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Comparison of molecular markers for determining the viability and infectivity of Cryptosporidium oocysts and validation of molecular methods against animal infectivity assay

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SUMMARY

Background: Globally, disinfectants are widely used to intervene in the dissemination of Cryptosporidium oocysts. However, extensive investigations of oocyst inactivation by various disinfectants are not feasible due to the limitations imposed by animal infectivity methods. Molecular techniques provide an alternative strategy; however, non-metabolic genes have been used as markers for determining viability/infectivity.
Methods: In this study we used amyloglucosidase (AG) – a metabolic protein – as a marker to determine viability/infectivity of Cryptosporidium. Oocysts were exposed to 6% hydrogen peroxide for 2 min. Samples were analyzed by cell culture polymerase chain reaction (CC-PCR) using PCR primers specific for heat shock protein 70 (hsp70) and AG. Both target genes were amplified with the same level of intensity.
Results: Based on the results it can be concluded that AG is a valid target for the study of environmental survival and for the evaluation of the efficacy of microbicides against Cryptosporidium using molecular and cellular assays. Comparison of the CC-PCR assay and mouse infectivity assay showed a fairly good correlation under these test conditions.
Conclusion: Results indicate that the CC-PCR assay presents a valid and cost-effective alternative to the mouse infectivity assay.
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1. Introduction

Cryptosporidium is one of the most common protozoan parasites causing diarrheal illness worldwide. Cryptosporidiosis cases are most prevalent in populations in Asia, Australia, Africa, and South America, and huge outbreaks have been reported in North America and Europe.[1]

Cryptosporidium is of major human health concern due to: (1) extreme resistance of oocysts to routine disinfectants, (2) low infective dose, (3) high risk of mortality in the immunocompromised population, and (4) possibility of zoonotic transmission.[2] Cryptosporidium infections can be prevented by eliminating or reducing infectious oocysts in the environment.[1] Good hygiene and personal care practices play a critical role in reducing the infectious diseases caused by a variety of etiological agents. The effectiveness of personal hygiene practices, such as the regular use of soap in hand washing, has been well documented for the control of infectious/communicable diseases caused by bacterial and viral agents.[3] Not much information is available on the effectiveness of personal care products for the removal/elimination/inactivation of parasites found on a variety of surfaces.

A number of assays have been described for differentiating viable/infectious oocysts from dead/non-infectious oocysts.[4,5] The mouse infectivity assay has been considered the gold standard for such studies;[6] other assays include in vitro excystation, dye exclusion, cell culture, fluorescence in situ hybridization, and reverse transcriptase polymerase chain reaction (RT-PCR) directed to mRNA.[4,5,7] The method of disinfection and type of disinfectant can impact the results of these assays. Therefore, the choice of assay is critical in studying the resistance of oocysts to different disinfection processes and their survival under different environmental conditions. The objectives of this study were (1) to compare the mouse infectivity assay with the RT-PCR assay, which is based on detection of metabolic genes expressed in the target oocysts, and (2) to develop a rapid method for studying the survival of Cryptosporidium oocysts dried on a variety of experimentally-contaminated prototypical carriers simulating environmental surfaces.

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2. Materials and methods

2.1. Parasites

Cryptosporidium parvum oocysts (Iowa isolate) were obtained from the Sterling Parasitology Laboratory, University of Arizona, Tucson, AZ. The oocysts were stored in antibiotic solution (100 μg/ml penicillin and 100 μg/ml gentamicin) containing 0.01% Tween 20. The concentration of oocysts in the stock and working solution was determined by direct count using a hemocytometer. The integrated cell culture RT-PCR (ICC-RT-PCR) assay was used to detect infectious oocysts, as previously described.[8]

2.2. Disinfection and excystation procedure

The hydrogen peroxide (H1009; Sigma) stock (30% in water) was further diluted in distilled water to achieve a 6% (vol/vol) solution. Cryptosporidium oocysts (10^6) were suspended in 500 μl of freshly prepared hydrogen peroxide dilution, and incubated at room temperature (24 °C) for 1, 2, 3, 4, and 5 min. No neutralizing agent was applied, and after the exposure time, oocysts were washed three times with distilled water (2500 × *g* for 5 min). The disinfectant exposure time was considered to be the time oocysts were actually in hydrogen peroxide, excluding the time spent on subsequent washing steps. For cell culture assay, oocysts were further treated with acidified (pH 2.0) Hank's balanced salt solution (AHBSS). Different bile salt components have been reported to enhance excystation and infection of cell monolayers.[9] Bile salts have been used for a long time in excystation formulations without an understanding of the nature of their action. A recent study has shown that bile salts induce apical organelle discharge, which is essential for the gliding motility of sporozoites.[10] In most Apicomplexa, gliding motility is critical for invasion of host cells by the invasive stages such as sporozoites.[11] Based on our experience, lower cell passage compensates for the difference between the excystation protocol with or without bile salts (un-published data). Cell surface membranes are known to significantly change with increasing numbers of cell passage.[12] This is very relevant for the cell culture-based Cryptosporidium infectivity assays, as the sialic acid of glycoconjugates on the host cell surface are known to facilitate excystation of *C. parvum*[13] and glycoconjugates on the cell surface are known to change during the course of differentiation and aging of cells.[14] We believe that there is a need to standardize cell culture-based infectivity assays, and cell passage number should be considered when comparing results from such studies.

For control treatment, oocysts were subjected to all of the same experimental procedures as the oocysts from the experimental groups, except that phosphate-buffered saline (PBS) was used instead of hydrogen peroxide.

2.3. Integrated cell culture and molecular assay

An integrated cell culture polymerase chain reaction assay (ICC-PCR) was used to detect infectious oocysts, as described by Di Giovanni et al.[8] Human ileocecal adenocarcinoma (HCT-8) cells (ATCC CCL-244) were grown in a maintenance medium: RPMI 1640 supplemented with 5% fetal bovine serum (FBS), 2 mM L-glutamine, 20 mM HEPES, and 10% Opti-MEM. Maintenance medium contained penicillin, streptomycin, and amphotericin. Cell culture assays were performed in 24-well plates, which were incubated at 37 °C under an atmosphere of 5% CO_2. After 24 h, the maintenance medium was removed and replaced with growth medium, which was similar to maintenance medium with the exception of an increased (10%) concentration of FBS.

Before disinfection, oocysts were washed twice with sterile PBS (pH 7.2) in sterile 1.5-ml microcentrifuge tubes by centrifuging at 10 000 × g, and the supernatant was completely removed. Oocysts were suspended in 1 ml of 6% hydrogen peroxide for 1, 2, 3, 4, and 5 min. After the specified time, disinfectant was removed by centrifuging at 10 000 × g, and oocysts were washed with sterile PBS (pH 7.2); concentrated oocysts were excysted using AHBSS and inoculated into confluent HCT-8 cells. The cells were washed 2 h later to remove parasites (non-excysted oocysts or non-infectious sporozoites) that had not invaded the monolayer.

The cells were harvested at 48 h post-inoculation and RNA was extracted using RNeeasy Kit (Qiagen, Valencia, CA) and subjected to RT-PCR analysis. The RT-PCR conditions involved an 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, MI, USA). The target products were quantified by image analyses of each band on the electrophoresis gel. The intensity of each band was analyzed using Scion Image 4.0.2 software.

2.4. Rationale for the selection of amyloglucosidase as a marker of infectivity

Apicomplexa protozoa are known to use amylopectin granules as an energy source during survival stages.[15–17] In this situation, they are totally reliant on endogenous sources of energy for sporulation to remain viable,[18] and the stored energy is used during the excystation process and release of infective stages.[16] Prolonged storage or exposure to environmental conditions results in the depletion of these energy reserves.[19,20] In a million oocysts of *Eimeria acervulina*, the amylopectin content decreased from 33.3 μg to 1.5 μg after 6 years of storage at 4 °C.[21] A steady decrease in the infectivity of *E. acervulina* oocysts over 3, 12 and 24 months of storage was observed, and complete loss of infectivity was noted after 6 years of storage,.[21]

The enzyme amyloglucosidase is perceived to play a critical role in the utilization of this stored energy for mobilizing the infective stages during cell invasion.[22] In this study, the metabolic gene for amyloglucosidase was used as a marker of infectivity because of the putative correlation with the level of amylopectin reserves and the infectivity status of the oocysts.

2.5. Mouse infectivity assay

For each treatment, four neonatal mice (6 days old) were inoculated by intrapharyngeal delivery of 10^4 oocysts of *C. parvum* in 30 μl. Mice used for 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 analyzed for Cryptosporidium oocysts. Six days after infection, mice were euthanized using chloroform. The ileal tissue was collected and processed to obtain the total DNA, as described by Jenkins et al.[7] Ileal DNA was analyzed for the presence of *C. parvum* DNA using primers specific for the heat shock protein 70 (hsp70) gene and AG. PCR products were analyzed as described earlier.

3. Results and discussion

3.1. Comparison of metabolic and non-metabolic genes to study viability and infectivity of oocysts

Cell culture PCR assays showed that *C. parvum* oocysts lost their viability/infectivity after treatment with hydrogen peroxide. Based

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