CPS 2024 RFP – FINAL PROJECT REPORT

Project Title:
Validation of novel DNA isolation procedures from limited numbers of *Cyclospora* oocysts

Project Period:
January 1, 2025 – December 31, 2025

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Objectives:

1. Evaluate >10 methods for efficient DNA extraction from *Cyclospora* oocysts.
2. Validate methods for concentrating oocysts from produce washes.

Funding for this project was provided partly through the CPS Campaign for Research.

FINAL REPORT

Summary of Findings and Recommendations

In recent years, *Cyclospora* has emerged as an important pathogen that threatens the produce industry with costly recalls and few detection and mitigation strategies. One of the primary challenges for detecting *Cyclospora*, and thus alerting the produce industry to a contamination event, is the lack of effective methods to isolate *Cyclospora* DNA. The current gold-standard approach to *Cyclospora* DNA requires specialized equipment and long processing times. In this work, we developed a rapid, cost-effective method for DNA isolation from *Cyclospora* that does not require specialized equipment. This method was effective at isolation and detection of *Cyclospora* DNA from < 10 oocysts/mL, and was capable of isolating *Cyclospora* DNA from fecal samples with similar efficiency to the UNEX system currently used by the Centers for Disease Control and Prevention (CDC) for molecular diagnostics. The new DNA isolation method developed in this work represents the first step in the development of a sensitive molecular diagnostic assay for *Cyclospora* that could serve as an early warning system for the produce industry.

Note: Objective 1 is the focus of this report. Our ability to secure sufficient numbers of oocysts to satisfactorily complete both Objectives 1 and 2 became impossible, largely influenced by the government shutdown. The majority of the oocysts we received were used to validate Objective 1, with a subset reserved for any suggested work stemming from manuscript review. While Objective 2 remains unfinished, Objective 1 briefly addresses the efficacy of the presented DNA isolation method on limited numbers of oocysts.

Abstract

Few methods for isolation of high quality *Cyclospora cayetanensis* DNA have been described and many require specialized equipment or long processing times. We sought to develop a rapid, cost-effective method for *Cyclospora* DNA isolation that reduces equipment burden. We first devised and validated a method using safe and abundant oocysts of *Eimeria acervulina*, a *C. cayetanensis* surrogate organism, before applying it to oocysts of *Cyclospora*. *Eimeria* oocysts were exposed in aqueous solution to compounds potentially disruptive to the protein, carbohydrate, and lipid moieties of the coccidian oocyst wall. Among these compounds, the organic base ethylenediamine (EDA), in conjunction with the anionic surfactant sodium dodecylsulfate (SDS), was effective in degrading the oocyst wall and releasing DNA for downstream applications. A chemical-only treatment regimen liberated DNA from unsporulated oocysts, but sporulated *E. acervulina* oocysts required an additional brief physical disruption step, which could be accomplished by vortexing oocysts for 15 seconds in a bead beating matrix tube. To assess the efficiency of EDA:SDS DNA isolation, we used PCR amplification of a 186-bp fragment of the *E. acervulina* β-tubulin gene. Sensitivity analysis indicated that this method allows PCR amplification of β-tubulin from DNA extracted from fewer than 10 oocysts/mL. Following protocol validation in *E. acervulina*, EDA:SDS extraction was assessed for isolation of *Cyclospora* DNA from clinical stool samples. To validate the utility of the protocol, PCR targeting the 8 loci that comprise the Centers for Disease Control and Prevention's inter-lab validated *Cyclospora* molecular genotyping protocol was conducted. Compared to the widely used UNEX DNA isolation method, the EDA:SDS protocol substantially reduces processing time and provides more than a 30-fold reduction in reagent costs, thereby lowering the barrier of entry for *Cyclospora* detection and diagnostics.

Background

Cyclospora cayetanensis is an apicomplexan parasite that causes cyclosporiasis, an enteric illness marked by prolonged, relapsing, watery and explosive diarrhea and associated gastrointestinal symptoms. Although historically considered endemic to tropical and subtropical regions, cyclosporiasis represents an emerging foodborne concern in industrialized countries, with seasonal outbreaks increasingly reported in North America and Europe.

The oocysts are resilient to environmental degradation and chemical disinfectants, complicating both containment and decontamination efforts. Since few *Cyclospora* oocysts are required for infection (Dixon et al., 2005), rapid and sensitive detection methods are needed. Detection and characterization would be aided by easy and cost-effective means to extract nucleic acids from their physically robust oocysts.

The primary challenge in isolating genetic material from *Cyclospora* and other coccidian parasites resides in breaking the hardy wall structures. The barriers that constitute the oocyst wall are composed of carbohydrates, lipids, and protein. Some coccidia, such as *Toxoplasma* and *Eimeria*, are enclosed by two walls while *Cryptosporidium* oocysts harbor only one. Within these walls, most coccidian parasites (excluding *Cryptosporidium*; Samuelson et al., 2013) produce a primary, clade-specific sugar polymer.

Acid-fast lipids are also structural components of the oocyst wall (Bushkin et al., 2013). Mai et al. (2009) identified the fatty acids (saponifiable lipids) palmitic acid, stearic acid, oleic acid, linoleic acid, behenic acid, lignoceric acid, and the non-saponifiable lipids cholestadiene, cholestane, and cholesterol as major lipid constituents of the *Eimeria* oocyst wall, with the total lipid fraction making up 1.4-7.6% of the wall content on a w/w basis. However, most of the oocyst wall appears proteinaceous (Stotish et al., 1978). While Stotish et al. (1978) reported an *E. tenella* oocyst wall composition of 67% peptide, 14% lipid, and 19% carbohydrate, more recent studies suggest the protein fraction accounts for greater than 90% of the wall content (Mai et al., 2009).

Among the various modalities to break the coccidian oocyst wall that have been published include rapid methods that obviate the requirement for specialized laboratory equipment and forego the cumbersome processes of serial centrifugation or phase separation. Physical disruption, chemical lysis via alkalizing agents, osmotic stress, thermal treatment, exposure to alcohols, and even essential oils have been used to "crack" *Eimeria*, *Toxoplasma*, and *Cryptosporidium* oocysts (see Gerhold et al., 2015; Millar et al., 2001; Belli et al., 2003; Remmal et al., 2011; Tang et al., 2018; Hagos et al., 2022). In comparison, few methods have been published and validated for *Cyclospora* wall rupture.

One widely used method is the UNEX method for DNA isolation (Qvarnstrom et al., 2018), which is proscribed by the CDC for molecular genotyping efforts of *C. cayetanensis* across affiliate labs. While the method is efficient, UNEX buffer is costly and a single UNEX extraction can take nearly 30 minutes. Therefore, we sought to develop a method that employs comparatively inexpensive reagents, and which reduces hands-on time.

Research Methods and Results: Objective 1

Methods

Eimeria acervulina oocyst propagation

HR308 broiler chicks (Longeneckers Hatchery, Elizabethtown, PA) were inoculated with *E. acervulina* oocysts following previously published methods. Oocyst laden chicken litter was mixed with tap water at a 1:5 ratio and agitated for 30 minutes using magnetic stirring. This mixture was filtered through

cheesecloth placed in a funnel and collected in a large beaker. Aliquots were provisioned into 50 mL conical tubes, and these suspensions were centrifuged at 935 × *g* for 10 minutes using a Thermo Scientific TX-1000 rotor (Thermo Scientific X Pro Series, r = 20.9 cm). The resulting pellets were subject to saturated NaCl (360 g/L) treatment and centrifuged again at 935 × *g* for 10 minutes. Finally, the suspensions were allowed to rest for 10 minutes at room temperature. The top fractions of approximately 1 mL were collected and washed with tap water, followed by centrifugation at 1460 × *g* for 15 minutes. Residual salt was removed via two subsequent wash steps.

E. acervulina oocyst treatment and PCR amplification

E. acervulina oocysts stored in 2.5% potassium dichromate were rinsed three times with distilled water to remove potassium dichromate. Oocysts were then suspended in distilled water at an initial testing concentration of 4.6×10^6 oocysts/mL. 100 µL of oocyst suspension was added to 100 µL of EDA and 100 µL of 1% SDS (EDA:SDS 1:1 v:v). Oocysts were then spun through an Epoch Biosciences DNA filter column on a Fisher Scientific AcuSpin Micro 17 (Ch. 500025PP rotor) at 14,000 × *g* for 1 minute. The column was then subject to DNA cleanup using Qiagen buffer AW1 and AW2. Briefly, 500 µL buffer AW1 was applied to the center of the column, and the column and collection tube were spun at 14,000 × *g* for 1 minute. The flowthrough was discarded and Qiagen buffer 500 µL AW2 was added to the column, followed by centrifugation for 1 minute at 14,000 × *g*. Flowthrough was discarded and the column was centrifuged for an additional minute at 14,000 × *g*. Distilled water was used to elute DNA from the column.
PCR amplification was performed using primers flanking a 186-bp fragment of the β-tubulin locus of *E. acervulina*. Amplification was carried out using 5 µL of template DNA under a program using a melting temperature of 58°C for 40 amplification cycles, and amplicons were visualized on 3% agarose gel stained with SYBR Safe.

C. cayetanensis oocyst treatment and PCR amplification

Cyclospora-positive clinical stool samples were similarly subject to EDA:SDS treatment. Briefly, 300 µL of stool were transferred into a clean microcentrifuge tube and mixed with an equal volume of phosphate-buffered saline (PBS, pH = 7.4). This mixture was centrifuged for 5 minutes at 14,000 × *g*. The supernatant was discarded, and the remaining material was treated with 200 µL EDA and 200 µL 1% SDS. The mixture was vortexed and placed in a Power Bead Pro tube (Zymo), followed by bead beating for 1 minute at 6.0 m/sec or vortexing for 15 seconds. The tube was centrifuged for 5 minutes at 14,000 × *g* to settle SDS foam. The supernatant was transferred to an Epoch Biosciences DNA filter column and centrifuged for 1 minute at 14,000 × *g*, following which, treatment with Qiagen buffers AW1 and AW2, and elution with water was performed as above.

qPCR

The efficiency of DNA isolation was compared between the EDA:SDS method and the UNEX method by quantitative real-time PCR. *Cyclospora* oocysts from two clinical stool samples, a positive control sample spiked with oocysts, and a stool sample containing no oocysts were each extracted using EDA:SDS method as described above or the UNEX method (Qvarnstrom et al., 2018) and subject to qPCR of the 18S locus using primers and probes published previously (Verweij et al., 2003), with modifications. First, Probe281T was labeled on the 5' end with FAM instead of HEX and used a black hole quencher on the 3' end. A reaction targeting the internal control plasmid was also included in this mix, using primers IC-F and IC-R and the HEX-labeled probe IC-P2 (**Table 1**). Cycling parameters included 1 cycle of 50°C for 2 minutes followed by 95°C for 2 minutes, after which 40 cycles of 95°C for 15 sec and 67°C for 1 min were carried out using a QuantStudio 3 thermocycler and associated software.

Sensitivity test

A culture of *E. acervulina* enumerated to 4.6×10^6 oocysts/mL was used as a stock, from which serial 1:10

dilutions were prepared in distilled water. The diluted suspensions were subject to EDA:SDS extraction as above, using the one-minute bead beating approach, and used as input template for PCR amplification of β-tubulin. The resultant amplicons were visualized on a 3% agarose gel stained with SYBR safe.

Results

An initial screen revealed several candidate compounds with oocyst disrupting action, according to the successful PCR amplification of target DNA. Representative results are given in **Figure 1**, which shows that permanganate and persulfate ions (**Fig. 1A, lanes 1, 2**), formalin (**Fig. 1A, lane 5**), and 1% Triton X 100 + H_2O_2 were moderately successful, as evidenced by weak to moderate bands on gel electrophoresis. Among the various compounds and combinations tested, treatments with the basic compounds NaOH, KOH, and EDA were capable of reliably and reproducibly isolating DNA for amplification (**Fig. 1B**). Of these three, we used EDA (**Fig. 1A, lane 11**) to treat unsporulated oocysts for various contact times: 0 min (brief exposure before spinning through the column), 1 min, 15 min, and 30 min (**Fig. 1C**). Weak amplification of the β-tubulin locus resulted following even brief treatment (0 min) with EDA.
To evaluate the sensitivity of the isolation method, we prepared a 1:10 serial dilution of unsporulated oocysts, starting with a concentration of 4.6×10^6 oocysts/mL; each member of the series was extracted with EDA:SDS. Subsequently, these isolations were subject to PCR amplification of β-tubulin (**Fig. 1D**, representative of three replicates). The results of this experiment indicate that DNA isolation using EDA isolated amplifiable DNA from fewer than 10 oocysts/mL.

While unsporulated oocysts were sensitive to a handful of chemical-only treatments, sporulated oocysts proved resistant to wall dissolution via strictly chemical means, requiring substantially extended treatment times to produce β-tubulin amplicons. In contrast to unsporulated oocysts, which were sensitive to a 15-minute exposure of NaOH, KOH, or EDA (**Fig. 1B**), sporulated oocysts required contact for up to 4 hours to isolate their DNA for successful PCR amplification (**Fig. 2A**). A brief physical disruption step overcame the need for prolonged chemical exposure to liberate DNA from sporulated oocysts. Initially, sporulated oocysts were treated with test compounds for 4 hours followed by bead beating. This method produced amplicons from various treatments (including combinations of EDA, NaOH, KOH, Triton, and SDS, as well as acetone and BME) (**Fig. 2A**). Of the chemical combinations tested in conjunction with physical disruption, EDA + KOH + SDS, EDA + NaOH + SDS, and EDA + SDS proved most successful (**Fig. 2A, lanes 10, 11, 16**). Furthermore, contact time with EDA:SDS could be reduced to less than 1 minute when followed by bead beating (**Fig. 2B**). Even more surprising, vortexing the EDA:SDS and oocysts in the PowerBeadPro tube for 15 seconds proved effective at rupturing oocysts after EDA:SDS exposure, yielding robust and reproducible β tubulin amplification (**Fig. 2C**, representative of three experiments).

Cyclospora oocysts derived from clinical stool samples were extracted using the EDA:SDS method. First, primers targeting CDS 1 were used to validate EDA:SDS extraction of *Cyclospora* from stool. Band intensity appeared more intense for the UNEX extracted samples than for the EDA:SDS extracted samples, but both methods supported robust amplification (**Fig. 3A**). Finally, primers for the eight targets of the CDC NGS panel were employed for PCR amplification. Seven of eight markers were obtained, which satisfies the CDC criteria of ≥5 bands for genotyping, indicating that EDA:SDS succeeds to extract DNA for genotyping *C. cayetanensis* (**Fig. 3B**). The UNEX isolation from these samples yielded eight of eight PCR bands and was successfully genotyped in the 2025 season. The difference may stem from the inclusion of a Zymo inhibitor cleanup in the UNEX protocol. We used no such cleanup step for EDA:SDS. Inhibitor cleanup might conceivably facilitate amplification of 8^{th} product after EDA:SDS extraction. Quantitative realtime PCR was used to assess the efficiency of EDA:SDS vs. UNEX extraction by targeting the 18S rRNA gene from *C. cayetanensis*. **Fig. 3C** shows that EDA:SDS was less efficient than UNEX isolation via qPCR under the ΔΔCt calculation.

Outcomes and Accomplishments

We evaluated a battery of chemical compounds for their utility as agents to rupture coccidian oocyst walls. These included weak and strong acids and bases, detergents, reducing agents, organic solvents, peroxides, amino acid derivatives and organic amines, and alcohols. Mixing ethylenediamine (EDA) with 1% SDS for DNA extraction proved fruitful with unsporulated oocysts and subsequently with sporulated *E. acervulina* oocysts; efficient amplification from sporulated oocysts (bearing an additional impervious structure in the form of the sporocyst wall) required a brief physical disruption process, which could be accomplished with a 15-second vortex step. Following validation using *E. acervulina*, the method proved efficacious for extracting DNA from *Cyclospora* oocysts isolated from positive clinical stool samples. The method supported amplification of seven of the eight targets of the genotyping panel used by CDC and affiliate labs to aid outbreak traceback.

We submit that EDA:SDS DNA isolation lowers the barrier of entry for *Cyclospora* detection by reducing equipment burden, reagent costs, and processing time. Compared to the UNEX method of DNA isolation, EDA:SDS extraction reduces costs by greater than 30-fold and reduces hands-on time approximately 6-fold. Since few oocysts are required for infection and disease, we sought to ascertain the limit of detection using EDA:SDS coupled with conventional PCR amplification of the β-tubulin locus. In our hands the method showed sensitivity that allowed successful amplification from input material harboring fewer than 10 oocysts/mL. While EDA:SDS extraction is less efficient than UNEX, ongoing analysis of sequence data quality from EDA:SDS and UNEX isolated specimens suggests that EDA:SDS is suitable and appropriate for downstream molecular analyses.

APPENDICES

Publications and Presentations

Publication: (Manuscript in preparation) “Ethylenediamine and sodium dodecylsulfate treatment destabilizes *Eimeria acervulina* and *Cyclospora cayetanensis* oocyst walls” (submitting to Parasitology Research)

Presentation & Poster: Validation of novel DNA isolation procedures from limited numbers of *Cyclospora* oocysts, CPS Research Symposium, 2025, La Jolla, CA

Budget Summary

This project was awarded $57,900 in research funds. All funds have been expended, with the exception of the travel funds to be used for the 2026 CPS Research Symposium.

Table and Figures

Table 1. Primers used in this study. 186F/R are original to this work. Ea: *Eimeria acervulina*; Cc: *Cyclospora cayetanensis*.

Target	Primer name	Primer sequence
Eaβ-tubuin	186F	5' - TGGAATGGGTACGCTGCTTA -3'
	186R	5' - AGCGCCTCGTTGTCAATAAC -3'
CcCDS-1	GT1-F	5' - CTCCTTGCTGCTCAGAACGA -3'
	GT1-R	5' - CAAGAGAGGAGCAGTGGCAA -3'
CcCDS-2	GT2-F	5' - TGCAAACTACTAAGGGCGCA -3'
	GT2N-R	5' - CGCCTTCTCTTGAGCCTTGA -3'
CcCDC-3	GT3-F	5' - AATCGAATCGGTGCAGTGCTTA -3'
	GT3N-R	5' - GACTGAACGTGTGAGAGGGG -3'
CcCDS-4	GT4-F	5' - GTAGATGGGTCCTTGAAGGCT -3'
	GT4N-R	5' - CAGACGCCTAAGGAACCGAA -3'
CcHC378	HC378F	5' - CCCCTGCCTTGTTCTTGGTGAA -3'
	HC378R	5' - CCGGCGACACAGAGGTACC -3'
CcHC360i2	HC360i2F	5' - CCCATTACGCCGCATAGAGT -3'
	HC360i2R	5' - GCATTGCAAAGCCAGTCAGC -3'
CcMT Junction	CycloMT5732F	5' - GTCGTTACACCATTCATGCAG -3'
	CycloMT6266R	5' - CTTTCAAAGTAACCATCAAGCCT -3'
CcMSR	Cyclo_MSR_15F	5' - GGACATGCAGTAACCTTTCCG -3'
	Cyclo_MSR_688R	5' - AGGAAAGGTTAACCGCTGTCA -3'
Cc18s	Cyclo250F	5' - TAGTAACCGAACGGATCGCATT -3'
	Cyclo350R	5' - AATGCCACGGTAGGCCAATA -3'
	Cyclo281T	5' - FAM-CCGGCGATAGATCATTCAAGTTTCTGACC-BHQ -3'
internal control	IC-F primer	5' - ACCGTCATGGAACAGCACGTA -3'
	IC-R primer	5' - CTCCCGCAACAAACCCTATAAAT -3'
	IC-P2 probe	5' - HEX-CCACTGCTAAAGGTAGCCCACGTC-BHQ -3'

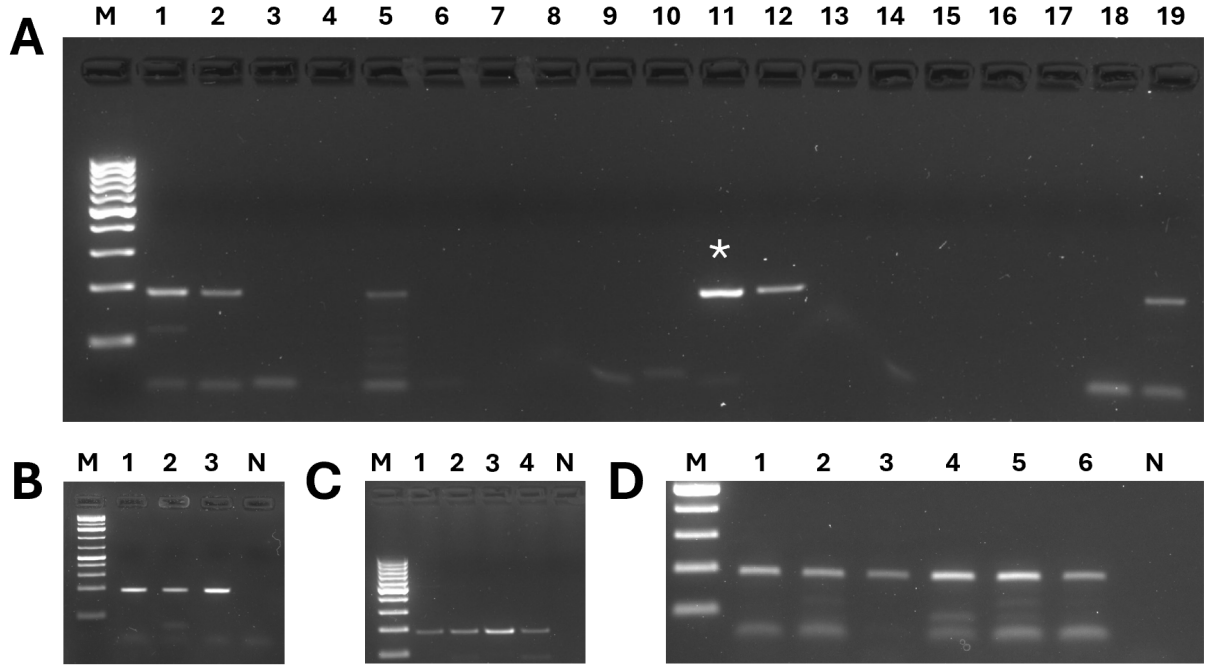

Figure 1. *B-tubulin* amplicons obtained from DNA isolated from unsporulated oocysts. Oocysts were treated with one or a combination of chemical agents. **A:** 60-minute contact time **M.** marker **1.** permanganate ion **2.** persulfate ion **3.** H_2O_2 **4.** propionic acid **5.** formalin **6.** 0.1m periodate ion (PI) **7.** methanol **8.** lactic acid (LA) **9.** NaOH + LA **10.** NaOH + PI **11.** EDA + SDS 12. EDA + KOH **13.** EDA + LA **14.** 1% Triton + acetone **15.** 1% Triton + PI **16.** 1% Triton + methanol, 60m **17.** 1% Triton + LA **18.** 1% Triton + chloroform **19.** 1% Triton + H_2O_2 **B:** 15-minute treatment using three downselected compounds **1.** EDA **2.** NaOH **3.** KOH **4.** negative **C: M.** marker **1.** brief contact (0 min) **2.** 1 min **3.** 15 min **4.** 30 min **5.** Negative. **D.** A 1:10 dilution series of *E. acervulina* DNA isolated using EDA:SDS method and used as template for beta-tubulin amplification; Lane 1: 10^{-1}, 2: 10^{-2}, 3: 10^{-3}, …, 6: 10^{-6}, N: negative.

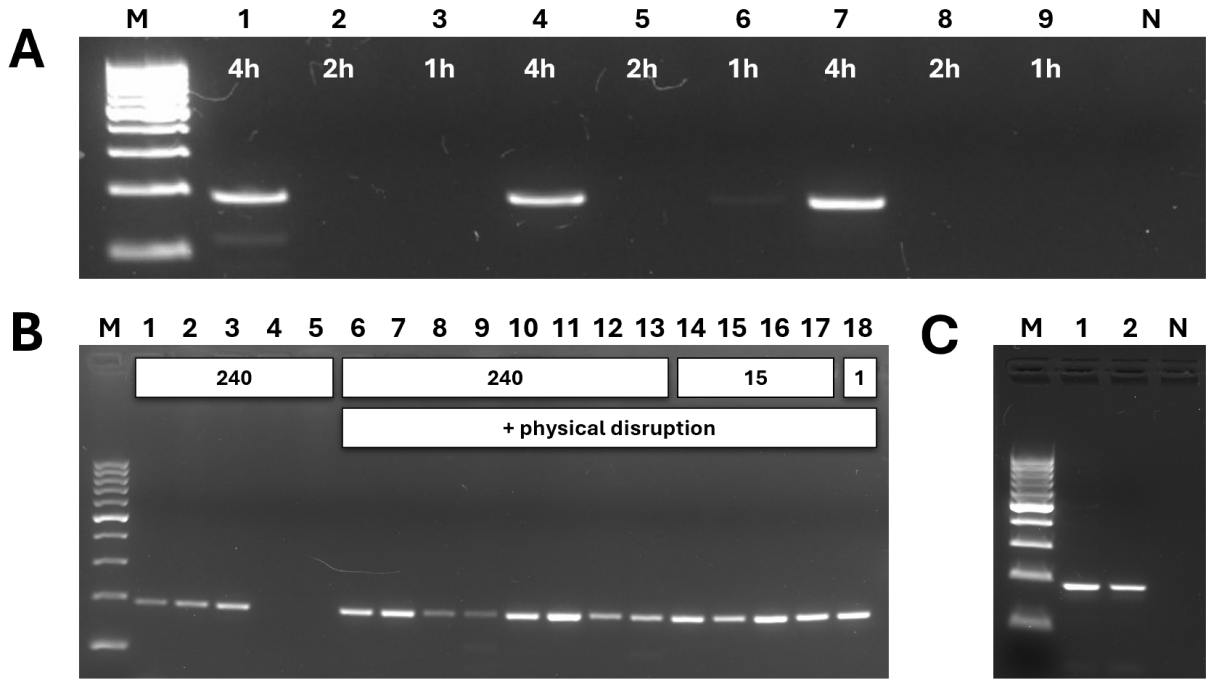

Figure 2. Chemical exposure followed by physical disruption yields successful amplification of the *B-tubulin* locus. **A.** Sporulated oocysts were treated with NaOH (Lanes 1-3), KOH (Lanes 4-6), or EDA (Lanes 7-9) for 4h, 2h, or 1h. **B.** Sporulated oocysts were treated with various compounds or combinations of compounds for 240, 15, or 1 minute(s) (indicated by white boxes). Physical disruption was employed for samples 6-18. **1.** EDA + β-mercaptoethanol (BME) **2.** EDA + NaOH + SDS + Triton **3.** EDA + KOH + SDS + Triton **4.** EDA + acetone + SDS + Triton **5.** NEGATIVE **6.** EDA + NaOH + SDS + Triton **7.** EDA + KOH + SDS + Triton **8.** EDA + acetone + SDS + Triton **9.** EDA + BME + SDS + Triton **10.** EDA + NaOH + SDS **11.** EDA + KOH + SDS **12.** EDA + acetone + SDS **13.** EDA + SDS **14.** KOH + SDS **15.** SDS **16.** EDA + SDS **17.** NaOH + SDS **18.** EDA + SDS. **C.** Sporulated oocysts were treated with EDA:SDS for 1 minute and subject to bead beating for one minute (lane 1) or a 15-second vortex (lane 2).

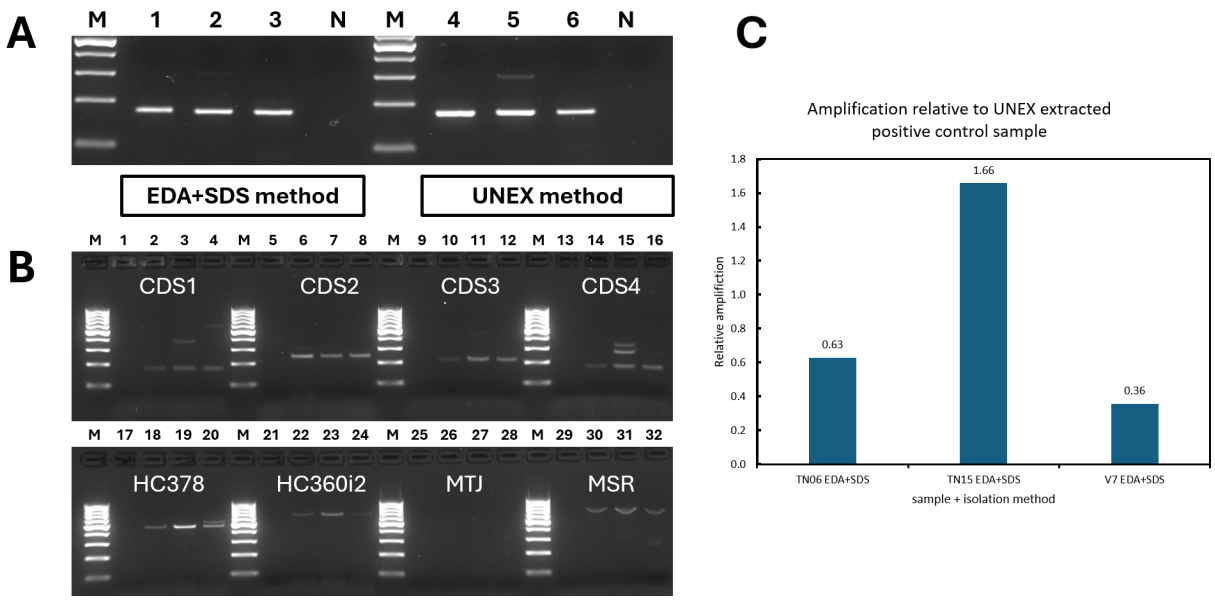

Figure 3. EDA:SDS DNA isolation from *Cyclospora* oocysts. A. CDS1 was amplified from the clinical samples TN06 and TN15 using either the EDA:SDS or UNEX method (**lanes 1, 2, 4, 5**) and SVD07 positive control (**lanes 3, 6**); M: Marker; N: negative. **B.** Seven of eight amplicons in the CDC targeted next generation sequencing panel were successfully amplified following EDA:SDS extraction of *Cyclospora*-positive clinical stool samples. **C.** Quantitative realtime PCR amplification results for 18 seconds from EDA:SDS isolated oocysts, expressed in comparison to the same PCR on UNEX isolated oocysts.

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