Inactivation of *Cryptosporidium* Oocysts for Use in Immunological and Molecular Assay Applications

Poster LB-5115

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Background

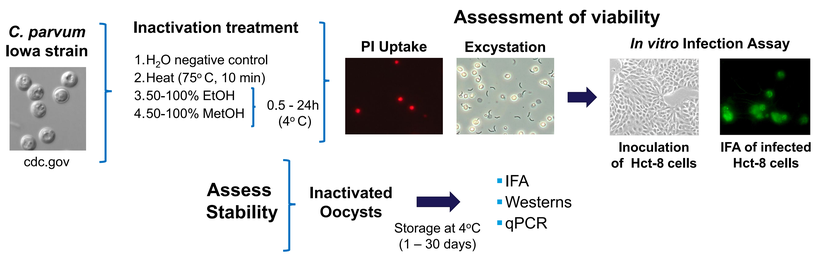
- *Cryptosporidium* spp. are obligate, intracellular parasites that can cause life-threatening diarrhea among children and immunocompromised adults [1, 2].
- Infection with the parasite is transmitted orally by thick-walled oocysts that can contaminate, persist, and resist disinfection in water and food [3].
- Previous studies reported the inactivation of oocysts by disinfectants such as sodium hypochlorite, peroxides, ozone, formaldehyde, and ammonia [4, 5]. The regular use of effective concentrations of these chemicals to produce inactivated oocysts in the laboratory is limited due to safety concerns and possible impact on the integrity of parasite antigens and nucleic acids.
- Molecular assays are replacing conventional methods for the detection of *Cryptosporidium* and other intestinal parasites in clinical and environmental samples [6, 7].
- There are limited studies on laboratory methods of oocyst inactivation that retain the properties of *Cryptosporidium* antigens and nucleic acids. Inactivated oocysts can be subsequently used in downstream research applications such as assay development performed under BSL1 conditions.

Objectives

- Evaluate simple methods of inactivation of *Cryptosporidium* oocysts that can be readily applied in the laboratory.
- Examine the loss of oocyst viability *in vitro* and determine the utility of non-viable oocysts stored for long periods of time in immunological and molecular assays.

Experimental Approach

Fig. 1. Inactivation of *Cryptosporidium parvum* oocysts



- Oocysts of *C. parvum* Iowa strain (5 x 10^6, Sterling Labs, University of Arizona) were treated with increasing concentrations of ethanol or methanol (50-100%) for 30 min to 24 h at 4° C. Oocysts suspended in water or inactivated by heat (75° C, 10 min) were used as negative or positive controls of inactivation, respectively.
- Viability assays based on propidium iodide (PI) permeability, *in vitro* excystation of sporozoites, and infection of the Hct-8 cell line (ATCC® CCL-244™) were used to evaluate the effectiveness of the treatments.
- Inactivated oocysts recovered from the most effective treatments were tested in immunological (IFA, westerns) and molecular (qPCR) assays to determine their utility as reference reagents. The stability of inactivated oocysts in these assays was examined following long-term storage at 4° C.

Results

Fig. 2. Propidium iodide incorporation into *C. parvum* oocysts

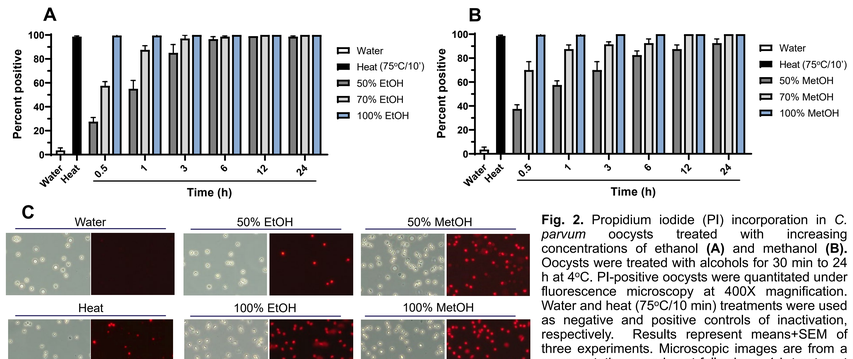


Fig. 2. Propidium iodide (PI) incorporation in *C. parvum* oocysts treated with increasing concentrations of ethanol **(A)** and methanol **(B).** Oocysts were treated with alcohols for 30 min to 24 h at 4°C. PI-positive oocysts were quantitated under fluorescence microscopy at 400X magnification. Water and heat (75°C/10 min) treatments were used as negative and positive controls of inactivation, respectively. Results represent means+SEM of three experiments. Microscopic images are from a representative experiment following a 1 h treatment with ethanol and methanol **(C).**

Fig. 3. Excystation of *C. parvum* sporozoites following inactivation treatments

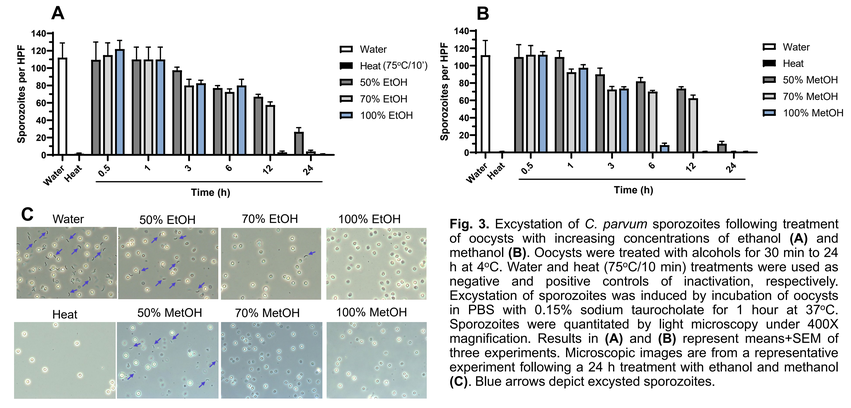


Fig. 3. Excystation of *C. parvum* sporozoites following treatment of oocysts with increasing concentrations of ethanol **(A)** and methanol **(B).** Oocysts were treated with alcohols for 30 min to 24 h at 4°C. Water and heat (75°C/10 min) treatments were used as negative and positive controls of inactivation, respectively. Excystation of sporozoites was induced by incubation of oocysts in PBS with 0.15% sodium taurocholate for 1 hour at 37°C. Sporozoites were quantitated by light microscopy under 400X magnification. Results in **(A)** and **(B)** represent means+SEM of three experiments. Microscopic images are from a representative experiment following a 24 h treatment with ethanol and methanol **(C).** Blue arrows depict excysted sporozoites.

Fig. 4. Infectivity of *C. parvum* sporozoites following inactivation treatments

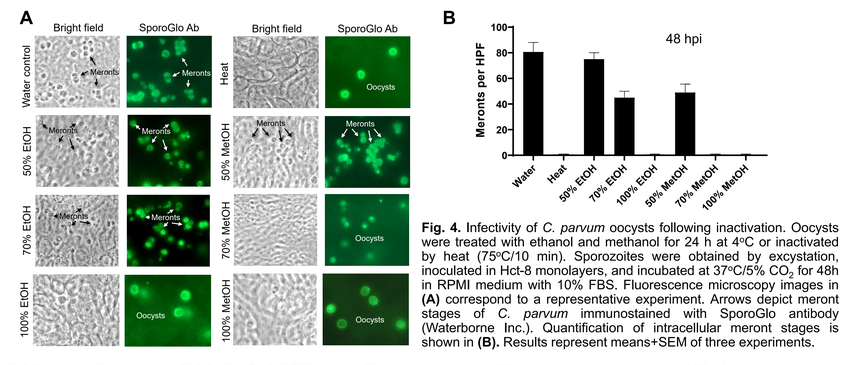


Fig. 4. Infectivity of *C. parvum* oocysts following inactivation. Oocysts were treated with ethanol and methanol for 24 h at 4°C or inactivated by heat (75°C/10 min). Sporozoites were obtained by excystation, inoculated in Hct-8 monolayers, and incubated at 37°C/5% CO_2 for 48h in RPMI medium with 10% FBS. Fluorescence microscopy images in **(A)** correspond to a representative experiment. Arrows depict meront stages of *C. parvum* immunostained with SporoGlo antibody (Waterborne Inc.). Quantification of intracellular meront stages is shown in **(B).** Results represent means+SEM of three experiments.

Fig. 5. Antigenicity of inactivated *C. parvum* oocysts

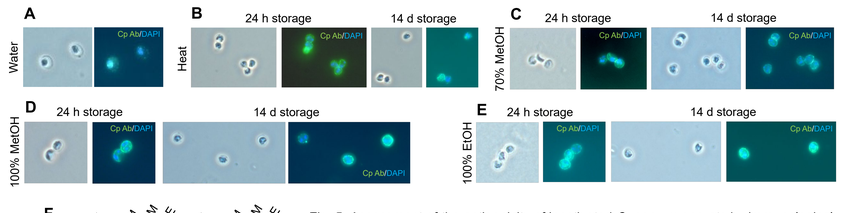


Fig. 5. Assessment of the antigenicity of inactivated *C. parvum* oocysts by immunological assays. Oocysts were suspended in water **(A)**, inactivated by heat (75°C/10 min, **B**), or treated for 24 h at 4°C with methanol **(C and D)**, or ethanol **(E)**. Following inactivation, oocysts were stored at 4°C for 24 h and 14 days. Oocysts were subsequently immunostained with SporoGlo antibodies and images were acquired by fluorescence microscopy at 1000X magnification. Untreated and inactivated oocysts stored for up to 30 days at 4°C were also subjected to SDS-PAGE (10^8 oocysts loaded per lane) and Western blots were performed with rabbit polyclonal antibodies to the *C. parvum* surface antigen CpAlp854 **(F).** A prominent band of ~50kD was observed in all treatments, even after 30 days of storage, however, higher signals were detected in untreated oocysts (Water control) and oocysts inactivated by 70% methanol (70%M). L, protein ladder.

Fig. 6. Utility of inactivated *C. parvum* oocysts in qPCR

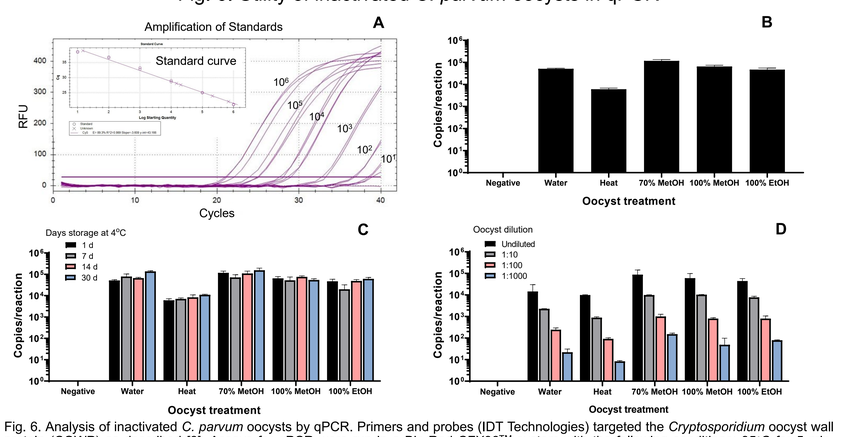


Fig. 6. Analysis of inactivated *C. parvum* oocysts by qPCR. Primers and probes (IDT Technologies) targeted the *Cryptosporidium* oocyst wall protein (COWP) as described [8]. Assays for qPCR were run in a Bio-Rad CFX96™ system with the following conditions: 95°C for 5 min, followed by 40 cycles of 95°C for 5 sec and 60°C for 30 sec. Standard curves were generated by using serial dilutions of a quantified *C. parvum* DNA (ATCC® PRA-67DQ™) **(A)**. Five µl of untreated and inactivated oocyst samples ($2.5x10^5$ oocysts) were examined directly in qPCR and the detection of gene copy numbers was determined by extrapolating the Cq values with those from the standard curves. Results in **(B)** show the detection and quantification of *C. parvum* DNA in oocysts inactivated by methanol and ethanol (24 h, 4°C) or heat. Results in **(C)** represent qPCR assays performed with inactivated oocysts stored for up to 30 days at 4°C. Results in **(D)** show the performance of the qPCR assay using serial dilutions of inactivated oocysts that had been stored for 30 days.

Summary

- We present a convenient and simple method of inactivation of *Cryptosporidium* oocysts that can be readily used in the laboratory.
- Oocysts inactivated by 70% methanol for 24 h exhibit complete loss of viability *in vitro* and retain antigenicity, even after long term storage at 4°C.
- Oocysts inactivated by 70% methanol, or 100% ethanol are suitable for use as reference reagents in qPCR-based assays.

References

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