

RESEARCH

Open Access



Stable expression of mucin glycoproteins GP40 and GP15 of *Cryptosporidium parvum* in *Toxoplasma gondii*

Muxiao Li¹, Xiaohua Sun¹, Haoyu Chen¹, Na Li¹, Yaoyu Feng¹, Lihua Xiao¹ and Yaqiong Guo^{1*}

Abstract

Background *Cryptosporidium* spp. are common protozoa causing diarrhea in humans and animals. There are currently only one FDA-approved drug and no vaccines for cryptosporidiosis, largely due to the limited knowledge of the molecular mechanisms involved in the invasion of the pathogens. Previous studies have shown that GP60, which is cleaved into GP40 and GP15 after expression, is an immunodominant mucin protein involved in the invasion of *Cryptosporidium*. The protein is highly O-glycosylated, and recombinant proteins expressed in prokaryotic systems are non-functional. Therefore, few studies have investigated the function of GP40 and GP15.

Methods To obtain recombinant GP40 with correct post-translational modifications, we used CRISPR/Cas9 technology to insert *GP40* and *GP15* into the *UPRT* locus of *Toxoplasma gondii*, allowing heterologous expression of *Cryptosporidium* proteins. In addition, the Twin-Strep tag was inserted after *GP40* for efficient purification of GP40.

Results Western blotting and immunofluorescent microscopic analyses both indicated that GP40 and GP15 were stably expressed in *T. gondii* mutants. GP40 localized not only in the cytoplasm of tachyzoites but also in the parasitophorous vacuoles, while GP15 without the GPI anchor was expressed only in the cytoplasm. In addition, a large amount of recTgGP40 was purified using Strep-TactinXT supported by a visible band of ~ 50 kDa in SDS-PAGE.

Conclusions The establishment of a robust and efficient heterologous expression system of GP40 in *T. gondii* represents a novel approach and concept for investigating *Cryptosporidium* mucins, overcoming the limitations of previous studies that relied on unstable transient transfection, which involved complex steps and high costs.

Keywords *Cryptosporidium parvum*, *Toxoplasma gondii*, GP40, Glycoprotein, Expression

Background

Cryptosporidium spp. primarily infect the intestinal epithelial cells of humans and a variety of animals, causing diarrhea and associated disease [1]. They are transmitted through the fecal-oral route [2], and they cause cryptosporidiosis, which is the second leading cause of

childhood diarrhea in middle and low-income countries and a major cause of mortality in individuals with HIV/AIDS [3]. Nitazoxanide is the only FDA-approved drug against human cryptosporidiosis, and there is no available vaccine against this infection [4].

An established strategy for the identification of potential drug targets or vaccine candidates in *Cryptosporidium* involves the exploration of surface antigens that participate in the recognition, attachment, and invasion of host epithelial cells, as well as the investigation of their interactions with the host [5]. A family of mucin glycoproteins is involved in the invasion of *Cryptosporidium* into host cells [6]. These mucins

*Correspondence:

Yaqiong Guo
guoyq@scau.edu.cn

¹ State Key Laboratory for Animal Disease Control and Prevention, South China Agricultural University, Guangzhou 510642, China



undergo significant O-linked glycosylation modifications, which have been shown to be crucial for their proper folding and function [7]. Among them, GP60 emerges as one of the most prominent mucin proteins in the invasion of *Cryptosporidium* [8]. This protein is cleaved into GP40 and GP15 after its expression, and GP40 remains on the parasite surface by binding to GP15, which has a GPI anchor [9, 10]. GP40 has > 30 O-linked glycosylation sites, while GP15 has a few such sites. In 2000, several research groups reported the discovery of the gene encoding GP60. However, few studies have investigated its function. This is mainly due to the challenges associated with conducting in-depth studies of mucins using recombinant proteins expressed in *Escherichia coli*, which have no biological function [9, 11–13].

Heterologous expression of *Cryptosporidium* mucins in *Toxoplasma gondii* probably provides recombinant proteins with the correct post-translational modifications. *Toxoplasma gondii* and *Cryptosporidium* belong to the phylum Apicomplexa, and *T. gondii* is amenable to in vitro cultivation [14]. In addition, well-established genetic manipulation techniques such as CRISPR/Cas9 have been applied to *T. gondii* [15]. In 2003, the transient expression of recombinant GP40/15 was achieved using the RH Δ hxgprt strain of *T. gondii*, and mass spectrometry analysis confirmed that the recombinant GP40/15 had the same O-linked glycosylation as the native GP40/15 [16]. The researchers further improved the stability of the system by modifying the expression plasmid, enabling the successful affinity purification of recombinant GP40 [17]. These suggest that the heterologous expression system of *T. gondii* can be utilized for the expression and production of *Cryptosporidium* mucins.

Despite the incorporation of the episomal shuttle sequence to mitigate plasmid loss associated with transient expression of foreign proteins in *T. gondii*, the plasmid remains susceptible to loss during the passage, freezing, and recovery of parasites [18]. In addition, the high cost of HA-tag affinity purification poses a challenge to the production of recombinant proteins for biological studies. In view of these limitations, we employed CRISPR/Cas9 technology to insert GP40 and GP15 into the UPRT locus of *T. gondii*, respectively [15]. In addition, the Twin-Strep tag was used for protein purification, which offers improved purification efficiency at a more affordable cost [19–21]. As a result, a more robust and efficient heterologous expression system of *T. gondii* was established, providing a novel approach and concept for the study of *Cryptosporidium* mucins.

Methods

Parasite strains

The IIdA20G1-HLJ isolate of *Cryptosporidium parvum* was originated from a dairy calf in Heilongjiang (HLJ) Province, China, in 2018 [22]. The oocysts of IIdA20G1-HLJ were initially recovered from a fecal sample from the calf by gradient centrifugation, maintained by passage in IFN- γ knockout (GKO) mice, and stored at 4 °C in PBS containing antibiotics for < 3 months prior to use in the study [23, 24]. The RH Δ ku80 strain of *T. gondii* was obtained from Dr. Bang Shen at Huazhong Agricultural University in 2021. It was continuously passaged in human foreskin fibroblast (HFF, ATCC SCRC-1041, Manassas, VA, USA) monolayers and frozen in liquid nitrogen for long-time storage. Prior to use in the study, the RH Δ ku80 strain was recovered from the liquid nitrogen and passaged in HFF monolayers. After 2–3 days of culture, the fresh tachyzoites of the RH Δ ku80 strain in the HFF culture were harvested by differential centrifugation.

HFF cell culture and infection of *T. gondii*

The HFF cells were used for in vitro cultivation of *T. gondii*. The cells were seeded in flasks (T25 and T175) or plates (24-well and 96-well) and cultured in DMEM high-glucose medium (Thermo, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS; Thermo), 1% L-glutamine (Sigma Aldrich, St. Louis, MO, USA), and 1% antibiotic antimycotic solution (Sigma Aldrich) at 37 °C in a 5% CO₂ atmosphere until they reached approximately 80% confluence. Tachyzoites of the RH Δ ku80 strain or the mutants generated in the study were allowed to infect cells in confluent cultures in DMEM high-glucose medium supplemented with 2% FBS [25].

Plasmid construction

A specific CRISPR/Cas9 plasmid (pSAG1-Cas9-TgU6-sgTgUPRT) was obtained from Dr. Bang Shen, Huazhong Agricultural University, in 2021 [15]. Homology repair template plasmids capable of overexpressing CpGP40 or CpGP15 were generated by Gibson assembly cloning using the ClonExpress MultiS One Step Cloning Kit (Vazyme, Nanjing, China) according to the procedure. To construct repair plasmids, GP40 (633 bp) and GP15 Δ gpi (228 bp) were first amplified from the genomic DNA extracted from the *C. parvum* IIdA20G1-HLJ isolate. Subsequently, a portion of the UPRT 5'-homologous (1178 bp), 3'-homologous (994 bp), TgGRA1 promoter (451 bp), and TgGRA2 3'UTR (398 bp), were amplified from the genomic DNA of *T. gondii*, respectively. The DHFR*-TS (3513 bp) and pUC19 (2769 bp) backbone

was amplified from the *pUC19-loxp-DHFR*-amp* plasmid provided by Dr. Bang Shen in 2021. After the plasmid *TgUPRT 5'UTR-CpGP40-DHFR*-TgUPRT 3'UTR* was completed by Gibson assembly cloning with the sequences amplified, the Twin-Strep and 6 × HA tags were inserted into the C-terminus of GP40 or GP15 using the same approach to finally generate the plasmid *TgUPRT 5'UTR-CpGP40-Twinstrep-6×HA-DHFR*-TgUPRT 3'UTR* or *TgUPRT 5'UTR-CpGP15-6×HA-DHFR*-TgUPRT 3'UTR*. Subsequently, these homology repair plasmids were then used as templates in PCR amplifications to obtain enough linearized homologous templates for transfection. The primers used (*pGRA1-Cpgene-TerGRA2-DHFR*-F/R*) are listed in Additional file 1: Table S1.

Generation of the RH Δ ku80 *Tguprt-Cpgp40* and *Tguprt-Cpgp15 Δ gpi* mutants

Fresh tachyzoites (2×10^7 per transfection) of the harvested RH Δ ku80 strain were resuspended in 250 μ l transfection buffer cytomix; 7.5 μ g CRISPR/Cas9 plasmids and 1.5 μ g linearized homology repair templates were added to the mixture containing tachyzoites and cytomix, which was then transferred to a cuvette (BioRad, Hercules, CA, USA) and electroporated twice (1600 V, 25 μ F, 50 Ω) using a BTX Gemini system (BTX, Holliston, MA, USA). The transfected tachyzoites were allowed to infect HFF cells. After about 18 h of culture, 1 μ M pyrimethamine (Life Technologies, Carlsbad, CA, USA) and 10 μ M floxuridine (Thermo) were added to the culture medium for selection of the mutants. After 2–3 passages, 0–1 of tachyzoite obtained by limiting dilution was allowed to infect cells cultured in 96-well plates to obtain single colony. PCRs targeting three different sites of each mutant were used to confirm the correct integration of templates as previously described [26]. Briefly, DNA was extracted from the culture of each mutant using the TIANamp Blood DNA Midi Kit (Tia-gen Biotech, Beijing, China), and used as the template in the PCR analyses. The three sites of each mutant amplified by PCR are shown in Fig. 1b, and the primers used are listed in Additional file 1: Table S1.

Immunofluorescence verification of rec*TgGP40* and rec*TgGP15*

The expression of *C. parvum* GP40 and GP15 Δ gpi in *T. gondii* was initially verified by immunofluorescence assay (IFA). For the IFA analysis of the RH Δ ku80 *Tguprt-Cpgp40* mutant and the *Tguprt-Cpgp15 Δ gpi* mutant, HFF cells were infected with 10^5 tachyzoites of these two mutants, respectively. After 16 h of culture at 37 °C with 5% CO₂, the HFF cells were fixed with 4% paraformaldehyde for 15 min, permeabilized with 0.1% Triton X-100

at 37 °C for 10 min, and blocked with 1% BSA at 37 °C for 20 min. The following primary antibodies were diluted in 0.1% BSA for staining: mouse anti-HA (MBL Beijing Biotech, Beijing, China) at a 1:500 dilution, mouse anti-GP60 (purified rabbit pAbs) at 1:800 dilution. Cells were incubated with the antibodies for 40 min at 37 °C, followed by six washes with PBS. Alexa Fluor-conjugated secondary antibodies (Thermo) diluted 1:1000 and Hoechst diluted 1:2000 in 0.1% BSA were added to the coverslips and incubated at 37 °C for 20 min. The cells were then washed six times with PBS and mounted on slides using Vectashield. The stained slides were examined using a Zeiss Axioskop Mot 2 fluorescence microscope (Carl Zeiss, Oberkochen, Germany). Images were manipulated using ZEN microscopy software (Carl Zeiss, Oberkochen, Germany).

Western blot analysis of rec*TgGP40* and rec*TgGP15*

The expression of *C. parvum* GP40 in *T. gondii* was further verified by Western blot analysis using rabbit anti-GP60 (purified rabbit pAbs). The RH Δ ku80 *Tguprt-Cpgp40* mutant (10^5 tachyzoites) was grown in HFF cells cultured in DMEM medium for 24 h. All parasites were then purified and collected by differential centrifugation, and total proteins of the parasites were extracted using RIPA lysis buffer (Thermo) by incubation at 4 °C. The lysis was centrifugated, and 50 μ l of the supernatant was used for Western blot analyses. The PVDF membrane was blocked in TBST (0.05% Tween 20 in TBS) containing 1% BSA and then incubated with the primary antibodies. The following primary antibodies were used and diluted in TBST containing 0.1% BSA: mouse anti-HA (MBL Beijing Biotech, Beijing, China) at 1:1000 dilution; rabbit anti-GP60 (purified rabbit pAbs) at 1:800 dilution. After thoroughly washing with TBST, the membrane was then incubated with HRP-conjugated antibodies (Beyotime, Shanghai, China) diluted 1:3000 in TBST containing 0.1% BSA. After washing with TBST, the membrane was incubated with Immobilon Western Chemiluminescent HRP Substrate (Millipore, Massachusetts, USA) and analyzed using a Chemstudio imaging system (Analytik Jena, Jena, Germany).

Purification of rec*TgGP40*

Strep-TactinXT resin (IBA GmbH, Göttingen, Germany) was used to purify rec*TgGP40*-Twinstrep-HA as shown in Fig. 4a. In this procedure, the RH Δ ku80 *Tguprt-Cpgp40* mutant was grown in HFF cells cultured in DMEM medium for 24 h. The cultures were then lysed with RIPA lysis buffer and centrifuged at 16,000g and 4 °C for 10 min to obtain the supernatant. The Strep-Tactin column with resin was equilibrated with wash buffer (100 mM Tris/HCl pH 8.0, 150 mM NaCl, 1 mM

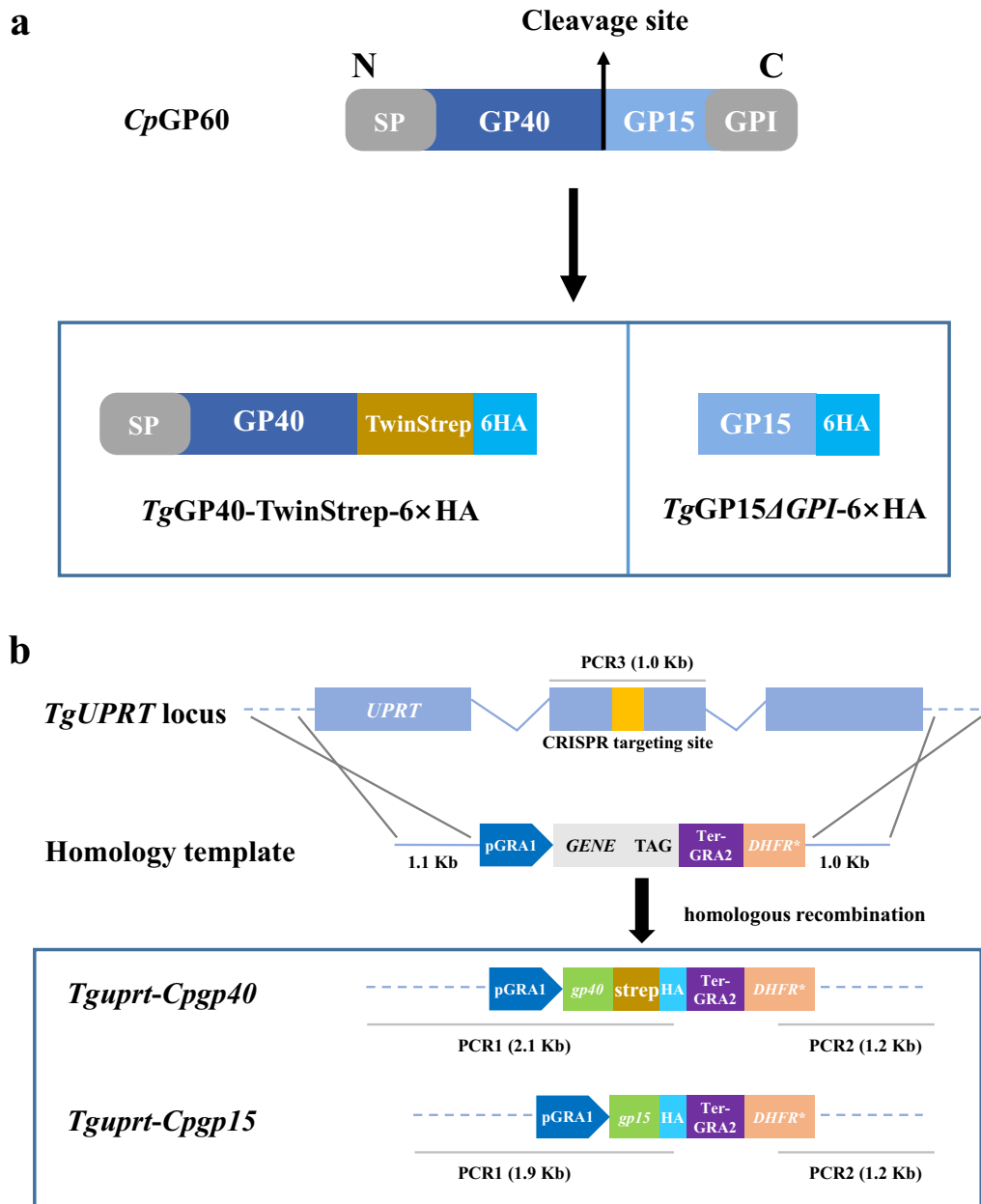


Fig. 1 Strategy used in the heterologous expression of GP40 and GP15 of *Cryptosporidium parvum* in *Toxoplasma gondii*. **a** The *C. parvum* GP60 is composed of GP40 and GP15, with a signal peptide at the N-terminus, a GPI anchor at the C-terminus, and a furin cleavage site RSRR between the two cleavage products. In this study, GP40 was truncated and fused to the Twinstrep-6xHA tag for expression, retaining the signal peptide of GP40 while removing the furin cleavage site RSRR. GP15 without GPI was fused to the 6xHA tag for expression. **b** Schematic illustration of the CRISPR/Cas9 strategy used to generate RHΔku80 mutant (*Tguprt-Cpgp40* and *Tguprt-Cpgp15Δgpi*) by inserting *CpGP40-Twinstrep-6xHA-DHFR** (pyrimethamine-resistant DHFR) or *CpGP15Δgpi-6xHA-DHFR**. The transfection of the sgUPRT together with an amplicon containing a *CpGP40-Twinstrep-6xHA-DHFR** (*CpGP15Δgpi-6xHA-DHFR**)-expressing cassette flanked by homology regions to *UPRT* was used to generate the gene insertion. The orange bar in *UPRT* gene represents the region targeted by the sgRNA

EDTA) and then combined with the supernatant. After the supernatant had completely entered the column, the column was successively washed with wash buffer and elution buffer (wash buffer containing 2.5 mM desthiobiotin). The eluate was further concentrated by dialysis,

and 100 μl of the concentrated eluate was used for identification of protein bands by SDS-PAGE, Western blot, and liquid chromatography/mass spectrometry (LC/MS).

Results

Construction of RH Δ ku80 *Tguprt-Cpgp40* mutant and *Tguprt-Cpgp15* Δ *gpi* mutant

For the expression of *CpGP40* and *CpGP15* in *T. gondii*, we attempted to insert the *GP40* and *GP15* into the *UPRT* locus of the RH Δ ku80 strain using CRISPR/Cas9 (Fig. 1b). To facilitate the purification of the recombinant protein, GP40 was truncated by removing the RSRR site, and a TwinStrep-6 \times HA tag was added to the C-terminus of GP40 (Fig. 1). The HA tag was used for the identification of the mutants, and the Twin-Strep tag was used for purification of the recombinant proteins. The RH Δ ku80 *Tguprt-Cpgp40* mutant and the RH Δ ku80 *Tguprt-Cpgp15* mutant were obtained after selection of pyrimethamine and floxuridine, and limiting dilution. In PCR analyses of the three sites of the mutants, expected bands were present in the PCR1 and PCR2 analyses of the RH Δ ku80 *Tguprt-Cpgp40* mutant and RH Δ ku80 *Tguprt-Cpgp15* mutant, while no band was observed in the PCR1 and PCR2 analyses of the RH Δ ku80 strain (WT). This confirmed that *CpGP40* and *CpGP15* were correctly inserted into the *UPRT* locus. In the PCR3 analysis, the sequence covering the CRISPR targeting site (the *UPRT* locus) was amplified in the RH Δ ku80 strain (WT). In contrast, no bands were present in the PCR3 analysis of the RH Δ ku80 *Tguprt-Cpgp40* mutant and the RH Δ ku80 *Tguprt-Cpgp15* mutant, which confirmed that the *UPRT* was knockout in the two mutants (Fig. 2a).

Expression of GP40 in fusion with the Twin-Strep tag and HA tag

GP40 was regulated by activation of the *TgGRA2* promoter, resulting in its significant overexpression. In Western blot analysis of the lysates of RH Δ ku80 *Tguprt-Cpgp40* mutant, a ~50 kDa protein was recognized by HA monoclonal antibody, Strep monoclonal antibody, and GP40 monoclonal antibody, respectively (Fig. 2b). Therefore, the protein recognized was considered rec*TgGP40*-TwinStrep-6 \times HA. In addition, the size of rec*TgGP40*-TwinStrep-6 \times HA is comparable to that of the reported native GP40 with post-translational modification, indicating O-linked glycosylation of the recombinant protein. In the IFA analysis, *CpGP40* was co-localized with the Twin-Strep tag and HA tag, respectively, further supporting the fusion of *CpGP40* with the tags in *T. gondii* (Fig. 3a, b).

Distribution of rec*TgGP40* and rec*TgGP15* in *T. gondii*

IFA analysis of the RH Δ ku80 mutant (*Tguprt-Cpgp40* and *Tguprt-Cpgp15* Δ *gpi*) showed that rec*TgGP40*-TwinStrep-6 \times HA was distributed in both the cytoplasm of *T. gondii* and parasitophorous vacuole (Fig. 3a, c), while

rec*TgGP15*-6 \times HA without GPI was only distributed in the cytoplasm (Fig. 3a). Because rec*TgGP40* has a signal peptide, it can be secreted outside the tachyzoites and into the parasitophorous vacuole. However, rec*TgGP15* has neither signal peptide nor GPI anchor, so it can only be distributed in the cytoplasm. This suggested two potential approaches for subsequent purification of rec*TgGP40*. Freshly egressed tachyzoites can be lysed and used for purification of recombinant proteins. Alternatively, cells can be lysed directly prior to parasite egress, and the lysate supernatant can be collected for purification of the recombinant proteins.

Purification of rec*TgGP40*

IFA and Western blot analyses both indicated that rec*TgGP40*-TwinStrep-6 \times HA was abundantly expressed in the cytoplasm of the RH Δ ku80 *Tguprt-Cpgp40* mutant (Figs. 3a, 4b). Therefore, freshly egressed tachyzoites of the mutant were collected and subjected to lysis for protein purification. Rec*TgGP40*-TwinStrep-6 \times HA was purified using Strep-TactinXT through the Twin-Strep tag (Fig. 4a) and then concentrated by dialysis. In SDS-PAGE and Western blot analyses of the concentrated solution, several bands were present including one of ~50 kDa (Fig. 4b). In the corresponding Western blot analysis, a strong band of ~50 kDa was recognized by HA monoclonal antibody (Fig. 4c). Because the size of the band was comparable to the predicted size of rec*TgGP40*, we supposed the recombinant protein is rec*TgGP40*. In the mass spectrometry analysis of the band, seven peptide sequences yielded were compatible to the GP60 sequence of IIdA20G1-HLJ isolate (Protein ID: AZJ53484.1), which confirmed the *CpGP40* identity of the ~50 kDa band (Table 1). However, probably because of its low expression, rec*TgGP15* was not detectable in Western blot, and therefore could not be purified.

Discussion

GP60 is one of the most prominent mucin proteins in *Cryptosporidium* and is involved in the invasion of the parasites. Upon expression, it is cleaved into GP40 and GP15, with the former containing several O-linked glycosylation sites. In this study, we employed CRISPR/Cas9 technology to insert *GP40* and *GP15* into the *UPRT* locus of *T. gondii*. The *T. gondii* mutants generated can stably express GP40 and GP15. The recombinant GP15 without the GPI anchor localized in the cytoplasm of *T. gondii*. In contrast, the recombinant GP40 localized in the tachyzoite cytoplasm and parasitophorous vacuole and probably underwent O-linked glycosylation in *T. gondii* like that in *Cryptosporidium*. Using Strep-TactinXT, we recovered a large amount of

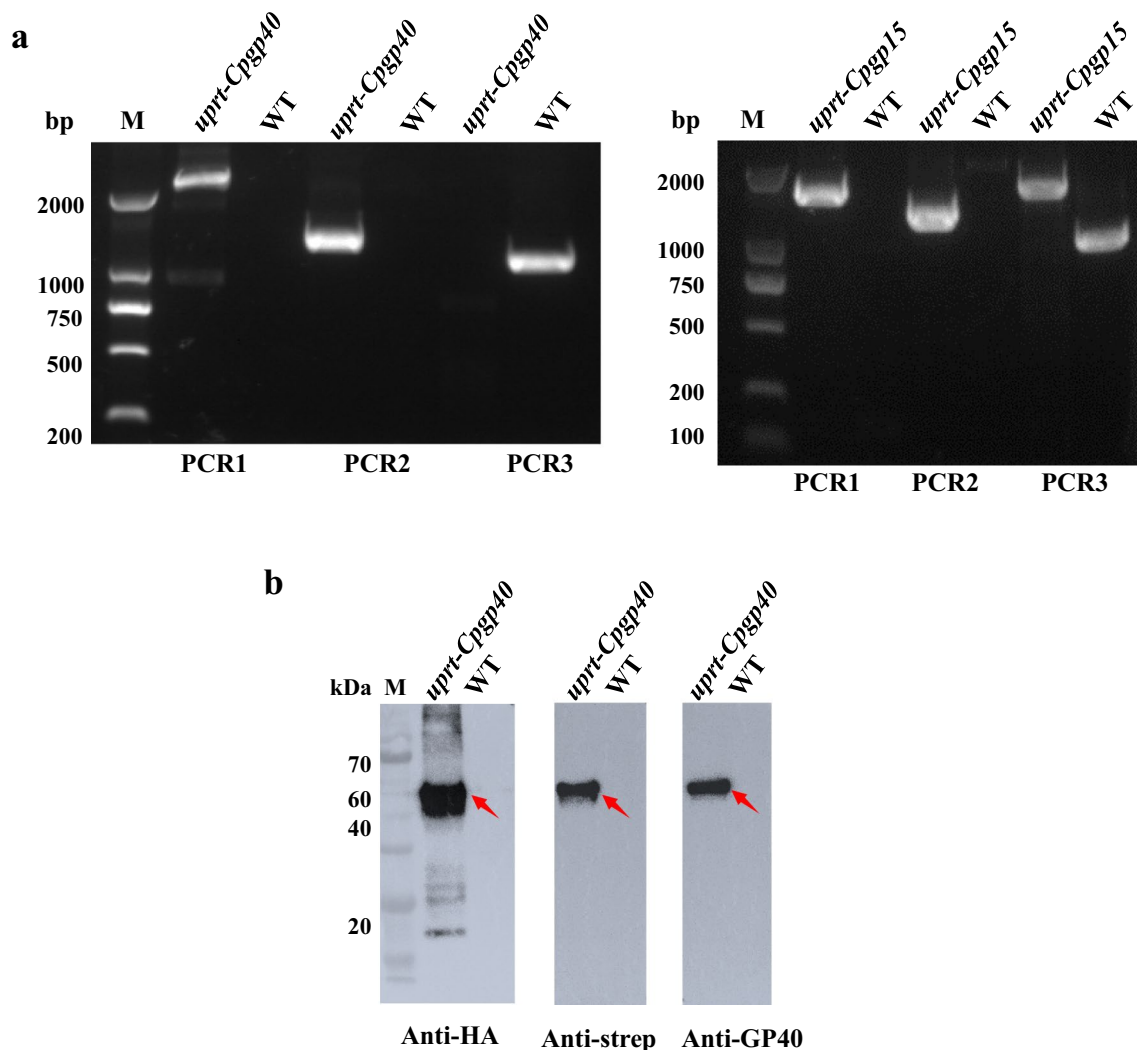


Fig. 2 Verification of the correct integration and expression of the GP40 and GP15 Δ GPI of *Cryptosporidium parvum* in *Toxoplasma gondii*. **a** Diagnostic PCR confirming the homologous integration and gene disruption in a representative clone compared to the parental line RH Δ ku80. PCR1 and PCR2 provide evidence of homologous integration based on products amplified between the *CpGP40-Twinstrep-6 \times HA-DHFR* (or *CpGP15-6 \times HA-DHFR*) gene and regions in the *UPRT* locus that lie outside of the targeting amplicon. PCR3 amplifies a 1.0-kb fragment in wild-type (WT) parasites that is lost because of the insertion of *CpGP40-Twinstrep-6 \times HA-DHFR* (or *CpGP15 Δ gpi-6 \times HA-DHFR*). **b** Expression of *CpGP40-Twinstrep-6 \times HA in *Tguprt-Cpgrp40* mutant determined by Western blotting. *CpGP40* was detected with mouse anti-HA, rabbit anti-Strep, and rabbit anti-GP60, with the WT control*****

(See figure on next page.)

Fig. 3 Verification of the expression of the GP40 and GP15 Δ GPI of *Cryptosporidium parvum* in *Toxoplasma gondii* by immunofluorescence assay. **a** *CpGP40* and *CpGP15 Δ GPI* expressed in *T. gondii*. *T. gondii* RH Δ ku80 tachyzoites were transfected with pGRA1-*CpGP40* or pGRA1-*CpGP15 Δ gpi* and allowed to infect HFF monolayers. IFA was performed 24 h after infection with anti-GP60 and -HA followed by Alexa Fluor-488-conjugated and 594-conjugated secondary antibodies. The far left panel shows the DIC field, and the far right panel shows a merged fluorescence image of the same field. DAPI-stained nuclei are in blue, Alexa Fluor-488 is in green, and Alexa Fluor-594 is in red. **b** *CpGP40* expressed in *T. gondii*. IFA was performed 24 h after infection with anti-GP60 and -Strep followed by Alexa Fluor-488-conjugated and 594-conjugated secondary antibodies. **c** High-power images of the IFA to show the fine localization of the GP40 expression. *CpGP40* proteins expressed in *T. gondii* are localized to the parasitophorous vacuole. Scale bars = 5 μ m

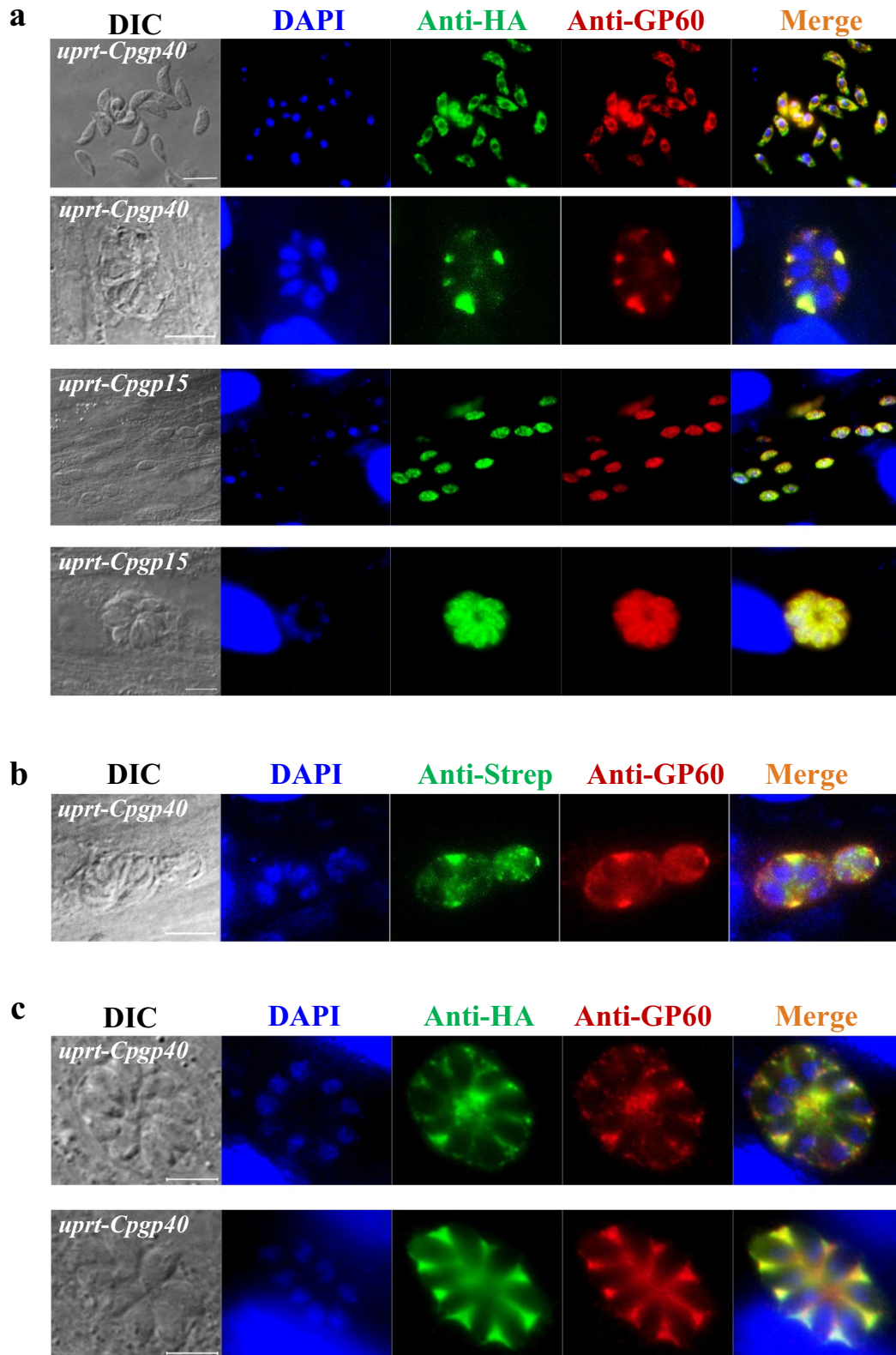


Fig. 3 (See legend on previous page.)

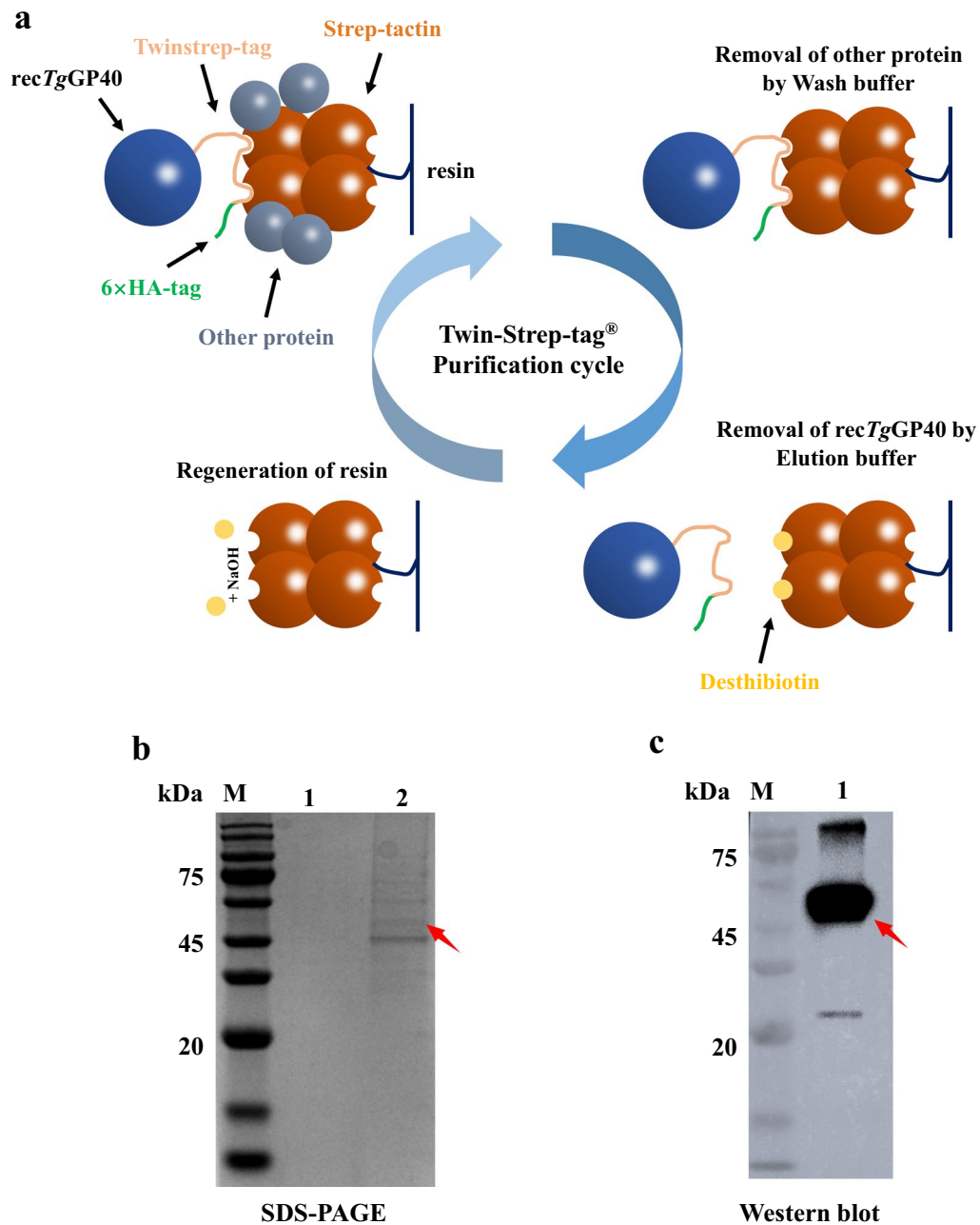


Fig. 4 Production and purification of GP40 of *Cryptosporidium parvum* in *Toxoplasma gondii*. **a** Purification process of GP40 in *T. gondii* using Strep-TactinXT. The parasite lysis supernatant was coupled to a Strep-Tactin resin column. Due to the low affinity of Strep-Tactin for non-specific interactions, other proteins were readily washed away even under mild physiological conditions. The purified recTgGP40 was eluted by the introduction of low concentrations of desthiobiotin. This step, based on competitive displacement, enhances specificity while maintaining the overall buffer conditions (such as pH and ionic strength) unaltered. As a result, highly purified recTgGP40 was obtained and the function of the target protein was preserved; 10 mM NaOH solution was used to regenerate the Strep-Tactin column. **b** The purified recTgGP40 protein was visualized by Coomassie Brilliant Blue staining of SDS-PAGE gels (sodium dodecyl sulfate–polyacrylamide gel electrophoresis): Lane 1: purified recTgGP40 before concentration; lane 2: purified recTgGP40 after concentration. The red arrow indicates the recTgGP40 glycoprotein band. **c** The purified protein was analyzed by Western blotting with mAb HA. Lane 1: recTgGP40 after purification

Table 1 Peptides of recTgGP40 matching CpGP40 obtained by LC/MS

Start–end	Numbers	Sequence
70–121	1	TGEEVGNPQSEGQDGKGDTEETEDNQTESTVS-QNTPAQTEGTTTETTEAAPK
122–142	3	KECGTSFVMWFGEGVVASLK
143–162	2	CGDYTMVYAPEKDKTDPAPR
163–183	2	YISGEVTSVTFEKQESTVTIK
184–218	3	VNNVEFSTLSTSSSPTENSGSAGQVPSEFGPASK
219–233	2	GSSTQLWVSHQPFEK
234–253	2	GGSGGGSGGSAWVSHQPFEK

recTgGP40, which should be useful for further investigation of *Cryptosporidium* mucins.

The mutants of *T. gondii* generated using CRISPR/Cas9 technology are capable of stable expression of *Cryptosporidium* proteins. *Toxoplasma gondii* can be easily cultured and genetically manipulated in vitro, making it a suitable choice for overexpressing proteins of *C. parvum*, which also belongs to Apicomplexa. Previously, *Cryptosporidium* GP60 has been expressed heterologously in *T. gondii* [16]. Subsequently, other *Cryptosporidium* mucins, P23 and Muc4, were expressed in *T. gondii* [27, 28]. However, in these studies, the heterogenous proteins were expressed through the plasmids transiently transfected into *T. gondii*, and the plasmids could be lost during the passages [29, 30]. In the current study, the heterologous genes of *Cryptosporidium* were integrated into the chromosome of *T. gondii* using CRISPR/Cas9 technology. GP40 was overexpressed in the RH Δ ku80 Tguprt-Cpgp40 mutants as demonstrated by Western blot and IFA analyses. Therefore, this approach has an improved stability and reliability for the overexpression of target genes of *Cryptosporidium*.

The recTgGP40 is probably glycosylated in a manner analogous to native expression of the protein. Native CpGP40 contains a signal peptide and several O-linked glycosylation sites [9, 12]. Signal peptides play crucial roles in directing proteins to the endoplasmic reticulum and Golgi apparatus for post-translational processing, including glycosylation [31, 32]. Therefore, the signal peptide of CpGP40 was retained during the overexpression in *T. gondii* in this study. The similarity in the size of recTgGP40 and native CpGP40 suggested the glycosylation of the recombinant protein in *T. gondii*. In addition, recTgGP40 was distributed in the cytoplasm of tachyzoites and the parasitophorous vacuoles, indicating secretion of the protein under the direction of the signal peptide. This is inconsistent with the surface localization of GP40 in *C. parvum* and transiently

transfected *T. gondii* [16, 33]. The disruption of surface localization of recTgGP40 is probably due to the absence of GP15, which is associated with GP40 and attached to the membrane.

GP15 without a GPI anchor was only expressed in the cytoplasm. GPI is a common structural motif in apicomplexans, serving as anchors of some proteins for the attachment to cell membrane [34, 35]. In a previous study, GP15 was expressed in *T. gondii* with and without the GPI anchor [16]. The result showed that GP15 was unable to localize to the membrane of tachyzoites in absence of the GPI anchor. In IFA analysis, we observed recTgGP15 in the cytoplasm rather than on the surface of tachyzoites. Therefore, the localization of GP15 on the cell membrane is indeed GPI-dependent, at least within the heterologous expression system.

Combined with the Twin-Strep-based purification, the heterologous expression system established here allowed a high yield of purified recTgGP40 at a low cost. Because *C. parvum* is difficult to propagate in vitro and in vivo, native GP40 can only be isolated in limited amounts from purified *C. parvum* oocysts. The amount of recombinant GP40 expressed by transiently transfected *T. gondii* was limited, which was only detectable by silver staining [17]. Similarly, the heterologous expression of other *C. parvum* mucin proteins in *T. gondii*, such as P23 and Muc4, resulted in poor yields of recombinant proteins [27, 28]. These could be due to the loss of the plasmid containing the target genes and complicated purification procedure using a HA tag. In this study, stable heterologous expression of GP40 was achieved by inserting GP40 into the chromosome of *T. gondii* using CRISPR/Cas9 technology. In addition, a Twin-Strep tag was fused to the C-terminal of GP40 for purification. Compared to the purification using a HA tag, the purification using the Twin-Strep tag was achieved by specific binding between the Strep-tag and the Strep-Tactin affinity chromatography resin, which was more efficient and cost effective [28, 36]. The high efficiency of the heterologous expression and Strep-based purification was supported by a visible band of ~ 50 kDa in SDS-PAGE (Fig. 4b), which was confirmed to be recTgGP40 by LC/MS analysis.

Conclusions

In conclusion, we have established a heterologous expression system of *Cryptosporidium* mucin glycoproteins in *T. gondii* using CRISPR/Cas9 technology. The system allowed stable expression of GP40 and GP15 of *C. parvum* in *T. gondii*. In addition, we fused GP40 with a Twin-Strep-tag and HA tag, and a significant amount of recTgGP40 was purified using Strep-TactinXT resin at an affordable cost. Therefore, the novel approach and

concept present here should be useful for the further study of *Cryptosporidium* mucins.

Abbreviations

GP60	60 kDa glycoprotein
GP40	40 kDa glycoprotein
GP15	15 kDa glycoprotein
GPI	Glycosyl phosphatidyl inositol
UPRT	Uracil phosphoribosyl transferase
CRISPR	Clustered regularly interspaced short palindromic repeats
Cas9	CRISPR-associated protein 9
PVDF	Polyvinylidene fluoride
TBST	Tris-buffered saline with Tween-20
IFA	Immunofluorescence assay

Supplementary Information

The online version contains supplementary material available at <https://doi.org/10.1186/s13071-024-06159-y>.

Additional file 1: Table S1. Primers used in this study.

Acknowledgements

We thank Dr. Bang Shen of Huazhong Agricultural University for providing the RHΔku80 strain, pSAG1-Cas9-TgU6-sgTgUPRT plasmid, and pUC19-loxp-DHFR*-amp plasmid.

Author contributions

YG and LX conceived and designed the experiments. ML, XS, and HC performed the experiments. ML and NL participated in the data analysis. ML, YF and YG wrote the manuscript. All authors read and approved the final manuscript.

Funding

This work was supported by National Natural Science Foundation of China (31972697 and 32273032), Double First-Class Discipline Promotion Project (2023B10564003), and 111 Project (D20008).

Availability of data and materials

All data are included as tables and figures within the article.

Declarations

Ethics approval and consent to participate

The experimental animals were housed and treated in accordance with the Regulations for the Administration of Affairs Concerning Experimental Animals of the People's Republic of China. All experiments were conducted under the approval of the Laboratory Animals Research Center of Guangdong Province and South China Agricultural University (approval number: 2021C005).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Received: 5 December 2023 Accepted: 24 January 2024

Published online: 15 February 2024

References

1. Checkley W, White AC Jr, Jaganath D, Arrowood MJ, Chalmers RM, Chen XM, et al. A review of the global burden, novel diagnostics, therapeutics, and vaccine targets for *Cryptosporidium*. *Lancet Infect Dis*. 2015;15:85–94.
2. Xiao L. Molecular epidemiology of cryptosporidiosis: an update. *Exp Parasitol*. 2010;124:80–9.
3. Darabus G, Lupu MA, Mederle N, Darabus RG, Imre K, Mederle O, et al. Epidemiology of *Cryptosporidium* infection in Romania: a review. *Microorganisms*. 2023;11:1793.
4. Caravado MA, White AC Jr. Treatment of cryptosporidiosis: nitazoxanide yes, but we can do better. *Expert Rev Anti Infect Ther*. 2023;21:167–73.
5. Guerin A, Striepen B. The biology of the intestinal intracellular parasite *Cryptosporidium*. *Cell Host Microbe*. 2020;28:509–15.
6. Pinto DJ, Vinayak S. *Cryptosporidium*: host–parasite interactions and pathogenesis. *Curr Clin Microbiol Rep*. 2021;8:62–7.
7. Martinez-Ocana J, Maravilla P, Olivo-Diaz A. Interaction between human mucins and parasite glycoproteins: the role of lectins and glycosidases in colonization by intestinal protozoa. *Rev Inst Med Trop Sao Paulo*. 2020;62:e64.
8. Gilchrist CA, Campo JJ, Pablo JV, Ma JZ, Teng A, Oberai A, et al. Specific *Cryptosporidium* antigens associate with reinfection immunity and protection from cryptosporidiosis. *J Clin Invest*. 2023;133:e166814.
9. Cevallos AM, Zhang X, Waldor MK, Jaison S, Zhou X, Tzipori S, et al. Molecular cloning and expression of a gene encoding *Cryptosporidium parvum* glycoproteins gp40 and gp15. *Infect Immun*. 2000;68:4108–16.
10. Wanyiri JW, O'Connor R, Allison G, Kim K, Kane A, Qiu J, et al. Proteolytic processing of the *Cryptosporidium* glycoprotein gp40/15 by human furin and by a parasite-derived furin-like protease activity. *Infect Immun*. 2007;75:184–92.
11. Winter G, Gooley AA, Williams KL, Slade MB. Characterization of a major sporozoite surface glycoprotein of *Cryptosporidium parvum*. *Funct Integr Genomic*. 2000;1:207–17.
12. Strong WB, Gut J, Nelson RG. Cloning and sequence analysis of a highly polymorphic *Cryptosporidium parvum* gene encoding a 60-kilodalton glycoprotein and characterization of its 15- and 45-kilodalton zote surface antigen products. *Infect Immun*. 2000;68:4117–34.
13. Priest JW, Kwon JP, Arrowood MJ, Lammie PJ. Cloning of the immunodominant 17-kDa antigen from *Cryptosporidium parvum*. *Mol Biochem Parasitol*. 2000;106:261–71.
14. Kim K, Weiss LM. *Toxoplasma gondii*: the model apicomplexan. *Int J Parasitol*. 2004;34:423–32.
15. Shen B, Brown KM, Lee TD, Sibley LD. Efficient gene disruption in diverse strains of *Toxoplasma gondii* using CRISPR/CAS9. *mBio*. 2014;5:e0111414.
16. O'Connor RM, Kim K, Khan F, Ward HD. Expression of Cpgp40/15 in *Toxoplasma gondii*: a surrogate system for the study of *Cryptosporidium* glycoprotein antigens. *Infect Immun*. 2003;71:6027–34.
17. O'Connor RM, Wanyiri JW, Wojczyk BS, Kim K, Ward H. Stable expression of *Cryptosporidium parvum* glycoprotein gp40/15 in *Toxoplasma gondii*. *Mol Biochem Parasitol*. 2007;152:149–58.
18. Black MW, Boothroyd JC. Development of a stable episomal shuttle vector for *Toxoplasma gondii*. *J Biol Chem*. 1998;273:3972–9.
19. Xiong J, He J, Xie W, Hinojosa E, Ambati CSR, Putluri N, et al. Rapid affinity purification of intracellular organelles using a twin strep tag. *J Cell Sci*. 2019;132:jcs235390.
20. Rai J, Pemmasani JK, Voronovsky A, Jensen IS, Manavalan A, Nyengaard JR, et al. Strep-tag II and Twin-Strep based cassettes for protein tagging by homologous recombination and characterization of endogenous macromolecular assemblies in *Saccharomyces cerevisiae*. *Mol Biotechnol*. 2014;56:992–1003.
21. Dammeyer T, Timmis KN, Tinnfeld P. Broad host range vectors for expression of proteins with (Twin-) Strep-tag, His-tag and engineered, export optimized yellow fluorescent protein. *Microb Cell Fact*. 2013;12:49.
22. Li N, Zhao W, Song S, Ye H, Chu W, Guo Y, et al. Diarrhoea outbreak caused by coinfections of *Cryptosporidium parvum* subtype IIdA20G1 and rotavirus in pre-weaned dairy calves. *Transbound Emerg Dis*. 2022;69:e1606–17.
23. Jia R, Huang W, Huang N, Yu Z, Li N, Xiao L, et al. High infectivity and unique genomic sequence characteristics of *Cryptosporidium parvum* in China. *PLoS Negl Trop Dis*. 2022;16:e0010714.
24. Guo Y, Li N, Lysen C, Frace M, Tang K, Sammons S, et al. Isolation and enrichment of *Cryptosporidium* DNA and verification of DNA purity for whole-genome sequencing. *J Clin Microbiol*. 2015;53:641–7.

25. Portes JA, De Souza W. Development of an *in vitro* system to study the developmental stages of *Toxoplasma gondii* using a genetically modified strain expressing markers for tachyzoites and bradyzoites. *Parasitol Res*. 2019;118:3479–89.
26. Xia N, Ye S, Liang X, Chen P, Zhou Y, Fang R, et al. Pyruvate homeostasis as a determinant of parasite growth and metabolic plasticity in *Toxoplasma gondii*. *mBio*. 2019;10:e00898-19.
27. Shirafuji H, Xuan X, Kimata I, Takashima Y, Fukumoto S, Otsuka H, et al. Expression of P23 of *Cryptosporidium parvum* in *Toxoplasma gondii* and evaluation of its protective effects. *J Parasitol*. 2005;91:476–9.
28. Paluszynski J, Monahan Z, Williams M, Lai O, Morris C, Burns P, et al. Biochemical and functional characterization of CpMuc4, a *Cryptosporidium* surface antigen that binds to host epithelial cells. *Mol Biochem Parasitol*. 2014;193:114–21.
29. Liu M, Ji S, Rizk MA, Adjou Moumouni PF, Galon EM, Li J, et al. Transient transfection of the zoonotic parasite *Babesia microti*. *Pathogens*. 2020;9:108.
30. Soldati D, Boothroyd JC. Transient transfection and expression in the obligate intracellular parasite *Toxoplasma gondii*. *Science*. 1993;260:349–52.
31. von Heijne G. The signal peptide. *J Membr Biol*. 1990;115:195–201.
32. Owji H, Nezafat N, Negahdaripour M, Hajiebrahimi A, Ghasemi Y. A comprehensive review of signal peptides: Structure, roles, and applications. *Eur J Cell Biol*. 2018;97:422–41.
33. O'Connor RM, Wanyiri JW, Cevallos AM, Priest JW, Ward HD. *Cryptosporidium parvum* glycoprotein gp40 localizes to the sporozoite surface by association with gp15. *Mol Biochem Parasitol*. 2007;156:80–3.
34. Gerold P, Schofield L, Blackman MJ, Holder AA, Schwarz RT. Structural analysis of the glycosyl-phosphatidylinositol membrane anchor of the merozoite surface proteins-1 and -2 of *Plasmodium falciparum*. *Mol Biochem Parasitol*. 1996;75:131–43.
35. Wang Y, Yin H. Research progress on surface antigen 1 (SAG1) of *Toxoplasma gondii*. *Parasit Vectors*. 2014;7:180.
36. Schmidt TG, Skerra A. The Strep-tag system for one-step purification and high-affinity detection or capturing of proteins. *Nat Protoc*. 2007;2:1528–35.

Publisher's Note

Springer Nature remains neutral with regard to jurisdictional claims in published maps and institutional affiliations.