Title: CryptoPMA-PCR: A Molecular-Based Technique for Genotyping Viable Cryptosporidium Oocysts Using Propidium Monoazide to Prevent the Amplification of DNA from Non-Viable Oocysts

Running title: Detection of live Cryptosporidium oocysts

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Cryptosporidium is an important waterborne protozoan parasite that can cause severe diarrhea and death in the immunocompromised. Current methods to monitor for Cryptosporidium oocysts in water are microscopy-based USEPA Methods 1622 and 1623. These methods assess total levels of oocysts in source waters, but do not determine oocyst viability or genotype. Recently, propidium monoazide (PMA) has been used in conjunction with molecular diagnostic tools to identify species and assess the viability of bacteria. The goal of this study was the development of a Cryptosporidium propidium monoazide-polymerase chain reaction (CryptoPMA-PCR) assay that includes PMA treatment prior to PCR analysis in order to prevent the amplification of DNA from dead oocysts. The results demonstrated that PMA penetrates only dead oocysts and blocks amplification of their DNA. The CryptoPMA-PCR assay can also specifically detect live oocysts within a mixed population of live and dead oocysts. More importantly, live oocysts and not dead oocysts were detected in raw waste or surface water samples spiked with Cryptosporidium oocysts. This proof of concept study is the first to demonstrate the use of PMA for pre-PCR treatment of Cryptosporidium oocysts. The CryptoPMA-PCR assay is an attractive approach to specifically detect and genotype viable Cryptosporidium oocysts in the water, which is critical for human health risk assessment.
**INTRODUCTION**

*Cryptosporidium* is a protozoan parasite infecting many animals and humans. Infection occurs following ingestion of oocyst-contaminated food, drinking or recreational waters. It can also be transmitted from human to human or animal to human (13). Typically, it causes a self-limiting diarrheal disease in immunocompetent individuals, but can become severe and even lead to death in the immunocompromised (e.g., AIDS patients, elderly, and infants) (51). This parasite has been detected in many drinking water sources and is considered an important waterborne contaminant (52). The *C. hominis*-linked outbreak in Milwaukee, WI, USA in 1993, which involved over 400,000 infections and at least 54 deaths, is a clear example of a cryptosporidiosis outbreak due to oocyst contaminated drinking water (24, 27). There are currently twenty valid species and over forty genotypes of this parasite. *C. canis, C. felis, C. hominis, C. meleagris, C. muris, C. parvum, C. suis*, and the cervine, chipmunk, rabbit, and horse genotypes have been shown to cause disease in humans (13, 14, 36, 38). Of these, *C. parvum* and *C. hominis* cause over 95% of the reported cases of human cryptosporidiosis (52).

To reduce health risks posed by this parasite, the USEPA promulgated Long Term 2 Enhanced Surface Water Treatment Rule (LT2). Under this rule, drinking water utilities that use surface water or ground water under the direct influence of a surface water as their primary drinking water source are required to monitor their source waters for *Cryptosporidium* oocysts using USEPA Methods 1622 or 1623 (43-45). These microscopic-based methods produce total counts of live and dead *Cryptosporidium* oocysts in water samples without distinguishing species or genotypes that can infect humans from those that cannot. Results are then used to determine the need for additional water treatment in order to reduce the public health burden of waterborne
cryptosporidiosis. These methods can overestimate the concentration of oocysts infectious for humans in water supplies and health risks posed by waterborne exposures to this parasite.

Many genotyping methods have been used to identify anthroponotic and zoonotic Cryptosporidium species in water supplies (52). These include the use of PCR-restriction fragment length polymorphism (PCR-RFLP), single-strand conformational polymorphism (SSCP), PCR-randomly amplified polymorphic DNA (PCR-RAPD) (15, 54), and PCR-DNA sequencing (54). Despite being instrumental in illustrating the immense diversity of Cryptosporidium spp. present in the environment, these methods cannot distinguish viable from non-viable oocysts.

Alternative approaches that do distinguish viable from non-viable oocysts have been developed. For example, rodent models of infection are used to measure the infectivity of this parasite (3, 23, 29) but this approach is time consuming, labor intensive, and expensive. In addition, it can only determine infectivity of a select number of Cryptosporidium species. More rapid alternative techniques that do not use animal models include cell culture (37, 39) and in vitro excystation assays (23). The cell culture assay, however, is also limited in its range. It can only be used to detect viable C. andersoni, C. hominis C. parvum, C. meleagridis and C. muris oocysts but not viable oocysts of other Cryptosporidium species (1, 8, 19). In vitro excystation assays can overestimate oocyst viability, particularly if oocysts have been treated with various disinfectants (6, 7, 16). Other techniques use vital dye staining approaches like 4',6-diamidino-2-phenylindole (DAPI), propidium iodide (PI) and SYTO®-9. These approaches can also be unreliable and can overestimate the total number of viable oocysts in water samples, especially if the water was chemically disinfected (6, 7, 9, 21). Other more recent approaches utilize molecular-based techniques to assess oocyst viability. Fluorescence in situ hybridization (FISH)
(5, 10, 20, 26, 40, 47), Nucleic Acid Sequence Based Amplification (NASBA) (4), reverse-
transcriptase PCR (RT-PCR) (17, 22, 50), and an integrated cell culture/PCR assay (12, 25) all
show promise in providing a rapid and relatively inexpensive viability assay for
*Cryptosporidium*. However, it still remains to be determined if these methods can detect all
*Cryptosporidium* species and genotypes found in the environment.

It has been demonstrated recently that propidium monoazide (PMA) or ethidium
monoazide (EMA) treatment, in conjunction with molecular methods, can be used for selective
detection and genotyping of viable bacteria (30, 34) and fungi (48). PMA and EMA are new
photoactive vital dyes that can preferentially penetrate dead cells, or cells with damaged or
permeabilized cell membranes, but not viable cells with intact cell membranes. Once inside the
cell, these molecules then intercalate into the DNA and become covalently bound to DNA upon
exposure to bright white light. This photoactivation process results in the formation of a stable
DNA-PMA/EMA complex that renders the DNA inaccessible for PCR amplification (31, 33).

However, Nocker and colleagues have reported that EMA can also penetrate live bacteria and
suggested that PMA is more effective at discriminating live from dead bacteria (31). In this
paper, we developed and evaluated the use of PMA to selectively block PCR amplification of
DNA from dead *Cryptosporidium* oocysts. Following PMA treatment, the SSU rRNA and hsp 70
genes were PCR amplified and sequenced to determine the genotypes of these oocysts. This
“CryptoPMA-PCR” approach was then successfully applied to selectively detect viable oocysts
in a mixed suspensions of viable and dead oocysts that were spiked into various environmental
matrices. This new Crypto-PMA-PCR assay, which allows genotyping and viability
determination, has a potential to substantially improve the data on waterborne exposures to
*Cryptosporidium* and enhance the validity of human risk assessment.
MATERIALS AND METHODS

Cryptosporidium oocysts. *C. parvum* oocysts (Iowa isolate, Harley Moon strain) were purchased from Waterborne, Inc., and propagated in C57BL/6 mice at the U.S. EPA facility in Cincinnati, OH as previously described (28). Oocysts were purified from the feces by sieving, step sucrose gradients, and cesium chloride purification (3). Purified oocysts were then suspended in reagent grade water containing 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco, Gaithersburg, MD) and stored at 4 °C. *C. muris* oocysts (RN66), originally obtained from Dr. U. Iseki, (Osaka University, Medical School, Osaka, Japan), were propagated in CF-1 female mice, purified from the feces by sieving and sucrose flotation, and stored at 4 °C in 0.01% Tween-20 containing 100 U penicillin/mL and 100 µg streptomycin/mL as described by Miller, et. al. (28). For all experiments, oocysts were used within three months of isolation with the exception of intentionally aged oocysts that were kept at ambient temperature (20-22 °C) for 14 months in the presence of Tween-20 and antibiotics as described above.

Heat inactivation. 2 ml Costar natural clear microcentrifuge tubes (Corning Inc., Corning, NY) with oocyst suspended in PBS were heat treated in a water bath at 70 °C for 30 min. Oocyst infectivity was determined by cell culture as described below.

PMA treatment of oocysts. Propidium monoazide (PMA) (Biotium, Inc., Hayward, California) was received lyophilized and reconstituted with 20 % DMSO to a stock concentration of 20 mM and aliquoted in 5 µL volume and stored at 4 °C for no longer than 2 months. The PMA stock solution was diluted with PBS to final concentrations ranging from 5 µM to 150 µM depending
on the experiment. Oocysts were incubated with PMA for 5-30 min in a 2 ml Costar natural clear microcentrifuge tube (Corning Inc.) with vortexing for 10 sec every 5 min. Samples were then exposed to a Cosmobeam 800-W halogen light source (Cosmolight, Rome, Italy) at a distance of 20 cm while horizontal in ice for photo-induced cross-linking of PMA to oocyst DNA. Light exposure time ranged between 1 to 5 min depending on the experiment. Exposures were in 30 sec increments with 1 min rest in between exposures to avoid damaging the oocysts.

**Flow sorting of C. parvum samples.** A Fluorescence Activated Cell Sorter (FACS VantageSE, Beckton Dickinson, Palo Alto, CA) equipped with CloneCyt software was used for enumerating oocysts (49). Oocysts were gated by forward and side scatter (FSC and SSC) with FSC and SSC measured linearly with a gain of 1, SSC set at 399 V, and FSC threshold set at 32 V, reflecting their size and shape. This gate was used for live and heat-killed oocysts. Sorted oocysts between $1 \times 10^4$ and $1 \times 10^6$ were collected in sterile 1.5 mL microfuge tubes containing 0.5 mL of sterile PBS.

**Infectious foci detection and in vitro cell culture of Cryptosporidium oocysts.** Human ileocecal adenocarcinoma cells (HCT-8) were purchased from the American Type Cell Culture (ATCC CCL 244, Manasas, VA) and maintained as previously described (11). Briefly, HCT-8 cells were grown as monolayers in 75-cm$^2$ cell culture flasks in maintenance media consisting of RPMI 1640 medium (Gibco, Gaithersburg, MD), 5% fetal bovine serum (Hyclone, Logan, UT), 100 U/mL penicillin and 100 µg/mL streptomycin (Gibco) at 37ºC with 5% CO$_2$. For infectivity experiments, cells were grown on 25 mm coverslips in six-well Costar tissue culture plates.
(Corning, Inc.) to 80-85% confluency. Cells were then washed three times with PBS and fresh maintenance media was added prior to the addition of oocysts.

Live or heat-killed *C. parvum* oocysts were pretreated with 5% sodium hypochlorite (Sigma-Aldrich, St. Louis, MO) for 5 min on ice, followed by three washes with PBS prior to inoculation. 1 x 10⁶ *C. parvum* oocysts were then added to the coverslips as previously published (39). At 24 and 72 h post-inoculation, coverslips containing infected cells were washed 3 times in ice-cold PBS and fixed with 4% paraformaldehyde for 10 min. Cells were then permeabilized with 0.25% Triton X-100 (Sigma) for 10 min, followed by a blocking step using fresh 1% BSA in PBS. *Vicia villosa* lectin (VVL) (Vector Laboratories, Burlingame, CA) was used to detect parasites as previously described (18).

**Genomic DNA extraction, PCR amplification and sequencing.** Genomic DNA from 1 x 10⁴-1 x 10⁵ PMA treated and untreated live and heat-killed oocysts were isolated using the MasterPure DNA Purification Kit in accordance with manufacturer’s protocol (Epicentre Biotechnologies, Madison, Wisconsin). Briefly, oocysts resuspended in tissue and cell lysis solution were subjected to five freeze/thaw cycles followed by an overnight Proteinase K digestion at 55 °C. Samples were then treated with RNase and protein precipitation reagent. After the removal of cell debris by centrifugation, genomic DNA was precipitated with isopropanol, followed by two 75% ethanol wash steps, and then resuspended in 35 μL of nuclease-free water (Invitrogen, Grand Island, NY).

Primers that amplified a 346-bp length of the *C. parvum* hsp70 gene, as described by Di Giovanni et. al. (12), were used to determine presence or absence of amplifiable DNA following PMA treatment. Sequences were as follows: forward primer, 5’-TCC TCT GCC GTA CAG
GAT CTC TTA-3’ and reverse primer, 5’-TGC TGC TCT TAC CAG TAC TCT TAT CA-3’.

PCR conditions consisted of an initial denaturation at 95 °C for 10 min followed by 30 cycles of 95 °C for 30 sec, 60 °C for 1 min, 72 °C for 2 min with a final extension step at 72 °C for 10 min.

An 834 bp portion of C. parvum SSU rRNA or 824 bp portion of C. muris SSU rRNA were also amplified to determine presence or absence of amplifiable DNA following PMA treatment using the following previously published sequences: forward primer 5’-GGA AGG GTT GTA TTT ATT AGA TAA AG-3’ and reverse primer 5’-AAG GAG TAA GGA ACA ACC TCC A-3’ (53). PCR conditions for the SSU rRNA reactions were as follows: an initial denaturation step at 95 °C for 4 min followed by 30 cycles at 94 °C for 45 sec, 58 °C for 45 sec and 72 °C for 1 min, with a final extension step at 72 °C for 10 min. All 40 μL PCR reactions contained 2.5 U AmpliTaq Gold, 4 mM MgCl₂, 1X PCR buffer 2 (Applied Biosystems), 1 μM of each primer and 2 μL of genomic DNA. 1μL of the PCR product was visualized using the DNA 1000 kit for the Agilent 2100 bioanalyzer (Agilent Technologies, Palo Alto, CA).

For sequence analysis, PCR fragments were purified with the QIAquick PCR Purification Kit (QIAGEN, Valencia, California) and sent to Cincinnati Children’s Hospital Medical Center DNA Core facility for sequencing. The Lasergene 7 software package was used to analyze DNA sequences.

Analysis of Cryptosporidium oocysts by immunofluorescence. Oocysts were stained using Crypt-a-Glo fluorescent antibody staining kit in accordance to manufacturer’s instruction (Waterborne, New Orleans, LA). Microscopic examinations were performed with a Zeiss Axiphot2 epifluorescence microscope (Carl Zeiss) equipped with bright field, DIC, and
epifluorescence optics. The oocysts stained with FITC-conjugated antibody were observed using a FITC filter set, while oocysts with PMA-labeled DNA were observed using a Rhodamine/Texas-Red filter set. Photomicrographs were taken using AxioVision (Carl Zeiss) and analyzed with Adobe Photoshop (Adobe, San Jose, CA).

**Detection of live and dead oocysts in a mixed sample.** FACS sorted oocysts of live or heat-killed *C. parvum* or *C. muris* were mixed at defined ratios of 0:100 (0:10,000 oocysts), 1:99 (100:9,900 oocysts), 10:90 (1,000:9,000 oocysts), 50:50 (5,000:5,000 oocysts), 90:10 (9,000:1,000 oocysts), 99:1 (9,900:100 oocysts), or 100:0 (10,000:0 oocysts), respectively, treated with PMA and then exposed to an 800W halogen light. Total genomic DNA from the oocyst mixture was then extracted and analyzed using a single round of PCR amplification of the *SSU rRNA* and *hsp70* gene followed by sequencing as described previously (53).

**Detection of viable oocysts in environmental samples.** 20 L water samples were collected in carboys from the Ohio River. Water turbidity was 17 Nephelometric Turbidity Units (NTU). Samples were filtered in the laboratory using a portable gear pump and Filta-Max compressed foam filters (IDEXX Laboratories, Westbrook, Maine). Filters were eluted into 500 mL conical centrifuge tubes using a Filta-Max xpress automatic Pressure Elution Station in accordance with the manufacturer’s instructions and Method 1623 (in 2007 USEPA accepted the use of Filta-Max™ xpress as a sample processing option for Methods 1622 and 1623) (44). The eluate was centrifuged at 3,000 × g for 15 min. The supernatant was then carefully aspirated and discarded. The centrifuged pellet (2 mL volume) was resuspended in 50 mL PBS and divided into five 10
mL aliquots containing 0.4 mL packed pellet each. These aliquots were dispensed into 12 mL flat-sided Leighton tubes.

For raw wastewater samples, 50 mL of raw wastewater was divided into five 10 mL aliquots each containing 58 mg of solids and transferred into flat-sided 12 mL Leighton tubes.

$1 \times 10^5$ live or heat-killed *C. parvum* oocysts were then spiked into each tube containing resuspended Ohio River packed pellet or raw wastewater. Oocysts were separated from the sample matrix using a Dynabeads ImmunoMagnetic Separation (IMS) kit, which employs magnetic beads covalently coupled to anti-*Cryptosporidium* antibody (Invitrogen Dynal AS, Oslo, Norway) in accordance with the manufacturer’s guidelines and method 1622. After separation from the sample matrix, the oocyst-bead complexes were resuspended in 200 µL PBS and treated with PMA as described above.
RESULTS

Microscopic analysis of live and dead *C. parvum* oocysts treated with PMA

Live or heat-killed oocysts, which were incubated with 25 μM PMA for 10 min, were stained with FITC-conjugated anti-*Cryptosporidium* antibody (Crypt-a-Glo) and analyzed using fluorescence microscopy (Fig. 1). Following PMA treatment, live oocysts remained impermeable to PMA and did not stain red (Fig. 1B), whereas heat-killed oocysts stained bright red (Fig. 1E). Figure 1C and F are overlain images of Crypt-a-Glo stained oocysts (Fig. 1A and D) and PMA (Fig. 1B and E). Dead oocysts (Fig. 1F) that are both Crypt-a-Glo and PMA positive appear yellow. Further analysis revealed that less than 10% of oocysts which were not treated with heat were positive for PMA, while greater than 95% of heat-killed oocysts were PMA positive. The observed PMA staining of some untreated oocysts seen in Fig. 1B was likely due to the natural attrition of oocysts that occurs over time and/or oocysts killed during the purification process. To confirm that PMA negative and PMA positive oocysts were indeed live or dead, respectively, we performed a cell culture infectivity analysis of these oocysts using HCT-8 cells. PMA negative oocysts infected HCT-8 cells easily as indicated by the presence of multiple infectious foci at 24 h post-infection which increased in numbers at 72 h post-infection. In contrast, no infectious foci were detected at both time points when cells were inoculated with heat-killed oocysts that previously stained positive for PMA (data not shown).

CryptoPMA-PCR assay to detect live but not dead *Cryptosporidium* oocysts

To determine optimal PMA concentrations for live/dead oocyst discrimination using PCR, oocysts were stained with PMA concentrations from 0.5 to 150 μM. 1 x 10^4 live or heat-
killed oocysts were incubated with PMA for 10 min followed by light exposure for 2 min. This was followed by DNA extraction and PCR amplification of the SSU rRNA gene (Fig. 2A). Our results revealed that PMA concentration as high as 150 μM had no noticeable inhibitory effects on the detection of live oocysts by PCR as indicated by the presence of a PCR product of the expected size with similar band intensity as compared with PCR products detected from live untreated oocysts (Fig. 2A, Lanes 2 and 3-6). By contrast, PMA treatment as low as 0.5 μM resulted in a marked reduction of detectable PCR products as compared to heat-killed oocysts which were not treated with PMA (Fig. 2A, Lane 7 and 8). More remarkable is the complete loss of amplicons from dead oocysts incubated with 5, 50, or 150 μM PMA (Fig. 2A, Lanes 9-11).

To determine if this CryptoPMA-PCR assay can also work on another Cryptosporidium gene locus used for genotyping, the hsp70 gene was also tested. As shown in Figure 2B, the hsp70 gene was easily amplified from live untreated C. parvum oocysts and was also detected with no marked inhibition of PCR amplification in live C. parvum oocysts treated with PMA (Fig. 2B, Lanes 2-6). PCR products were also detected from untreated dead oocysts, albeit with slightly reduced band intensity (Fig. 2B Lane 7). In contrast, treatment with as little as 0.5 μM PMA (Fig. 2B, Lane 8) resulted in an almost complete loss of the hsp70 PCR product. Treatment with higher PMA concentrations up to 150 μM resulted in undetectable levels of PCR products (Fig. 2B Lanes 9-11). These results demonstrate that SSU rRNA and hsp70 genes, two gene loci commonly used for genotyping Cryptosporidium spp. (15), are useful targets for this CryptoPMA-PCR detection assay. More importantly, pre-treatment of Cryptosporidium oocysts with PMA resulted in the selective PCR amplification of the SSU rRNA or hsp70 genes from live oocysts and the concomitant inhibition of PCR amplification of these genes from dead oocysts.
Optimizing PMA incubation times and photoactivation conditions for the CryptoPMA-PCR assay

To optimize PMA incubation time, C. parvum oocysts were incubated on ice for 5 min or 30 min with 150 μM PMA followed by a 2 min exposure to bright light, a photoactivation step required to covalently bind PMA to DNA (Fig. 3) (31, 33). Incubation of live oocysts with PMA for 5 min (Fig. 3, Lanes 3 and 12) or 30 min (Fig. 3, Lanes 4 and 13) followed by bright light exposure did not have an appreciable inhibitory effect on amplifying the SSU rRNA or hsp70 gene. Conversely, the PMA molecule readily penetrated and intercalated into DNA of heat-killed oocysts as seen by fluorescence microscopy (Fig. 1E) resulting in the loss of detectable SSU rRNA or hsp70 PCR amplicons at 5 min (Fig. 3, Lanes 6 and 15) or 30 min (Fig. 3, Lanes 7 and 16) incubation times. These results revealed that incubation of oocysts for at least 5 min allowed sufficient time for PMA to cross several complex barriers (oocyst wall, sporozoite cell and nuclear membranes) and intercalate within the DNA structure to prevent amplification of genomic DNA from dead oocysts. Incubation of live oocysts with PMA for up to 30 min did not prevent amplification of their DNA nor did it result in any observable reduction in PCR products detected as compared with live untreated oocysts (Fig. 3, Lanes 2-4 and 11-13).

To minimize excessive heating of the oocysts, heat-induced oocyst excystation, or light-induced DNA damage during the PMA photoactivation step, light exposure times between 1 min to 5 min were evaluated. Figure 4 illustrates that a 1 min exposure to bright light (800 W) was sufficient to photoactivate PMA and prevent amplification of both target genes from heat-killed oocysts (Fig. 4, Lanes 9 and 23) with no observable reduction of PCR products detected from live oocysts (Fig. 4, Lanes 3 and 17). Exposure times up to 4 min also had no detectable
inhibitory effects on amplifying the SSU rRNA or the hsp70 gene in live oocyst samples; however, a 5 min light exposure showed a slight reduction in detectable SSU rRNA but not the hsp70 amplicons from live oocysts (Fig. 4, Lane 7 vs. 21). Based on these experiments, oocysts were incubated with 150 μM PMA for 5 min followed by a 2 min light exposure time in all the experiments described below.

Molecular genotyping of live Cryptosporidium oocysts in a mixture of live and dead oocysts of different species

To determine if this CryptoPMA-PCR assay can be applied for the detection and genotyping of live Cryptosporidium oocysts in a mixed population containing different Cryptosporidium species, live or heat-killed C. muris oocysts were mixed with live or heat-killed C. parvum at defined ratios (Table 1). These samples were then treated with PMA followed by PCR reactions amplifying the SSU rRNA region. Detected amplicons were then sequenced to identify the species. Results in Table 1 demonstrate that in 2 of 3 experiments performed, live C. parvum oocysts were detected when mixed with dead C. muris at a 10:90 C. parvum: C. muris ratio. Additional experiments also revealed that only C. parvum DNA was detected in all three experiments when live C. parvum and dead C. muris oocysts were mixed at a 50:50 ratio. No C. muris SSU rRNA sequences were detected when dead C. muris oocysts were analyzed. Similarly, when ratios of 50:50, or 10:90 live C. muris to heat-killed C. parvum oocysts, respectively, were analyzed, only the C. muris SSU rRNA sequence was detected in all six experiments performed. The ability of this CryptoPMA-PCR assay to detect 100 live C. muris oocysts in the presence of 9,900 heat-killed C. parvum oocysts (1:99 ratio) in one of three experiments performed is also notable.
Application of the CryptoPMA-PCR assay for the detection of live oocysts in environmental matrices

To determine if the CryptoPMA-PCR assay is suitable for the detection and genotyping of live oocysts in complex matrices, samples of untreated water from the Ohio River and raw wastewater samples from a municipal sewage treatment plant were each spiked with live or dead *C. parvum* oocysts. These matrices were chosen because they contain debris and dissolved compounds (Fig. 5A), which may not be completely separated from oocysts by the IMS procedure and can physically block entry of PMA into oocysts, sequester PMA away from the oocysts and/or mask light exposure during the photoactivation step. There was nearly a complete loss of the SSU rRNA PCR product from heat-killed PMA treated oocysts spiked into the raw surface water (Fig. 5B Lane 5). Similarly, a marked reduction in the amount of amplicons was observed when analyzing the PMA treated heat-killed oocysts, which were spiked in the raw wastewater (Fig. 5B, lane 10). The presence of a faint band in the raw wastewater sample may be a result of the presence of excess free DNA in the sample binding PMA and/or masking effects of debris present in the matrix preventing the complete removal and/or inhibition of PCR amplification of spiked dead oocysts.

Using CryptoPMA-PCR assay to determine viability of *Cryptosporidium* oocysts stored at ambient temperatures

Previous studies by Jenkins and others have shown that long term storage of oocysts at ambient temperatures resulted in the loss of oocyst infectivity due to natural oocyst attrition as a consequence of time and temperature (22). To determine if the CryptoPMA-PCR assay can be
used to discriminate viable *Cryptosporidium* oocysts from oocysts that lost viability as a result of long-term storage at room temperature, $1 \times 10^4$ live, heat-killed, or aged (stored at 20-22 °C for 14 months) oocysts were analyzed as described above. PCR amplification of the *SSU rRNA* gene from DNA equivalent to 100 live or heat-killed oocysts in the absence of PMA pre-treatment resulted in a distinct band (Fig 6A, lanes 2 and 4). No difference in band intensity was observed with the addition of PMA in live oocysts, while no PCR product was observed in heat-killed oocysts treated with PMA (Fig 6A, lanes 3 and 5).

Furthermore, when the *SSU rRNA* region was amplified from DNA equivalent to 10 oocysts, amplicons were still detected in both live oocysts samples with or without PMA or heat-killed oocysts in the absence of PMA (Fig. 6B, lanes 9-11). When the *SSU rRNA* gene was PCR amplified from 100 aged oocysts, a distinct, albeit faint band, was detected in the absence of PMA, which was completely abolished upon the addition of PMA (Fig 6A, lanes 6 and 7 respectively). No amplicons were detected from ten PMA-treated heat-killed oocysts (Fig. 6B, lane 12), or ten aged oocysts in the presence or absence of PMA (Fig. 6B, lanes 13 and 14, respectively).

These results demonstrate that the CryptoPMA-PCR assay is effective at preventing the amplification of dead oocysts in a sample that has been kept at ambient temperatures for 14 months, conditions that oocysts can be exposed to in the natural environment. Moreover, these results also demonstrate the single round of PCR used for the CryptoPMA-PCR is able to detect DNA equivalent to 10 live oocysts.
DISCUSSION

Current methods for determining viability employ cell culture or rodent models of infection (37). However, cell culture-based and animal bioassay approaches have inherent limitations. For example, it is not known if all species and genotypes of Cryptosporidium can grow in cell culture (2). Little is known about the susceptibility of rodents to infection with many species and genotypes of this parasite that can contaminate water supplies (42). Vital dyes like PI, DAPI and SYTO®-9 have been used as rapid alternatives to animal bioassays or cell culture assays in determining viability of oocysts, but results from these approaches can be variable and offer limited genotyping capabilities (6, 7, 9, 21, 29). PMA and EMA represent a new generation of vital dyes that contain a photoreactive azide group that can covalently bind to nucleic acids following photolysis. And unlike PI, which can diffuse in and out of oocysts (6, 7), photoactivated PMA or EMA forms a stable complex with nucleic acids that does not diffuse out of oocysts, (31). Studies have shown however, that EMA can overestimate the amount of viable bacteria (31). Thus this study used PMA as a pre-PCR treatment for the CryptoPMA-PCR assay. An advantage of the Crypto-PMA-PCR assay is that it is a rapid approach that does not involve cell culture. In addition, this approach allows for downstream genotyping following PMA treatment.

This proof of concept study is the first to demonstrate that heat-killed and oocysts that were inactivated by long-term storage at room temperature were permeable to PMA, whereas live oocysts were impermeable to it. Since PMA can cross multiple parasite membranes to intercalate and covalently bind to Cryptosporidium genomic DNA it can be used as a pre-PCR treatment that renders dead oocysts undetectable by PCR analysis. For example, in the absence of a PMA labeling step, the SSU rRNA or hsp70 genes from live, heat-killed, or inactivated by
long-term storage Cryptosporidium oocysts were both amplified, whereas these genes were only
detected from live Cryptosporidium oocysts when the PMA labeling step was performed. As
illustrated in Figure 6 and Table 1, this method was able to detect relatively low numbers of live
oocysts (10-100 oocysts) in a pure preparation as well as in a mixture with at least 9,900 dead
oocysts. Conversely, these target genes were not detected from oocysts exposed to room
temperature for fourteen months or heat-killed oocysts at doses up to 10,000 oocysts further
demonstrating the efficiency of PMA to prevent amplification of DNA from dead
Cryptosporidium oocysts. This study builds upon previous findings by Nocker and others on the
use of PMA to selectively detect and genotype viable bacteria and extends the application of
PMA treatment to a more complex parasite (32). In this study, a single round PCR was effective
at detecting DNA from as few as 10 live oocysts (Fig. 6). We anticipate that combining the
PMA staining step with a more sensitive genotyping approach, such as nested PCR, will enable
the detection of a single live oocyst in environmental samples (15, 53). However, great care
should be taken with nested PCR since this approach can also increase a risk of false positive
results, particularly when environmental samples are analyzed. Additional experiments are also
warranted to further evaluate the utility of PMA for the differentiation of live and dead oocysts
using other molecular-based detection methods not described here.

The addition of PMA was effective at preventing the amplification of DNA from oocysts
that were killed by exposure to heat or long-term storage at room temperature suspended in
distilled water (Fig. 6). However, a faint band was detected in oocyst-free sewage samples that
were spiked with heat-killed oocysts. This result could be due to the presence of high levels of
indigenous extracellular DNA in waste water matrix, which can sequester free PMA molecules
away from oocysts, thereby reducing the effectiveness of PMA in inhibiting amplification of
DNA from dead oocysts. Two recent studies by Varma and others also reported similar findings when using PMA to detect bacteria in wastewater indicating the difficulty in using PMA on wastewater matrices (46). Alternatively, photoactivation of PMA could have been prevented through a “masking” effect by debris and contaminants that may have carried over during the IMS step. It is also possible that the beads themselves can interfere with the photoactivation process. However, PCR amplification of the SSU rRNA gene from live oocysts treated with PMA and photoactivated in the presence of at least $10^5$ magnetic beads had no effects on the levels of PCR amplicons detected (data not shown). To minimize the effects of indigenous DNA, suspended solids, and dead microorganisms on PMA staining of dead Cryptosporidium oocysts in environmental samples, adding excess amounts of PMA as well as increasing light exposure should be considered as potential strategies. Nevertheless, this study demonstrated that the CryptoPMA-PCR assay was an improvement to the specificity of current PCR techniques used to detect live Cryptosporidium oocysts seeded in turbid waters, like raw surface water samples (Figure 5B).

The mode of action of PMA is still not completely understood. Previous studies by Nocker and colleagues demonstrated that once PMA intercalates into and becomes covalently bound to DNA of bacteria, the PMA-DNA complex becomes insoluble and is subsequently removed during DNA extraction procedures (31). These scientists also hypothesized that bound PMA may affect Taq polymerase-based PCR amplification of the target DNA (32). Studies using EMA to differentiate live from dead L. monocytogenes bacteria have suggested that the duration of light exposure of DNA in the presence of EMA can inhibit PCR amplification of target genes (41). Whether PMA has similar effects as EMA remains to be determined. Despite our limited knowledge of its mode of action, PMA treatment is very effective at preventing the
amplification of the hsp70 and SSU rRNA genes from heat-killed Cryptosporidium oocysts.

Interestingly, during the optimization of the photoactivation process, we observed that exposure to light for at least 5 min resulted in a marked decrease in the signal intensity of the SSU rRNA amplicon, but not of the hsp 70 gene. This may be attributed to light-induced DNA strand breaks leading to decreased signal intensity for the SSU rRNA amplicon. These results were also seen in previous studies done in other eukaryotic cells (35). The SSU rRNA amplicon is longer than the 346 bp hsp 70 amplicon (346 bp) may explain why it might have a lower probability of light induced strand breaks. Since 2 min of light exposure did not result in any appreciable decrease in signal intensity for either SSU rRNA or hsp 70 amplicons from live Cryptosporidium oocysts, we chose this duration of light exposure for all further experiments performed in this study. Nonetheless, great care should also be taken when optimizing this approach to detect other microorganisms.

Here, we have developed the CryptoPMA-PCR technique that is very effective at specifically detecting and genotyping live oocysts seeded in distilled water samples and also has a potential to specifically detect viable oocysts in environmental water samples. Unlike RT-PCR, which is labor intensive and requires advanced technical skills to manipulate messenger RNA (a very labile molecule that easily degrades), the CryptoPMA-PCR assay uses DNA as its template, which makes this technique more versatile and user-friendly. The CryptoPMA-PCR can be modified to use other types of molecular genotyping techniques, such as nested PCR, single strand conformational polymorphisms, or multi-locus sequence typing. The use of quantitative real-time PCR with PMA pre-treatment may also enable quantitation of viable pathogenic Cryptosporidium oocysts in environmental samples.
Future research efforts should also focus on determining sensitivity and versatility of PMA to distinguish live from dead oocysts that have been treated with different methods of disinfection (e.g. UV, chlorine, ozone, and natural sunlight). Previous studies have demonstrated that real-time PCR plus PMA treatment could not be used to differentiate UV-inactivated bacteria from viable ones suggesting that PMA pre-treatment may also be limited to preventing PCR amplification of DNA from oocysts that were treated with heat or chemicals (e.g., chlorine, ozone, aldehydes, and alcohols) that make the oocyst wall permeable for PMA (33). More importantly, standardization and inter-laboratory performance comparison of this assay, using different water matrices spiked with low numbers of oocyst typically found in environmental samples, must be performed in order to evaluate the practical applicability of this approach for water quality monitoring.

This CryptoPMA-PCR approach is a novel tool that combines genotyping and viability determination. After further refinement and validation, this method may be used in source tracking studies or for generating improved data on waterborne exposures to Cryptosporidium and assessing health risks posed by this parasite.
ACKNOWLEDGEMENTS

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AUTHOR CONTRIBUTIONS

CCB and ENV conceived and designed the experiments. CCB, SMG, MWW, EAV, and AIE performed the experiments. CCB and ENV analyzed the data. CCB, ENV, SMG, MWW, EAV, and AIE contributed reagents/materials/analysis tools. CCB and ENV wrote the paper.
REFERENCES


**FIGURE LEGENDS**

**Figure 1.** Microscopic analyses distinguishing live from dead *C. parvum* oocysts using PMA.

(A-C) Live or (D-F) heat-killed (70 °C, 20 min) *C. parvum* oocysts treated with PMA and stained with Crypt-a-Glo. Crypt-a-Glo labeled oocysts (green) are shown in A and D, PMA staining (red) of the same oocysts is shown in B and E, while C and F represent overlain images with Crypt-a-Glo and PMA (yellow) staining. Bar = 10 μm.

**Figure 2.** Effects of different PMA concentrations on detecting live and heat-killed *C. parvum* oocysts. Live or heat-killed *C. parvum* oocysts were incubated with 0.5, 5, 50 or 150 μM PMA for 10 min followed by a 2 min light exposure. This was followed by the extraction of genomic DNA and PCR amplification with primers targeting the (A) SSU rRNA or (B) hsp 70 genes. PCR products were analyzed using an Agilent Bioanalyzer 2100. M: Marker; - : No PMA control; Δ: 70 °C, 30 min treated oocysts; C: PCR control that lacks template.

**Figure 3.** Effects of PMA incubation time on detecting live and heat-killed *C. parvum* oocysts.

Live or heat-killed *C. parvum* oocysts incubated with 150 μM PMA for either 5 or 30 min and then exposed to light for 2 min. Genomic DNA was purified as described in the Materials and Methods. Amplification of a portion of the hsp70 (Lanes 2-9) or SSU rRNA (Lanes 11-18) genes was performed using PCR. Gel electrophoresis analysis was performed using an Agilent Bioanalyzer 2100. M: Marker, - : No PMA control, Δ: 70 °C treated oocysts, C: PCR control that lacks template.
Figure 4. Effects of the duration of exposure to light after PMA treatment on detecting live and heat-killed *C. parvum* oocysts. Live or heat-killed *C. parvum* oocysts were incubated with 150 μM PMA for 5 min and exposed to light for 1, 2, 3, 4, or 5 min. Genomic DNA was purified the *hsp70* (Lanes 1-14) or *SSU rRNA* (Lanes 15-28) genes were amplified by PCR. Gel electrophoresis analysis was performed using an Agilent Bioanalyzer 2100. M: Marker, - : No PMA control, Δ: 70 ºC treated oocysts, C: PCR control that lacks template.

Figure 5. Detection of *C. parvum* oocysts in Ohio River and raw wastewater samples using CryptoPMA-PCR. (A) Environmental samples spiked with oocysts prior to immunomagnetic separation, PMA treatment and PCR: resuspended 0.4 mL pellet from raw surface water concentrate (1) and raw wastewater (2). (B) *SSU rRNA* PCR amplicons from live and dead oocysts spiked in raw surface water or raw wastewater. Amplification of a portion of the *SSU rRNA* gene was performed using PCR (Lanes 2-10). Gel electrophoresis analysis was performed using an Agilent Bioanalyzer 2100. M: Marker, - : No PMA, +: PMA added, Δ: 70 ºC treated oocysts, B: Blank sample that did not receive oocyst spike. Data represent one of two independent experiments performed.

Figure 6. Differentiation of live *C. parvum* oocysts from heat-killed oocysts (Δ) and oocysts inactivated by long-term storage at room temperature (Aged) using CryptoPMA-PCR. Live (lanes 2, 3, 9 and 10), heat-killed (lanes 4, 5, 11 and 12) or aged (lanes 6, 7, 13 and 14) *C. parvum* oocysts were incubated with 150 μM PMA for 5 min and then exposed to light for 2 min (lanes 3, 5, 7, 10, 12, and 14). Genomic DNA was purified as described in the Materials and Methods. *SSU rRNA* PCR amplicons from live, dead or aged oocysts with an oocysts equivalent.
of (A) 100 or (B) 10 were visualized using an Agilent Bioanalyzer 2100. M: Marker, - : No
PMA, +: PMA added, Δ: 70 °C treated.
Figure 1

Crypt-a-Glo

PMA

Crypt-a-Glo + PMA

Live

Heat-killed
A. 

\[
\begin{array}{c|c|c|c|c|c|c}
\text{LIVE} & \text{Δ} \\
\hline
\text{PMA (μM)} & M & - & 0.5 & 5 & 50 & 150 \\
\text{SSU rRNA} & & & 0.5 & 5 & 50 & 150 \text{C} \\
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 & 11 & 12 \\
\end{array}
\]

B. 

\[
\begin{array}{c|c|c|c|c|c|c}
\text{LIVE} & \text{Δ} \\
\hline
\text{PMA (μM)} & M & - & 0.5 & 5 & 50 & 150 \\
\text{hsp70} & & & 0.5 & 5 & 50 & 150 \text{C} \\
1 & 2 & 3 & 4 & 5 & 6 & 7 & 8 & 9 & 10 & 11 & 12 \\
\end{array}
\]
Figure 3

Incubation (min) | M | - | 5 | 30 | Δ | - | 5 | 30 | C | 5 | 30

hsp70

SSU rRNA
**Figure 4**

- **LIVE**
- **Δ**

**Light (min)**

<table>
<thead>
<tr>
<th>M</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
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- **hsp70**

1. 2. 3. 4. 5. 6. 7.

- **SSU rRNA**

15. 16. 17. 18. 19. 20. 21.

22. 23. 24. 25. 26. 27. 28.
Figure 5

A. 1 2

B. Raw surface water
   | LIVE | Δ |
   | M   |   |
   | 1   | - |
   | 2   | + |
   | 3   | - |
   | 4   | + |
   | 5   | + |
   | 6   | B |

Raw wastewater
   | LIVE | Δ |
   | M   |   |
   | 7   | - |
   | 8   | + |
   | 9   | - |
   | 10  | + |
   | 11  | B |

SSU rRNA
A. 100 oocysts

<table>
<thead>
<tr>
<th>M</th>
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<th>Δ</th>
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<tr>
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<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
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</table>

B. 10 oocysts

<table>
<thead>
<tr>
<th>M</th>
<th>Live</th>
<th>Δ</th>
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</thead>
<tbody>
<tr>
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<td></td>
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<td></td>
</tr>
<tr>
<td>10</td>
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SSU rRNA
TABLE 1: Molecular genotyping of *C. muris* (Cm) and *C. parvum* (Cp) in a mixture of live and heat-killed oocysts using CryptoPMA-PCR*

<table>
<thead>
<tr>
<th>Oocyst</th>
<th>Ratio</th>
<th>Experiment</th>
<th>Live oocysts detected</th>
<th>Dead oocysts detected</th>
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<tbody>
<tr>
<td><em>C. parvum</em></td>
<td>0:100</td>
<td>-</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td><em>C. muris</em></td>
<td>1:99</td>
<td>-</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>10:90</td>
<td>Cp</td>
<td>2/3</td>
<td>0/3</td>
</tr>
<tr>
<td><em>C. muris</em></td>
<td>50:50</td>
<td>Cp Cp</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
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<td>100:0</td>
<td>Cp Cp Cp</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td><em>C. muris</em></td>
<td>0:100</td>
<td>-</td>
<td>0/3</td>
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<td><em>C. parvum</em></td>
<td>1:99</td>
<td>Cm</td>
<td>1/3</td>
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<tr>
<td><em>C. muris</em></td>
<td>10:90</td>
<td>Cm Cm Cm</td>
<td>3/3</td>
<td>0/3</td>
</tr>
<tr>
<td><em>C. parvum</em></td>
<td>50:50</td>
<td>Cm Cm Cm</td>
<td>3/3</td>
<td>0/3</td>
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<tr>
<td><em>C. muris</em></td>
<td>100:0</td>
<td>Cm Cm Cm</td>
<td>3/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

*SSU rRNA and hsp70 genes were amplified and sequenced.*