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# Comparison of freeze-thaw cycles for nucleic acid extraction and molecular detection of *Cryptosporidium parvum* and *Toxoplasma gondii* oocysts in environmental matrices

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ABSTRACT

Freeze-thaw DNA extraction methods and PCR primers were compared to optimize detection of *Cryptosporidium parvum* and *Toxoplasma gondii* oocysts in different matrices. Increasing FT cycles did not increase parasite DNA detection, and primers targeting the 18S ssrRNA gene yielded the most sensitive detection of *C. parvum* oocysts.

*Cryptosporidium* spp. and *Toxoplasma gondii* are globally ubiquitous zoonotic parasites with worldwide distribution (Bahia-Oliveira et al., 2017; Thompson et al., 2016). Exposure can occur by ingestion of oocysts that can survive in the environment for weeks to years (Bahia-Oliveira et al., 2017; Robertson, 2007), posing the risk of infection to people and animals through contaminated water or food, including shellfish (Robertson, 2007; Willis et al., 2013; Elmore et al., 2012). Oocysts are resistant to chemical disinfectants and require additional processes such as filtration or settling for physical removal (Willis et al., 2013).

Detection of oocysts in environmental and clinical samples has been described utilizing light and fluorescence microscopy (Mosteo et al., 2016; Bigot-Clivot et al., 2016), direct and indirect fluorescence antibody staining (Miller et al., 2005a, 2006; Hohweyer et al., 2013), and molecular methods (e.g., conventional and quantitative PCR) (Bigot-Clivot et al., 2016; Miller et al., 2005a; Adell et al., 2014; Staggs et al., 2015). PCR can further facilitate molecular characterisation of pathogens needed for investigating transmission across environmental matrices and hosts (Miller et al., 2005a; Hohweyer et al., 2013).

The robust nature of protozoan parasite oocysts poses challenges for nucleic acid extraction needed in molecular assays (Hohweyer et al., 2013). Freeze-thaw (FT) cycles are commonly used to disrupt oocyst walls prior to DNA extraction using commercial kits that employ silica-membrane spin columns (Miller et al., 2005a, 2005b; Hohweyer et al., 2013; Molini et al., 2007; Giangaspero et al., 2005; Giangaspero et al., 2014; Fayer et al., 2003). However, reported methods for oocyst DNA extraction using FT cycles vary widely (Bigot-Clivot et al., 2016; Miller et al., 2005a; Adell et al., 2014; Staggs et al., 2015; Miller et al., 2005b; Molini et al., 2007; Giangaspero et al., 2005; Giangaspero et al., 2014; Fayer et al., 2003; Villena et al., 2004; Fayer et al., 2002; Wells et al., 2015). To achieve deep freezing, storage at −80 °C (Bigot-Clivot et al., 2016; Miller et al., 2005a; Molini et al., 2007; Giangaspero et al., 2014; Villena et al., 2004), or immersion in liquid nitrogen (Adell et al., 2014; Staggs et al., 2015; Giangaspero et al., 2005) or a dry ice/ethanol slurry (Robertson, 2007) have been reported. Duration of these freeze cycles ranged from 3 to 60 min (Bigot-Clivot et al., 2016; Miller et al., 2005b; Molini et al., 2007). Reported approaches for thawing include incubation at room temperature (Villena et al., 2004), 55 °C (Schwab and Mcdevitt, 2003), 70 °C (Staggs et al., 2015), 80 °C (Giangaspero et al., 2005; Giangaspero et al., 2014), 95 °C (Bigot-Clivot et al., 2016), or in boiling water (Miller et al., 2005a; Adell et al., 2014) for durations of 3–15 min (Bigot-Clivot et al., 2016; Miller et al., 2005b; Molini et al., 2007; Giangaspero et al., 2014). The number of FT cycles also varies, with studies reporting 1 cycle (Adell et al., 2014), 3 cycles (Molini et al., 2007; Giangaspero et al., 2005; Giangaspero et al., 2014; Schwab and Mcdevitt, 2003), 5 cycles (Staggs et al., 2015; Fayer et al., 2003; Fayer

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et al., 2002), 6 cycles (Bigot-Clivot et al., 2016), or 10 cycles (Wells et al., 2015). Utility of FT may also differ for different environmental matrices, including water and foods (Miller et al., 2005a; Molini et al., 2007; Krusor et al., 2015). Therefore, it is important to investigate the efficacy of extraction in matrices that can serve as exposure routes for infection.

To address the lack in reported studies comparing different FT cycles for DNA extraction from protozoan oocysts, this investigation was designed to test the efficiency of oocyst DNA extraction following various numbers of FT cycles applied on a simple matrix (water or PBS), or whole mussel homogenate (*Mytilus californianus*). The sensitivity of *C. parvum* detection in water was further investigated using three different PCR primer sets, following similar spiking experiments previously reported for *T. gondii* (Shapiro et al., 2015).

Type II *T. gondii* (M4 strain (Gutierrez et al., 2010)) oocysts were produced in experimentally-infected cats as previously described (Fritz et al., 2012). *C. parvum* (Iowa isolate) oocysts were obtained from experimentally-infected calves (University of Arizona Cryptosporidium Production, 2018). Parasite stock solutions were enumerated using a hemocytometer chamber under light microscopy at 400× magnification. For *T. gondii*, triplicate serial dilutions of 0, 1, 10, 100, and 1000 oocysts were prepared in either 100 μL of sterile deionized (DI) water or mussel homogenate samples. Mussel homogenates were prepared by blending entire mussel tissue with equal volume of peptone water (Adell et al., 2014). Because results for *T. gondii* suggested similar DNA extraction efficiency from these two matrices (Kruskal-Wallis test, $P > .05$ (Table 1)), FT cycles were compared for *C. parvum* in 100 μL sterile phosphate-buffered saline (PBS), with triplicate dilutions of 0, 10, 100, 1000, and 10,000 oocysts. The relevant matrix (PBS, water, or mussel homogenate) not spiked with oocysts, and a negative control containing only extraction reagents, served as negative controls. Spiked samples and negative controls were subjected to one, three, or six FT cycles consisting of 4 min in liquid nitrogen followed by 4 min in boiling water. The remainder of the DNA extraction process utilized the Qiagen DNeasy® Blood & Tissue Kit, with final DNA eluted using 50 μL 95 °C ultrapure water with 10% AE buffer (QIAGEN Inc., Toronto, ON, Canada) (Adell et al., 2014).

**Table 1**
Detection of *Toxoplasma gondii* oocysts extracted from water and mussel tissue homogenate, and *Cryptosporidium parvum* oocysts extracted from phosphate-buffered saline (PBS) using one, three, or six freeze-thaw (FT) cycles.

| *Protozoan parasite* | # Oocysts per 100 μL sample | PCR target | Matrix | # Detected[a]/# Tested | | |
|---|---|---|---|---|---|---|
| | | | | FT cycles | | |
| | | | | 1 | 3 | 6 |
| *T. gondii* | 0 | B1 (Shapiro et al., 2015; Grigg and Boothroyd, 2001) | Deionized water | 0/3 | 0/3 | 0/3 |
| | 1 | | | 0/3 | 0/3 | 0/3 |
| | 10 | | | **1/3** | 0/3 | 0/3 |
| | 100 | | | 3/3 | **3/3** | **3/3** |
| | 1000 | | | 3/3 | 3/3 | 3/3 |
| | | | | **1** | **3** | **6** |
| *T. gondii* | 0 | B1 (Shapiro et al., 2015; Grigg and Boothroyd, 2001) | Mussel tissue homogenate | 0/3 | 0/3 | 0/3 |
| | 1 | | | **1/3** | 0/3 | 0/3 |
| | 10 | | | 2/3 | 0/3 | **2/3** |
| | 100 | | | 3/3 | **3/3** | 3/3 |
| | 1000 | | | 3/3 | 3/3 | 3/3 |
| | | | | **1** | **3** | **6** |
| *C. parvum* | 0 | gp60 (Iqbal et al., 2012) | Phosphate buffered saline (PBS) | 0/3 | 0/3 | 0/3 |
| | 10 | | | **1/3** | 0/3 | **2/3** |
| | 100 | | | 3/3 | **2/3** | 3/3 |
| | 1000 | | | 3/3 | 3/3 | 2/3 |
| | 10,000 | | | 3/3 | 3/3 | 3/3 |

[a] All PCR reactions used 5 μL template representing 1/10th of the 50 μL eluted DNA from each sample; thus, (oo)cysts per reaction are 1/10th of spiked oocyst numbers; Numbers in bold represent the lowest number of oocysts detected when tested in triplicate for a given matrix and number of FT cycles.

**Table 2**
Mastermix components, thermocycler conditions, and primer sequences used for detection of *Toxoplasma gondii* and *Cryptosporidium parvum*. All reactions were carried out in a 50 μL volume. Negative controls included extraction reagents with no sample added, PCR mastermix containing PCR-grade water instead of DNA template, as well as mastermix with no template added. DNA extracted from *T. gondii* tachyzoite pellets (RH strain) obtained through parasite propagation in cell culture and from 10,000 *C. parvum* oocysts served as positive controls for PCR.

| Parasite | Locus | Master mix | Thermocycler | Primers | Reference(s) |
|---|---|---|---|---|---|
| *T. gondii* | B1 | 1× PCR Buffer ($Mg^{2+}$), 300 μM of each dNTP, 500 nM of each primer, 0.4 μg/μl BSA, 1.5 U Taq polymerase, and 5 μL (external) or 2 μL (internal) DNA template. | Pre-PCR 3 min at 94 °C; 35 cycles of: denaturation at 95 °C for 40s, annealing at 58 °C (external) or 59 °C (internal), extension at 72 °C for 90s; post-PCR at 72 °C for 4 min. | External forward: TGTTCTGTCCTATCGCAACG<br>External reverse: ACGGATGCAGTTCCTTTCTG<br>Internal forward: TCTTCCCAGACGTGGATTTC<br>Internal reverse: CTCGACAATACGCTGCTTGA | Shapiro et al. (2015); Grigg and Boothroyd (2001) |
| *C. parvum* | gp60 | 1× PCR Buffer ($Mg^{2+}$), 200 μM of each dNTP, 500 nM of each primer, 0.4 μg/μl BSA, 1.5 U Taq polymerase and 5 μL (external) or 2 μL (internal) DNA template. | Pre-PCR 5 min at 94 °C; 40 cycles of: denaturation at 94 °C for 30s, annealing at 55 °C for 45 s, extension at 72 °C for 60s (external) or 45 s (internal); post-PCR at 72 °C for 10 min. | External Forward: ATGAGATTGTCGCTCATTATC<br>External Reverse: TTACAACACGAATAAGGCTGC<br>Internal Forward: GCCGTTCCACTCAGAGGAAC<br>Internal Reverse: CCACATTACAAATGAAGTGCC | Iqbal et al. (2012) |
| *C. parvum* | 18S ssrRNA | 1× PCR Buffer ($Mg^{2+}$), 200 μM of each dNTP, 500 nM of each primer, 0.4 μg/μl BSA, 1.5 U Taq polymerase and 5 μL (external) or 2 μL (external) DNA template. | Pre-PCR 3 min at 94 °C; 35 cycles of: denaturation at 95 °C for 40s, annealing at 58 °C (external) or 59 °C (internal) for 40s, extension at 72 °C for 90s; post-PCR at 72 °C for 4 min. | External Forward: TTCTAGAGCTAATACATGCG<br>External Reverse: CCCATTTCCTTCGAAACAGGA<br>Internal Forward: GGAAGGGTTGTATTTATTAGATAAAG<br>Internal Reverse: AAGGAGTAAGGAACAACCTCCA | Xiao et al. (2000) |
| *C. parvum* | 18S ssrRNA | 1× PCR Buffer ($Mg^{2+}$), 200 μM of each dNTP, 200 nM of each primer, 0.4 μg/μl BSA, 1.5 U Taq polymerase and either 5 μL (external) or 2 μL (internal) DNA template. | Pre-PCR 3 min at 94 °C; 35 cycles of: denaturation at 95 °C for 40s, annealing at 58 °C (external) or 60 °C (internal) for 40s, extension at 72 °C for 90s; post-PCR at 72 °C for 4 min. | External forward (358-F): CGGGTAACGGGGAATTAGGG<br>External reverse (1141-R): TCAGCCTTGCGACCATACTC<br>Internal forward (153-F): TGGAATGAGTTAAGTATAAACCCCT<br>Internal reverse (695-R): GCTGAAGGAGTAAGGAACAACC | Present study (m18S) |

**Table 3**
Detection of *Cryptosporidium parvum* DNA using primer sets targeting the gp60 protein or 18S ssrRNA gene. DNA was extracted from oocysts in PBS using a single FT cycle.

| # Oocysts per 100 µL PBS | # Detected[a]/# Tested | | |
|---|---|---|---|
| | Primers | | |
| | gp60 | Xiao 18S | m18S |
| 0 | 0/3 | 0/3 | 0/3 |
| 10 | **1/3** | **2/3** | 0/3 |
| 100 | 3/3 | 3/3 | **2/3** |
| 1000 | 3/3 | 3/3 | 3/3 |
| 10,000 | 3/3 | 3/3 | 3/3 |

[a] All PCR reactions used 5 µL template representing 1/10th of the 50 µL eluted DNA from each sample; thus, (oo)cysts per reaction are 1/10th of spiked oocyst numbers; Numbers in bold represent the lowest number of oocysts detected for a given primer set when tested in triplicate.

Extracted *T. gondii* and *C. parvum* DNA was amplified via PCR using nested primer sets targeting the B1(Krusor et al., 2015; University of Arizona Cryptosporidium Production, 2018) and gp60 genes (Grigg and Boothroyd, 2001), respectively (Table 2). DNA extracted from *T. gondii* tachyzoite pellets (RH strain) obtained through parasite propagation in cell culture and from 10,000 *C. parvum* oocysts served as positive controls for PCR.

A single FT cycle yielded the highest sensitivity of *T. gondii* detection in water at one oocyst/reaction, with 10 oocysts/reaction detected following three or six FT cycles. When extracted from mussel tissue homogenate, detection was achieved for a single oocyst spiked in 100 µL of matrix (0.1 oocyst/reaction) following one FT cycle, ten oocysts/reaction following three FT cycles, and one oocyst/reaction following six FT cycles (Table 1). For *C. parvum*, both one and six FT cycles yielded parasite detection at one oocyst/reaction in PBS, with a 100 oocysts/reaction detected following three FT cycles. All replicates extracted from 100 or more oocysts using one FT cycles tested positive, while one replicate with 1000 oocysts extracted with six FT cycles did not amplify (Table 1). All negative controls tested negative by PCR for both parasites.

To compare different primer sets and PCR methods for detection of *C. parvum* DNA following 1 FT cycle, three assays were compared; one targeting the *gp60* gene (Iqbal et al., 2012), and two targeting the *18S ssrRNA* gene: commonly used primers designed by Xiao et al. (2000) and a recently-designed primer set termed m18S (Table 2), intended for multiplex PCR for simultaneous detection of various parasites. DNA extracted from 10,000 oocysts served as a positive control for PCR.

Highest assay performance for *C. parvum* DNA amplification was observed with the 18S primer set published by Xiao et al. (2000) with 1 oocyst/reaction detected in 2/3 replicates (Table 3). The gp60 assay resulted in 1/3 replicates amplifying at 1 oocyst/reaction, while the m18S assay detected oocysts at 10 oocysts/reaction. All replicates containing 100 or more oocysts/reaction tested positive using all primer sets, while all negative controls tested negative (Table 3).

This study is the first to systematically compare different FT cycles for extraction of DNA from *T. gondii* and *C. parvum* oocysts in diverse matrices. Under the specific temperatures (liquid nitrogen/boiling water) and durations (5 min each) tested here, increasing numbers of FT cycles did not enhance oocyst DNA detection and may have resulted in decreased sensitivity due to degradation of DNA with repeated FT cycles. The validation of a single FT cycle for efficient extraction of oocyst DNA should aid surveillance studies that aim to screen large number of environmental samples in a timely manner, with minimal cost and personnel time (Carey et al., 2004). Future investigations should further compare how different temperature settings used in FT procedures compare for optimized protozoan parasite DNA extraction and detection.

For *C. parvum*, nested PCR primers designed by Xiao et al. (2000) targeting the 18S ssrRNA gene yielded the highest sensitivity, validating its use for screening environmental samples that typically harbor low (but epidemiologically significant) concentrations of parasites. While qPCR has been advocated as most sensitive and specific for nucleic acid detection (Forootan et al., 2017), other reports have demonstrated conventional PCR to be more sensitive for *T. gondii* detection in fresh and marine waters (Shapiro et al., 2010). Therefore, molecular assays must be tested in parallel for targeted pathogens in specific sample types to establish a robust comparison for aiding surveillance for pathogen contamination in diverse environmental matrices.

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