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Enrichment of *Cryptosporidium parvum* from *in vitro* culture as measured by total RNA and subsequent sequence analysis

Jessica C. Kissinger^{a,b,c}, Karen E. Hermetz^{a,+}, Keith M. Woods^{d,*}, and Steve J. Upton^d

^aCenter for Tropical and Emerging Global Diseases, University of Georgia, Athens, GA 30602, USA

^bDepartment of Genetics, University of Georgia, Athens, GA 30602, USA

^cInstitute of Bioinformatics, University of Georgia, Athens, GA 30602, USA

^dDivision of Biology, Kansas State University, Manhattan, KS 66506, USA

Abstract

Cryptosporidium parvum is an apicomplexan parasite that infects a wide range of hosts including humans. Due to the parasite's quasi-intracellular, intermembrane location on the host cell, it is difficult to purify parasites from *in vitro* and *in vivo* infections for molecular studies. We have developed a method to greatly enrich *in vitro* *C. parvum* merozoites from host cells. The efficiency of the protocol was assessed with *C. parvum* (KSU-1 isolate) parasites of different developmental stages isolated following a synchronized infection of HCT-8 host cells. Total RNA was extracted from the samples and used to evaluate the quantity of host cell contamination in enriched parasite fractions. The quality of the RNA was verified using an Agilent BioAnalyzer. cDNA libraries of RNA isolated from 24 and 48 h *C. parvum in vitro* preparations isolated via this protocol were sequenced at the Broad Institute via an NIH Microbial Sequencing (GSCID) Contract.

Cryptosporidium sequences comprised 30% of the cDNA reads, demonstrating significant enrichment.

Graphical abstract

Corresponding Author: Jessica C Kissinger, Coverdell Center, University of Georgia, 500 D.W. Brooks Drive, Athens, GA 30602, +1-706-542-6562, jkissing@uga.edu.

Current address: Emory University School of Public Health Atlanta GA 30322

Last known address: University of Nebraska Medical Center, Omaha NE 68198

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Keywords

Cryptosporidium; merozoite; tissue culture; enrichment; *in vitro*

1. Introduction

Parasites of the genus *Cryptosporidium* are zoonotic and have been shown to be the second leading cause of diarrhea in infants in many countries [1, 2]. *Cryptosporidium* is spread via the oral fecal route in water and food [3]. *Cryptosporidium* parasites have proven difficult to culture continuously *in vitro* leading to the use of animal models (cow and immunosuppressed mouse) for propagation and study [4-6]. Although *C. parvum* can infect human ileocecal (HCT-8) cells and other intestinal epithelial cell lines *in vitro*, it does not complete its life cycle and produce infective oocysts when cultured in a typical cell culture monolayer [7]. Following *in vitro* infection of a host cell monolayer, *Cryptosporidium parvum* progresses through asexual (type I and II merozoites) and sexual (microgamont and macrogamont) life stages, yet no viable infective oocysts are produced [8].

The molecular biology of these asexual and sexual life stages is poorly understood because each stage exists as only a fraction of the overall *Cryptosporidium* infection, and these fractions are dwarfed in size by the host cell. For reference, the infectious form of *Cryptosporidium*, the oocyst, is roughly spherical, contains four sporozoites and is $\sim 5 \mu\text{M}$ in diameter or approximately $65 \mu\text{M}^3$ [5]. For reference, the volume of a fibroblast is $2000 \mu\text{M}^3$ and a HeLa cell is $3000 \mu\text{M}^3$ [9]. To facilitate the study of *Cryptosporidium* life cycle stages that occur following host-cell infection, an enrichment protocol was developed to isolate post-infection (non-sporozoite) stages from infected host cells. Enrichment is further complicated by the cellular localization of *C. parvum* parasites in quasi-intracellular, intermembrane location on the host cell which suggests that *C. parvum* parasites, unless released as merozoites or gamonts, might pellet with cellular membrane fractions. In this study, cellular membrane fractions were avoided and the focus was on enrichment of liberated, or free, merozoite stages. Enriched fractions were subjected to RNA isolation and subsequent sequence generation. Based on the data obtained, at 24 and 48 h post-infection time points, merozoites were easily enriched. However, enrichment at 72 h post infection, was limited. Parasite RNA yield was increased by using a large number of tissue culture flasks (4-8 T75) and high multiplicity of infection (MOI).

When this enrichment protocol for *in vitro C. parvum* developmental stages was developed in late 2005, only limited EST data (567 EST reads total) from non- *in vitro/in vivo* stages, e.g. sporozoites, were available for *C. parvum* [10]. Shortly thereafter, 27,498 EST reads for *C. muris* RN66 were generated from oocysts (Da Silva *et al.*, 2008, unpublished) and 10,110 full-length ESTs from *C. parvum* HNJ-1 oocysts (Watanabe *et al.*, 2009, unpublished) were deposited in the GenBank EST archive [11]. The EST sequence data described here, were generated from *C. parvum* KSU-1 sporozoites and *in vitro* enriched life cycle stages (24-48 h post-infection) and they were submitted to the GenBank in 2009. Like many EST studies, they were shared with the community but were unpublished.

RNA-Seq data are now being generated from *in vitro* or *in vivo* *C. parvum* infections (Hehl *et al.*, unpublished, CryptoDB; Widmer unpublished, CryptoDB) [12-14], but the efficiency of recovery of post-infection parasites stages is very poor, ranging from 1-4% of total transcripts (Sateriale, personal communication; [15]. The protocol described here provides yields of up to 30% parasite transcripts. Given the large number of new researchers entering the *Cryptosporidium* field this enrichment protocol is being shared to help facilitate research on the biology of this important pathogen.

2. Materials and Methods

2.1 Parasites and host cells utilized

Cryptosporidium parvum parasites were the KSU-1 strain maintained at Kansas State University. Oocysts less than 3 months of age were used. Host cells were Human colon ileocecal cells, HCT-8 [HRT18] (ATCC® CCL244™ American Type Culture Collection, Rockville, MD).

2.2 Media utilized

Infection media: RPMI 1640 (Thermo Fisher) with 10% Opti-MEM (Gibco), 10% heat inactivated Fetal Bovine Serum (HyClone) and 2mM L-glutamine (Thermo Fisher).

Growth Media: RPMI 1640, 10% Opti-MEM (Gibco), 50 mM glucose, 35 µg/ml ascorbic acid, 2.0 µl/ml folic acid, 4.0 µg/ml 4-amino benzoic acid, 2.0 µg/ml calcium pantothenate, 250 µM sodium butyrate (Alfa Aesar Cat# A11079), 250 µM sodium propionate (Alfa Aesar Cat# A17440), 20 µM beta-mercaptoethanol, 0.02% w/v bovine bile salts. The pH is adjusted to 7.4.

2.3 Oocyst sterilization

C. parvum KSU-1 oocysts were sterilized by treatment with 10% bleach (sodium hypochlorite) in ddH₂O for 10 min at room temperature as described previously [16, 17]. The oocysts were then washed twice with PBS and resuspended in 980 µl of infection media. A small aliquot of resuspended oocysts was then counted at a 1:100 dilution in H₂O on a hemocytometer to determine their concentration.

2.4 Sporozoite excystation for use in tissue culture monolayer infections

Oocysts were added to infection media supplemented with 100 µl of a freshly-prepared 2.5% bovine bile salt solution (Sigma, B-3883; syringe filtered (0.22 µm filter)) to reach a total volume of 5 ml and induce excystation. If tissue culture monolayers were to be infected, 10⁷ oocysts were used for cultures to be harvested at 24 h, 10⁶ for harvests at 48 h and 10⁵ for harvests at 72 h). 5 ml of excystation preparation per T75 flask to be infected were prepared. Six to Twelve T75 flasks per time period (24, 48 or 72 h) were prepared. The excystation mixture was added to flasks as described below. An extra aliquot (not added to a flask) was prepared in 1 ml of infection media and used to monitor excystation progress at 37 °C.

2.5 In vitro tissue culture

HCT-8 cells were seeded in T75 flasks (a 1:2 split) the day before the planned *in vitro* infection and allowed to grow until they reached approximately 90% confluency at 37°C with 5% CO₂ in Growth Media [18, 19]. When the HCT-8 cells were almost confluent, the tissue culture growth media was removed and replaced with 5 ml of the oocyst excystation mixture described above and the flasks were incubated at 37 °C with 5% CO₂ for 2 h. Any remaining oocysts or free sporozoites were removed after the 2 h infection period via media removal and 2 washes with 5 ml of pre-warmed PBS. 20 ml of infection media was then added to each flask and the flasks were incubated at 37°C with 5% CO₂. Infected monolayers were harvested at 24, 48 and 72 h post-infection. The target infection rate for 24 h *in vitro* cultures was an MOI of 15 *C. parvum* parasites per host cell.

2.6 Harvest and enrichment of post-infection parasites

C. parvum post-infection parasites were harvested by removal of the media from the infected tissue culture monolayer followed by a rinse with 5 ml of PBS to remove cell debris. A Pasteur pipet was used to remove any remaining media. 4 ml of trypsin/EDTA (Gibco) was added to the monolayer and the flasks were returned to 37 °C for 15-20 min or until the cells detached from the flask surface.

In preparation for a subsequent step, Percoll reagent (Sigma P1644) was warmed to room temperature and used to prepare 1 ml of a 30% Percoll solution in PBS. 0.5 ml of the solution was placed into each of two, 2 ml low adhesion/retention microfuge tubes and they were set aside at room temperature.

The trypsinized cells were collected in a 50 ml conical tube (6 flasks/tube) and an equal volume of ddH₂O was added. The cells were pelleted by centrifugation at 350 × *g* for 10 min. The supernatant was removed and 0.5 ml PBS was added without resuspension. The HCT-8 cell pellet + PBS solution was then drawn up through a 22 gauge needle and dispensed into a fresh 2 ml low adhesion microfuge tube. The dispensed HCT-8 and PBS mixture was then drawn and ejected two more times through the same 22 gauge needle to further disrupt the cells. The total volume was then brought up to 1.5 ml in the 2.0 ml tube with the addition of PBS. 0.5 ml of room temperature Percoll was added to the tube make a total of 2ml and the tube was vigorously mixed with a vortex for 10 sec. 1 ml of this mixture was then carefully layered over each of the pre-prepared room temperature 0.5 ml 30% Percoll cushions by dripping the mixture slowly down the side of the tubes. The layered tubes were then centrifuged for 5 min at 10,000 × *g* with a “soft stop” to not disturb the Percoll gradient. The *Cryptosporidium* parasites form a faint cloud in the bottom 100 µl fraction of the tube gradient. This layer can be difficult for the untrained eye to see. All cells floating on the upper layers and along the sides of the tube were carefully removed with aspiration down to the level of the parasite “cloud”, beginning around the 100 µl mark. While being careful to not touch the sides of the tube (to avoid host cell contamination) the parasite fraction from the two tubes were transferred into a single new tube. 1 ml of PBS was added to resuspend the parasite fraction and the tube was then centrifuged for 3 min at 2,000 × *g* to pellet the *C. parvum* parasites. The pellet was used immediately for RNA extraction.

2.7 Sporozoite excystation for generating purified sporozoites

When purified sporozoites (oocyst-free) were needed for RNA preparation, the excystation volume (Section 2.4) was reduced to 2 ml. Excysted sporozoites were pelleted by centrifugation at $5,000 \times g$ for 3 min. Following removal of the liquid supernatant, the parasite pellet was carefully resuspended in 0.5 ml PBS. The suspension was then carefully over-layered on 0.5 ml of a 10% Percoll in PBS solution. The tube was then centrifuged for 5 min at $5,000 \times g$. Following centrifugation, the sporozoites are located in the pellet and the oocyst walls are located at the interface. The upper phase and interphase were removed. The remaining supernatant over the sporozoite pellet was carefully removed. The sporozoite pellet was resuspended in 1 ml PBS and centrifuged again at $5,000 \times g$ for 3 min. The supernatant was removed and the sporozoites were carefully resuspended in 0.5 ml PBS and the suspension was carefully layered onto 0.5 ml of 40% Percoll in PBS solution. The layered tube was centrifuged at $5,000 \times g$ for 5 min. Under these conditions, the sporozoites are now located at the interface and the pellet contains unexcysted oocysts. The upper phase was carefully removed. The sporozoites at the interface were carefully removed and resuspend in 1 ml PBS and pelleted by centrifugation at $5,000 \times g$ for 3 min. The supernatant was removed and the sporozoite pellet was immediately utilized for RNA preparation.

2.8 RNA and cDNA library preparation

Excysted sporozoites, 24 h or 48 h *C. parvum* enriched pellets from *in vitro* purifications (4 T75 flasks per time point) were resuspended in RLT buffer (Qiagen RNeasy kit 74104) and total RNA was purified according to manufacturer's directions and assessed on an Agilent Bioanalyzer (Santa Clara, CA) using their Agilent RNA 6000 Nano Kit and ladder. Two excystation preparations followed by sporozoite purification yielded at total of 65 μg of total RNA for construction of a sporozoite library. Yields were variable for the *in vitro* purifications and ranged between 0.5 μg and 3 μg per preparation and bioanalyzer RNA integrity Numbers (RIN) of 0 to 10. Higher values of RIN are preferable and indicate greater integrity of 18S and 28S RNA. The 24 h sample that was used for library preparation contained 2.2 μg of total RNA with a RIN of 5.3 and the 48 h sample had 3.08 μg of total RNA and a RIN of 9.2.

Total RNA samples were provided to Express Genomics (Frederick, MD) for cDNA library generation. The vector was pExpress-1 and the Eco RV and Not I sites were utilized. The libraries were not strand-specific. One normalized library (highly-redundant mRNAs removed via digestion of rapidly self-hybridized cDNA to maximize the recovery of sequence diversity) was prepared from excysted sporozoites with an average insert size of 2.1 kb. 15,000 inserts were bi-directionally sequenced generating ~30,000 paired-end Sanger sequences. The 24 and 48 h *in vitro* total RNA preparations were combined and one non-normalized (no mRNAs removed) nano-scale library was created. The average insert size was 500 bp. Approximately 65,000 Sanger single-read only sequences were generated due to the short insert size. The cDNA libraries were sequenced by the Broad Institute (Cambridge, MA) using Sanger sequencing.

3. Results and Discussion

3.1 Culture and purification protocol

A protocol was developed that permitted the enrichment of *C. parvum* developmental stages from *in vitro* culture in HCT-8 cells (Figure 1). HCT-8 host cells reached an average multiplicity of infection, MOI of 14 as determined by light microscopy at 24 h (Figure 2). It was possible to visually follow the parasites through the purification protocol up until lysis for RNA preparation (Figure 2). The size of the large and small subunit rRNA molecules are different in *C. parvum* and humans. *C. parvum* bands are smaller, especially for the LSU rRNA. As a result, an electrophoretic analysis of total RNA permitted a quick determination of the quality of an RNA preparation (Figure 3). Agilent's Bioanalyzer technology permitted analysis of very small volumes of total RNA to visualize the ratios of LSU and SSU rRNA bands for *C. parvum* with respect to host and quantify the total RNA and its integrity. As can be seen in Figure 3, the results were variable with enrichments working the best for 24 and 48 h post-infection samples. The two best *in vitro* total RNA preparation time points, indicated by an "*" in Figure 3 (24 h (2.2 µg/4 flasks) and 48 h (3.08 µg/4 flasks)), were selected for cDNA and library generation. Excysted sporozoites (Figure 3, *ExSp lane) yielded 65 µg of total RNA that was used for the generation of a normalized sporozoite library.

3.2 Sequence generation

29,571 *C. parvum* KSU-1 sporozoite paired-end reads were generated and 63,580 24-48 h reads were generated from two cDNA libraries. Analysis of the reads from the pooled 24-48 h library, revealed that 39,049 reads represented genes encoded in the human nuclear genome and 5,350 reads were derived from human mitochondrial RNA leaving a total of 19,181 reads from the 24-48 h *in vitro* developmental stages of *C. parvum*, a 30% recovery rate. Considering the difference in cell volume, this is a considerable enrichment.

The 19,181 *C. parvum* KSU-1 ESTs were assembled with cap3 [20] and resulted in 3,644 transcript assemblies. At the time, this was a three-fold increase in the number of available transcript assemblies. These transcripts, when mapped to the *C. parvum* genome sequence, revealed a number of introns that were missing from annotated genes and validated a large number of predicted genes. The mapped sequences also suggest the presence of many overlapping transcripts. This finding is not unexpected given the very compact genome sequence with an estimated size of only 9.1 Mb. Confirmation of overlapping transcripts can only be verified with strand-specific RNA sequence data. If this protocol were to be utilized today to generate mRNA for RNA-Seq analysis, the sequence information obtained would be both quantitative and more complete.

An assembly of the available *C. parvum* oocyst and sporozoite sequences prior to the addition of the data described here yielded only 1,261 EST assemblies, about 1/3 of all predicted genes. Clustering of the EST sequence data generated in this study yielded 3,644 EST assemblies, only 650 of which overlapped with the previous EST assemblies, demonstrating the value added by including post-infection life cycle stages. 399 of the new EST assemblies mapped to the genome sequence but did not overlap with any currently

annotated gene, suggesting many genes had been missed in the initial annotation. This finding was understandable as almost no expression data for *C. parvum* was available at the time. 114 EST assemblies provided evidence of introns that were either missed or improperly located in the existing annotation.

As the sporozoite EST library presented here was normalized, analyses of expression levels were not possible. However, in 2012 a brute force effort led by another group determined the relative expression levels for 3,302 genes at 2-72 h post-infection time points via semi-quantitative real-time PCR [21].

Yields are best at the 24-48 h post-infection time point suggesting success in capturing merozoites. However, Figures 2b and 2c demonstrate that the major stage that is enriched is merozoites that have not yet been released. It is hoped that modifications to the protocol presented here might lead to enrichment of micro- and macrogametocytes by altering the Percoll gradient or centrifugation conditions as gametes should be formed by 48 h.

It is important to note that comparison of Human and *Cryptosporidium* rRNA bands in total RNA preparations did not reflect the levels of host contamination observed in the mRNA sequence results. rRNA band comparisons suggested a host contamination rate of ~10% (Figure 3). However, the mRNA sequence results revealed a 70% host contamination rate, so users are cautioned to get host rRNA levels as low as possible. This said, a 30% recovery rate for *C. parvum* *in vitro* represents significant enrichment, but primarily of merozoite stage parasites. RNA preparations from 72 h infections were poor in quality (Figure 3).

3.3 Data dissemination

All sequence data generated in this project were submitted to the NCBI GenBank EST and Trace Archive repositories. *C. parvum* KSU-1 sporozoite sequences (LIBEST_024208) have accession numbers GH611761.1 - GH582316.1. Pooled 24-48 h *C. parvum* KSU-1 sequences (LIBEST_025356) have accession numbers GT555582.1 - GT494278.1. Additionally, they were submitted to CryptoDB.org (<http://cryptodb.org>).

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Highlights

- A new method is presented for the enrichment of 24-48 h *in vitro* *Cryptosporidium parvum* parasites.
- EST sequences derived from an enriched *C. parvum* fraction revealed that 30% of the sequences obtained originated from the parasite.
- In the absence of enrichment, only 1-4% of RNA sequences from *in vitro* culture represent the parasite.

Figure 1. *Cryptosporidium* oocyst sterilization, excystation, sporozoite and *in vitro* parasite purification workflows

Two workflow scenarios are presented. Following oocyst sterilization, sporozoites are excysted and purified for total RNA extraction (grey workflow) or sporozoites are excysted and used to infect HCT-8 monolayers in T75 flasks for subsequent parasite harvest and enrichment (blue workflow). This figure is a schematic only and additional information is provided in the detailed materials and methods.

Figure 2. Microscopic images of *Cryptosporidium* pre- and post-enrichment

(a) 24 h post infection *C. parvum* KSU-1 infection of Human HCT-8 cells demonstrating high multiplicity of infection. *C. parvum* parasites are in the focal plan with the monolayer below. (b & c) microscopic images of an aliquot of enriched *C. parvum* KSU parasites 48 h post-infection. At 48 hours, type I and type II meronts, free merozoites and few macrogametes are observed.

Figure 3. Bioanalyzer electrophoresis analysis of various total RNA preparations

Total RNA from 24-, 48- or 72-hour enriched fractions from *in vitro* *C. parvum* culture in Human colon ileocecal (HCT-8) cells; L = RNA ladder, size in bp; *C. parvum* excysted and purified sporozoites (ExSp) were used as a control for *C. parvum* SSUrRNA and LSUrRNA size); Hs = human total RNA (prepared from uninfected HCT-8 cells) to use as a control for host cell total RNA contamination by following the differently sized SSU- and LSUrRNA bands; * = total RNA preparations used to generate cDNA libraries; SSUrRNA = Small Subunit ribosomal RNA; LSUrRNA = Large Subunit ribosomal RNA.