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RNA-Seq analysis during the life cycle of *Cryptosporidium parvum* reveals significant differential gene expression between proliferating stages in the intestine and infectious sporozoites

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ABSTRACT

Cryptosporidium parvum is a major cause of diarrhoea in humans and animals. There are no vaccines and few drugs available to control *C. parvum*. In this study, we used RNA-Seq to compare gene expression in sporozoites and intracellular stages of *C. parvum* to identify genes likely to be important for successful completion of the parasite's life cycle and, thereby, possible targets for drugs or vaccines. We identified 3774 protein-encoding transcripts in *C. parvum*. Applying a stringent cut-off of eight fold for determination of differential expression, we identified 173 genes (26 coding for predicted secreted proteins) upregulated in sporozoites. On the other hand, expression of 1259 genes was upregulated in intestinal stages (merozoites/gamonts) with a gene ontology enrichment for 63 biological processes and upregulation of 117 genes in 23 metabolic pathways. There was no clear stage specificity of expression of AP2-domain containing transcription factors, although sporozoites had a relatively small repertoire of these important regulators. Our RNA-Seq analysis revealed a new calcium-dependent protein kinase, bringing the total number of known Calcium-dependent protein kinases (CDPKs) in *C. parvum* to 11. One of these, CDPK1, was expressed in all stages, strengthening the notion that it is a valid drug target. By comparing parasites grown in vivo (which produce bona fide thick-walled oocysts) and in vitro (which are arrested in sexual development prior to oocyst generation) we were able to confirm that genes for oocyst wall proteins are expressed in gametocytes and the proteins stockpiled rather than generated de novo in zygotes. RNA-Seq analysis of *C. parvum* revealed genes that are expressed in a stage-specific manner and others whose expression is required at all stages of development. The functional significance of these can now be addressed through recent advances in transgenics for *C. parvum*, and may lead to the identification of viable drug and vaccine targets.

1. Introduction

Cryptosporidium is a genus of apicomplexan parasites recognised as one of the four main causes of diarrhoea in young children and, hence, a major contributor to early childhood mortality worldwide (Kotloff et al., 2013; Striepen, 2013; Checkley et al., 2015; Ryan and Hijjawi, 2015; Certad et al., 2017. Furthermore, Cryptosporidia are significant causes of potentially fatal diarrhoea in AIDS patients (Morgan et al., 2000). They are also extremely problematic in livestock, causing profuse diarrhoea and considerable economic losses in young calves and lambs in industrialised agriculture (Joachim et al., 2003; O'Handley and Olson, 2006).

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Although nearly 20 species and genotypes of *Cryptosporidium* have been recorded in humans (Ryan and Hijjawi, 2015), the vast majority of cases associated with diarrhoea in humans are caused by *Cryptosporidium hominis* or *Cryptosporidium parvum* (Certad et al., 2017) Whilst the former appears to be largely human-specific, the latter is also a significant cause of disease in cattle and, thus, a parasite of substantial zoonotic concern (Abeywardena et al., 2015).

Numerous developmental and molecular peculiarities indicate that *Cryptosporidium* spp. are more closely related to a primitive group of apicomplexans, the gregarines, than to coccidian parasites such as *Toxoplasma gondii* or *Eimeria* spp. (reviewed recently (Ryan and Hijjawi, 2015)). However, the developmental life cycles of, for example, *C. hominis* and *C. parvum,* are superficially similar to classic enteric coccidian development. Thus, infection follows a faecal-oral route and begins with the ingestion of sporulated oocysts. Sporozoites are released in the small intestine shortly after ingestion, invade enterocytes and form intracellular parasitophorous vacuoles at the apical surface of host cells, but remain extracytoplasmic. Within these, type I meronts develop, forming eight merozoites. Merozoites released from type I meronts either repeat this proliferative step or progress to type II meronts, containing four merozoites. The latter transform into the sexual stages, i.e., macrogametocytes, whose prominent wall-forming bodies can be readily detected in thin section transmission electron micrographs, or microgametocytes containing bullet-shaped microgametes, which are more difficult to identify. Fertilisation leads to the formation of oocysts that sporulate in the intestine (endogenous sporulation) without forming sporocysts and are immediately infectious upon shedding. There is an unresolved controversy about the ability of *Cryptosporidium* to replicate outside of a host organism (Clode et al., 2015).

There are no vaccines or chemoprophylactics to prevent infection with *Cryptosporidium* and very few chemotherapeutic options for its treatment (Checkley et al., 2015). New opportunities to uncover targets for drugs are inherent in the availability of genome sequence databases for *C. parvum* (Abrahamsen et al., 2004) and *C. hominis* (Abrahamsen et al., 2004) and the development of technologies for the genetic manipulation of the former parasite (Vinayak et al., 2015). The *Cryptosporidium* genome has several unique features: it is markedly reduced (at around 9 million bases and <4,000 genes) compared with other enteric apicomplexans (e.g., ~52 million bases and >9,000 genes for *Eimeria tenella*, ~63 million bases and >8,000 genes for *T. gondii*), displaying streamlined metabolic pathways, minimal modes of energy production and an inability to synthesise essential building blocks for amino acid, purine, and fatty acid synthesis. It lacks an apicoplast, due to secondary reduction, and has a reduced mitochondrion (mitosome) without a role in energy metabolism (Keeling, 2004). Thus, *C. parvum* relies heavily on nutrient acquisition from the host (Abrahamsen et al., 2004; Xu et al., 2010) and, arguably, its commensal bacterial flora for intracellular survival. All these features of *Cryptosporidium* result in many of the unique anti-apicomplexan drug targets being absent. Moreover, the complexity and limitations of the available, reliably reproducible and highly productive in vitro cultivation protocols, as well as the lack of robust small animal models for reproducing the entire *Cryptosporidium* life cycle, make new target identification and validation somewhat challenging.

Comparative RNA-Seq analysis can be exploited to uncover critical molecules and pathways in enteric parasite development; we have recently used such approaches to highlight unique expression profiles in the development of *T. gondii* in the cat small intestine (Walker et al., 2015) and *E. tenella* in the caeca of chickens (Walker et al., 2015). In this study, we use similar strategies to compare *C. parvum* stages prepared from the small intestinal epithelium of experimentally infected calves with parasites propagated in vitro. The latter proliferate efficiently as merozoites but they apparently lack the ability to differentiate to oocysts in culture, and can be considered arrested in sexual development. Hence, we used comparative analysis of gene expression as a means to identify factors likely to be relevant for successful sexual development and oocyst formation. In contrast to most intestinal apicomplexans, sporulation ensues without delay in *Cryptosporidium*, resulting in excretion of infectious oocysts containing fully developed sporozoites, which can be considered a stable developmental end point in terms of their mRNA complement until they invade a new host cell to develop into first generation merozoites. Thus, RNA-Seq data of sporozoites from purified oocysts completes the full characterisation of developmental gene expression during the *Cryptosporidium* life cycle.

2. Materials and methods

2.1. Preparation of parasites and RNA

Oocysts of a field strain of *C. parvum*, IPZ:CH-Crypto_K6769, were purified from faeces using a protocol published previously (Meloni and Thompson, 1996). The purity and identification of these oocysts as *C. parvum* "cattle genotype" was confirmed using PCR and direct sequencing of a fragment of the 18S rDNA using published methods (Ward et al., 2002). Oocysts of *C. parvum* IPZ:CH-Crypto_K6769 were stored in PBS with antibiotic/antimycotic solution (Sigma-Aldrich, Switzerland) and passaged regularly through calves purchased from farms in the Canton of Zurich (Switzerland) at 1–3 days of age prior to experimental infections; calves were confirmed to be *Cryptosporidium*-free prior to experimental inoculation by modified Ziehl-Neelsen stain and by coproantigen detection (Bio-X Diagnostics®). The calves were housed in an experimental animal facility of the Institute of Parasitology of the University of Zürich, Switzerland. Individual calves were housed in pens of ~9 m^2 on a litter of straw and sawdust over a rubber mat on a concrete base. The pens were cleaned daily. Calves were fed ad libitum with a commercial milk substitute (UFA200) supplied by an automatic dispenser. Water and hay were also provided ad libitum.

Biological material for RNA extraction and RNA-Seq analysis was generated using in vivo and in vitro stages as well as freshly excysted sporozoites. For the former, a calf was inoculated orally with 2.5×10^9 oocysts and euthanised 47 h p.i. (day 2). Subsequently, another calf was inoculated orally with 1.2×10^9 oocysts and sacrificed 93 h p.i. (day 4). The small intestine was immediately removed and chilled on ice. All subsequent processing of tissue was done using pre-chilled solutions in a 4 °C cold room. Each small intestine was divided into five segments of equal length. In step 1, all segments were rinsed with PBS and then filled with HBSS (w/o Mg^{2+}/Ca^{2+})/2 mM DTT/5 mM EDTA, ligated and incubated for 15–30 min. The HBSS wash fraction was then collected and stored on ice for parasite enrichment. In step 2, the five segments were each divided into two parts. Part 1 was filled with HBSS (w/o Mg^{2+}/Ca^{2+})/5 mM EDTA, ligated and incubated for another 15–20 min. The liquid was collected and added to the HBSS wash collected in Step 1. Part 2 was filled with 80 ml of trypsin-EDTA solution (Sigma, 3924, diluted 1:1 with PBS) and incubated for approximately 25 min. Trypsin was inactivated by adding 40 ml of FCS and this liquid was also added to the HBSS wash collected in Step 1. The combined material was pelleted and then mixed with RLT buffer (guanidine thiocyanate lysis buffer) (supplemented with 10 µl of 2-mercaptoethanol/ml buffer, RNeasy® Mini- or Midikit, QIAGEN). The resulting lysate was passed eight times through a 20-gauge needle fitted to an RNase-free syringe.

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