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Research paper

The *Cryptosporidium parvum* gp60 glycoprotein expressed in the ciliate *Tetrahymena thermophila* is immunoreactive with sera of calves infected with *Cryptosporidium* oocysts

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ABSTRACT

Cryptosporidium parvum is a protozoan parasite of the phylum Apicomplexa responsible for cryptosporidiosis in calves, a disease that causes significant diarrhea and impairs gain of body weight, generating important production losses. As to now, no effective drugs or vaccines are available for the treatment or prevention of bovine cryptosporidiosis. Several reports suggest that development of a vaccine to prevent cryptosporidiosis is feasible, but relatively few vaccine candidates have been characterized and tested. The most prominent *C. parvum* antigen is gp60, an *O*-glycosylated mucin-like protein tethered to the parasite membrane by a glycosylphosphatidylinositol (GPI) anchor. Gp60 has been shown to be involved in essential mechanisms for the survival of *C. parvum*, such as recognition, adhesion to, and invasion of host cells. This work was aimed at expressing gp60 in *Tetrahymena thermophila*, a ciliated protozoon with numerous advantages for the heterologous expression of eukaryotic proteins, as a first approach for the development of a recombinant vaccine for bovine cryptosporidiosis. *T. thermophila*-expressed gp60 localized to the protozoon cell surface and oral apparatus, and partitioned into the Triton X-114 detergent phase. This indicates that the protein entered the reticuloendothelial system of the ciliate, and suggests it contains a GPI-anchor. Homogenates of gp60-expressing *T. thermophila* cells were recognized by sera from calves naturally infected with *C. parvum* demonstrating their immunoreactivity. In summary, the heterologous expression of gp60, a *C. parvum*-encoded GPI-anchored protein, has been successfully demonstrated in the ciliate *T. thermophila*.

1. Introduction

Cryptosporidium spp. are protozoan parasites of the phylum Apicomplexa responsible for cryptosporidiosis, a disease that causes intense diarrhea (Tzipori and Ward, 2002). Cryptosporidia can infect a wide range of vertebrate host species (Šlapeta, 2013). In cattle *C. parvum* is the most important species and causes diarrhea in young calves, generating significant economic losses related to low performance and production (Olson et al., 2004).

As to now, no effective drugs or vaccines are available for the treatment or prevention of bovine cryptosporidiosis. Halofuginone lactate is the only licensed treatment for cryptosporidiosis in calves, but it does not completely prevent or cure the disease (De Waele et al., 2010). Regarding vaccines, several attempts to develop an effective vaccine have been made, including the use of killed *C. parvum* oocysts (Jenkins et al., 2004). Although some developments showed partial success under experimental conditions, none of them was effective under field conditions (Mead, 2014). A few antigens have been explored as vaccine candidates against bovine cryptosporidiosis. Most studies have focused on the immunogenic potential of specific proteins involved in the attachment and invasion of host cells, such as the *O*-glycosylated mucin-like gp60 (also known as gp40/15) (Cevallos et al., 2000; Priest et al., 2001). Post-translational proteolytic cleavage of gp60 generates two fragments of 15 and 40 kDa, respectively; gp15, tethered to the parasite membrane by a glycosylphosphatidylinositol (GPI) anchor and gp40, non-covalently associated to gp15 (Fig. 1).

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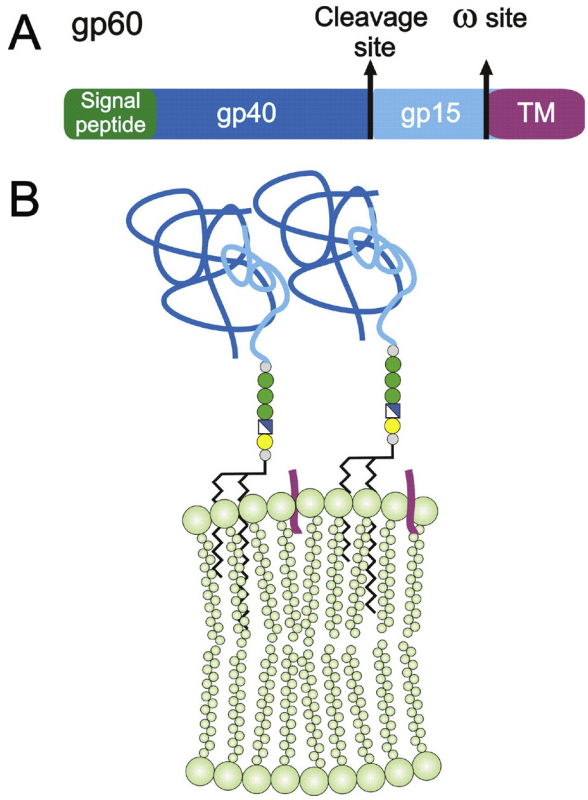

Fig. 1. A. Schematic representation of the *Cryptosporidium parvum*-gp60 (gp40/15) protein. **B.** Membrane topology of gp60, a GPI-anchored protein attached to the exoplasmic leaflet of the membrane lipid bilayer. In blue the gp40 mature protein is associated to the gp15 mature protein (light blue) which is attached to a hypothetical GPI anchor. Green circle: mannose; blue-white square: glucosamine; yellow circle, grey circle, and black lines: phosphatidylinositol (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

Antibodies against gp60 are generated in different *C. parvum*-infected animal species, including humans, suggesting that this antigen is a potential immunotherapeutic target (Mead, 2014). Importantly, nasal immunization of pregnant goat dams with a gp15-recombinant DNA vaccine showed a significant reduction of oocyst excretion in the offspring. Furthermore, a monoclonal antibody against gp15 showed protective immunity in experimentally infected mice (Jenkins et al. 1993; Sagodira et al. 1999). This characteristic strongly suggests that the gp60 antigen is essential for the survival of *C. parvum* and consequently, justifies its expression as a first step for a future development of a recombinant vaccine.

A GPI anchored-antigen consists of a hydrophilic protein covalently attached to a glycolipid. The precursor protein contains an N-terminal signal peptide that directs it to the endoplasmic reticulum (ER), and a C-terminal GPI anchor signal, which is cleaved at a specific position known as omega site, after which a GPI molecule also synthesized in the ER is transferred to the protein. The protein reaches the cell surface through the cell secretory system, where it remains anchored to the external leaflet through the GPI molecule (Chatterjee and Mayor, 2001).

Different types of organisms have been used to express recombinant surface proteins (Georgiou et al., 1997; Kondo and Ueda, 2004). In this study, the free-living non pathogenic organism *Tetrahymena thermophila* was used as eukaryotic expression system. *T. thermophila* belongs to the phylum Ciliophora, a group of protozoans which, together with dinoflagellates and Apicomplexa, form the monophyletic lineage of Alveolates (Adl et al., 2012). Ciliates are characterized by the presence of cilia, nuclear dimorphism and the use of conjugation for sexual replication (Lynn, 2010). The development of numerous tools for the genetic manipulation of *T. thermophila* have made this protozoon a widely used model organism for the study of a number of fundamental processes, including major discoveries, such as the nature of telomeres, telomerase, and self-splicing RNA (Ruehle et al., 2016). *T. thermophila* can be easily grown in inexpensive culture media to high cell densities (up to 2.2×10^7cells/ml) and cultures are amenable for scaling up, facilitating the expression of recombinant antigens (Elguero et al., 2018). Additionally, due to the hundreds of cilia that expand the cell surface, *T. thermophila* has the advantage to express surface proteins in large quantities (Gaertig et al., 1999). Some protozoan surface antigens have been successfully expressed in *T. thermophila,* including the immobilization antigen variant B protein (I antigen) of the ciliate *Ichthyophthirius multifiliis* and the circumsporozoite protein (CSP) of the apicomplexan *Plasmodium falciparum*. These antigens represent vaccine candidates against fish white spot disease (Gaertig et al., 1999) and human malaria (Peterson et al., 2002), respectively.

In this study at hand, *C. parvum* gp60 was recombinantly expressed in *T. thermophila* and its immunoreactivity has been evaluated against sera of calves with cryptosporidiosis.

2. Material and methods

2.1. Strains and culture conditions

T. thermophila strain CU428 mpr1-1/mpr1-1 (mp-s, VII) and CU427 [chx1-1/chx1-1 (VI, cy-s)] was acquired from the Tetrahymena Stock Center, Cornell University and used for subsequent transformation assays. Cells were grown in 250 ml Erlenmeyer flasks, containing 30 ml SPP medium of the following composition (wt/vol): 1% proteose-peptone (Oxoid, United Kingdom), 0.1% yeast extract (Merck, Germany), 0.2% glucose (Merck, Germany), and 0.003% iron citrate (Sigma-Aldrich). Flasks were subcultured by 1:10 every 24 h. Cultivation was carried out in a rotary shaker (150 rpm) at 30 °C. When indicated, paromomycin (Sigma-Aldrich) was added to a final concentration of 100 µg/ml together with 1 µg/ml of $CdCl_2$. For determination of the cell density, cells were treated with 1% trichloroacetic acid and counted in duplicates in a Neubauer chamber.

2.2. Design of constructs and T. thermophila transformation

The complete coding sequence of the *gp60* gene (UniProtKB ID: Q9NDH6) of *C. parvum* strain Iowa II, was synthesized with optimized codons (GenBank ID: MK135785) for the expression in *T. thermophila* (Genscript, USA) and was designated rgp60Tt. The sequence was cloned into the *T. thermophila* rDNA -based pICY -GTW vector (Cole et al., 2008), between *Bsi*WI and *Xho*I sites downstream of the $CdCl_2$ -inducible MTT1 promoter (Shang et al., 2002), permitting its controlled expression. Twenty µg of the resulting pICY-rgp60 plasmid were used for conjugative electrotransformation of 5×10^6 pre-starved mixed *T. thermophila* cell populations for 8.5 and 9.5 h using a BTX model ECM630 electroporator set to 250 V, 125 m F, 25 ohms as described previously (Gaertig and Gorovsky, 1992). The pICY-rgp60 vector is converted into a palindromic molecule and amplified to approx. 9000 copies during the development of the macronuclei, thus allowing for high-level expression of the gp60 sequence. After conjugation, cells were allowed to recover for 20–24 h in growth medium and transformants were selected in SPP medium containing 100 µg/ml paromomycin sulfate (Sigma).

2.3. Recombinant expression of gp60 in Escherichia coli and generation of polyclonal murine sera

For the detection of the rgp60Tt protein, a polyclonal murine antiserum was produced as described by Tomazic et al. (2018). Briefly, the hydrophilic region of the gp60 protein was expressed in *E. coli* and

designated rgp60Ec. The strain *Rosetta 2DE3 pLacI* was transformed with pRSETC/gp60 (Thermo Scientific) and positive clones were grown and induced in LB medium supplemented with ampicillin (100 µg/ml) and chloramphenicol (34 µg/ml) at 37 °C to OD of 0.6 with 1 mM isopropyl-D-thiogalactopyranoside (Promega, USA) for 4 h. The cell pellet was lysed and sonicated in PNLB Buffer (50 mM K_2HPO_4, 400 mM NaCl, 100 mM KCl, 10% V/V glycerol, 0.5% V/V Triton- X100, 10 mM imidazole). Inclusion bodies were solubilized with Binding Buffer (8 M Urea, 20 mM sodium phosphate buffer (pH 7.8), 500 mM NaCl, 20 mM imidazole) and purified by immobilized metal affinity chromatography in Ni-agarose (Qiagen). The protein was eluted with 400 mM imidazole, dialyzed against PBS, and concentrated by lyophilization to 0.4 mg/ml. For subsequent immunizations, groups of 8–10 week-old Balb/c mice (National University of La Plata, UNLP) were inoculated subcutaneously with 30 µg of purified rgp60Ec emulsified in Freund's incomplete adjuvant 1:1 v/v or PBS, pH 7.4. At day 14, a booster of 30 µg purified recombinant protein in Freund's incomplete adjuvant was administered subcutaneously, followed by a second booster at day 28. At 42 days, sera were collected and stored at −20 °C until use.

2.4. Phase partitioning of membrane proteins and gp60 identification

Membrane proteins from cilia and cell body were fractionated by extraction in Triton X-114, as described by Peterson et al. (2002). Briefly, 10^6 cells were suspended in 800 µl 20 mM Tris/HCl, pH 7.5/ 2% v/v Triton X-114/ 300 mM NaCl/ 10 mM PMSF. Samples were centrifuged at 16,000 x g for 10 min and the supernatant was warmed at 30 °C for 5 min, layered on 300 µl of a sucrose cushion and incubated at 30 °C for 10 min. Detergent and aqueous phases were separated by centrifugation at 400 x g for 5 min at room temperature (RT). Cells were deciliated as described by Calzone and Gorovsky (1982).

The identification of gp60 was performed by nano-Liquid Chromatography-Mass Spectrometry (nLC-MS). Bands were excised from the polyacrylamide gel and were treated to obtain tryptic and chymotryptic peptides. These peptide samples were then separated by reverse phase chromatography *via* an Accucore TM Easy-Spray C18 analytical column (Thermo Fisher Scientific, USA) coupled to an EASY-nLCTM 1000 system. The mass spectra of peptides were acquired in an Orbitrap nLC/MS. Data analysis was performed using the Thermo Proteome Discoverer program (Thermo Fisher Scientific).

2.5. Immunofluorescence analysis

T. thermophila cells were fixed with 4.2% paraformaldehyde in HEPES buffer (50 mM HEPES, pH 7.0), for 10 min. After being washed with ice-cold HEPES buffer 3 times, the fixed cells were treated with blocking solution (1% BSA in TBS buffer) for 15 min, and incubated for 30 min in blocking solution containing anti-rgp60Ec primary mouse polyclonal antibodies at a 1:400 dilution. Cells were washed 3 times for 5 min in TBS buffer containing 0.1% BSA and then incubated for 30 min with goat anti-mouse–Alexa Fluor 488 secondary antibody (A11001; Invitrogen) at a 1: 200 dilution in blocking solution. All incubations were performed at RT. After an additional wash with blocking solution and two washes in HEPES buffer, the cells were mounted with Trolox anti-bleaching solution (Sigma-Aldrich). Digital images were collected using a Carl Zeiss Axio Imager M2 fluorescence microscope, with differential interference contrast (DIC) optics.

2.6. Collection of calf oocysts and sera

Twelve calves (*Bos taurus*) (12–28 days old) were identified as positive for *Cryptosporidium* sp. infection. Oocysts detected in feces by microscopic examination of fecal smears at 100× magnification after staining with the modified Ziehl- Neelsen technique were purified by sucrose flotation (s.p. 1.18) as described in OIE Terrestrial Manual (2012) and were typed as *C. parvum* by PCR-RFLP (Tomazic et al., 2013). Blood samples were withdrawn from the jugular vein of calves for 12–28 days after birth, incubated at 37 °C for 1 h, and then centrifuged at 800 *g* at 4 °C for 20 min. Sera were collected and stored at −20 °C until use. Sample collection followed the International Guiding Principles for Biomedical Research Involving Animals, written by the Council for International Organizations of Medical Sciences (CIOMS) and the International Council for Laboratory Animal Science (ICLAS) Ginebra 2012.

2.7. Immunoblotting

Calf sera were used to probe the immunoreactivity of the rgp60Tt protein by western blotting (WB). Total proteins of a homogenate of *T. thermophila* expressing the rgp60Tt antigen (clone G1) were separated by 12% SDS-PAGE and transferred to a nitrocellulose membrane, which was cut into strips. Blot strips were incubated overnight at 4 °C with sera from *C. parvum*-infected calves at a 1:20 dilution in 1% gelatin in TBS containing 0.05% Tween-20. As secondary antibody, goat anti-bovine IgG conjugated to horseradish peroxidase (KPL, USA) (1:1000) was applied for 1 h at RT. Subsequently, blot strips were washed once with TBS containing 0.1% Tween-20, twice with TBS containing 0.05% Tween-20, and once with TBS. All washes were performed for 10 min at RT. Reactions were developed using 3,3′-diaminobenzidine (Sigma, USA) as substrate and observed within 5 min. As negative control, serum from a newborn calf before its first feeding with colostrum was used.

3. Results

3.1. gp60 expression in T. thermophila

An episomal strategy of overexpression through the use of an autonomously replicating rDNA -based vector was chosen for gp60 expression in *T. thermophila*. The *gp60* sequence was optimized for *T. thermophila* codon usage frequency and ligated to the pICY-GTW plasmid containing the cadmium-inducible MTT1 promoter. The resulting vector, pICY-rgp60, was introduced into cells and clones resistant to paromomycin were selected. The transformation efficiency was 624 transformants/ ug.

The recombinant gp60 protein (rgp60Tt) of 2 PCR-positive *T. thermophila* clones (G1 and G2) was detected by WB in lysates using murine anti-rgp60Ec-antibodies. The rgp60Tt protein migrated as a band of 42 kDa but lower molecular weight bands of 27 kDa and 38 kDa were additionally observed (Fig. 2A). Analysis of the protein tryptic digest of the 42 kDa and 27 kDa bands by liquid chromatography tandem-mass spectrometry (LC-MS/MS) identified peptide fragments of both gp15 and gp40 (Fig. 2B). Optimal expression was achieved after 8 h of cadmium chloride induction (Fig. 2A).

3.2. Recombinant gp60Tt is membrane-associated and localizes to the oral apparatus

To determine the localization of rgp60Tt, its presence in different cell fractions, its partitioning into the Triton X-114 detergent phase, and the immunofluorescence labeling pattern were evaluated.

As shown in Fig. 2C, the rgp60Tt protein was detected by WB analysis primarily in the membrane fraction whereas a smaller amount could be demonstrated in the ciliary fraction. Localization of the rgp60Tt was further analyzed by indirect immunofluorescence without permeabilization, demonstrating that surface labeling of oral structures was exclusively observed in cadmium-induced recombinant *T. thermophila* clones (Fig. 3).

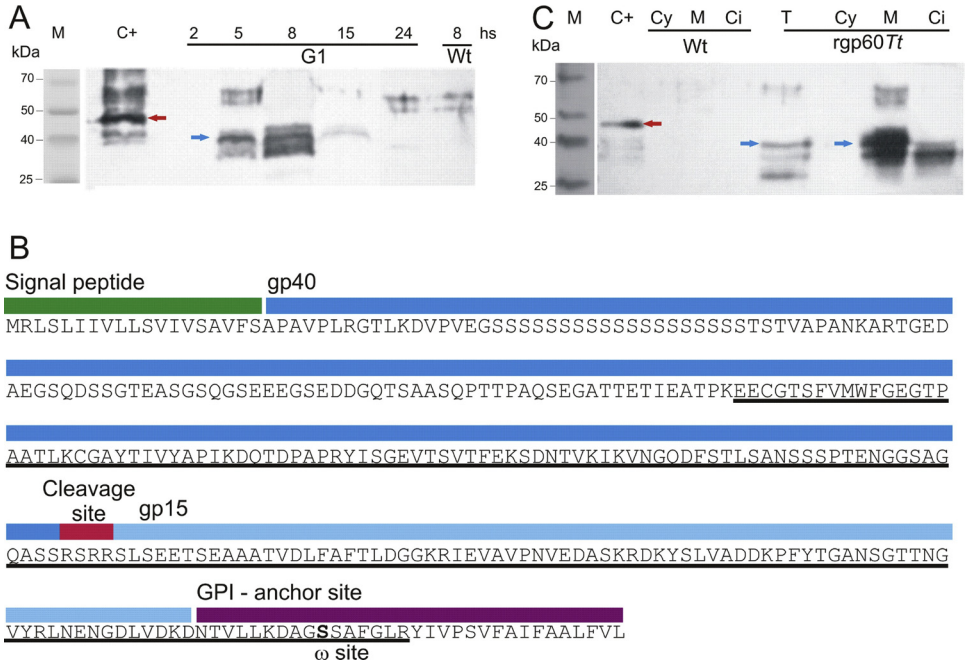

Fig. 2. A. Western blot analysis of total protein extracts of a recombinant *T. thermophila* clone expressing gp60 (rgp60Tt) at different times after induction. Each sample was loaded with 50 µg of total protein. C+: protein extract of recombinant *E. coli* expressing gp60. WT: Wild-type strain of *T. thermophila*. Molecular weights were calculated by Gel Analyzer software (Lazar and Lazar 2010) **B.** gp60 protein sequence expressed in *T. thermophila*, showing the peptides with high and medium probability (black underline) identified by LC/MS/MS of the excised SDS-PAGE 42 kDa band. **C.** Western blot analysis of different cellular fractions of *T. thermophila* WT and recombinant rgp60Tt strains. Each sample was loaded with 50 µg of total protein as measured by BCA assay. Cy: cytoskeleton; M: membrane extracted in Triton X-114; Ci: isolated cilia; T: total cellular protein. C+: protein extract of recombinant *E. coli* expressing gp60.

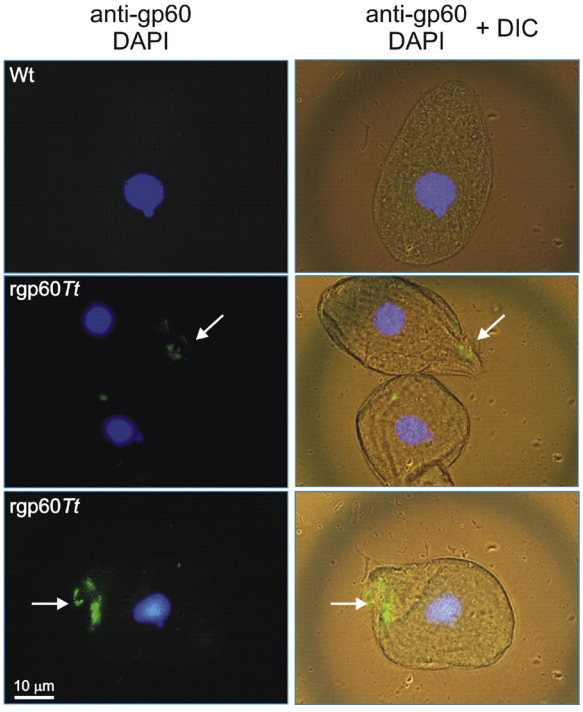

Fig. 3. Localization of rgp60Tt. Immunofluorescence of *T. thermophila* WT and rgp60Tt strains using anti-rgp60Ec murine antibodies. Localization of rgp60Tt to oral structures (cilia, membranelles and/or undulating membrane) can be observed only in recombinant clones (white arrows). Macro and micro nucleus are stained with DAPI.

3.3. Immunoreactivity of recombinant T. thermophila-expressed gp60 antigen

In order to evaluate the immunoreactivity of the rgp60Tt protein against sera of *C. parvum*-infected calves, a homogenate of recombinant *T. thermophila*- clone G1 was probed with twelve bovine sera. Calves were diagnosed as positive for *Cryptosporidium* sp. infection by microscopic examination of fecal smears. As shown in Fig. 4B, calf sera reacted with a band with an apparent molecular weight of 42 kDa, which is consistent with the size observed using murine anti-rgp60Ec (Fig. 4A). Of the twelve tested calf sera, one showed a very high signal intensity (line 7), five a medium (line 5, 8, 9, 10 and 11) and four low signal intensity (line 2, 3, 4 and 6). Only 2 sera did not react against rgp60Tt (line 1 and 12). Serum from a newborn calf before its first feeding with colostrum did not react with rgp60Tt. Additionally, no sera reacted with a homogenate of wildtype (WT) *T. thermophila* (Fig. 4B). These results indicate that the gp60 antigen expressed in *T. thermophila* is immunoreactive since it is specifically recognized by IgG antibodies generated by calves after *C. parvm* infection.

4. Discussion

In this study, the *C. parvum* gp60 has been expressed in *T. thermophila*. The ciliate, is one of the most studied and characterized microorganism within the Alveolata monophyletic lineage. Several significant contributions have been generated from this model organism in various areas such as cell and molecular biology, and it received important recognition in the biotech area as well (Ruehle et al., 2016; Elguero et al., 2018). Regarding the use of *T. thermophila* as an expression system, proteins from a broad range of organisms have been successfully expressed, including enzymes (Aldag et al., 2011), antibodies (Calow et al., 2016), antigens (Gaertig et al., 1999) and vaccine candidates (Peterson et al., 2002; Jayaram et al., 2010). The result obtained in this study extends the spectrum of organisms, in particular of the apicomplexan lineage, from which an antigen has been chosen for recombinant expression in *T. thermophila*.

The genus *Tetrahymena*, but also other ciliate genera, have a particular codon usage and also differ in their genetic code in that UAA and UAG triplets encode glutamine instead of stop codons (Tourancheau et al., 1995). Accordingly, in the *C. parvum* gp60 coding sequence (EMBL: AY048666), the stop triplet UAA was replaced by UGA, and the remaining codons were optimized with regard to the codon usage frequency of *T. thermophila*.

The unexpected SDS-PAGE migration of rgp60Tt observed in this study may suggest a dissimilar glycosylated form and/or proteolytic degradation of the glycoprotein. This pattern was evidenced in the native *C. parvum* gp60 protein, which is predicted to encode an acidic 34 kDa mucin-like glycoprotein. However, it displayed an apparent molecular weight of 60 kDa as judged by SDS-PAGE (Strong et al.,

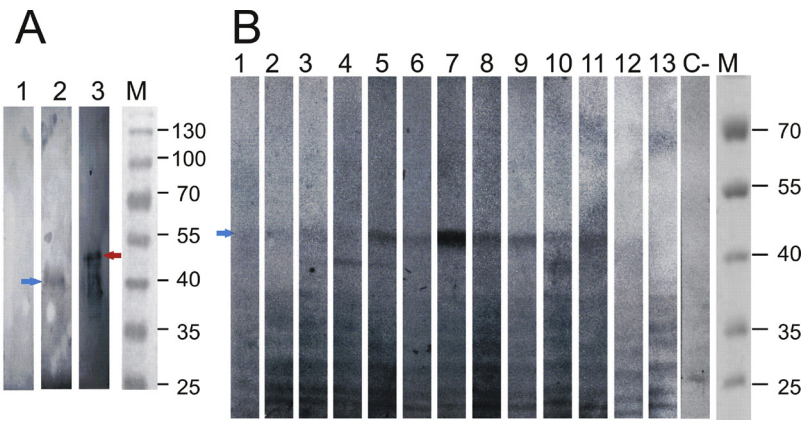

Fig. 4. Immunoreactivity of rgp60Tt. **A.** Western blot performed with murine anti- rgp60Ec serum against homogenates of *T. thermophila* WT (1) or recombinant rgp60Tt strains (2). Positive control with purified rgp60Ec protein (3). An apparent molecular size at 42 kDa is observed in homogenates of recombinant *T. thermophila* cells (blue arrow) whereas 50 kDa is observed in rgp60Ec (red arrow). **B.** A homogenate of *T. thermophila* rgp60Tt strain (clone G1) was incubated in immunoblots with twelve sera from 11 to 28-day old calves naturally infected with *C. parvum* (1–12). 13: Negative control corresponding to serum from a newborn calf before its first feeding with colostrum. C-: Negative control corresponding to a *T. thermophila* wildtype homogenate following incubation with calf sera. A band of 42 kDa is observed in calves 2–11, which coincides with the apparent size observed in A (blue arrow). Molecular weights were calculated by Gel Analyzer software (Lazar and Lazar, 2010) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.).

2000). It has been shown that gp60 is proteolytically cleaved by a furin-like protease to yield glycopeptides gp40 and gp15, which showed apparent molecular weights of 40 kDa and 15 kDa, respectively (Wanyiri et al., 2007). The dissimilar sizes observed could be due to different reasons: a high glycosylation pattern (36 sites of O-linked plus 1 site of N-linked glycosylation), the hydrophobicity of the C-terminal peptide or the high content of acidic amino acid residues (pI 445) (Graceffa et al., 1992; Rath et al., 2009; Shi et al., 2012). A difference between the calculated and apparent molecular weight of rgp60Tt was also observed for the recombinant protein expressed in *E. coli* (Tomazic et al., 2018).

The observation that rgp60Tt partitioned into Triton X-114-detergent phase is in agreement with the expectation for GPI-anchored antigens (Ko and Thompson, 1995). The localization of rgp60Tt on the membrane surface and the oral apparatus of the ciliate (Fig. 3) is in agreement with the presence of a GPI-anchor in the recombinant protein. GPI-anchored proteins have been extensively studied in *T. thermophila*, particularly the SerH surface antigen encoded by this ciliate (Bolivar and Guiard-Maffia, 1989) but also others in which the GPI-anchor has been attached during heterologous expression such as the I antigen of the ciliate *I. multifiliis*, the CSP of the apicomplexan *P. falciparum*, and the human alkaline phosphatase (Gaertig et al., 1999; Peterson et al., 2002; Aldag et al., 2011). In all these studies, labeling of GPI-anchored proteins appears in the surface of the ciliate with a strong signal in the oral region.

The rgp60Tt expressed in *T. thermophila* was localized at the surface membrane, showing that both, the GPI anchor and the N-terminal signals, were appropriately recognized. This result is in agreement with the surface localization of the gp60 protein expressed in the apicomplexan *Toxoplasma gondii* (O'Connor et al., 2003, 2007). Analysis of the protein tryptic digest of the 42 kDa and 27 kDa bands by liquid chromatography tandem-mass spectrometry (LC-MS/MS) of rgp6Tt, allowed us to identified in each bands, peptides fragments of both gp15 and gp40 cleavage products (Fig. 2B), suggesting that the protein remained uncleaved when it was expressed in *T. thermophila.* These results are in contrast to those reported in *T. gondii* which showed that the protein is partially processed to gp40 and gp15.

Bioinformatic analysis of the furin-like protease cpSUB1 involved in mediating gp60 cleavage (GenBank: EAK90066) showed that no furin-like protease homolog is encoded in the ciliate lineage (Wanyiri et al., 2007) whereas 2 furin-like protease homologs have been identified in *T. gondii.* Depending on the objective, it might be important to consider that rgp60 remains uncleaved when *T. thermophila* is used as an expression system in future studies. In the case that processing of gp60 is desired, the furin-like protease could be cotransfected and over-expressed. Corresponding strategies have been developed for *T. thermophila* to express the 1, 2-mannosidase from *Entamoeba histolytica* with a modified pattern of glycosylation (Colussi and Taron, 2011) and also to co-express the antibody light and heavy chain using the promoters metallothionein 1 and 5, respectively, which are both regulated with the same inducer (Calow et al., 2016).

Finally, the immunoreactivity of the *T. thermophila*-expressing recombinant gp60 with sera of calves naturally infected with *C. parvum* shows the feasibility to use this ciliate to produce immunoreactive antigens. The immunity against cryptosporidia seems to be dependent on both humoral and cellular responses of the host's immune system; therefore more studies are needed to conclude that gp60 expressed in the ciliate is suitable as a vaccine candidate. Although bioinformatic tools such as NetMHC 4.0 Server (Lundegaard et al., 2008) have predicted several peptide-MHC class I binders, suggesting a potential T cell response, future *in vivo,* and *in vitro* studies such as vaccine trials, and T-cell activation assays and characterization of the involved T cell subpopulations, respectively are needed for the development of a recombinant vaccine against this intracellular pathogen.

5. Conclusion

The present work showed the production of the recombinant antigen gp60 from *C. parvum* in the ciliate *T. thermophila,* which was localized at the ciliate cell surface and oral apparatus. We have demonstrated that it is a feasible expression system that can be used for protozoan parasites, extending the spectrum of organisms from which an antigen has been chosen for recombinant expression in *T. thermophila*. Moreover, the immunoreactivity of rgp60Tt has been demonstrated, being the first step in a future development of a recombinant vaccine against *C. parvum*.

CRediT authorship contribution statement

María E. Elguero: Data curation, Formal analysis, Writing - review & editing. **Mariela L. Tomazic:** Data curation, Data curation, Formal analysis, Writing - review & editing. **María G. Montes:** Data curation, Formal analysis, Writing - review & editing. **Mónica Florin-Christensen:** Formal analysis, Conceptualization, Funding acquisition, Investigation, Supervision, Writing - original draft, Writing - review & editing. **Leonhard Schnittger:** Formal analysis, Conceptualization, Funding acquisition, Investigation, Supervision, Writing - original draft, Writing - review & editing. **Alejandro D. Nusblat:** Formal analysis, Conceptualization, Funding acquisition, Investigation, Supervision, Writing - original draft, Writing - review & editing.

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References

Adl, S.M., Simpson, A.G.B., Lane, C.E., Lukeš, J., Bass, D., Bowser, S.S., Brown, M.W., Burki, F., Dunthorn, M., Hampl, V., Heiss, A., Hoppenrath, M., Lara, E., Le, Gall L., Lynn, D.H., McManus, H., Mitchell, E.A.D., Mozley-Stanridge, S.E., Parfrey, L.W., Pawlowski, J., Rueckert, S., Shadwick, L., Schoch, C.L., Smirnov, A., Spiegel, F.W., 2012. The revised classification of eukaryotes. J. Eukaryot. Microbiol. 59, 429–493. https://doi.org/10.1111/j.1550-7408.2012.00644.x.

Aldag, I., Bockau, U., Rossdorf, J., Laarmann, S., Raaben, W., Herrmann, L., Weide, T., Hartmann, M.W.W., 2011. Expression, secretion and surface display of a human alkaline phosphatase by the ciliate *Tetrahymena thermophila*. BMC Biotechnol. 11, 11. https://doi.org/10.1186/1472-6750-11-11.

Bolivar, I., Guiard-Maffia, J., 1989.) Cellular localization of the SerH surface antigen in *Tetrahymena thermophila*. J. Cell. Sci. 94, 343–354.

Calow, J., Behrens, A.J., Mader, S., Bockau, U., Struwe, W.B., Harvey, D.J., Cormann, K.U., Nowaczyk, M.M., Loser, K., Schinor, D., Hartmann, M.W.W., Crispin, M., 2016. Antibody production using a ciliate generates unusual antibody glycoforms displaying enhanced cell-killing activity. MAbs 8, 1498–1511. https://doi.org/10.1080/19420862.2016.1228504.

Calzone, F.J., Gorovsky, M.A., 1982. Cilia regeneration in Tetrahymena. A simple reproducible method for producing large numbers of regenerating cells. Exp. Cell Res. 140 (2), 471–476.

Cevallos, A.M., Bhat, N., Verdon, R., Hamer, D.H., Stein, B., Tzipori, S., Pereira, M.E.A., Keusch, G.T., Ward, H.D., 2000. Mediation of *Cryptosporidium parvum* infection in vitro by mucin-like glycoproteins defined by a neutralizing monoclonal antibody. Infect. Immun. 68, 5167–5175. https://doi.org/10.1128/IAI.68.9.5167-5175.2000.

Chatterjee, S., Mayor, S., 2001. The GPI-anchor and protein sorting. C Cell Mol Life Sci 58, 1969–1987. https://doi.org/10.1007/PL00000831.

Cole, E.S., Anderson, P.C., Fulton, R.B., Majerus, M.E., Rooney, M.G., Savage, J.M., Chalker, D., Honts, J., Welch, M.E., Wentland, A.L., Zweifel, E., Beussman, D.J., 2008. A proteomics approach to cloning fenestrin from the nuclear exchange junction of *Tetrahymena*. J. Eukaryot. Microbiol. 55, 245–256. https://doi.org/10.1111/j.1550-7408.2008.00337.x.

Colussi, P., Taron, C., 2011. Production of Glycoproteins in Genetically Modified Ciliates. WO2011119498A1. .

De Waele, V., Speybroeck, N., Berkvens, D., Mulcahy, G., Murphy, T.M., 2010. Control of cryptosporidiosis in neonatal calves: use of halofuginone lactate in two different calf rearing systems. Prev. Vet. Med. 96, 143–151.

Elguero, M.E., Nudel, C., Nusblat, A., 2018. Biotechnology in ciliates: an overview. Crit. Rev. Biotechnol. https://doi.org/10.1080/07388551.2018.1530188.

Gaertig, J., Gao, Y., Tishgarten, T., Clark, T.G., Dickerson, H.W., 1999. Surface display of a parasite antigen in the ciliate *Tetrahymena thermophila*. Nat. Biotechnol. 17, 462–465. https://doi.org/10.1038/8638.

Gaertig, J., Gorovsky, M.A., 1992. Efficient mass transformation of *Tetrahymena thermophila* by electroporation of conjugants. Proc. Natl. Acad. Sci U S A 89, 9196–9200. https://doi.org/10.1073/pnas.89.19.9196.

Georgiou, G., Stathopoulos, C., Daugherty, P.S., Nayak, A.R., Iverson, B.L., Curtiss, R., 1997. Display of heterologous proteins on the surface of microorganisms: from the screening of combinatorial libraries to live recombinant vaccines. Nat. Biotechnol. 29–34.

Graceffa, P., Jancsó, A., Mabuchi, K., 1992. Modification of acidic residues normalizes sodium dodecyl sulfate-polyacrylamide gel electrophoresis of caldesmon and other proteins that migrate anomalously. Arch. Biochem. Biophys. 297 (1), 46–51.

Jayaram, J., Papoyan, A., Bisharyan, Y., Cassidy-Hanley, D., Zhang, X., Colussi, P., Appleton, J.A., Gagliardo, L., Clark, T.G., 2010. An alternative platform for rapid production of effective subunit vaccines. BioPharm Int. 23, 6–13.

Jenkins, M., Fayer, M., Tilley, M., Upton, S., 1993. Cloning and expression of a cDNA encoding epitopes shared by 15- and 60-kilodalton proteins of Cryptosporidium parvum sporozoites. Infect. Immun. 61 (6), 2377–2382.

Jenkins, M., Higgins, J., Kniel, K., Trout, J., Fayer, R., 2004. Protection of calves against cryptosporiosis by oral inoculation with gamma-irradiated *Cryptosporidium parvum* oocysts. J. Parasitol. 90, 1178–1180. https://doi.org/10.1645/GE-3333RN.

Ko, Y.G., Thompson, G.A., 1995. Purification of Glycosylphosphatidylinositol-Anchored Proteins by Modified Triton X-114 Partitioning and Preparative Gel Electrophoresis. Anal. Biochem. 224, 166–172. https://doi.org/10.1006/abio.1995.1024.

Kondo, A., Ueda, M., 2004. Yeast cell-surface display - Applications of molecular display. Appl. Microbiol. Biotechnol. 64, 28–40.

Lazar, I., Lazar, I., 2010. Gel Analyzer 2010a: Freeware 1D Gel Electrophoresis Image Analysis Software.

Lundegaard, C., Lamberth, K., Harndahl, M., Buus, S., Lund, O., Nielsen, M., 2008. NetMHC-3.0: accurate web accessible predictions of human, mouse and monkey MHC class I affinities for peptides of length 8–11. Nucleic. Acids Res. 36, 509–512.

Lynn, D.H. (Ed.), 2010. The Ciliated Protozoa. Springer Netherlands, Dordrecht.

Mead, J.R., 2014. Prospects for immunotherapy and vaccines against Cryptosporidium. Hum. Vaccines Immunother. 10, 1505–1513.

O'Connor, R.M., Kim, K., Khan, F., Ward, H.D., 2003. Expression of Cpgp40/15 in *Toxoplasma gondii*: a surrogate system for the study of cryptosporidium glycoprotein antigens. Infect. Immun. 71, 6027–6034. https://doi.org/10.1128/IAI.71.10.6027-6034.2003.

O'Connor, R.M., Wanyiri, J.W., Wojczyk, B.S., Kim, K., Ward, H., 2007. Stable expression of *Cryptosporidium parvum* glycoprotein gp40/15 in *Toxoplasma gondii*. Mol. Biochem. Parasitol. 152, 149–158. https://doi.org/10.1016/j.molbiopara.2007.01.003.

OIE, 2012. OIE manual of diagnostic tests and vaccines for terrestrial animals. Chapter 2.9.4. Cryptosporidiosis, 7th ed. ISBN 978-92-9044-878-5.

Olson, M.E., O'Handley, R.M., Ralston, B.J., McAllister, T.A., Thompson, R.C.A., 2004. Update on *Cryptosporidium* and *Giardia* infections in cattle. Trends Parasitol. 20, 185–191.

Peterson, D.S., Gao, Y., Asokan, K., Gaertig, J., 2002. The circumsporozoite protein of *Plasmodium falciparum* is expressed and localized to the cell surface in the free-living ciliate Tetrahymena thermophila. Mol. Biochem. Parasitol. 122, 119–126. https://doi.org/10.1016/S0166-6851(02)00079-8.

Priest, J.W., Xie, L.T., Arrowood, M.J., Lammie, P.J., 2001. The immunodominant 17-kDa antigen from *Cryptosporidium parvum* is glycosylphosphatidylinositol-anchored. Mol. Biochem. Parasitol. 113, 117–126. https://doi.org/10.1016/S0166-6851(00)00386-8.

Rath, A., Glibowicka, M., Nadeau, V.G., Chen, G., Deber, C.M., 2009. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. Proc Natl Acad Sci 106, 1760–1765. https://doi.org/10.1073/pnas.0813167106.

Ruehle, M.D., Orias, E., Pearson, C.G., 2016. *Tetrahymena* as a unicellular model eukaryote: genetic and genomic tools. Genetics 203, 649–665.

Sagodira, S., Iochmann, S., Mevelec, N., Dimier-Poisson, I., Bout, D., 1999. Nasal immunization of mice with Cryptosporidium parvum DNA induces systemic and intestinal immune responses. Parasite Immunol. 21 (10), 507–516.

Shang, Y., Song, X., Bowen, J., Corstanje, R., Gao, Y., Gaertig, J., Gorovsky, M.A., 2002. A robust inducible-repressible promoter greatly facilitates gene knockouts, conditional expression, and overexpression of homologous and heterologous genes in *Tetrahymena thermophila*. Proc Natl Acad Sci 99, 3734–3739.

Shi, Y., Mowery, R.A., Ashley, J., Hentz, M., Ramirez, A.J., Bilgicer, B., Slunt-Brown, H., Borchelt, D.R., Shaw, B.F., 2012. Abnormal SDS-PAGE migration of cytosolic proteins can identify domains and mechanisms that control surfactant binding. Protein Sci. 21, 1197–1209. https://doi.org/10.1002/pro.2107.

Šlapeta, J., 2013. Cryptosporidiosis and *Cryptosporidium*species in animals and humans: A thirty colour rainbow? Int. J. Parasitol. 43, 957–970.

Strong, W.B., Gut, J., Nelson, R.G., 2000. Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zoite surface antigen products. Infect. Immun. 68, 4117–4134. https://doi.org/10.1128/IAI.68.7.4117-4134.2000.

Tomazic, M.L., Maidana, J., Dominguez, M., Uriarte, E.L., Galarza, R., Garro, C., Florin-Christensen, M., Schnittger, L., 2013. Molecular characterization of *Cryptosporidium* isolates from calves in Argentina. Vet. Parasitol. 198, 382–386. https://doi.org/10.1016/j.vetpar.2013.09.022.

Tomazic, M.L., Rodriguez, A.E., Lombardellic, J., Poklepovich, T., Garro, C., Galarza, R., Tiranti, K., Florin-Christensen, M., Schnittger, L., 2018. Identification of novel vaccine candidates against cryptosporidiosis of neonatal bovines by reverse vaccinology. Vet. Parasitol. 264, 74–78. https://doi.org/10.1016/j.vetpar.2018.11.007.

Tourancheau, A.B., Tsao, N., Klobutcher, L.A., Pearlman, R.E., Adoutte, A., 1995. Genetic-code deviations in the ciliates - evidence for multiple and independent events. EMBO J. 14, 3262–3267.

Tzipori, S., Ward, H., 2002. Cryptosporidiosis: biology, pathogenesis and disease. Microbes Infect. 4, 1047–1058.

Wanyiri, J.W., O'Connor, R., Allison, G., Kim, K., Kane, A., Qiu, J., Plaut, A.G., Ward, H.D., 2007. Proteolytic processing of the *Cryptosporidium* glycoprotein gp40/15 by human furin and by a parasite-derived furin-like protease activity. Infect. Immun. 75, 184–192. https://doi.org/10.1128/IAI.00944-06.