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Short communication

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

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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.

## 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 ~5 μM in diameter or approximately 65 $\mu M^3$ [5]. For reference, the volume of a fibroblast is 2000 $\mu M^3$ and a HeLa cell is 3000 $\mu M^3$ [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

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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 remained 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 to 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

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

Infection media: Growth media plus f 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 $ddH_2O$ 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 $H_2O$ 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, $10^7$ oocysts were used for cultures to be harvested at 24 h, $10^6$ for harvests at 48 h and $10^5$ 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% $CO_2$ 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% $CO_2$ 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% $CO_2$. 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 $ddH_2O$ 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 2 ml and the tube was vigorously mixed with a vortex for 10 s. 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 2000 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 5000 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 5000 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