Development and comparison of ECC-rtPCR with *in vivo* method to determine the viability and infectivity of Giardial cysts

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ABSTRACT

Globally, *Giardia* is one of the major cause of diarrheal illnesses and rapid diagnostic methods differentiating infectious cysts are critical for developing intervention strategies through contaminated surfaces, food and water. This is currently hampered by lack of *in vitro* model. We evaluated mRNA expression in trophozoites and their attachment to CaCo2 (C2bb) cell monolayer and changes in trans-cellular resistance as an indicator of *Giardia* viability and infectivity. Heat shock mRNA in *Giardia* cysts and variant specific protein (VSP) mRNA in trophozoites were quantified by RT-PCR. When compared with neonatal mice infectivity, the attachment of trophozoites to cell monolayer, expression of VSP and change in the trans-cellular resistance, the infectivity directly correlated with infectivity in neonatal mice. This study highlights the use of molecular method combined with electrophysiological analysis of cell culture (ECC-rtPCR) post-trophozoite's attachment for assessing viability and infectivity of environmental isolates of *Giardial* cysts.

INTRODUCTION

Giardia is a common cause of gastrointestinal infections in humans and many non-human mammals. Giardiasis is reported in both developing and developed countries; however its incidence is generally higher in underdeveloped and low-income countries. In developed countries, approximately 5% of the cases of gastroenteritis are caused by *Giardia*. In the US alone annual cases of Giardiasis are estimated at 45,000[1].

The *Giardia* parasite can extensively proliferate in the intestine of infected subject thus releasing millions of cysts in the feces of infected host. *Giardia* infections are acquired through fecal-oral route often through ingestion of contaminated water and 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[2].

Infection with *Giardia* during early childhood has been reported to be responsible for impaired cognitive function and poor thrivability [3, 4]. Despite the health significance of its incidence, *Giardia* has been largely ignored as public health threat during last century, and finally it was included in the 'Neglected Diseases Initiative' in 2004 [5]. In 2001, the U.S. Environmental Protection Agency established a method to detect *Giardia* cysts in aquatic environmental samples [6]. This is a multistep assay using *Giardia* specific antibody and fluorescence microscope. However, this method is not able to determine the viability/infectivity of *Giardia* cysts.

Information on the viability/infectivity of cysts is a critical factor in establishing the public health significance of environmentally prevalent *Giardia* cysts[7]. Several molecular-based methods have been reported for assessing the viability of *Giardia* cysts[8, 9]. Animal infectivity has been considered as gold standard to determine the infectivity of *Giardia* cysts[10].

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

RESULTS AND DISCUSSION

Comparison of viability and infectivity of cysts

Throughout these experiments fresh cysts stocks were used because stock age can significantly impact the infectivity of *Giardia muris* cysts (Dr. Ramalingam, personal communication). Mice infectivity assays showed that *Giardia muris* cysts infectivity is reflected by the viability status (% excystation) of the cysts stock (Table 1). The data show that cysts viability should be considered even before conducting infectivity assays because non-viable fraction of the stock may impact the infective dose and result in a biased data even by animal infectivity, the gold standard for such studies.

Table 1. Relation between the viability of *Giardia muris* cysts on the mice infectivity

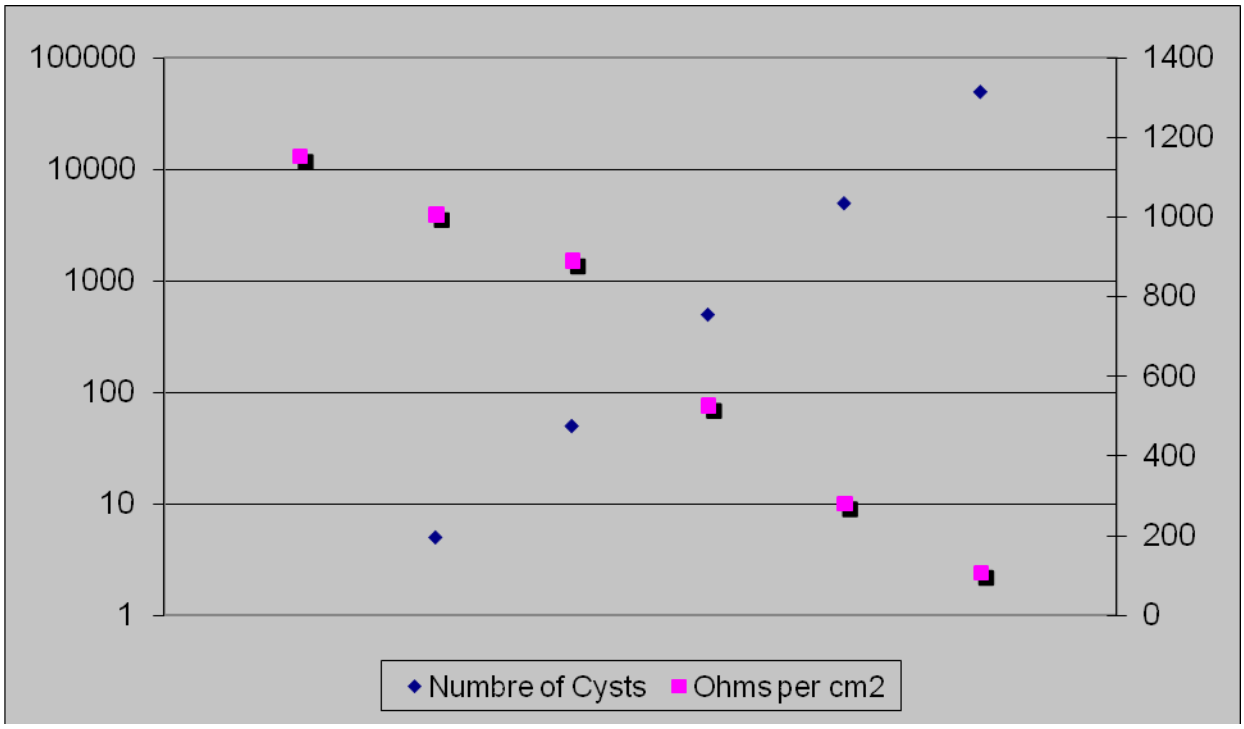
Experiment	Mice infectivity (ID50)	% Excystation
3	1.15	98
3	4.2	91

Impact of infection process on the transmonolayer resistance

Experiments were performed to investigate the impact of *Giardia muris* infection of C2bb monolayer and 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 (Fig. 1). In non-infected monolayer no change in the trans-monolayer resistance was observed. 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, which enable trophozoite colonization of intestinal endothelium, which is associated with reduction in the height of brush border microvilli[11]. These changes in intestinal endothelium and subsequent compromization of gut epithelia likely change the trans-membrane resistance. This change in trans-membrane resistance may be correlated with the parasite infection. The CaCo2 (C2bb) cell line used in this study is known to make brush border membranes and has been used for studying the pathogen attachment to the host intestinal endothelium[12, 13, 14]. Studying the resistance across the cell monolayer with brush border

membrane along with a gene expression profile may enhance the validity of CC-rtPCR method for infectivity determination.

Figure 1. Dose-dependent change in the transmonolayer resistance of CaCo2 cells after infection with *Giardia muris*



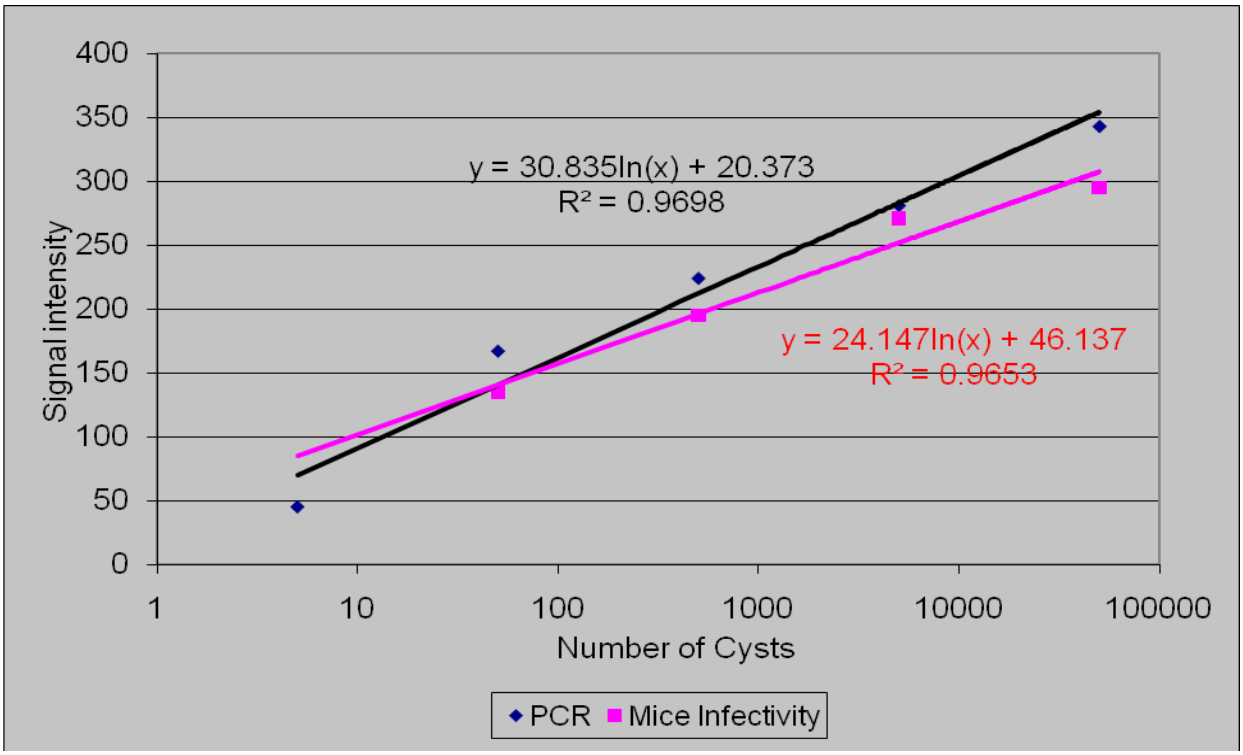
Comparison of mice infectivity assay with ECC-rtPCR to study the infectivity of cysts

The infectivity of *Giardia muris* cysts were determined by mice infectivity assay and level of VSP mRNA expression and results are presented in Figure 2. The experiment was focused on comparing the cysts infectivity determination by mice 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 ECC-rtPCR assay and mice infectivity assay are fairly correlated under these test conditions. Different assays have been used to compare viability and infectivity of *Cryptosporidium* oocysts and *Giardia* cysts[10, 15].

Therefore, data collected as percent infectivity for each set of assays. Similar decline in cysts 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 ECC-rtPCR assay and mice infectivity assay are fairly correlated under these test conditions. Different assays have been used to compare viability and infectivity of Cryptosporidial oocysts and Giardial cysts. Traditionally neonatal mouse infectivity has been considered as the gold standard and as the most sensitive assay for determining the infectivity of Giardial cysts and Cryptosporidial oocysts. Correlation of gene expression with infectivity assays has been debated[10]; however, the new ECC-rtPCR assays include recording the changes in trans-monolayer resistance, which is indication of the infectivity process. These changes in trans-monolayer resistance reflect the impaired integrity of cell membrane which may be manifested as diarrhea in the animal infectivity. The results of present study show that ECC-rtPCR assay to be slightly more sensitive than mice infectivity assay. To our knowledge this the first time such approach has been applied to compare infectious Giardial cysts *in vitro* and *in vivo.* The methodology developed here can be applied to study diagnostic pathophysiology and infectious traits of other microbial systems (protozoa, bacteria, viruses).

Figure 2. Comparison of mice infectivity assay with ECC-rtPCR to study the infectivity of cysts (CC-PCR is based on the VSP expression in trophozoites attached to monolayer in transwell chambers)



METHODS

Parasites stocks and excystation

In the present study *Giardia muris* cysts were used as surrogate of *Giardia duodenalis* or *Giardia intestinalis.* The U.S.EPA has accepted *Giardia muris* as an acceptable substitute for *Giardia* species infecting humans[16]. The *Giardia* muris cysts were obtained from Dr Shivaji Ramalingam (Oregon Health Sciences University, Portland, Oregon). Upon receipt, the stocks of

cysts were stored at 4°C until used for assays. Before the experiment, cysts stocks were washed with and re-suspended in fresh phosphate buffer saline (PBS). *In vitro* excystation was performed in two phases as previously described[17].

RNA extraction

Dynabeads® mRNA Direct Kit (Invitrogen) was used for the extraction of mRNA from *Giardia* cysts. Cysts stocks were diluted to desired concentrations using 10-fold serial dilution of PBS. To confirm the accuracy of dilution process, an aliquot from each was counted using Hemocytometer. To induce hsp70 mRNA transcription, cysts were exposed to 42C for 20 min. immediately, followed by six cycles of freeze thaw. The freeze-thawed sample was processed using Dynabeads® mRNA Direct Kit according to manufacturer instruction.

RT-PCR

The RT-PCR conditions involved RT 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, UV transillumination, and image capture using a Kodak camera (Biophotonics, Ann Arbor, Mich). The target products were quantified by image analyses of each band on electrophoreses gel. The primers used for RT-PCR are reported in the Table 2.

Table 2. Primers for RT-PCR assays

Gene	Primer Name	Primer Sequences	Product (pb)
Heat Shock	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	

Cell culture and CC-PCR

Caco-2 cells (C2bb) (human colonic adenocarcinoma) were maintained in 75-cm2 cell culture flasks (Corning) as recommended by American Type culture collection (ATCC). For trans-monolayer electrical resistance measurement, monolayers were grown in Transwell chambers and washed in fresh cell culture media with 0% FBS and incubated at 37°C for 20min. Monolayer was infected with excysted cysts of *Giardia muris*. Excystation was performed as previously described[17].

The trans-monolayer electrical resistance was measured by connecting electrode to the apical and basal reservoirs of the transwell chambers. Effect of *Giardia* infection on cell monolayer was studied by measuring the resistance for non-infected monolayer and monolayers infected with specified number of cysts. Trophozoites were harvested from transwell chamber and proceeded for RT-PCR as described earlier.

Mouse Infectivity Assay

For each treatment, four neonatal mice (6 day old) were inoculated by intra pharyngeal delivery of specified numbers (10^2-10^4) cysts of *G. muris* in 30µL. Mice used for infectivity assays were

handled in accordance with the protocols approved by in-house Animal Care and Use Committee. From the infected mice, 5 and 6 days post-infection, feces were collected and analyzed for *Giardia* cysts. Six days after infection, mice were euthanized using chloroform. The ileal tissue was collected and was processed to collect total DNA as described by Jenkins[15]. Ileal DNA was analyzed for the presence of *G. muris* DNA by using primers specific for HSP 70 gene. PCR products were analyzed as described earlier[10].

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