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A Simple Alcohol-based Method of Oocyst Inactivation for Use in the Development of Detection Assays for *Cryptosporidium*

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ABSTRACT

Cryptosporidium spp. are obligate, intracellular parasites that cause life-threatening diarrhea among children and immunocompromised adults. Transmission occurs by the fecal-oral route following ingestion of thick-walled oocysts that can contaminate, persist, and resist disinfection in water and food. Sodium hypochlorite, peroxides, ozone, formaldehyde, and ammonia are suitable disinfectants against *Cryptosporidium* oocysts. Effective concentrations of these chemicals can be toxic and not practical for downstream research use of non-viable oocysts. Oocyst inactivation approaches such as UV light, heat, and treatments with ethanol or methanol are generally more accessible for routine lab use, yet their applicability in *Cryptosporidium* assay development is limited. The aims of this study were to evaluate methods of inactivation of *Cryptosporidium* oocysts that can be readily applied in the laboratory and test the utility of whole inactive oocysts in quantitative PCR (qPCR). Experiments were performed on *C. parvum* oocysts subjected to heat (75 °C/10 min) or treated with increasing concentrations of ethanol and methanol over time. Viability assays based on propidium iodide (PI) staining, *in vitro* excystation, and infection of the Hct-8 cell line were used to evaluate the efficacies of the treatments. Excystation of sporozoites was not impaired with 24 h exposures of oocysts to 50% ethanol or methanol, even though significant PI incorporation was observed. Concentrations of ≥70% of these chemicals were required to completely inhibit excystation and infection of Hct-8 cells *in vitro*. Inactivated oocysts stored for up to 30 days at 4 °C retained cyst wall integrity and antigenicity as observed by light microscopy and immunofluorescence. Moreover, non-viable oocysts applied directly in qPCR assays of the COWP gene were useful reference reagents for the identification and quantification of *Cryptosporidium* in spiked water samples. In summary, we have established a practical approach to inactivate *C. parvum* oocysts in the laboratory that is suitable for the development of detection or diagnostic assays targeting the parasite.

1. Introduction

Cryptosporidium spp. are obligate, intracellular parasites that infect the digestive and respiratory tracts of humans and other vertebrates (Gibson and Striepen, 2018; O'Leary et al., 2021). Infections of humans caused by the parasite result in self-limiting diarrhea in immunocompetent individuals, but life-threatening disease in immunocompromised patients (Khalil et al., 2018; Pinto and Vinayak, 2021). Transmission of *Cryptosporidium* to susceptible hosts occurs *via* the ingestion of thick-walled, environmentally resistant oocysts

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that can contaminate water and food sources (Gibson and Striepen, 2018; Pinto and Vinayak, 2021). Previous studies reported the use of UV light (Adeyemo et al., 2019) and disinfectants such as sodium hypochlorite (Venczel et al., 1997), peroxides (Quilez et al., 2005), ozone (Biswas et al., 2003), formaldehyde, and ammonia (Petersen et al., 2021) as suitable approaches for the inactivation of oocysts. The regular use of effective concentrations of these chemicals to produce inactivated *Cryptosporidium* oocysts in the laboratory is inadequate due to safety concerns and possible impact on the integrity of parasite antigens and nucleic acids, thus obstructing the development of detection assays. Contrary to this, inactivation methods such as heat (Fayer, 1994) and alcohol treatments (Weir et al., 2002) are usually more manageable for routine use in the laboratory, yet their evaluation in the development of molecular assays for *Cryptosporidium* oocysts remains limited.

Molecular assays such as qPCR have been replacing traditional methods for the detection of *Cryptosporidium* and other intestinal parasites in clinical and environmental samples (Adeyemo et al., 2018; Haque et al., 2007; Staggs et al., 2013). However, there are limited studies on laboratory procedures of oocyst inactivation that examine the retention of the assayable properties of *Cryptosporidium* antigens and nucleic acids. Such inactivated oocysts can be subsequently used in downstream research applications such as assay development performed under Biosafety Level-1 (BSL-1) conditions.

The objectives of this study were to evaluate methods of inactivation of *Cryptosporidium* oocysts that can be readily applied in the laboratory and to and examine the benefits of using whole inactive oocysts as reference reagents in qPCR. We examined the efficacies of ethanol and methanol, two reagents that are commonly available in most laboratories and well-known to preserve protein and nucleic acid integrity in treated cells. Using increasing concentrations of these chemicals over time, we report the optimal conditions that result in the complete inactivation of oocysts as assessed by *in vitro* infectivity assays. Long-term refrigeration of non-viable oocysts did not result in observable defects in cyst wall morphology and antigenicity. Moreover, whole inactive oocysts were useful in the detection and quantification of *Cryptosporidium* in spiked water samples by qPCR.

2. Materials and methods

2.1. Chemicals and medium reagents

Ethanol, methanol, propidium iodide (PI), and antibiotics were purchased from Sigma (St. Louis, MO, USA). RPMI 1640 medium, fetal bovine serum, PBS, and molecular grade (MG) water were obtained from ATCC® (Manassas, VA, USA).

2.2. Cryptosporidium isolate

Oocysts of *Cryptosporidium parvum* IOWA were obtained from the *Cryptosporidium* Production Laboratory, University of Arizona (https://acbs.arizona.edu/cryptosporidium-production-laboratory). Oocysts were stored at 4 °C in antibiotic solution (0.01% Tween 20, 100 U of penicillin, and 100 μg/ml of gentamicin) and were less than 4 weeks old when used in inactivation experiments.

2.3. Inactivation of oocysts

Oocysts were centrifuged for 5 min at 6000 *g*, washed with sterile MG water, and resuspended in 100 μl MG water at a concentration of 5×10^6 oocysts per tube for each inactivation treatment. Oocyst counts were performed using a hemocytometer under light microscopy at 400× magnification. Oocyst samples were treated with increasing concentrations of ethanol or methanol (50–100%) for 30 min to 24 h at 4 °C. Oocysts suspended in MG water or inactivated by heat (75 °C for 10 min) were used as negative or positive controls of inactivation, respectively. We reasoned that a prolonged 10 min exposure to 75 °C assured complete inactivation of oocysts based on a previous report by Fayer (1994). Following each treatment, oocysts were washed twice in sterile MG water and examined for viability and infectivity as described below.

2.4. Assessment of oocyst viability and infectivity

2.4.1. Viability assay based on PI permeability

Oocysts (5×10^6) were resuspended in 100 μl of a 1:2500 solution of PI in PBS. Samples were incubated for 30 min at room temperature in the dark and examined at 400× magnification using a Zeiss Axioskop® fluorescence microscope (Zeiss, Oberkochen, Germany) coupled to a UV light source. The proportions of PI-positive and PI-negative oocysts were determined by counting a minimum of 200 oocysts for each treatment.

2.4.2. Viability assay based on in vitro excystation of sporozoites

Oocysts (5×10^6) were resuspended in 100 μl of excystation buffer consisting of 0.15% sodium taurocholate in PBS. Samples were incubated for 1 h at 37 °C and examined by light microscopy at 400× magnification for the presence of excysted sporozoites. The numbers of sporozoites were counted in a minimum of 100 high power fields (HPF) for each treatment.

2.4.3. Infectivity assay based on infection of the Hct-8 cell line (ATCC® CCL-244™)

Oocysts (5×10^6) were resuspended in 100 μl of excystation buffer and incubated for 1 h at 37 °C. Excysted sporozoites were washed twice in PBS by centrifugation at 800 *g* and inoculated into Hct-8 cell cultures growing in 8-well chamber slides (Corning Inc., Corning, NY, USA). Cultures were incubated at 37 °C/5% CO_2 in RPMI 1640 medium supplemented with 1 mM sodium pyruvate and

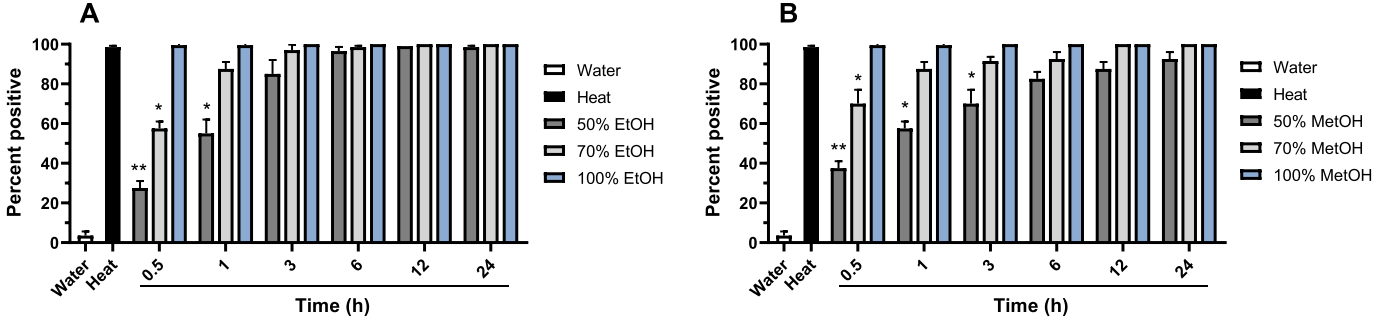

Fig. 1. PI incorporation in *C. parvum* oocysts treated with increasing concentrations of ethanol (A) and methanol (B). Oocysts were treated with the chemicals for 30 min to 24 h at 4 °C. PI-positive oocysts were quantitated under fluorescence microscopy at 400× magnification. Water and heat (75 °C/10 min) treatments were used as negative and positive controls of inactivation, respectively. Results represent means ± standard deviations of three experiments. *, $P < 0.05$; **, $P < 0.01$ as compared to heat-treated oocysts.

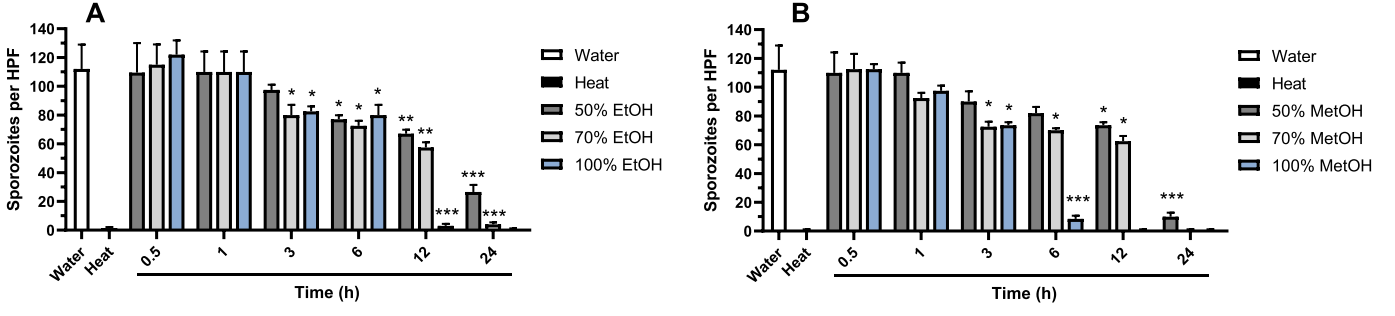

Fig. 2. Excystation of *C. parvum* sporozoites following treatment of oocysts with increasing concentrations of ethanol (A) and methanol (B). Oocysts were treated with the chemicals for 30 min to 24 h at 4 °C. Water and heat (75 °C/10 min) treatments were used as negative and positive controls of inactivation, respectively. Excystation of sporozoites was induced by incubation of oocysts in PBS with 0.15% sodium taurocholate for 1 h at 37 °C. Sporozoites were quantitated by light microscopy under 400× magnification. Results represent means ± standard deviations of three experiments. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ as compared to water-treated oocysts.

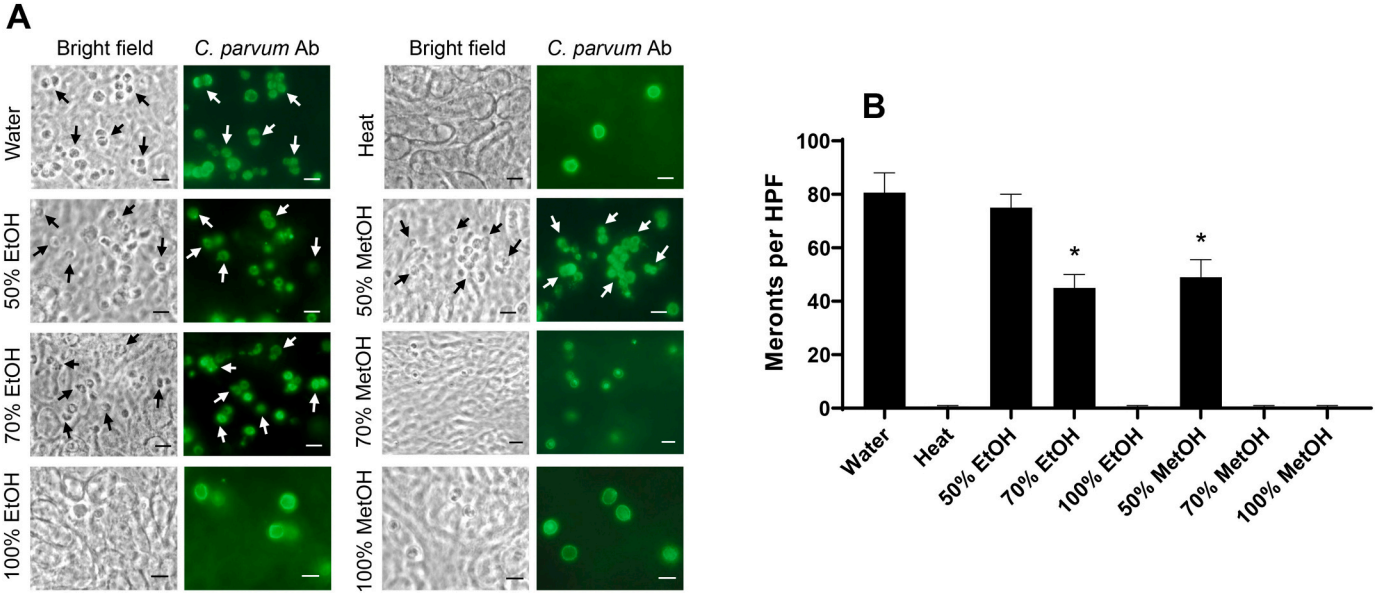

Fig. 3. Infectivity of *C. parvum* oocysts following inactivation. Oocysts were treated with increasing concentrations of ethanol and methanol for 24 h at 4 °C or inactivated by heat (75 °C/10 min). Sporozoites were obtained by excystation, inoculated in Hct-8 monolayers, and incubated at 37 °C/5% CO_2 for 48 h in RPMI 1640 medium with 10% FBS. Fluorescence microscopy images in (A) correspond to a representative experiment of three performed. Arrows depict meront stages of *C. parvum* immunostained with a FITC-conjugated *C. parvum* antibody. Bar, 5 μm. Quantification of intracellular meront stages is shown in (B). Results represent means ± standard deviations of three experiments. *, $P < 0.05$ as compared to Hct-8 cells inoculated with water-treated oocysts.

10% heat inactivated FBS. Cells were examined after 48 h for the presence of intracellular developmental forms of *C. parvum* by immunofluorescence assay (IFA) as described below.

2.5. IFA

Hct-8 cells were washed with PBS and fixed in methanol for 10 min. Fixed cells were washed with PBS and blocked in 3% bovine serum albumin (BSA) in PBS for 1 h. Cells were incubated for 1 h at room temperature with a 1:200 dilution of FITC-labeled rat anti-*C. parvum* sporozoite polyclonal antibody (Sporo-Glo™, Waterborne Inc., New Orleans, LA, USA). Cells were washed three times with PBS, suspended in mounting medium (ThermoFisher, Waltham, MA, USA) and visualized under 1000× magnification by fluorescence microscopy. The numbers of meronts observed in infected Hct-8 monolayers were counted in a minimum of 100 high power fields (HPF) for each treatment. Microscopic images were captured using a digital camera and the Zen Imaging Software (Zeiss, Oberkochen, Germany).

Where indicated, suspensions of viable and inactivated oocysts were also examined by IFA. For this purpose, oocysts were blocked in 0.5 ml of 3% BSA/PBS for 1 h. Oocysts were incubated for 1 h at room temperature with a 1:200 dilution of the FITC-labeled rat anti-*C. parvum* polyclonal antibody and washed twice with PBS by centrifugation at 6000 *g*. Oocyst pellets were resuspended in mounting medium with DAPI nuclear counterstain (ThermoFisher) and visualized by fluorescence microscopy.

2.6. DNA extraction

Procedures and reagents for DNA extraction were obtained from the DNeasy® Blood & Tissue Kit (Cat No. 69506, Qiagen, Hilden, Germany). Untreated and inactivated oocysts suspended in 1 ml of MG water at a concentration of 5×10^6 per ml were centrifuged for 5 min at 6000 *g*. The pellets were resuspended in 0.2 ml of DNeasy® buffer AL containing 20 µl of proteinase K (Qiagen) and incubated for 3 h at 52 °C. Subsequent steps were followed according to the Purification of Total DNA from Animal Blood or Cells Protocol (DNeasy® Blood & Tissue Handbook, Qiagen). DNA samples were eluted with 100 µl of DNeasy® buffer AE in the last step of the protocol and frozen at -20 °C.

2.7. qPCR

Primers and probe used in qPCR were purchased from IDT Technologies (Coralville, IA, USA) and targeted the *Cryptosporidium* oocyst wall protein (COWP) as described (Haque et al., 2007). The sequences of the forward and reverse primers (5′ → 3′) were CAAATTGATACCGTTTGTCCTTCTG and GGCATGTCGATTCTAATTCAGCT, respectively. The sequence of the Cy5-labeled probe was TGCCATACATTGTTGTCCTGACAAATTGAAT (Haque et al., 2007). qPCR assays were performed in a Bio-Rad CFX96™ System (Bio-Rad Laboratories, Hercules, CA, USA) using 25 µl reaction mixtures containing 1 µM of each primer, 250 nM of probe, 1× Bio-Rad SsoFast™ Probe Supermix (Bio-Rad Laboratories), 5 µl of untreated or inactivated whole oocyst sample (equivalent to 2.5×10^5 oocysts per reaction), or 5 µl of oocyst DNA. Cycling conditions were 95 °C for 5 min, followed by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Standard curves were generated by using tenfold serial dilutions of a quantified *C. parvum* DNA (ATCC® PRA-67DQ™). Oocyst samples and standards were tested in duplicate. The relative fluorescence unit (RFU) baseline threshold was calculated using the Bio-Rad CFX Manager™ 3.1 Software. Gene copy numbers were determined by extrapolating the quantification cycle (Cq) values with those from the standard curves.

2.8. Statistical analysis

Data from oocyst viability and infectivity assays were collected from three experiments. In each experiment, microscopic counts were performed in duplicate for PI-positive oocysts (Fig. 1), excysted sporozoites (Fig. 2), and intracellular meronts (Fig. 3). Data was subsequently analyzed using GraphPad Prism 8 (GraphPad Software, Inc., San Diego, CA, USA) to calculate means and standard deviation measurements. Where indicated, results were subjected to analysis of variance followed by the Tukey's multiple-comparison test. A P value of <0.05 was used to determine statistical significance. Samples from qPCR assays were tested in duplicate and results were plotted using GraphPad from a representative experiment of three performed.

3. Results

3.1. Effects of ethanol and methanol on PI permeability and excystation

Ethanol and methanol treatments of *C. parvum* oocysts resulted in concentration- and time-dependent increases in PI permeability as shown in Fig. 1. Treatment with 100% of each chemical for 30 min caused ≥99% of oocysts to test positive for PI incorporation (Fig. 1A–B). Similar results were observed after 12 h of treatment with 70% of ethanol or methanol. Permeability to PI was less efficient with 50% concentrations of ethanol and methanol, even after 24 h of incubation.

The release of sporozoites from oocysts by *in vitro* excystation was also affected in a concentration- and time-dependent fashion following treatment with ethanol and methanol. As shown in Fig. 2, a 24 h treatment with 100% ethanol was required to inhibit excystation to completion. Similar observations were observed after 12 h with 100% methanol. Complete inhibition of excystation by 70% methanol was only observed after oocysts were treated for 24 h (Fig. 2B).

3.2. Evaluation of oocyst infectivity in Hct-8 cells

To determine whether loss of excystation correlated with loss of infectivity, we performed experiments in cell culture. Oocysts that had been treated for 24 h with ethanol or methanol were subjected to excystation and examined for infectivity in Hct-8 cells as determined by the presence of intracellular meront stages after 48 h of infection. As shown in Fig. 3, and, in correlation with our excystation studies, oocysts that had been treated for 24 h with 100% ethanol and ≥ 70% methanol were non-infectious to Hct-8 cells (Fig. 3A-B). Only a partial loss of oocyst infectivity was observed following treatments with 50–70% ethanol and 50% methanol as indicated by the presence of intracellular meronts in infected Hct-8 cells (Fig. 3A-B). Heat-inactivated oocysts used as a control for complete loss of infectivity stained strongly with the FITC-conjugated *C. parvum* antibody and showed no evidence of parasite infection in Hct-8 cells (Fig. 3A). Oocyst immunostaining was also observed following inactivation by 100% ethanol or methanol (Fig. 3A). Separate experiments assessing the retention of oocyst antigenicity after inactivation showed immunostaining by the FITC-conjugated *C. parvum* antibody even after 14 d (Fig. 4) and 30 d (Data not shown) of storage of inactivated oocysts at 4 °C.

3.3. Evaluation of inactivated oocysts in qPCR assays

We assessed the utility of whole inactivated oocysts as reference reagents for the identification and quantification of *Cryptosporidium* in spiked water samples by qPCR. Plotting of calibration curves based on a dilution series of quantified *C. parvum* DNA standard (ATCC® PRA-67DQ™) showed a sensitivity of detection of our COWP qPCR assay of approximately 10 DNA copies (Fig. 5A). Samples of 2.5×10^5 untreated and inactivated oocysts were examined directly by qPCR without a previous DNA extraction step (Fig. 5B-C). To assess the sensitivity of the COWP assay, reactions included 1:10 serial dilutions of oocysts in water. As shown in Fig. 5B, qPCR detected *C. parvum* COWP directly in both untreated and inactivated oocysts, even at the highest 1:1000 dilution examined (~250 oocysts per reaction). Of note, copy numbers of COWP detected in methanol-inactivated oocysts were nearly two-fold and four-fold higher compared to untreated and heat-inactivated oocysts, respectively. Storage of inactivated oocysts for 30 days at 4 °C did not result in significant decreases in COWP detection compared to oocysts that had been stored for 24 h before qPCR (Fig. 5C).

We also examined the detection of COWP in DNA samples extracted from untreated and inactivated oocysts that had been stored for 24 h and 30 days at 4 °C. Extractions were performed from 5×10^6 oocysts for each treatment and 5 µl of DNA were run in qPCR. In general, similar measurements of COWP copy numbers were observed between DNA samples from oocysts stored for 24 h or 30 days (Fig. 6). A decrease from approximately 1.3×10^5 to 8.2×10^4 copies detected per reaction was observed between the two time points only in DNA samples from ethanol-inactivated oocysts (Fig. 6). Prior extraction of DNA from untreated oocysts enhanced the detection of COWP by nearly two-fold compared to qPCR performed directly in whole oocysts (Fig. 5). A similar effect was not observed in oocysts inactivated by methanol or ethanol, supporting their direct use in qPCR assays without a DNA extraction step. Copy numbers of COWP detected in DNA from heat-inactivated oocysts were nearly two-fold lower compared to untreated and alcohol-inactivated oocysts (Fig. 6).

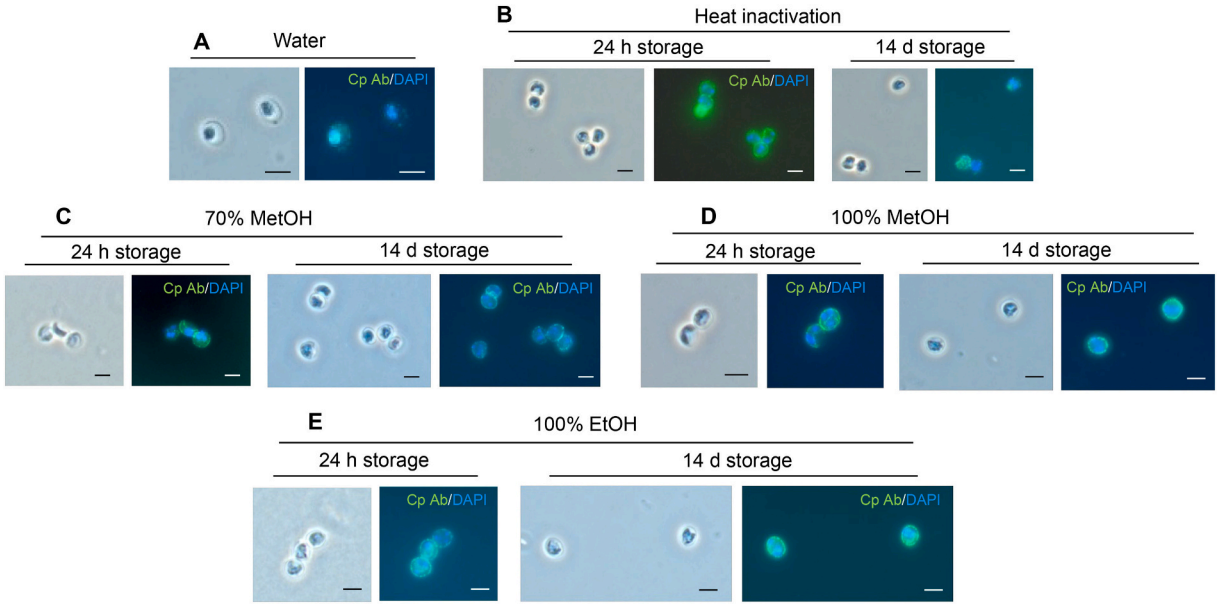

Fig. 4. Assessment of the antigenicity of inactivated *C. parvum* oocysts by IFA. Oocysts were suspended in water (A), inactivated by heat (75 °C/10 min, B), or treated for 24 h at 4 °C with methanol (C and D), or ethanol (E). Following inactivation, oocysts were stored at 4 °C for 24 h and 14 days. Oocysts were subsequently immunostained with a FITC-conjugated *C. parvum* antibody and suspended in mounting medium with DAPI nuclear counterstain. Images were acquired by fluorescence microscopy at 1000× magnification. Bar, 5 µm.

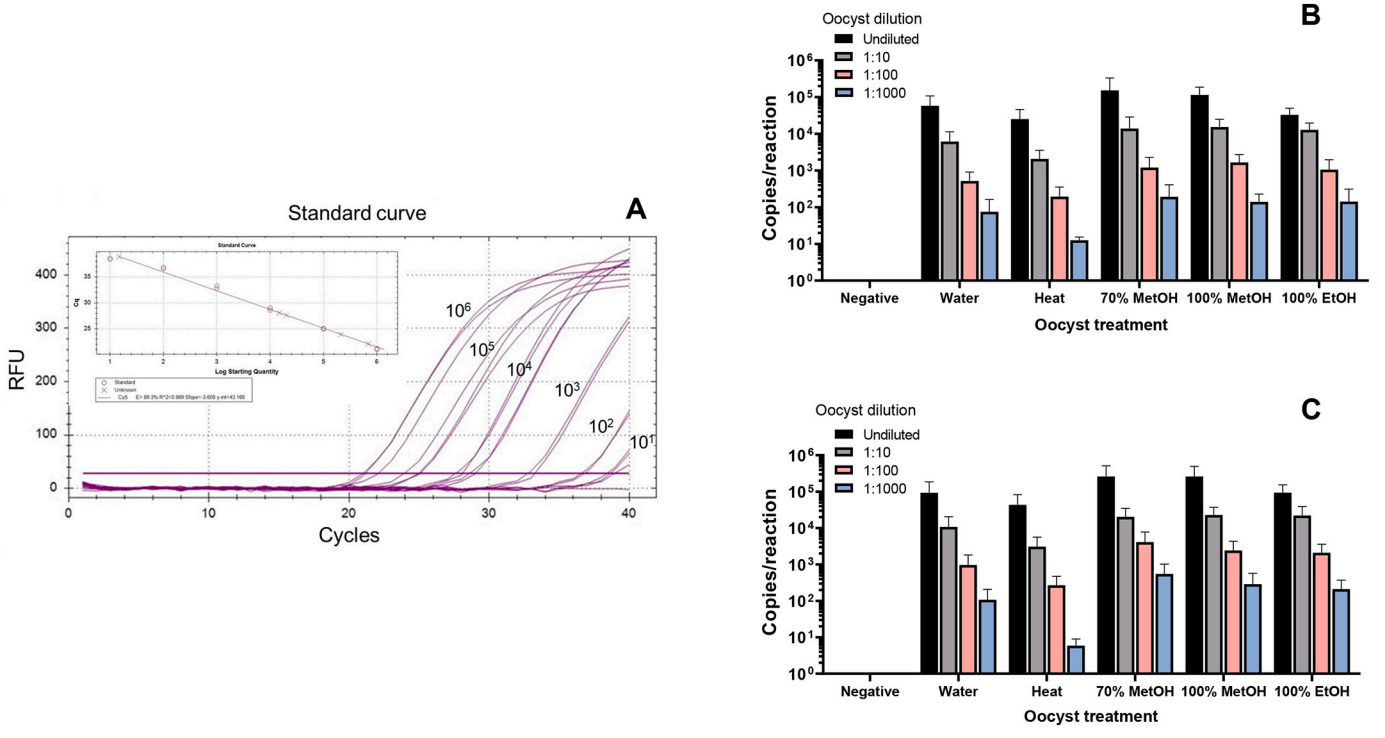

Fig. 5. Analysis of inactivated *C. parvum* oocysts by qPCR. Primers and probes targeted the *Cryptosporidium* oocyst wall protein (COWP) as described in Materials and Methods (Haque et al., 2007). (A) Example of standard curve generated by using six diluted linear DNA standards (10^6 to 10) of a quantified *C. parvum* DNA (ATCC® PRA-67DQ™). RFU, relative fluorescence units. Five µl of untreated and inactivated oocyst samples 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. (B) Detection and quantification of *C. parvum* DNA in tenfold dilutions of inactivated oocysts that had been stored for 24 h at 4 °C. Undiluted samples consisted of 2.5×10^5 oocysts per reaction. (C) qPCR assay performed with tenfold dilutions of inactivated oocysts that had been stored for 30 days at 4 °C. Undiluted samples consisted of 2.5×10^5 oocysts per reaction. Results correspond to a representative experiment of three performed. Bars represent the means ± standard deviations of each sample tested in duplicate.

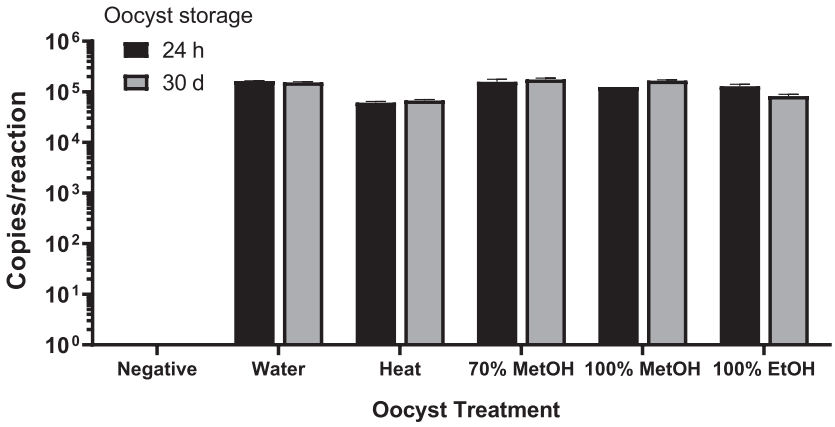

Fig. 6. COWP qPCR analysis of DNA samples extracted from inactivated *C. parvum* oocysts stored for 24 h and 30 days at 4 °C. DNA extractions were performed from 5×10^6 oocysts for each treatment. Five µl of each DNA sample was run in qPCR and the detection of COWP gene copy numbers was determined by extrapolating the Cq values with those from the standard curves. Results correspond to a representative experiment of three performed. Bars represent the means ± standard deviations of each sample tested in duplicate.

4. Discussion

In the present study, we examined the efficacies of ethanol and methanol-based treatments for the inactivation of *Cryptosporidium* oocysts. We chose these reagents due to their accessibility to most laboratories, moderately low cost, and the preservation of protein antigenicity and nuclear components in treated cells, which are important in downstream development of molecular assays. A complete loss of oocyst viability as determined by excystation assays and infection of Hct-8 cells required incubations of oocysts for 24 h with 100% ethanol and 70–100% methanol. Such treatments did not visually affect the antigenicity of oocysts as determined by IFA or the utility of inactivated oocysts as controls in a COWP qPCR assay, even after long-term storage at 4 °C. Of note, qPCR detected fewer copies of COWP in heat-inactivated oocysts compared to untreated or alcohol-inactivated oocysts, even though the input number of oocysts in the assay were the same. Detection levels of COWP were also lower in DNA extracted from heat-inactivated oocysts compared to the other treatments. It is unlikely that these observations result from deficiencies in DNA content given the high proportion of heat-inactivated oocysts staining positively with PI. Further studies are warranted to assess the potential for small losses of DNA during the extraction process or issues regarding DNA degradation (*i.e.*, sample quality) in oocysts inactivated by heat. Importantly, given that heat inactivation in the present study was restricted to 75 °C for 10 min, it would be informative to determine the qPCR utility of oocysts that had been subjected to a range of temperatures and times previously reported to result in loss of viability (Fayer, 1994).

Our results showing the inefficacy of short-term inactivation treatments of oocysts with ethanol or methanol are consistent with previous reports. Weir et al. (2002) reported that a 33 min exposure of *C. parvum* oocysts to 70% ethanol or 37% methanol resulted in only a ~ 50% decrease in infectivity of Hct-8 cells. In our study, the effects caused by 50–70% concentrations of ethanol or methanol, combined with exposure times of up to 6 h, were sufficient to cause gradual increases in the permeability of the oocyst wall without resulting in marked changes in sporozoite excystation. To completely block excystation, our conditions required 24 h treatments of oocysts with 100% ethanol or ≥ 70% methanol. Accordingly, these conditions were detrimental to the viability of sporozoites and prevented the infection of Hct-8 cells. Even though animal bioassays using neonatal mice have been considered the "gold standard" for evaluating *Cryptosporidium* oocyst infectivity, the alternative use of *in vitro* cell culture for the assessment of parasite growth is widely established to determine the presence of infectious oocysts (Monis et al., 2014; Woolsey et al., 2019).

There are limitations in this study that could be addressed in future research. First, treatments with ethanol and methanol were only performed at 4 °C. The time required to inactivate oocysts by these chemicals is likely dependent on temperature. In a previous study by Prescott and Fricker (1999) *C. parvum* oocysts were heated at 70 °C for 20 min in 50% ethanol. This temperature results in complete inactivation of oocysts (Fayer, 1994) and the permeabilization of the oocyst wall by ethanol allowed the direct detection of *Cryptosporidium* 18S rRNA in whole oocysts using an *in situ* reverse transcription assay (Prescott and Fricker, 1999). A second limitation in our study was the assessment of infectivity by IFA and enumerating the presence of meronts in Hct-8 cells at a single 48 h time point. We used this approach as a qualitative and semi-quantitative means to confirm the inactivation of oocysts following 24 h incubations with 100% ethanol and > 70% methanol (Figs. 2 and 3). Previous studies have used the focus detection method (FDM) to quantitate infectious oocysts in water samples (Slifko et al., 1997). This IFA-based method detects infectious foci in the host cell monolayer that represent meronts and other intracellular developmental stages of *Cryptosporidium*. The principle of FDM is that a single focus of infection is caused by the localized infection of nearby cells by sporozoites released from a single oocyst followed by the subsequent infection of neighboring cells by merozoite stages (Slifko et al., 1997). The accuracy of the FDM depends on the excystation of sporozoites upon oocyst contact with the monolayer and the prevention of the spread of sporozoites to other areas, which could lead to multiple foci from a single oocyst (King et al., 2011). Future studies should include the FDM as an approach to measure the proportions of infectious oocysts left in samples treated for different time periods with ethanol and methanol. In addition, FDM will also be useful in

executing time series experiments confirming the absence of early and late life cycle stages intracellularly following the inoculation of Hct-8 cells with inactivated oocysts.

As molecular assays have become more commonplace in the diagnosis of parasitic infections, both conventional and qPCR have been developed for the detection of *Cryptosporidium* in environmental and clinical samples (Haque et al., 2007; Staggs et al., 2013). One key requirement for qPCR is the use of reference reagents or controls diluted at different concentrations to generate standard curves (Woolsey et al., 2019). In *Cryptosporidium* qPCR assay development, such controls include serial dilutions of template DNA extracted from oocysts (Keegan et al., 2003; Yang et al., 2014), serial dilutions of oocysts followed by DNA extraction (Garvey et al., 2010; Koken et al., 2013), or dilutions of plasmids with cloned qPCR targets (Benamrouz et al., 2012; Shahiduzzaman et al., 2009). For the purposes of identifying and quantifying oocysts from clinical or environmental samples, the use of controls prepared from serial dilutions of a known quantity of oocysts is useful. Importantly, the availability of inactivated oocysts that are stable after long-term storage at 4 °C, as reported in the current study, is critical for diagnostic assay development by laboratories qualified to operate under BSL-1 conditions only.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgments

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