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Short communication

Identification of novel vaccine candidates against cryptosporidiosis of neonatal bovines by reverse vaccinology

M.L. Tomazic[a,b], A.E. Rodriguez[b,1], J. Lombardelli[c,1], T. Poklepovich[a,b], C. Garro[b], R. Galarza[d], K. Tiranti[c], M. Florin-Christensen[a,b,e], L. Schnittger[a,b,e,*]

[a] Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina
[b] Instituto de Patobiología Veterinaria, (CICVyA), Instituto Nacional de Tecnología Agropecuaria (INTA), Hurlingham, Argentina
[c] Departamento de Patología Animal, Universidad Nacional de Río Cuarto, CONICET, Córdoba, Argentina
[d] EEA-Rafaela, Instituto Nacional de Tecnología Agropecuaria (INTA), Santa Fe, Argentina
[e] Facultad de Ciencias exactas, químicas y naturales (FCEQN), Universidad de Morón, Morón, Argentina

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ABSTRACT

The apicomplexan protozoan *Cryptosporidium parvum* is an important causative agent of diarrhea of neonatal bovines. Vaccination has been proposed as an advantageous strategy against cryptosporidiosis of calves since besides protection against disease it has also the potential to prevent dissemination of infective oocysts into the environment. Antigens anchored to the parasite surface via glycosylphosphatidylinositol (GPI) are implicated in host cell attachment and invasion and represent promising vaccine candidates. A reverse vaccinology approach was employed to (i) identify the GPI-anchored proteome of *C. parvum* using available web-based bioinformatic tools and (ii) characterize previously unrecognized novel vaccine antigens. Altogether, 14 putative GPI-anchored proteins could be determined of which CpH1 and CpSUB2 as well as GP60 were further characterized. Sequencing and comparison of GP60, CpH1, and CpSUB1 alleles amplified from different geographic isolates showed a high degree of conservation. All three antigens were recombinant expressed and immunoblotted using sera of 12 *Cryptosporidium*-infected calves sampled at age periods 1–11 and 12–28 days after birth. Specific antibody reactions against the studied antigens were detected in all analyzed calves, demonstrating their immunreactivity and expression, and recognition *in vivo* at an early stage of host infection. Besides the acknowledged GP60 vaccinogen, the presented reverse vaccinology approach reveals the additional vaccine candidates CpH1 and CpSUB1 for inclusion into a subunit vaccine formulation.

1. Introduction

Cryptosporidium sp. infects the gastrointestinal tract of a wide range of vertebrates, including livestock animals and humans. In neonatal calves, *C. parvum* causes considerable economic losses as infections are commonly associated with intense diarrhea leading to impaired growth, a decreased performance and production, and often animal death (Tomazic et al., 2018; Garro et al., 2016). Furthermore, neonatal calves are considered a major reservoir of the zoonotic *C. parvum* posing a major human public health risk (Wyatt et al., 2010). The parasite is transmitted via the fecal-oral route, i.e. the highly infective oocyst stage is excreted with the feces of infected animals and disseminated into the environment contaminating water and food, eventually leading to outbreaks (Shrivastava et al., 2017).

The only licensed drug for the treatment of cryptosporidiosis of calves is Halofuginone, but its efficacy is controversial (Trotz-Williams et al., 2011). No vaccines are available, though protection by vaccination is favorably regarded since on one hand, it would benefit animal health and on the other hand, avoid oocyst contamination of the environment. Several reports suggest that development of a vaccine to prevent cryptosporidiosis is feasible, but relatively few vaccine candidates have been characterized and tested (Mead et al., 1988; Boulter-Bitzer et al., 2007; Manque et al., 2011; Mead, 2014). One of these is the glycosylphosphatidylinositol (GPI) anchor membrane surface antigen GP60, which has been reported to neutralize parasite infection of the host cell *in vitro* (O'Connor et al., 2007).

* Corresponding author at: Instituto de Patobiología, Centro de Investigaciones en Ciencias Veterinarias y Agronómicas (CICVyA), Instituto Nacional de Tecnología Agropecuaria (INTA), Los Reseros y Nicolás Repetto s/n, Hurlingham, Argentina.
E-mail address: schnittger.leonhard@inta.gob.ar (L. Schnittger).
[1] Contributed equally.

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The identification and characterization of additional vaccine candidates to be tested in vaccine trials against bovine cryptosporidiosis is essential. GPI-anchored proteins are expressed in parasitic pathogens in large amounts and are involved in host cell attachment, adhesion, and invasion (Rodriguez et al., 2014). Importantly, GPI-anchored proteins have been shown to confer protection and are considered as promising vaccines against animal and human apicomplexan pathogens of importance such as *Babesia* and *Plasmodium*, respectively (Florin-Christensen et al., 2014). For example, a recombinant form of the GPI-anchored antigen Bd37 of *B. divergens* has been shown to confer total protection in gerbils against challenge with homologous or heterologous strains in terms of survival rates and absence of clinical signs (Delbecq et al., 2006; Hadj-Kaddour et al., 2007). Furthermore, secreted and membrane-bound forms of Bd37 expressed in *Trypanosoma theileri* resulted in long-term immunity against this antigen in bovines (Mott et al., 2011). In addition, the GPI-anchored circumsporozoite surface antigen is the backbone of the first approved vaccine against human malaria, designated RTS,S, which has an efficacy against severe malaria of 55.8% in children between 5 and 17 months (RTS, S Clinical Trials Partnership, 2015).

With regard to *Cryptosporidium*, the identification of immune targets and vaccine development is hampered by the absence of an *in vitro* system of parasite propagation. Thus, a reverse vaccinology approach using bioinformatic tools constitutes an attractive strategy to identify vaccine candidates. In the present study, we tested the hypothesis that the GPI-anchored proteome of *C. parvum* contains conserved immunoreactive antigens that may be suitable as vaccine candidates to be included in future immunotherapeutic trials.

2. Material and methods

2.1. Prediction and selection of GPI-anchored antigens and amino acid sequence conservation

The GPI anchored proteome was determined based on the *C. parvum* Iowa II genome as described (Table 1, Supplementary data; Rodriguez et al., 2014). Primers were designed to amplify encoding genes of selected antigens from genomic DNA of different GP60 subgenotypes of *C. parvum* isolates originating from the Provinces of Santa Fe, Córdoba, and/or Buenos Aires (Table 2, Supplementary data; Tomazic et al., 2013). PCR products were directly sequenced in both directions using the respective forward and reverse amplification primers (Macrogen, Seoul, South Korea). Nucleotide sequences were deposited in the GenBank™ database under accession numbers MF576522 to MF576541.

2.2. Protein expression and purification

Gene fragments of gp60 (892 bp), Cph1 (915 bp), and Cpsub2 (1007 bp) coding for hydrophilic protein regions were PCR amplified using amplification primers cgd6_1080sF: 5´-CATTGGATCCTAGTCTCCGCTG TATTCTCAG-3´ and cgd6_1080sR: 5´-CATTGGATCC**TTA**ATGTATCTG AGTCCAAAAGCAGAG-3´ for GP60; cgd1_660sF: 5´-CATTGGATCCCAA CAGCTCAAGATGTAATACTCG-3´ and cgd1_660sR: 5´-CATTGGATCC**T TA**AGACCTTAAAAGCAAAAGATGAATC-3´ for Cph1 and cgd6_3730AsF: 5´-CATTGGATCCGCATTTATTGGTACTGGATTTGATAC-3´ and cgd6_3730AsR: 5´-AGTTGAATTC**TTA**TTGTTGTTGTTGCTGTTG AGG-3´ for Cpsub2.

Amplicons were digested with *Bam*HI and *Eco*RI and directionally cloned into the pRSET vector (Thermo Scientific). After transformation of *E. coli* Rosetta 2DE3 pLacI strain and plating and growth on agarose medium, qualified recombinant clones were selected, cultured overnight, purified, and the correct reading frame of the insert verified by sequencing (Macrogen, Seoul, South Korea). Recombinant clones were grown in LB medium supplemented with ampicillin/chloramphenicol and induced with IPTG (Promega, USA). After centrifugation of lysed cellular preparations, recombinant products rCpH1, rCpSUB2, and rGP60 were each found in inclusion bodies (IB). IBs were solubilized by sonication and purified by Ni^{2+}-NTA-agarose affinity chromatography (Qiagen).

Proteins were eluted using as buffer 50 mM K_2HPO_4, 400 mM NaCl, 100 mM KCl, 10% glycerol, and, depending on the protein, different concentrations of imidazole were included (400 mM for rCpH1 and rGP60, and 1 M for rCpSUB2). Eventually the eluted material was dialyzed in PBS and water. Dialyzed proteins were concentrated by lyophilization and the purity and identity of the recombinant protein was examined by 12% SDS-PAGE stained with Coomassie brilliant blue and by Western blotting using a primary mouse anti-6xHis-monoclonal antibody (BD Pharmingen TM) and a secondary anti mouse-horseradish peroxidase-conjugated polyclonal antisera (Sigma, USA). The concentration was measured with the Micro BCA Protein Assay Kit (Thermo Scientific) following the manufacture recommendations, as well as by band density comparison in SDS-PAGE gels with known amounts of a BSA standard. Purified expressed polypeptides were stored at −20 °C until further use.

2.3. Sera collection and Western blot analysis

Blood samples from 12 calves naturally infected with *Cryptosporidium* sp. were taken from the jugular vein at 1 to 11 days of

Table 2
Immunoreactivity of sera from calves naturally infected with *Cryptosporidium* spp. against recombinant rCpGP60, rCpH1, and rCpSUB2.

	Age 1-11 days			Age 12-28		
Calf no	rGP60	rCpH1	rCpSUB2	rGP60	rCpH1	rCpSUB2
1	+	+	+	+	+	+
2	–	+	+	–	+	+
3	–	+	+	+	+	+
4	–	–	+	- [a]	+	+
5	+	+	+	+	+	+
6	+	+	+	+	+	+
7	–	+	+	+	+	+
8	–	+	+	+	+	+
9	+	+	+	+	+	+
10	+	+	+	+	+	+
11	+	+	+	+	+	+
12	+	+	+	+	+	–

[a] Sera reacted positive at 38 days of age.

Table 1
Predicted features and experimental data of *C. parvum* vaccine candidates.

Name	GenBank designation	Exons	Aminoacids expressed	MW (kDa)	Predicted function	Transcripts (h)[a]	Protein evidence	Conservation (%)	
								Antigen	B epi
GP60	XP_627480	1	14 - 310	33.5	N	12	Sp	97.8-99.7[b]	97.8-99.7[b]
CpH1	XP_628629	1	17 - 280	37.1	N	48	Sp	100.0	100.0
CpSUB2	XP_627713	1	730 -1006	252.7	Proteolysis	48; 72	Oo	99.8-100.0	100.0

[a] Maximal level of expression according to CryptoDB.
[b] The percentage variation includes the hypervariable region of GP60; MW: molecular weight; N: none; Sp: sporozoite; Oo: oocyst; B epi: B epitopes.

age (age period 1), and at 12 to 28 days (age period 2). From calf 3 and 4 additional blood samples were obtained from the jugular vein at day 38 and 52 (age period 3) (Table 3, Supplementary data). The serum component of blood samples were collected and stored at −20 °C. Sample collection followed accepted animal welfare guidelines.

Calves were fed with different colostrum pools and the failure of passive transfer of immunity (FPTI) was determined by refractometry in all sera taken during age period 1 (Elsohaby et al., 2015). Recombinant proteins were separated by 12% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were treated with blocking buffer, cut in strips (0.6 mm), and incubated with a 1:20 dilution of calf sera in blocking buffer. Strips were washed three times and finally incubated with horseradish peroxidase-conjugated goat anti-bovine IgG (KPL, USA) (1:1000) for 1 h at RT. Strips were washed four times at RT for 10 min and reactions were developed using 3, 3-diaminobenzidine (DAB) (Sigma, USA). As a negative control, serum from a newborn calf before its first feeding with colostrum and fetal bovine serum were used.

3. Results

3.1. Selection of potential vaccine candidates from the GPI-anchored proteome

The GPI-anchored proteome of *C. parvum* was predicted using a bioinformatic search strategy. The applied strategy consisted of three consecutive steps that identify essential features characteristic for GPI-anchored proteins: (i) a signal peptide, (ii) a GPI-anchor signal sequence, and (iii) a C-terminal transmembrane domain (Table 1, Supplementary data).

By this approach, GP60, the only *C. parvum* protein that has been experimentally shown to be GPI-anchored, has been identified and was included in this study. Besides GP60, two additional proteins, designated CpH1 and CpSUB2, were selected from the GPI-anchored proteome and analyzed as potential vaccine candidates (Fig. 1 and Table 1). CpH1 and CpSUB2 were each predicted by three GPI-anchor predictors (Table 1, Supplementary data). CpH1 has a calculated molecular weight of 37.1 kDa and its function is unknown (Heiges et al., 2006). The CpH1 allele sequence of the analyzed five isolates and the reference sequence (CryptoDB ID cgd1_660) showed 100% identity. In addition, six B cell epitopes found to be identical in all studied alleles were predicted (Fig. 2, Supplementary data).

CpSUB2 is encoded by a single exon and its predicted molecular weight is 252.7 kDa. As the gene is very long the following three fragments of the gene, which cover 37% of the translated protein, were independently analyzed: (i) the upstream (Ups) region from the subtilisin-like domain that encompasses amino acids 344 to 600; (ii) the subtilisin-like domain (SLD) encompassing amino acids 752 to 1032 (Fig. 3, Supplementary data); and (iii) a region downstream (SLD-downstream) from the conserved SLD that encompasses amino acid position 1477 to 1776 and includes the expressed sequence tag (EST) (Heiges et al., 2006). Sequence analysis of the Ups fragment revealed a single non-synonymous nucleotide mutation at position 1492 (*CAT* > *TAT*) resulting in an amino acid exchange at position 498 (His > Pro) (Table 1).

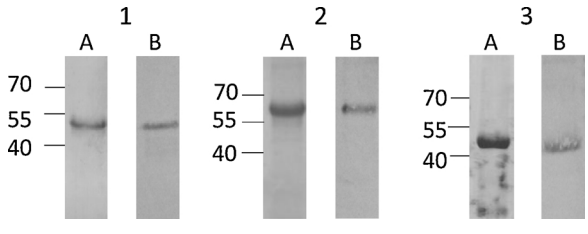

Fig. 1. Purification of *C. parvum* recombinant vaccine candidates. Recombinant parasite antigens rGP60 (1), rCpSUB2 (2), and rCpH1 (3) were separated by SDS-PAGE on 12% polyacrylamide gels and stained with Coomassie brillant blue (A) or transferred to nitrocellulose membranes and probed with anti-Histidine-monoclonal antibody (B) (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

Conserved B-cell epitopes were identified in the Ups, SLD, and in the SLD-downstream region of the analyzed CpSUB2. In the Ups region, seven B cell epitopes were identified and found to be identical between the studied isolates. Accordingly, in the SLD and SLD-downstream region four and seven conserved B cell epitopes were identified, respectively (Fig. 3, Supplementary data). Conservation of the identified B cell epitope imply that these regions are functionally important corroborating CpSUB2 as a vaccine candidate.

3.2. Protein expression and immunoreactivity of vaccine candidates

For all three expressed peptides the molecular weight as experimentally determined by SDS-PAGE did not correspond with the calculated molecular weight (Fig. 1, Table 1). rCpH1 showed a reaction with 11 calf sera at time period 1 and with all 12 calf sera at time period 2. rCpSUB2 was recognized by sera of all calves at time period 1 and by 11 calves at time period 2. In contrast, the peptide rGP60 reacted with 7 and 10 calves at time period 1 and 2, respectively; of the two sera that did not react at time period 2, one reacted eventually at time period 3, whereas a single serum showed no reaction at any of the tested time periods (Table 2). It is important to note that for the majority of calves infection was observed after sampling of sera (calf 2, 3, 5, 6, 7, 10, 11, 12) whereas from the remaining calves sera were sampled only 6 days (calf 1), 4 days (calf 8), 3 days (calf 9), and 2 days (calf 4) after infection. Thus, it can be concluded that observed antibody specificities have not been generated by calves as the time window between infection and antibody generations would have been too short (Table 3, Supplementary data).

In summary, antibody reaction against rCpH1 and rCpSUB2 could be observed early after calf birth (time point 1) for all or nearly all calves, respectively. For rGP60, this was also the case for seven animals, whereas an antibody response seems to have been elicited by four animals at a later time period 2, with one calf showing a reaction at a late time period 3. Thus all studied vaccine candidates showed immunoreactivity confirming the existence of B-cell epitopes that are conserved as suggested by sequencing of diverse geographic isolates (see Section 3.1).

4. Discussion

In order to identify novel vaccine antigens of *C. parvum* against bovine cryptosporidiosis, a reverse vaccinology approach was followed in this study. To this aim, surface antigens GP60, CpH1, and CpSUB2 identified as members of the GPI-anchored proteome of *C. parvum* were selected and characterized as potential vaccine candidates. GP60 has been experimentally demonstrated to be GPI anchored whereas CpSUB2 represents a homolog to subtilisins, which have been shown to be functionally important for host cell invasion. In contrast, no homolog to CpH1 could be identified. Interestingly, for the three expressed peptides the calculated molecular weight did not correspond with the apparent molecular weight, a phenomenon often observed in parasite surface proteins.

Importantly, we show that all three proteins are immunoreactive antigens that bear conserved B-cell epitopes suggesting their functional importance. For rGP60 this finding is in agreement with Priest et al., (2000), who reported GP60 as an immunodominant protein in humans. The detection of specific IgG antibodies against rGP60 in naturally infected calves is also in agreement with previous reports that demonstrate that GP60-specific antibodies are frequently identified in sera from convalescent humans or animals (Mead et al., 1988; Priest et al., 2006; Ajjampur et al., 2011; Allison et al., 2011).

GP60 is proteolytically cleaved into the GPI-anchored surface antigen GP15 and the noncovalently surface attached GP40. In previous studies it has been shown that the administration of a monoclonal anti-GP15 antibody to suckling mice experimentally infected with *C. parvum* reduced 75% of developmental stages and 68% of oocyst excretion 6 days post-infection (Tilley et al., 1991). Furthermore, it has been shown that specific anti-GP40 antibodies neutralized *in vitro*-infection of cultured cells (Cevallos et al., 2000). These findings suggest that passive immunization against cryptosporidiosis, should be able by application of neutralizing antibodies binding critical surface antigens which may include the novel predicted GPI-anchored antigens.

The presence of specific IgGs in the sera of infected animals directly after birth, i.e. 1–11 days of age, is indicative of the transfer of anti-*Cryptosporidium*-IgG via colostrum. FTPI can be estimated by refractometry of colostrum which measures the total protein content including the transferred IgG. With the exception of serum of calf 4, remaining sera sampled at the early time period 1 after calf birth, reacted positive against rCpH1 and rCpSUB2 including sera of calf 7, 9, and 12 that showed FTPI (Table 2). As outlined in section 3.2, specific antibodies against rCpH1 and rCpSUB2 must have been transferred by colostrum since calf 7 and 12 have been infected after sera sampling, whereas serum of calf 9 was sampled just 3 days after infection not enough to allow the generation of specific antibodies. This allows concluding that the observed antibody specificities are due to passive colostrum-mediated transfer of antibodies. However, the observation that colostrum of calves 7, 9, and 12 were able to transfer CpH1 and CpSUB2-specific antibodies although they present FTPI may be explained by the fact that refractometry allows only a rough estimation of the total IgG transferred and does not reliably predict the transfer of antibody specificities. The observation that specific anti-rCpH1 and anti-rCpSUB2 antibodies are consistently transferred via colostrum suggests that feeding calves with colostrum hyperimmune for these antigens might foster prevention of calf cryptosporidiosis. Future studies of the transfer of specific anti-rCpH1 and rCpSUB2 antibodies to calves and its association with a possible abrogation of oocyst excretion may reveal if these antibodies have a protective effect against infection.

In contrast, with respect to the antigen GP60 five of twelve calves (calf 2, 3, 4, 7, and 8) did not receive anti-GP60 antibodies via colostrum but seroconversion was observed for calf 3, 4, 7, and 8 not before day 12 and latest at day 38 after birth (Table 2; Fig. 1, Supplementary data). On one hand, this strongly suggests that calves, which have not received anti-GP60 antibodies via colostrum, are able to generate specific antibodies as has been previously reported (Tizard, 2017). On the other hand, colostrum-mediated transfer of anti-GP60 via immunization of dams may not to be a reliable strategy of passive immunization for this antigen. Instead, in this case passive immunity may be transferred by food supplementation with anti-GP60-IgY obtained by immunization of chicken as has recently been proposed (Askari et al., 2016). This strategy has been applied with success against viral but also bacterial infections that cause diarrhea of neonatal calves (Vega et al., 2015).

It is important to note that transfer of GP60-specific antibodies was observed in the remaining seven studied calves confirming a report that antibodies against GP60 are present in colostrum of cows immunized with *C. parvum* oocysts/sporozoites (Tilley et al., 1990). This somewhat contradictory result that 5 calves did and 7 did not show GP60-specific colostrum-mediated transfer may be explained by the fact that studied calves have received colostrum that originated from different colostrum pools and thus, differences in the composition of specific antibodies between these pools cannot be excluded. Alternatively, it is possible that *C. parvum*-infected dams show strong and consistent antibody responses against antigens CpH1 and CpSUB2, but a more inconsistent and lower antibody reaction against the antigen GP60.

As currently cultivation of *C. parvum* is not well established, the applied reverse vaccinology approach allowed to confirmed the previously known vaccine candidate GP60 and identifying novel GPI-anchored antigens CpH1 and CpSUB2 as potential vaccine candidates against *C. parvum*-infection.

Competing interests

The authors declare no competing interests in this study.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetpar.2018.11.007.

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