Gliding Motility Leads to Active Cellular Invasion by Cryptosporidium parvum Sporozoites


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We examined gliding motility and cell invasion by an early-branching apicomplexan, Cryptosporidium parvum, which causes diarrheal disease in humans and animals. Real-time video microscopy demonstrated that C. parvum sporozoites undergo circular and helical gliding, two of the three stereotypical movements exhibited by Toxoplasma gondii tachyzoites. C. parvum sporozoites moved more rapidly than T. gondii sporozoites, which showed the same rates of motility as tachyzoites. Motility by C. parvum sporozoites was prevented by latrunculin B and cytochalasin D, drugs that depolymerize the parasite actin cytoskeleton, and by the myosin inhibitor 2,3-butanedione monoxime. Imaging of the initial events in cell entry by Cryptosporidium revealed that invasion occurs rapidly; however, the parasite does not enter deep into the cytosol but rather remains at the cell surface in a membrane-bound compartment. Invasion did not stimulate rearrangement of the host cell cytoskeleton and was inhibited by cytochalasin D, even in host cells that were resistant to the drug. Our studies demonstrate that C. parvum relies on a conserved actin-myosin motor for motility and active penetration of its host cell, thus establishing that this is a widely conserved feature of the Apicomplexa.

The Apicomplexa is a large phylum of obligately intracellular parasites of medical and veterinary importance. The life cycles of all apicomplexans contain one or more haploid invasive stages and a diploid stage that is the result of a sexual cycle. Toxoplasma gondii, the causative agent of toxoplasmosis in neonates and the immunocompromised, has two tissue stages, tachyzoites and bradyzoites, within its life cycle. In all members of the phylum, the stage that emerges from oocysts after sexual recombination is termed a sporozoite. Sporozoites are also invasive and give rise to infection of intestinal enterocytes following oral ingestion by susceptible hosts. In Cryptosporidium spp., the sporozoite (contained within a thick-walled oocyst) is primarily responsible for transmission between hosts. Amplification of parasites within the host is the result of two cycles involving successive replication and invasion by merozoites and sporozoites (derived from thin-walled oocysts).

The members of the phylum Apicomplexa that commonly cause disease in humans include T. gondii; Plasmodium spp., the causative agents of malaria; and Cryptosporidium spp., agents of waterborne diarrhea that are particularly dangerous for those with immune deficiencies. Although the medical importance of Cryptosporidium spp. is well understood, the cellular and molecular mechanisms of infection by these organisms are poorly characterized, mostly due to their intractability. Cryptosporidium spp. are difficult to propagate in tissue culture, and no system for genetic study exists. Most of our understanding of the cellular biology of Cryptosporidium spp. is inferred from other members of the Apicomplexa, particularly T. gondii, which has been a model for this group. However, Cryptosporidium is an early-branching apicomplexan, more similar to gregarines (parasites of invertebrates) than to either Toxoplasma or Plasmodium, and thus may differ in fundamental features such as motility and invasion.

Cryptosporidium does not penetrate deeply into the cytosol of its host cell but rather rests on a pedestal of actin filaments that forms at the apical surfaces of epithelial cells of the gut. Enclosed with the host cell plasma membrane, Cryptosporidium remains intracellular but extracytoplasmic. Previous studies on the interaction of Cryptosporidium sporozoites with host cells reveal a substantial rearrangement of the host cell cytoskeleton following infection. This response stands in marked contrast to invasion by T. gondii, which is independent of host cell actin. Entry by T. gondii and Plasmodium spp. is extremely fast, occurring in less than 1 min. However, previous studies of Cryptosporidium infection examined the interaction only at 24 h or later after addition of parasites to host cells. As such, the events that lead to initial entry of Cryptosporidium into epithelial cells remain uncharacterized.

Apicomplexans are thought to advance upon a target host cell for invasion using a unique, active process termed gliding motility. Gliding motility by apicomplexans does not require shape changes like the crawling of amoebae, nor do these parasites have cilia or flagella, except in the microgamete life cycle stage. Instead, the motility of most stages appears to be driven by coupling the translocation of surface adhesins to an actin-myosin motor beneath the parasite plasma membrane.
(32). For *T. gondii* tachyzoites, genetic studies show that only parasite actin is required for cell invasion (9). Unlike mammalian cells, which retain 50% of their actin pool as filaments, *T. gondii* maintains the vast majority of its actin (97%) in the monomeric state (8). Induction of polymerization of actin with the drug jasplakinolide (JAS) causes *T. gondii* tachyzoites to form a projection of apical actin filaments (31). Treatment with JAS also increases the rate of *T. gondii* tachyzoite gliding; however, these movements are aberrant due to frequent direction reversals (36). Collectively, these findings indicate that polymerization of new actin filaments controls both the initiation and the directionality of motility.

In *T. gondii* tachyzoites, motility consists of three stereotypical behaviors: (i) circular gliding, which occurs only in a counterclockwise direction; (ii) upright twirling, which occurs only in a counterclockwise direction; and (iii) helical gliding, which uses a clockwise revolution to move the crescent-shaped parasite forward across its substrate (19). Gliding motility in other apicomplexans has been demonstrated by trail assays with *C. parvum* (2) and *Eimeria* spp. (16) as well as by video microscopy studies of gliding *Plasmodium berghei* (18) or beads moving along the surfaces of gregarines (25). While the mechanism and the stereotypical movements of gliding motility are believed to be conserved throughout the Apicomplexa (32), there are no published studies of the process in real time in Crypto-

**Materials and Methods**

*T. gondii* tachyzoite culture. *T. gondii* strain RH tachyzoites were propagated in human foreskin fibroblast monolayers as described previously (28). Parasites were obtained soon after host cell lysis. All cultures were tested with the Gen-

human foreskin fibroblast monolayers as described previously (28). Parasites 

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For all rate analyses, three separate experiments, each containing five examples of the type of motility under study from two or more separate dishes, were analyzed. The number of 360-degree revolutions per second was used to determine the twirling rate. The total distance traveled in a 10-s period of uninterrupted locomotion was used to determine rates for circular and helical gliding. Averages shown in tables are means ± SE and were analyzed for statistical significance by Student’s two-tailed t test.

RESULTS

*C. parvum* sporozoites form trails during gliding. Previous immunofluorescence studies indicate that *C. parvum* forms circular trails while gliding (2). To examine the pattern of trails formed by *C. parvum* on the substrates used here, we performed a gliding assay. *C. parvum* sporozoites were allowed to glide on PLL-coated coverslips and then were processed for immunofluorescence. Trail deposition was visualized using an antibody to the surface membrane protein p25. *C. parvum* sporozoites deposited trails that formed both circular and helical patterns (Fig. 1A and B), as previously reported for *T. gondii* tachyzoites.

Real-time analysis of *C. parvum* motility. Because the trail assays described above could not show the rate or pattern of gliding, we conducted real-time video microscopy studies to further examine motility by *C. parvum* sporozoites. We found that *C. parvum* exhibited similar motility to *T. gondii*. Although we could not identify episodes of upright twirling, *C. parvum* did exhibit counterclockwise circular movements (Fig. 1C; see also video 1) (all videos are available at http://www.sibleylab.wustl.edu/cptgmovies/) and directional helical gliding (Fig. 1D).
and Table 1; see also video 2). Notably, *C. parvum* sporozoites moved more rapidly than did *T. gondii* tachyzoites. The average gliding velocity for *C. parvum* was 5.1 μm/s, approximately three times the average for *T. gondii* tachyzoites (Table 1). Because helical gliding by *C. parvum* occurred rapidly (video 2), it was difficult to specify its precise phases in real time. However, frame-by-frame analysis of helical gliding by *C. parvum* (as shown in Fig. 1D) indicated that the steps involved were similar to those previously described for *T. gondii* (19).

In order to determine whether real-time gliding motility by *C. parvum* depended on actin and myosin, we next studied the effects of cytoskeletal inhibitors on gliding by *C. parvum* sporozoites. Motility by *C. parvum* sporozoites was completely blocked by actin-depolymerizing agents (CD and LatB) and by the myosin inhibitor BDM (data not shown). Treatment with the actin-polymerizing drug JAS completely prevented movement by *C. parvum* sporozoites, despite previous observations that it increases nonproductive motility by *T. gondii* tachyzoites (36).

In conclusion, although gliding motility by *C. parvum* and *T. gondii* clearly depended on stereotypical behaviors driven by an actin-myosin-based motor, there were differences between the two species in the rate of gliding and the responses to jasplakinolide.

**Gliding motility by *T. gondii* sporozoites is similar to that of tachyzoites.** We were interested in determining if the differences in motility seen between *C. parvum* sporozoites and *T. gondii* tachyzoites were due to species differences or the stage of the parasite. Therefore, for comparison, we analyzed motility by *T. gondii* sporozoites in real time using video microscopy. We found that sporozoite motility was strikingly similar to tachyzoite gliding by *T. gondii*. Identical stereotypical movements were used by tachyzoites and sporozoites: circular gliding (Fig. 2A; video 3), helical gliding (video 4), and clockwise twirling (Fig. 2B; video 5) (all videos are available at http://www.sibleylab.wustl.edu/cptgmovies/). Tachyzoites and sporozoites also moved at approximately the same velocity (Table 2). These movements depended on F-actin and myosin, as evidenced by the fact that the motility of *T. gondii* sporozoites was completely blocked by CD, LatB, and BDM (data not shown). In addition,

### Table 1. Gliding by *C. parvum* sporozoites

<table>
<thead>
<tr>
<th>Behavior</th>
<th><em>T. gondii</em> tachyzoites</th>
<th><em>C. parvum</em> sporozoites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Occurrence</td>
<td>Rate(^{bc}) (mean ± SE)</td>
</tr>
<tr>
<td>Twirling (clockwise)</td>
<td>+</td>
<td>0.37 ± 0.08</td>
</tr>
<tr>
<td>Circular gliding</td>
<td>+</td>
<td>0.98 ± 0.19</td>
</tr>
<tr>
<td>Helical gliding</td>
<td>+</td>
<td>1.64 ± 0.12</td>
</tr>
<tr>
<td>Rolling</td>
<td>Only with JAS</td>
<td>5.51 ± 0.67</td>
</tr>
</tbody>
</table>

\(^{a}\) +, behavior occurred; –, behavior did not occur.

\(^{b}\) For rate determination, three experiments containing five examples of each type of motility from at least two coverslips per experiment were analyzed. Rates are given as micrometers per second, except for twirling, for which revolutions per second are shown.

\(^{c}\) Rates for tachyzoite gliding are taken from reference 36.

\(^{d}\) *, significantly faster (P < 0.05) than *T. gondii* tachyzoites by Student’s two-tailed t test.

\(^{e}\) NA, not applicable.
TABLE 2. Rates of gliding motility by T. gondii sporozoites

<table>
<thead>
<tr>
<th>Behavior</th>
<th>Sporozoites</th>
<th>Tachyzoites,'a'</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Under control conditions</td>
<td>With 2 μM JASb</td>
</tr>
<tr>
<td>Twirling</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Clockwise</td>
<td>0.25 ± 0.04</td>
<td>0.37 ± 0.03c</td>
</tr>
<tr>
<td>Counterclockwise</td>
<td>NA</td>
<td>NDc</td>
</tr>
<tr>
<td>Circular gliding</td>
<td>1.37 ± 0.41</td>
<td>NDc</td>
</tr>
<tr>
<td>Helical gliding</td>
<td>1.65 ± 0.20</td>
<td>NA</td>
</tr>
<tr>
<td>Rolling</td>
<td>NA</td>
<td>3.50 ± 0.52d</td>
</tr>
</tbody>
</table>

For rate determination, three experiments containing five examples of each type of motility from at least two coverslips per experiment were analyzed. Rates are given as micrometers per second, except for twirling, for which revolutions per second are shown. NA, not applicable; ND, not determined.

For rate determination, three experiments containing five examples of each type of motility from at least two coverslips per experiment were analyzed. Rates are given as micrometers per second, except for twirling, for which revolutions per second are shown. NA, not applicable; ND, not determined.

a: For rate determination, three experiments containing five examples of each type of motility from at least two coverslips per experiment were analyzed. Rates are given as micrometers per second, except for twirling, for which revolutions per second are shown. NA, not applicable; ND, not determined.

b: *: significantly faster (P < 0.05) than under control conditions by Student’s two-tailed t test.

c: Rates for tachyzoite gliding are taken from reference 36.

d: Similar to rate for clockwise twirling.

e: Rarely occurring.

studies using high concentrations (2 μM) of JAS caused actin to accumulate at the apical ends of ~30% of treated sporozoites (Fig. 2C) and increased the rates of motility of both T. gondii tachyzoites and sporozoites (Tables 1 and 2). JAS-treated sporozoites were slightly more capable of carrying out stereotypical movements of gliding than were tachyzoites treated with similar drug concentrations. For example, sporozoites occasionally underwent one step in a cycle of helical gliding before changing direction or type of movement, while tachyzoites could only move back and forth (“roll”) in response to JAS. Importantly, however, both sporozoites and tachyzoites moved faster and were able to reverse direction after JAS treatment (Table 2).

Time lapse video analysis of Cryptosporidium invasion. To determine if motility by Cryptosporidium sporozoites was also involved in cellular invasion, we analyzed the interaction of freshly excysted sporozoites with epithelial cells in vitro. Two types of polarized epithelial cells were used in this analysis: KB100 cells, a human epithelial cell line, and MDCK cells, canine kidney epithelial cells. Time lapse video microscopy indicated that invasion by Cryptosporidium sporozoites was rapid, often occurring in ~30 s following initial contact with the host cell (Fig. 3A; video 6). However, in most cases, the parasite did not appear to enter the host cytosol but instead extended beyond the edge of the host cell (Fig. 3B; video 7). Individual sporozoites at times initially penetrated into the cytosol but then reversed direction and returned to the surface of the cell (Fig. 3C; video 8) (all videos are available at http://www.sibleylab.wustl.edu/cptgmovies/). Following invasion, a majority of parasites remained firmly attached but appeared to protrude from the cell surface. As previously observed for T. gondii (19, 36), invasion was always preceded by helical gliding while circular gliding did not lead to productive invasion (data not shown).

Rapid invasion leads to membrane engulfment at the cell surface. To determine whether attached and protruding parasites were actually within the host cell membrane, we used a latency assay to monitor their accessibility to antibodies to a cell surface antigen on the sporozoite. Following a short 5-min pulse for invasion, cells were lightly fixed and stained first with a MAb to the sporozoite surface antigen p25 and then with a red dye-conjugated secondary antibody. This step will stain extracellular parasites red, while internalized parasites will be protected from labeling. The monolayer was then permeabilized and restained with a MAb to the sporozoite surface antigen p25 followed by green dye-conjugated secondary antibodies that will label all parasites. The ratio of double-stained (red and green) (external) parasites to green only (internal) parasites provides a sensitive index for invasion. Analysis of Cryptosporidium sporozoites invading KB100 cells confirmed that approximately half of the parasites that were cell associated were internalized during this short invasion pulse (Fig. 4A). This rate of entry is roughly equal to that observed in invasion assays employing T. gondii (our unpublished observations). Internal parasites, as defined by their selective green staining, were often found to protrude from the edge of the cell (Fig. 4B), indicating that they were enclosed within the host membrane despite the appearance of being peripherally attached. At the points of parasite entry, there were no obvious changes in host cell actin as detected by phalloidin labeling (Fig. 4B).

Invasion by Cryptosporidium relies on the parasite’s actin cytoskeleton. Previous studies have shown that the invasion of host cells by T. gondii is blocked by CD, which also blocks parasite entry into host cells that are fully resistant to the effects of the drug (9). These findings established that invasion requires actin polymerization in the parasite. To test whether Cryptosporidium exhibited a similar requirement, we examined the rapid invasion of KB100 (wild-type) and CYT1 (CD-resistant) host cells by using the latency assay described above. The majority of Cryptosporidium sporozoites that were associated with KB100 and CYT1 cell monolayers were internalized after 5 min (Fig. 4A and B). However, their ability to enter both wild-type and CD-resistant host cells was almost completely blocked by CD (Fig. 4A and B). Inhibition of entry was evident in the two-color assay by the presence of parasites that remained attached to the cell but were exposed to externally added antibodies (red staining) (Fig. 4B). These results indicate that Cryptosporidium requires actin polymerization in the parasite to enter the protective environment of the host cell membrane.

DISCUSSION

Here we demonstrate that C. parvum sporozoites display gliding behaviors similar to those previously described for other apicomplexans. The motility of Cryptosporidium sporozoites was faster than that of either tachyzoites or sporozoites of T. gondii. Cryptosporidium motility was blocked by BDM, LatB, or CD, indicating requirements for both myosin activity and polymerized actin. Time lapse video microscopy revealed that gliding motility led to invasion of host cells but that this process differed significantly from invasion by T. gondii. While T. gondii tachyzoites invade a vacuole within the cytosol, Cryptosporidium sporozoites invaded at the cell periphery but were nonetheless enclosed within the host cell membrane. Host cell invasion required actin polymerization by the parasite but evidently not the host, indicating that a common mechanism drives invasion by diverse apicomplexan parasites.

Our studies using C. parvum are the first real-time characterizations of its motility. Gliding motility occurred in both
circular patterns, as described previously from static assays (2), and helical patterns, previously described for T. gondii. Helical, but not circular, gliding precedes cell entry by T. gondii (19, 28) and Plasmodium (35), and a similar relationship was also observed here for Cryptosporidium. During gliding, C. parvum exhibited movements similar to those exhibited by T. gondii, except that it was not observed to twirl on its posterior end. Perhaps this difference is due to the relative shape of the two organisms; C. parvum is about half the diameter of T. gondii. Consequently, it may lack the ability to generate the force necessary to pivot about the posterior end, or, alternatively, it may lack the rigidity necessary for standing upright. The importance of twirling in 3-dimensional settings has not been examined, and therefore it is uncertain if it plays any role in vivo.

Motility by C. parvum occurred almost three times faster than T. gondii motility under similar situations. Interestingly, this matches the enhanced speed with which T. gondii tachyzoites move after treatment with jasplakinolide (Table 2) (36). It also corresponds to the rate of purified TgMyoA sliding along actin filaments (20). Since TgMyoA is a nonprocessive motor without a clear method of activation (27), we have previously suggested that the rate-limiting step for gliding motility by apicomplexans is actin polymerization (36). The faster motility by Cryptosporidium sporozoites may indicate that they naturally have a higher proportion of polymerized actin, which is also consistent with their being less responsive to the effects of JAS.

C. parvum and Plasmodium sp. sporozoites move more rapidly than T. gondii tachyzoites (18), suggesting that this may be a general trait of sporozoites. However, when analyzed by time lapse video microscopy, T. gondii sporozoites moved at speeds comparable to those of tachyzoites, indicating that these differences are species specific, not stage specific. T. gondii sporozoites displayed the same stereotypical movements as tachyzoites despite minor variations in susceptibility to inhibitors. For example, T. gondii sporozoites were slightly less susceptible to JAS and were still able to carry out one cycle of helical gliding after treatment, while tachyzoites were unable to proceed forward (30).
Previous studies have established that sporozoites of *T. gondii* have a much greater capacity to migrate rapidly through host tissues than bradyzoites (10, 13). We have not examined the motility of bradyzoites in vitro but would expect their behaviors to be similar to those of tachyzoites, since they are able to invade similar types of cells in vitro. The enhanced dissemination ability of sporozoites suggests that additional factors must contribute to their rapid spread in vivo. Following infection in the gut, parasites must traverse the epithelial monolayer, cross the basement membrane, and migrate through the lamina propria in order to reach the circulatory system (4). While in vivo studies have shown that *T. gondii* can migrate deep into the intestinal submucosal tissue (5, 10, 13), it is unclear if parasites disseminate while extracellular or by gaining access into migrating leukocytes that then traffic to distant sites. In vitro studies have established that virulent strains of *T. gondii* have an enhanced capacity to migrate directly across biological barriers in vitro (3); however, this mechanism and an intracellular dispersal route are not mutually exclusive.

Although previous studies have suggested that F-actin is necessary for trail formation by *C. parvum* (17), these experiments relied solely on static assays. We have extended these observations to examine the behavior of *Cryptosporidium* sporozoites during both gliding and host cell invasion by using time lapse video microscopy. These observations reveal that *Cryptosporidium* utilizes a conserved process of gliding motility that also results in a unique mode of cell entry. Instead of penetrating into a vacuole within the host cell cytosol, the parasite becomes enveloped within a membrane-bound compartment while remaining at the cell periphery. A majority of parasites interacting with host cells during a short invasion pulse of 5 min were able to complete the entry process, as shown by their protection from antibody labeling. This mode of entry coincides with the development of *Cryptosporidium*, which proceeds within an apical protrusion from the host cell surface. Our studies using inhibitors and CD-resistant host cells reveal that the successful engulfment of the parasite requires actin polymerization on the part of the parasite, but evidently not by the host cell. Collectively, these findings indicate that host cell internalization is driven by the parasite actin cytoskeleton.

Our studies might appear to contradict earlier findings that indicate that *C. parvum* induces changes in host cell actin after infection (9, 10). However, our experiments have been conducted at a time point very different from those reported previously. We specifically examined the early events (5 min) in entry, while previous studies examined infection only after 24 h. We observed no change in host cell actin during invasion (9), while previous studies examined infection only after 24 h. We observed no change in host cell actin during invasion.
required to define the mechanisms that induce changes in host cell actin and to resolve the precise time frame when such changes are initiated.

The phylum Apicomplexa is highly diverse and contains some 5,000 species, only a few of which are studied. Cryptosporidium represents a fairly early branching member of this group, more closely related to gregarines than to Plasmodium or Toxoplasma (6). The conservation of an actin-based system for motility and cell invasion in Cryptosporidium indicates that this process is likely common to most members of the phylum. C. parvum possesses homologues to T. gondii actin and myosin that are 85% (22) and 57% identical, respectively (Table 3). Furthermore, TBLASTN searches of the CryptoDB (http://CryptoDB.org/) and NCBI databases identified clear homologues of myosin light chain kinase, a component of the myosin motor complex in T. gondii (21), and several actin polymerization-regulatory proteins from T. gondii, including actin-depolymerizing factor (ADF) (1), profilin, and capping protein (CapZβ) (Table 3). While these components have not been fully characterized in parasites, our results suggest that a common process of gliding motility is based on a relatively simple set of conserved cytoskeletal proteins in the Apicomplexa.

Gliding motility is an actin- and myosin-dependent process that is conserved throughout the life cycle and different species of the Apicomplexa. The actin dynamics in these parasites are also highly unusual, with a majority of actin being retained in a monomeric form (8). Due to their unique reliance on polymerization of new filaments for gliding (36), these organisms are acutely sensitive to the inhibitors of actin polymerization.

**ACKNOWLEDGMENTS**

We thank Michael White (Montana State University) for providing the cell culture media and equipment.

**REFERENCES**


**TABLE 3. Homologues of actin-myosin cytoskeleton genes in T. gondii and C. parvum**

<table>
<thead>
<tr>
<th>Gene product</th>
<th>Gene</th>
<th>Homologue in:</th>
<th>% Identity</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Actin</td>
<td>ACT1</td>
<td>T. gondii</td>
<td>88</td>
<td>0.0</td>
</tr>
<tr>
<td>Myosin A</td>
<td>MYOA</td>
<td>T. gondii</td>
<td>56</td>
<td>0.0</td>
</tr>
<tr>
<td>Myosin light chain kinase</td>
<td>MLC1</td>
<td>C. parvum</td>
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<td>7e−06</td>
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<tr>
<td>Capping protein β</td>
<td>CapZb</td>
<td>C. parvum</td>
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<td>1e−15</td>
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<td>C. parvum</td>
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<td>Profilin</td>
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<td>C. parvum</td>
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</tr>
</tbody>
</table>

*Comparison was done by BLASTP using T. gondii genes compared against GenBank by using default settings. Predicted protein found in Cryptosporidium hominis, the homologue in C. parvum was identified by TBLASTN against http://CryptoDB.org (CFpOVA III 2:345790-466126).*

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