCR

C2bb cells (Caco-2; human colonic adenocarcinoma) were maintained in 75-cm^2 cell culture flasks (Corning Costar, Cambridge, MA, USA), as recommended by the American Type Culture Collection (ATCC, Manassas, VA, USA). For the measurement of trans-monolayer electrical resistance, monolayers were grown in 24-mm Transwell chambers (Corning Costar) by adding approximately 1.6×10^5 cells per well. Cell monolayers were washed in fresh serum-free cell culture medium, incubated at 37 °C for 20 min, and infected with a series (5, 50, 500, 5000, and 50 000) of excysted cysts of *G. muris*. Excystation was performed as previously described.[27]

The trans-monolayer electrical resistance was measured by connecting an electrode to the apical and basal reservoirs of the transwell chambers (Corning Costar). The effect of *G. muris* infection on the cell monolayer was studied by measuring the resistance of non-infected monolayers and of monolayers infected with specified numbers of cysts. After measuring the trans-monolayer electrical resistance, inserts were removed from the wells and the medium carefully removed from the wells. The inserts were rinsed with Tris buffer, and 750 μl of trypsin–EDTA was added to dislodge the cells (along with trophozoites). Harvested cells were processed for RT-PCR as described previously.[28]

2.5. Mouse infectivity assay

For each treatment, four neonatal mice (6 days old) were inoculated by intrapharyngeal delivery of specified numbers (10^2–10^4) of *G. muris* cysts in a volume of 30 μl. Mice used for the infectivity assays were handled in accordance with the protocols approved by the in-house Animal Care and Use Committee. Feces were collected from the infected mice at 5 and 6 days post-infection, and were analyzed for Giardia cysts.[28] At 6 days after infection, the mice were euthanized using chloroform. The ileal tissue was collected and was processed to obtain the total DNA, as described by Jenkins et al.[25] Ileal DNA was analyzed for the presence of *G. muris* DNA using primers specific for the *hsp*70 gene. PCR products were analyzed as described previously.[28]

3. Results and discussion

3.1. Comparison of viability and infectivity of cysts

Throughout these experiments fresh cyst stocks were used, because stock age can significantly impact the infectivity of *G. muris* cysts (Dr Ramalingam, personal communication). Mouse infectivity assays showed that the infectivity of *G. muris* cysts was reflected by the viability status (% excystation) of the cyst stock (Table 2). The data show that cyst viability should be considered

Table 2
Relationship between the viability of *Giardia muris* cysts and infectivity in the mice

Number of replicates	Mouse infectivity (ID_{50})	% Excystation
3	1.15	98
3	4.2	91

ID_{50}, the dose at which 50% of expose subjects are infected exposed mice.

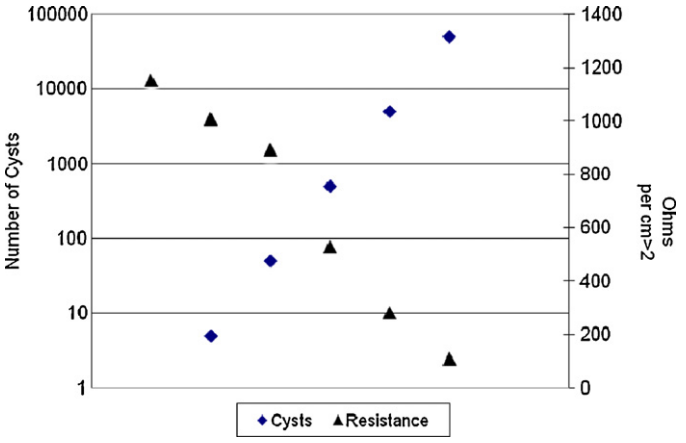

Figure 1. Dose-dependent change in the trans-monolayer resistance of Caco-2 cells after infection with *Giardia muris*.

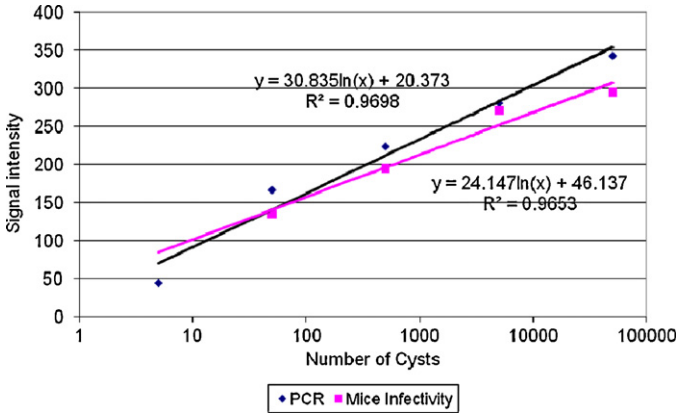

Figure 2. Comparison of the mouse infectivity assay with the ECC–RT-PCR to determine the infectivity of cysts (the cell culture PCR is based on the expression of VSP in trophozoites attached to the monolayer in transwell chambers).

even before conducting infectivity assays because a non-viable fraction of the stock may impact the infective dose and result in biased data, even from the animal infectivity method, the gold standard for such studies.

3.2. Impact of the infection process on the trans-monolayer resistance

Experiments were performed to investigate the impact of *G. muris* infection of a C2bb monolayer on trans-monolayer resistance; results are presented in Figure 1. Giardia-infected C2bb cell monolayers demonstrated a dose- and time-dependent change (inverse correlation) in the trans-monolayer resistance (Figure 1). No change in the trans-monolayer resistance was observed in the non-infected monolayer. The pathobiological basis for the profuse watery diarrhea associated with giardiasis is not well understood. Very limited information is available on the virulence factor involved in giardiasis. Some of the main factors of Giardia virulence identified so far are the ventral adhesive disc and surface lectins. These enable colonization of the intestinal endothelium, which is associated with a reduction in the height of the brush border microvilli.[29] These changes in the intestinal endothelium and the compromise of the gut epithelia likely change the trans-membrane resistance. This change in trans-membrane resistance may be correlated with the parasite infection. The C2bb cell line used in this study is known to make brush border membranes and has been used for the study of pathogen attachment to the host intestinal endothelium.[22–24] Studying the resistance across the cell monolayer with the brush border membrane, along with a gene expression profile, may enhance the validity of the ECC–RT-PCR method for the determination of infectivity.

3.3. Comparison of the mouse infectivity assay with ECC–RT-PCR to study the infectivity of cysts

The infectivity of *G. muris* cysts was determined by a mouse infectivity assay and the level of VSP mRNA expression; results are presented in Figure 2. The experiment was focused on comparing the determination of cyst infectivity by mouse infectivity and molecular methods. A good agreement between these two methods was noted. The *R*-value for these assays was calculated to be 0.878 (*R*-values greater than 0.9 are very high and likely to be significant). These results suggest that the ECC–RT-PCR assay and mouse infectivity assay are fairly well correlated under these test conditions.

Data were collected as the percent infectivity for each set of assays. A similar decline in cyst infectivity was recorded using both methods. The *R*-value for these assays was calculated to be 0.905 (*R*-values greater than 0.9 are very high and likely to be significant). These results suggest that the ECC–RT-PCR assay and mouse infectivity assay are fairly well correlated under these test conditions. Different assays have been used to compare viability and infectivity of Cryptosporidium oocysts and Giardia cysts.[15,25] Traditionally neonatal mouse infectivity has been considered the gold standard and most sensitive assay for determining the infectivity of Giardia cysts and Cryptosporidium oocysts. The correlation of gene expression with infectivity assays has been debated;[10] however, the new ECC–RT-PCR assay includes recording the changes in trans-monolayer resistance, which is an indication of the infectivity process. These changes in trans-monolayer resistance reflect the impaired integrity of the cell membrane, which may be manifested as diarrhea in the animal infection. The results of the present study show the ECC–RT-PCR assay to be slightly more sensitive than the mouse infectivity assay. To our knowledge this is the first time such an approach has been applied to compare infectious Giardia cysts in vitro and in vivo. The methodology developed here could be applied to study the diagnostic pathophysiology and infectious traits of other microbial systems (protozoa, bacteria, viruses).

Acknowledgements

Dr Alum's research is supported by Reckitt Benckiser, and JRR and MKI are engaged in R&D work at Reckitt Benckiser Inc., Montvale, NJ, USA.

Conflict of interest: No conflict of interest to declare.

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