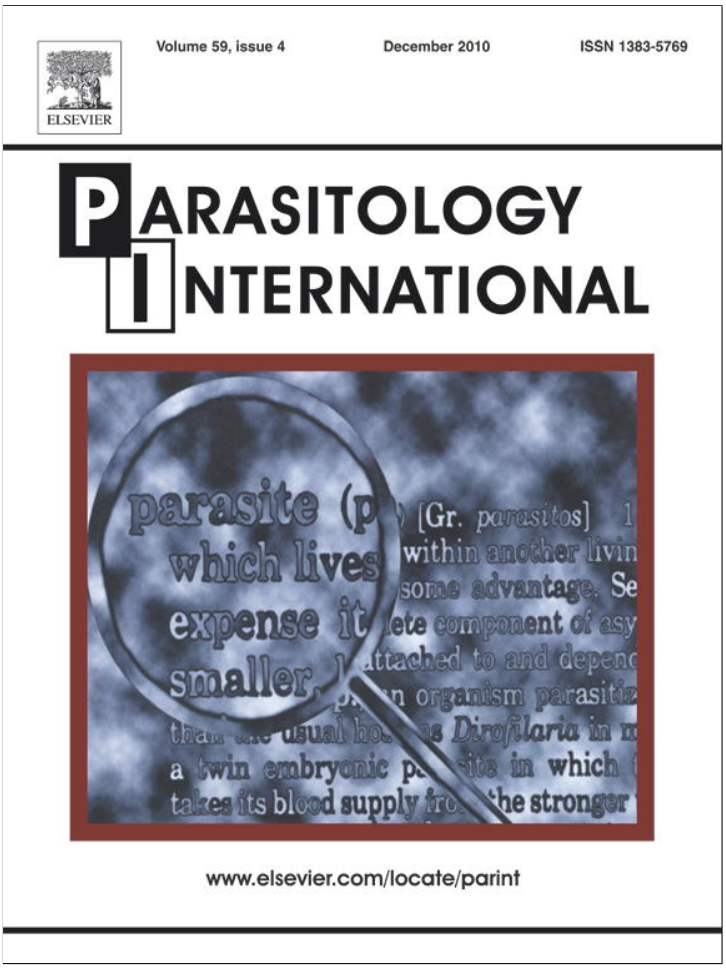

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The novel protein BboRhop68 is expressed by intraerythrocytic stages of *Babesia bovis*☆

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ABSTRACT

The apical complex of intracellular hemoparasites contains organelles like micronemes and rhoptries, specialized structures required for adherence and invasion of host cells. Several molecules discharged from rhoptries have been identified from *Plasmodium* spp., but only a single rhoptry associated protein-1 (RAP-1) has been characterized from *Babesia bovis*. *In silico* search of the *B. bovis* genome allowed to identifying a sequence homologous to the gene that encodes a *P. falciparum* rhoptry protein PfRhop148. The intron-less 1830 bp novel gene, predicted a 68 kDa protein, and it was highly conserved among different *B. bovis* strains and isolates. The deducted protein from the *B. bovis* T2Bo strain, named BboRhop68, showed two putative transmembrane domains, at least seven B-cell epitopes, and a well conserved DUF501 super family domain. The *bborhop68* gene was amplified, analyzed and compared among different *B. bovis* strains and isolates showing overall high sequence conservation. A fragment of *bborhop68* was expressed as a recombinant fusion protein (rBboRhop68). The mice anti-rBboRhop68 serum identified the novel protein in intraerythrocytic trophozoites and merozoites by WB and ELISA, but not in free merozoites. Sera from naturally and experimentally infected bovines also recognized BboRhop68, suggesting that it is expressed and immunogenic during *B. bovis* infection. Fluorescence microscopy analysis using anti-rBboRhop68 antibodies showed a rod structure associated to trophozoites and merozoites infected erythrocytes, but this pattern of reactivity was not observed in free merozoites. The BboRhop68 was also not detected in ELISA based on solubilized merozoites. Thus, at least three independent lines of evidence support differential expression of BboRhop68 in intraerythrocytic stages of *B. bovis* and its possible functional role immediately after *B. bovis* erythrocyte invasion. The results of this work suggest that BboRhop68 could be considered as a novel additional target for developing improved methods to control bovine babesiosis.

1. Introduction

Bovine babesiosis is caused by *Babesia bovis*, an apicomplexan hemoparasite, transmitted by the tick vector *Riphicephalus microplus*, which is endemic in tropical and subtropical areas of the world. The economic losses provoked to cattle production in Latin America have been estimated in 1370 million US dollars [1]. Babesiosis is characterized by anemia, sensorial depression and frequently death. Anemia and microvasculature alteration are largely the consequence of specific modifications provoked by *B. bovis* to the erythrocytes, causing sequestration to the endothelial cells and hemolysis [2,3]. A better understanding of the complete *B. bovis* asexual cycle is essential for developing improved methods to control bovine babesiosis. Since the erythrocytic cycle of hemoparasites has been extensively studied in *Plasmodium falciparum*, this specie is frequently used as a model for *B. bovis* [3,4]. The *B. bovis* zoites modify both the erythrocyte membrane during the invasion and before the parasite break out the cell. These pathogenic mechanisms likely require the involvement of the rhoptries, apical organelles that discharge proteins and lipids on the erythrocyte membrane during internalization and also probably during egression. Nevertheless, little is known about rhoptry protein involvement during zoites invasion of *B. bovis*, compared with *P. falciparum* [4,5]. Rhoptry proteins identified to date in *P. falciparum* are synthesized as larger precursors (pre–pro-proteins) with a signal sequence that is cleaved presumably in the endoplasmic reticulum [6]. Even though rhoptries disappear during the trophozoite stage [7],

☆ Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession numbers FJ215859–FJ215860–FJ215861–FJ215862.

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since presumably they are not required for cell division, some rhoptry pre-proteins have been identified during this intermediate stage. At least two *P. falciparum* proteins, RAMA and RAP-1, are synthesized as preproteins that are proteolytically processed within nascent rhoptries [8,9]. Although it is expected that several pre and mature rhoptry proteins are also required by *Babesia* spp. during the erythrocyte stages, only one rhoptry associated protein (RAP-1), has been characterized for *B. bovis* [10–13].

The protein PfRhop148 recently characterized for *P. falciparum* has been shown to have functional relevance in early rhoptry biogenesis [9,14,15]. *In silico* search at the *B. bovis* genome [16] allowed the localization of a single *PfRhop148* homologous gene. The aim of this work was the identification and analyzes of a *B. bovis* novel gene and the initial characterization and localization of the novel expressed protein.

2. Materials and methods

2.1. Babesia strains

B. bovis pathogenic strains BboS2P [17] and T2Bo [18], the *B. bovis* attenuated strain BboR1A [19], the biological clone Mo7 [20] and *B. bigemina* pathogenic strain BbiS2P [21] used in this work, were multiplied *in vitro*, using a micro aerophilous stationary phase culture method [22]. Trophozoites and merozoites enriched erythrocytes cultures were prepared based on a technique described previously [23].

2.2. Cattle field samples

Blood samples from *B. bovis*-naturally infected cattle from different endemic regions of Argentina were collected into 0.17 M sodium citrate solution. Twelve samples were selected for analyses after the bovine carrier status was confirmed using ELISA [17] and PCR [24].

2.3. BLAST analyses

The sequence of *P. falciparum* rhoptry protein PfRhop148 (accession number AAP14083) was used to *in silico* identify a similar coding region by performing a BLAST search on the *B. bovis* T2Bo strain genome (genome sequencing project http://www.vetmed.wsu.edu/research_vmp/babesia-bovis). The identified sequence was in turn used for a BLASTp search in the genomes of different apicomplexa parasites to perform an alignment of all homologous proteins using the CLUSTAL program.

2.4. B. bovis predicted protein

The deducted protein was analyzed looking for i) hydrophobic and hydrophilic regions using TmPred (http://searchluncher.bcm.tmc.edu); ii) B-cell epitopes using Antigen Design Tools (http://www.genscrip.com); iii) transmembrane domains using TMAP (EMBOSS package®), iv) tandem repeats regions using Tandem Repeat Finder (TRF) program [25].

2.5. PCR

Primers, Rho-F (5´ATGGACAATGCTGCCTCC 3´) and Rho-R (5´ TAATTTGT GATCTGATAG 3´) were designed to amplify and sequence the complete gene in the *B. bovis* strains described above. Additionally two other primers Rho3-F (5´GATTGGATCCATGCTCTCGATCTTCA3´) and Rho1-R (5´CCAATGAATTCTAGTTGGAAATGGGACAAG 3´), including BamH1 and EcoR1 enzyme restriction sites (underlined) were designed *ad hoc* to amplify a gene fragment used for cloning in frame into both a fusion expression and a sequencing vectors.

Genomic DNA (gDNA) was extracted from pelleted infected erythrocytes by standard phenol-chloroform-isoamyl alcohol method. The PCR mix included 0.2 mM dNTPs, 2.2 mM $MgCl_2$, 1.25 U Taq polymerase (Invitrogen), 0.8 mM of each primer and 0.05–0.1 μg/μl of DNA template. The PCR amplifications were carried out at 94 °C for 2 min; followed by 40 cycles at 94 °C for 1 min, 60 °C for 2 min, 72 °C for 1 min, and a final extension at 72 °C for 7 min. Amplification products were analyzed by electrophoresis in 1.5% agarose gels and visualized under UV light after ethidium bromide staining. *B. bigemina* gDNA and PCR reagents without DNA were included as negative controls.

2.6. Polymorphism analyses

To assess the polymorphism of the *B. bovis bborhop* among strains with different phenotypes and isolates from different geographic regions, the PCR products were cloned into the vector *pGEM*-T easy, following the manufacturer's instructions (Promega). The alignments and the distance analyzes of the sequences using Clustal W (Bioedit®) and distance MEGA® 4.0.1 methods respectively [26], were carried out on three clones for each strain.

2.7. cDNA analysis

Messenger RNA (mRNA) was isolated from BboS2P and BboR1A purified merozoites by using oligo(dT) affinity columns, following the manufacturer's instructions (Ambion). The cDNA synthesis and PCR amplification of *bborhop* were carried out using SuperScript III One-Step RT-PCR System with Platinum Taq DNA polymerase (Invitrogen) and the primers *Rho3-F* and *Rho1-R* described above. The cDNA synthesis was obtained at 50 °C for 30 min and PCR amplification was performed as described above, mRNA without reverse transcriptase and gDNA were included as negative and positive controls respectively. All cDNA amplification products were cloned into *pGEM*-T easy vector. Three clones corresponding to each of above mentioned strain were sequenced.

2.8. Protein extracts

Three protein extracts were prepared from suspensions of *in vitro*-multiplied *B. bovis* BboS2P strain (80% parasitemia). i) *B. bovis* infected red blood cells (iRBC) were washed three times in VyM buffer [27] by centrifugation at 2000×*g*, at 5 °C, for 10 min, and the pellet resuspended in 1 ml of cell lysis buffer (0.05 M Tris–HCl, 0.1 M/pH. 8, EDTA/0.1 M NaCl/2% SDS) containing a protein proteases inhibitor (HALT, Pierce). The lysate was incubated 30 min on ice and sonicated by 3 pulses for 5 s each (Virsonic 300, Virtis Co. Inc. USA). Normal red blood cells (nRBC) were similarly processed and included as control. ii) *B. bovis* merozoites (extracellular merozoites), were isolated using a Percoll (Sigma) gradient and resuspended in sodium citrate buffer (10 mM $NaH_2(C_3H_5O(COO)_3$/140 mM NaCl, pH. 7.05) [23]. One volume of cell lysis buffer was added, incubated 30 min on ice and sonicated by 3 pulses for 5 s each, and stored −20 °C until use.

2.9. Production of recombinant protein

To express the novel recombinant form of BboRhop fused to glutathione S-transferase (GST; 26 kDa), a 1156 bp PCR fragment obtained from BboS2P gDNA was digested with BamH1 and EcoR1 and cloned into *pGEX* 4T-3 (GE Healthcare) sites following the manufacturer protocol. After the verification of the proper in-frame position of the sequence, the *Escherichia coli* Rosetta (DE3) strain was transformed with *pGEX* 4T-3-*bborhop* sequence fragment. Bacteria were grown in Circle Grow® medium with 0.1 mg/ml ampicillin at 25 °C, in an orbital shaker at 200 rpm. To induce the expression of fusion protein, 1 mM isopropyl β-D-thiogalactoside (IPTG), was added to the culture when the OD_{600} achieved 0.8 absorbance value, and maintained for 18 h. Bacteria were harvested by centrifugation (4000×*g*, 4 °C, 20 min) and the pellet was suspended in cell lysis buffer (50 mM

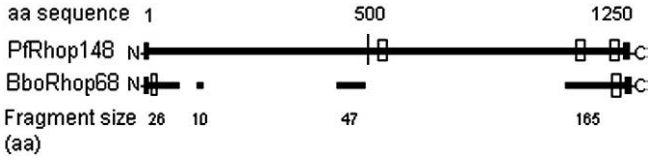

Fig. 1. Schematic comparison between the amino acids (aa) sequence of PfRhop148 (1250 aa) (ACR09853.1) from *Plasmodium falciparum* and BboRhop68 (610 aa) (XP001611661.1) from *Babesia bovis*. The homologous conserved stretches of 26 aa, 10 aa, 47 aa and 165 aa from BboRhop68 are represented by matching segments. Transmembrane domains are indicated by rectangles.

K_2HPO_4/400 mM NaCl/100 mM KCl/10% Glycerol/0.5% Triton X-100, pH 7.8) with addition of protease inhibitors cocktail (HALT, Pierce). The lysate was sonicated using 6 pulses, during 15 s each, and centrifuged (18,000×*g*, 4 °C, 15 min). The supernatant with the recombinant fusion protein was filtered through 0.22 μm membrane, and purified by affinity chromatography, using glutathione agarose beads (B-PER-GST Fusion Protein purification kit, Thermo Scientific). Agarose beads were washed with buffer containing 125 mM Tris/150 mM NaCl, pH.8, and the recombinant fusion protein was eluted using 50 mM reduced glutathione in 50 mM Tris–HCl buffer, pH.8. The presence of the solubilized fusion protein before and after chromatography was verified by SDS-PAGE followed by silver staining method [28].

2.10. Antibodies

To obtain specific antibodies against rBboRhop, five BALB/c mice were subcutaneously inoculated with 15 μg of purified recombinant fusion protein emulsified in incomplete Freund's adjuvant and boosted three times, every 15 days. Pre-immune sera were collected before the initial inoculation and the immune serum samples were collected 15 days after the final boost, and preserved at −20 °C.

2.11. Western blot analysis

The protein extracts (3–5 μg) from *B. bovis* iRBC, purified merozoites, recombinant fusion protein and nRBC were electrophoresed using 12% SDS-PAGE and transferred to 0.45 μm nitrocellulose membrane (BioRad). Membranes were blocked with 20% non-fat milk in TBS-T buffer (100 mM Tris–Cl, pH 7.5/150 mM NaCl/0.1% Tween-20), at 25 °C

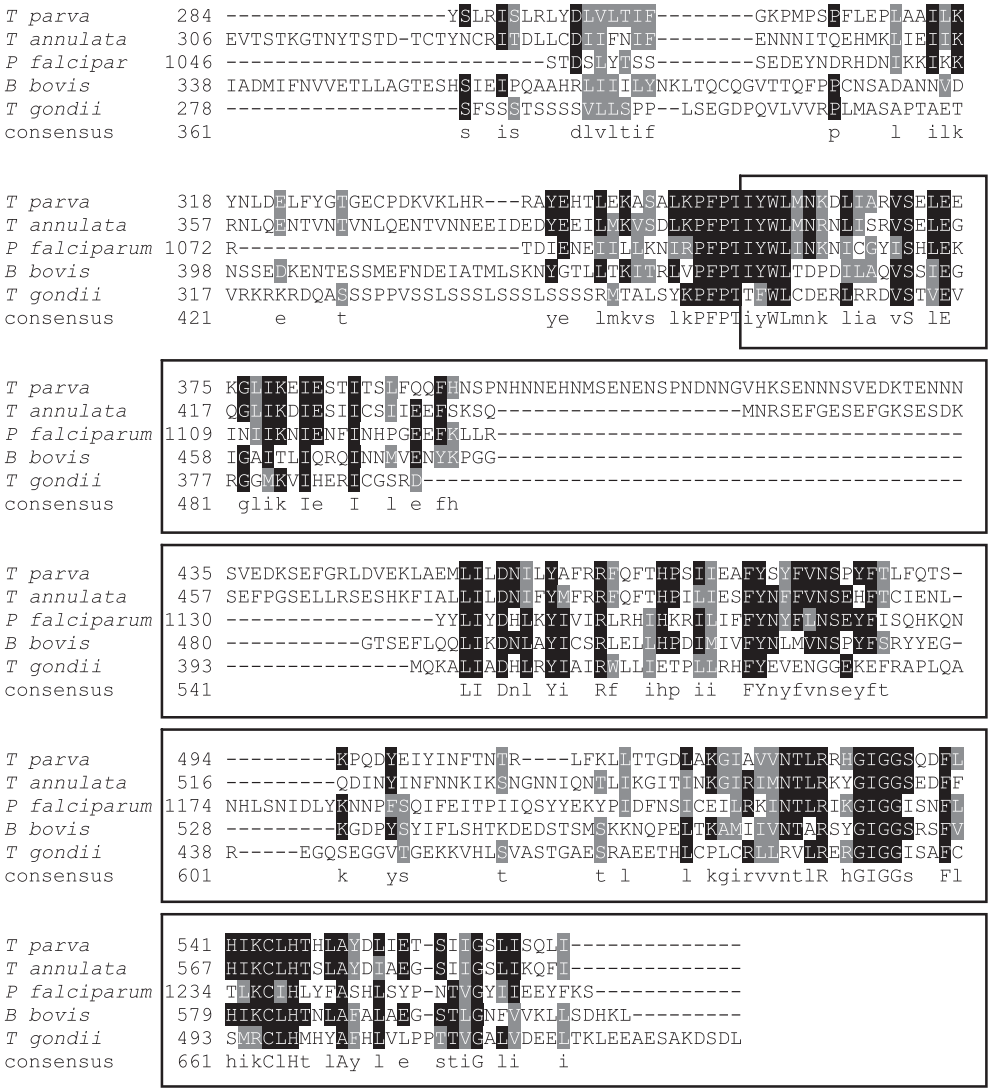

Fig. 2. Sequence comparison of amino acids located at the carboxyl-termini of the *B. bovis* BboRhop68 protein (XP001611661.1) and putative apicomplexan proteins bearing the DUF501 super family motif (box): *Theileria parva* (XP764987); *Theileria annulata* (XP952295.1); *Plasmodium falciparum* Rhop148 (ACR09853.1); and *Toxoplasma gondii* (XP002371291.1). Areas of amino acid identity among all three proteins have a black background; amino acids conserved between two of the proteins have a gray background, and variant amino acids have a white background. Deletions are indicated by dashed lines. A consensus sequence is shown at the bottom of the alignments.

during 1 h and washed. Strips of the membranes with the blotted proteins were incubated with different sera, at 25 °C during 45 min, under agitation. To identify native and recombinant BboRhop68, the following sera were used: i) murine sera against rBboRhop-GST; ii) sera from bovines experimental infected with the BboS2P strain ($n=2$; ID´s 6; 8) or the BboR1A strain ($n=1$; ID 5) iii) sera from cattle naturally infected with *B. bovis* ($n=3$; ID's 171; 181; 19). Pre-inoculation bovine and murine sera and a monoclonal antibody to RAP-1 (23/53.156) [29] were used as controls. All sera were diluted 1:100 in TBS-T containing 10% non-fat milk. Additionally a commercial rabbit anti-GST serum was used as expression control diluted 1:2000. Strips were incubated at 25 °C, during 45 min, washed with TBS-T and incubated with Protein A/G conjugated with alkaline phosphatase diluted 1:3000 (Pierce) or anti-mouse conjugated with alkaline phosphatase diluted 1:3000 (Pierce). After washing with TBS-T, blots were developed with BCIP/NBT color development substrate following the manufacturer protocol (S3771-Promega), until the reactions were observed.

2.12. Immunofluorescence antibody test (IFAT)

The antigens used for the IFAT were i) a suspension of *B. bovis* (BboS2P strain) trophozoites and merozoites iRBC, and ii) purified free merozoites. Suspensions of 8% parasitized erythrocytes were washed twice with 0.01 M PBS pH 7.2. Pellets were suspended in 1:3 of 0.5% normal horse serum/PBS, smeared on glass slides and dried at 37 °C for 30 min. Ten µl of mouse anti-fusion protein serum, mouse pre-immune serum, and a monoclonal antibody to RAP-1 (23/53.156) diluted 1:100 in PBS, were added inside each circle and incubated for 30 min at 37 °C. After three washes with PBS, slides were incubated with rabbit anti-mouse IgG FITC conjugate, diluted 1:300 (Sigma) at 37 °C, for 40 min. After two washes with PBS, and one with distilled water, smears were incubated with 0.01 mM ethidium bromide or 0.5 µM 4',6-diamidino-2-phenylindole (DAPI; Invitrogen) for 10 min, for the nuclei staining, and washed. Fluorescence was examined by regular UV light microscope (Eclipse 80i, Nikon) or with a confocal laser scanning fluorescent microscope (Digital Eclipse C1 Plus, Nikon).

2.13. ELISA procedure

100 µl containing protein extracts from a suspension of *B. bovis* iRBC, purified extracellular merozoites, nRBC and rBboRhop-GST fusion protein in coating buffer (0.05 M carbonate/bicarbonate, pH 9.6), was distributed and adsorbed to each 96 well flat-bottom plates (COSTAR-3590). The plates were sealed and incubated 1 h at 37 °C. The buffer was eliminated and replaced with 100 µl per well of blocking buffer (coating buffer with 10% of non-fat milk) and incubated for 30 min at 28 °C. The wells were then washed four times with PBS-T (0.01 M PBS/0.02% Tween 20). Mouse anti-fusion protein serum, mouse pre-immune serum, bovine anti- *B. bovis* serum and bovine pre-immune serum were diluted 1:20 in PBS/0.075 M EDTA-EGTA (pH 6.3) with 10% non-fat milk and incubated on a shaker for 30 min at 28 °C. After four washings, 100 µl of anti-mouse conjugated with peroxidase diluted 1:4000 (Sigma) or Protein A/G conjugated with peroxidase diluted 1:6000 (Pierce) were added and incubated on a shaker for 40 min at 28 °C. After four washings, 100 µl of 3% H_2O_2/0.04 M ABTS (2.2-azino-bis 3-ethylbenzthiazoline-6-sulphonic acid) were added as substrate/chromogen. A kinetic reading was determined at an optical density of 405 nm (OD_{405}) [30].

3. Results

3.1. BLAST search

A novel *B. bovis* gene of 1830 bp homologous to *PfRhop148* was identified after *in silico* BLAST-search of the *B. bovis* T2Bo genome, and it was denominated *bborhop68*. The sequence of the newly identified gene (accession number XM001611611), encodes for a putative protein of 610 amino acids (aa) with a molecular weight of 68 kDa, and a predicted isoelectric point (p*I*) of 6.39, denominated BboRhop68. Sequence alignment of BboRhop68 and PfRhop148 proteins showed significant conservation (blast score: $4e^{-08}$) with 31% of sequence identity and 48% of similarity in a ~165 aa long stretch located at the carboxyl-termini of both proteins (Fig. 1).

In addition, BLASTp search of the non-redundant protein database using the BboRhop68 protein sequence as a query, showed high similarity scores to PfRhop148 of 1250 aa (ACR09853.1) (pI: 8.72) and to proteins of unknown function from the apicomplexan parasites *Theileria parva* (XP764987) of 567 aa (pI: 6.30), *T. annulata* (XP952295.1) of 592 aa (predicted pI: 6.35), and *Toxoplasma gondii* (XP002371291.1) of 534 aa (predicted pI: 6.09). BLASTp analyses revealed the presence of a domain of unknown function 501 (DUF501) present in the carboxyl end of these parasites proteins (~aa 440 to 610). The alignment of the carboxyl-termini and the DUF501 super family motif of these proteins are shown in Fig. 2. The DUF501 motif [pfam04417] has been defined as a family of uncharacterized bacterial proteins. The closest sequence-related members to this gene family were *Petrotoga mobilis* SJ95 (YP_001568865), *Corynebacterium efficiens* (Q8FQS4), *Neptuniibacter caesariensis* (Q2BPY1), *Marine actinobacterium* PHSC20C1 (ZP_01130728) and *Thermotoga petrophila* RKU-1 (YP_001245247).

3.2. Bborhop68 sequence analysis

In silico secondary structure analysis of the predicted protein sequence of BboRhop68 derived from the *B. bovis* T2Bo strain showed a hydrophilic structure with two different putative transmembrane regions (aa 20–50 and 496–519) thus the conserved DUF501 domain is located in the putative C-terminal transmembrane region. Neither asparagine rich regions nor tandem repeat sequences such as those present in PfRhop148, or a canonical signal peptide were identified in BboRhop68.

A 385 aa fragment (aa 54–440) of BboRhop68 free of hydrophobic regions, including at least seven putative B-cell epitopes, was selected for polymorphism analysis and for the production of a recombinant fusion protein.

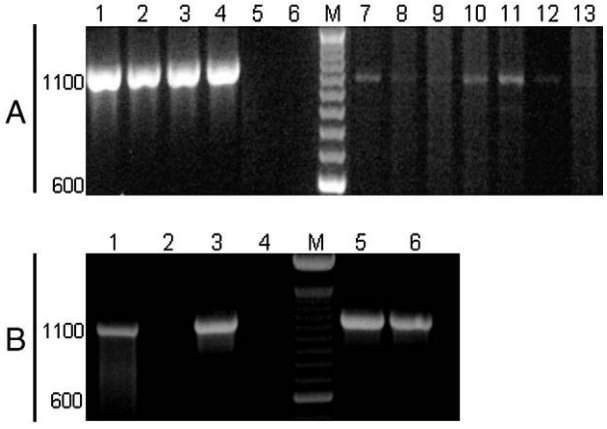

Fig. 3. PCR and RT-PCR analysis of the *bborhop68* gene. Panel A) Ethidium bromide-stained 1.5% agarose gel of PCR amplification products of the *bborhop68* fragment gene (1156 bp) from *Babesia bovis* geographically distant strains using the primers set *Rho3-F* and *Rho1-R*, BboS2P(Lane 1); BboR1A (lane 2); Mo7 (lane 3);T2Bo (lane 4), and from seven field isolates (lanes 7–13). No amplification product was obtained with *Babesia bigemina* sample (lane 5) and with no DNA sample (lane 6). Panel B) Ethidium bromide-stained 1.5% agarose gel of RT-PCR analysis of mRNA extracted from *B. bovis* BboR1A and BboS2P strains (lanes 1 and 3), negative controls without reverse transcriptase (lanes 2 and 4) and genomic DNA amplification control (lanes 5 and 6). M: molecular size marker (100 bp DNA Ladder, Invitrogen). The size of the markers (pb) is indicated on the left.

3.3. BboRhop68 characterization

A fragment of 1156 bp (159–1315 bp) of the *bborhop68* gene was amplified from the gDNA of T2Bo, Mo7, BboS2P and BboR1A *B. bovis* strains and from 7 out of 12 *B. bovis* field isolates, producing a band of expected size. No amplification product was obtained from *B. bigemina* sample (Fig. 3A). The alignment of the sequences of all strains compared showed that BboR1A lacked three contiguous nucleotides coding for the amino acid valine, located at the position 215 and showed a similarity of 97.4% and 99.9% with the strains BboS2P and T2Bo respectively. The lowest degree of similarity (96.9%) was observed between BboS2P and Mo7 *bborhop68* genes. The *bborhop68* sequence obtained from two *B. bovis* field samples (171 and 181) showed that 171 had a similarity of 96.6%, 99.3% and 97.8% with BboS2P, BboR1A and 181, respectively; and the later a similarity of 97.7%, 98.5% with BboS2P y BboR1A respectively. The alignment of predicted amino acid complete sequences (610 aa) from T2Bo, Mo7, BboR1A and BboS2P *B. bovis* strains, showed a total of 41 aa substitutions, 17 of them were conservative and 24 were variant.

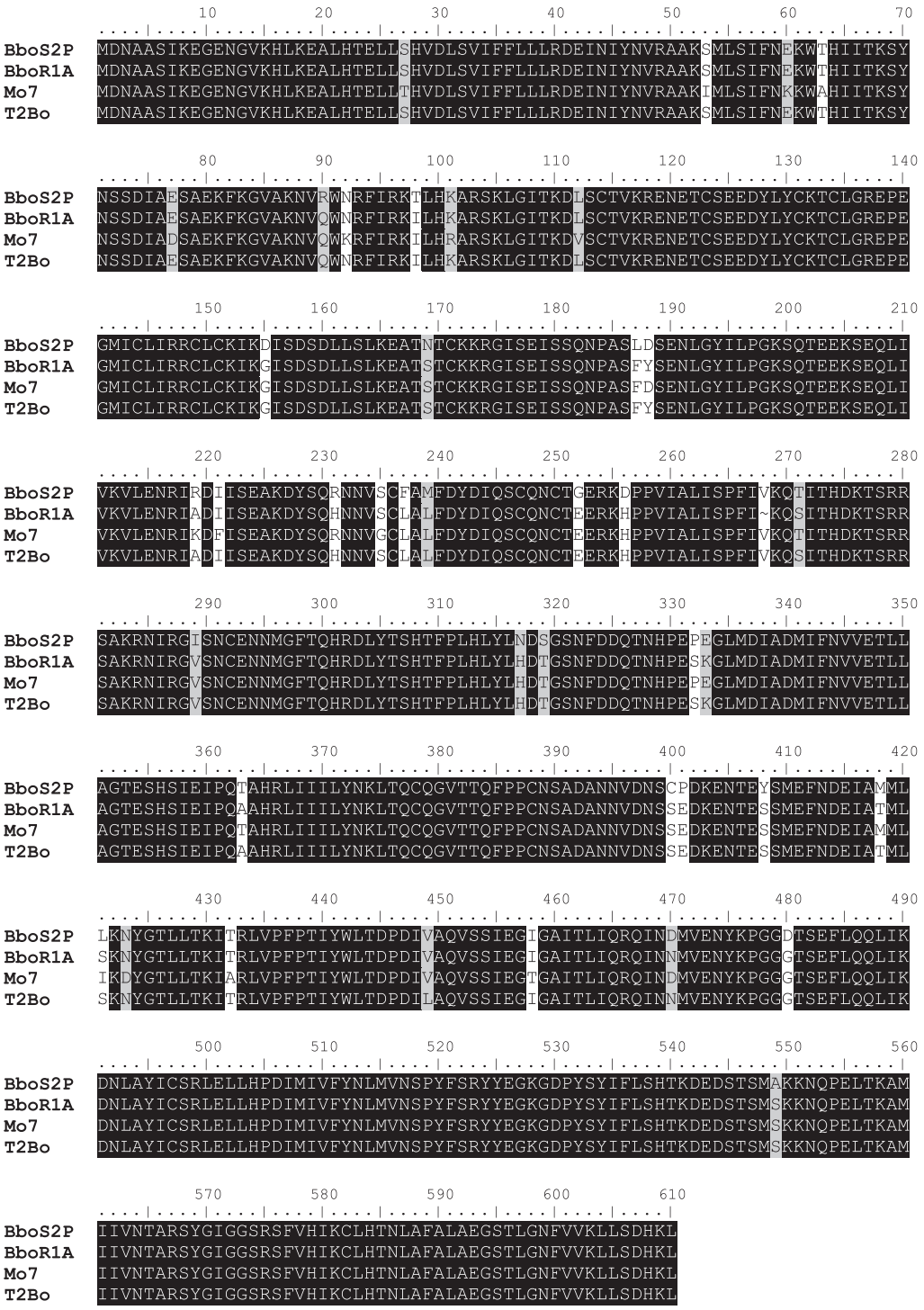

Fig. 4. Comparison of amino acid sequences of a fragment from BboRhop68 (total size 610 predicted amino acids) from T2Bo, BboS2P, BboR1A and Mo7 strains. Amino acid numbers are shown above the sequences. Areas of amino acid identity are enclosed in black background, conservative amino acid substitutions have a gray background, and variant amino acids have a white background.

The conservation of the DUF501 super family motif (~aa 440 to 610) was also identified in the predicted amino acid complete sequences from the same strains (Fig. 4).

3.4. Transcriptional analysis

Gene transcription analysis of *bborhop68* fragment performed on RNA extracted from blood stage *B. bovis* parasites using RT-PCR amplifications, resulted in the production of the expected 1156 bp product in the BboR1A and BboS2P strains (Fig. 3B; lanes 1 and 3). Identical size products were obtained by PCR amplification with the same set of primers utilized by RT-PCR using gDNA from the same strains (Fig. 3B; lanes 5 and 6), whereas no amplification resulted in the RT-PCR when the reverse transcriptase enzyme was omitted (Fig. 3B; lanes 2 and 4). In both strains, the sequence of the cDNA was 100% identical to the gene sequence obtained from gDNA (data not shown). Thus transcription of the *bborhop68* gene was found occurring in *B. bovis* strains regardless of their attenuated (BboR1A) or virulent (BboS2P) phenotypes.

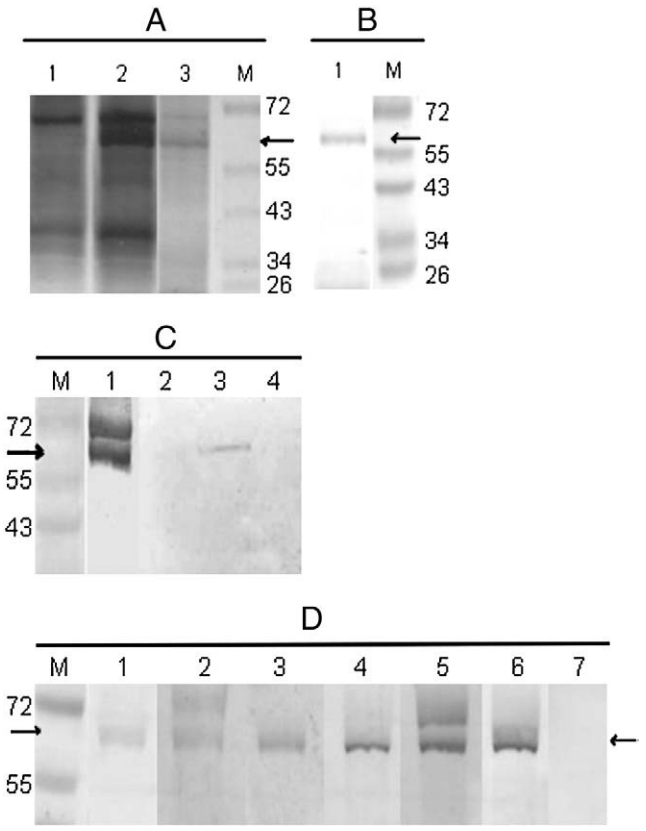

Fig. 5. SDS-PAGE and Western blot analysis of recombinant and native BboRhop68 protein. A fragment of *bborhop68* gene (1156 bp) from pathogenic BboS2P strain was cloned into vector *pGEX* 4 T-3 and expressed in *E. coli* Rosetta (DE3). A) Silver staining SDS-polyacrylamide gel. Protein electrophoresis of total cell lysates obtained before (lane 1) and after (lane 2) induction with IPTG; purified rBboRhop68-GST protein (lane 3). B) Western blot using anti-GST serum: rBboRhop68-GST protein (lane 1). C) Western blot using mouse polyclonal anti rBboRhop68-GST serum. Purified rBboRhop68-GST protein (lane 1); protein extract from BboS2P purified merozoites (lane 2); protein extract from BboS2P infected erythrocytes (lane 3); protein extract from normal erythrocytes (lane 4). Recombinant and native BboRhop68 are indicated with arrows. D) Western blot using purified rBboRhop68-GST protein (lanes 1 to 7). Immune sera from *B. bovis* infected cattle (ID's 171, 181 and 19) from endemic regions of Argentina (lanes 1, 2 and 3 respectively). Immune sera from two bovines (ID´s 6; 8) experimentally infected with BboS2P strain (lanes 4 and 5), and from a bovine (ID 5) experimentally infected with BboR1A strain (lane 6). No reaction was observed with a pre-immune serum from ID 6 (lane 7). M: molecular weight (kDa) markers (Page Ruler Prestained, Fermentas™). The weight of rBboRhop68 protein is indicated with arrows.

3.5. BboRhop68 expression

Recombinant BboRhop68-GST of 68 kDa, (42 kDa for the protein fragment and 26 kDa for GST) was identified in silver stained polyacrylamide gels, after IPTG induction and after purification by chromatography (Fig. 5A; lanes 2 and 3). An unspecific band of ~70 kDa was also present in the lanes where the products obtained before and after the IPTG induction were electrophoresed (Fig. 5A, lanes 1 and 2). The fragment of rBboRhop68-GST protein was specifically recognized by the anti-GST serum, having the expected size in the immunoblot (Fig. 5B; lane 1). Murine anti-rBboRhop68 antibodies specifically recognized the recombinant fusion protein (of 68 kDa) and a native *B. bovis* protein of ~68 kDa present in erythrocytic stages of *B. bovis* (Fig. 5C; lane 1 and 3). This serum also recognized the unspecific protein of ~70 kDa (Fig. 5C; lane 1), but was unable to recognize it in the *B. bovis* protein extract (Fig. 5C; lane 3). No reaction was observed with *B. bovis* purified merozoites and nRBC extracts in the immunoblots (Fig. 5C; lanes 2 and 4). Mouse pre-immune serum did not recognized any protein. The monoclonal antibody 23/53.156 recognized specifically the *B. bovis* RAP-1 (~55 kDa) in the protein extracts from *B. bovis* iRBC and purified merozoites, producing bands of similar intensity in both types of extracts (data not shown). Sera from cattle naturally infected with *B. bovis* and experimentally infected with the BboS2P and BboR1A strains also identified rBoRhop68-GST in the immunoblots (Fig. 5D; lanes 1 to 6) and no reaction was observed with a bovine preimmune serum (Fig. 5D; lane 7). Murine anti-BboRhop68 serum recognized the native protein in iRBC, with strong reactivity in the trophozoite and merozoites parasitized erythrocytes using IFAT (Fig. 6A) and confocal microscopy (Fig. 6B), whereas weak or no reactivity was observed in the free merozoites (not shown). The fluorescent reaction was observed as a single rod associated to trophozoites or as double rod, one associated to each early formed merozoites. The rods were observed in variable positions, relative to the ethidium bromide stained nucleus. No reactivity was observed with mouse pre-immune serum (Fig. 6C). The mAb 23/53.156 used as a control, reacted with the RAP-1 in an expected punctuate pattern (Fig. 6D).

Antigen recognition was detected by ELISA when the mouse anti rBboRhop68 serum was confronted with *B. bovis* iRBC lysate or the rBboRhop68, but no reaction was detected with identical lysates prepared from purified merozoites. Serum from cattle inoculated with *B. bovis* iRBC showed seroconversion with the rBboRhop68 (Fig. 7).

4. Discussion

The host cells invasion is a key step for apicomplexa hemoparasites like *B. bovis* and the neutralization of molecules associated to the apical organelles might be useful to hamper the cycle of the parasite to protect the host. Here it was possible to identify the novel protein BboRhop68 expressed by *B. bovis* during the erythrocytic stages. The *bborhop68* gene display a high degree of conservation (≥96.91% identity) among different *B. bovis* strains T2Bo, BboS2P, BboR1A, Mo7 and two field isolates. This sequence also showed a 38% homology to PfRhop148, a protein from *P. falciparum* associated with the rhoptries [14]. The BLASTP allowed revealing the presence of the region containing DUF501 super family motif as a common feature of both proteins and the conservation among distinct *B. bovis* strains. This region is also well conserved among homologous proteins in *T. parva*, *T annulata*, and *T. gondii*. Although the function of this motif is still unknown the conservation of the DUF501 motif in apicomplexa parasites and the presence in the genome of several prokaryotic organisms, allow to hypothesize that it has a functional role relevant for the microorganisms life cycle. In spite of striking differences between BboRhop68 protein and its homologous PfRhop148, represented by long stretches of asparagine, abundance of short tandem repeats, and a larger size, they share a well conserved region suggesting that these homologous proteins could be involved in a similar function. These features could

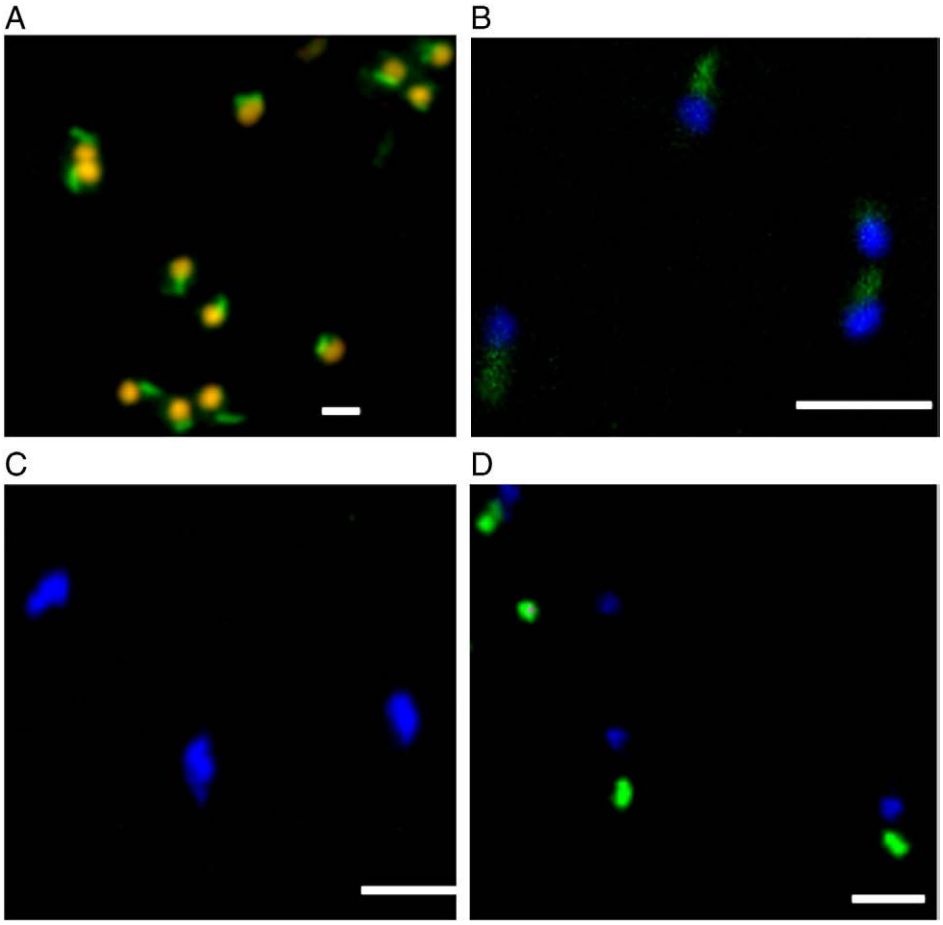

Fig. 6. Localization of BboRhop68 using smears of BboS2P infected erythrocytes by fluorescent microscopy. Immunofluorescent antibody test using mouse polyclonal anti rBboRhop68-GST serum (green) and parasite nuclei stained red with ethidium bromide (panel A). Confocal laser scanning microscopy. Mouse polyclonal anti rBboRhop68-GST serum (panel B); mouse pre-immune serum (panel C); monoclonal immune serum anti-RAP-1 (panel D). The green fluorescence corresponds to the antigen–antibody reaction and the blue fluorescence to the parasite nuclei stained with DAPI. Bars represent 4 μm.

be extended to *T. parva*, since it is phylogenetically closer to *B. bovis*, with a remarkably structural similarity to its genome and specifically to *bborhop68* gene [16].

The strong fluorescence of the novel protein, persistently associated to both *B. bovis* intraerythrocytic stages, suggests that it has a relevant function during the asexual multiplication.

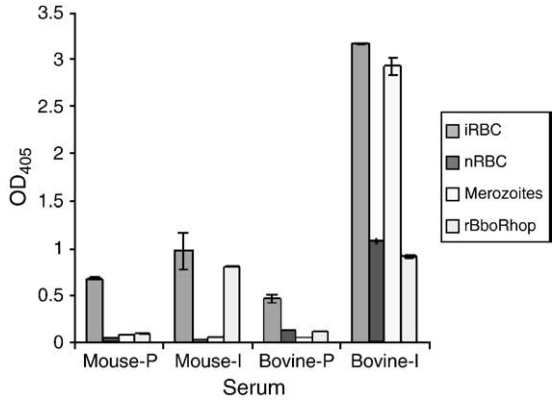

Fig. 7. Reactivity of mice anti rBboRhop68 serum and *Babesia bovis* infected cattle sera using ELISA. Antigens: infected erythrocyte protein extract (iRBC), non-infected erythrocyte protein extract (nRBC), free merozoite protein extract (Merozoites) and rBboRhop68-GST. The (x) axis represents the average optical density (OD_{405}) obtained with mouse pre-immune serum (Mouse-P), polyclonal mouse immune serum anti rBboRhop68-GST (Mouse-I), bovine pre-immune serum (Bovine-P) and bovine immune serum against *B. bovis* (Bovine-I). Bars indicate standard deviations of duplicate samples.

The rhoptry family proteins can be identified in spite of the absence of this organelle in trophozoites as it was observed for RAP-1, which was synthesized as a dense accumulation in the cytoplasm of trophozoite stage [31]. The *Plasmodium* proteins PfRhop148 and RAMA, associated with rhoptries biogenesis, are synthesized during the trophozoites stage, 16 h before the rhoptries synthesis [14,15,32]. Furthermore, rhoptry biogenesis occurs by sequential fusion of Golgi-derived vesicles which deliver protein cargo into the rhoptry lumen [32,33].

The lack of fluorescent rods associated to free parasites, was in agreement with the absence of reactivity of free merozoite lysates obtained in the Western blot and ELISA. Thus, the three distinct serologic tests used in this work support differential expression of BboRhop68 between the *B. bovis* intraerythrocytic stages and the free merozoites.

Importantly, the immunogenicity of the novel protein was established not only by the reactivity of sera from mice inoculated with the rBoRhop68 fragment, but also by the sera from *B. bovis* naturally and experimentally infected cattle, demonstrating that the novel protein is expressed and it is immunogenic during *B. bovis* cattle infection. Although those sera from mice and the sera from 2 of 6 bovines, recognized a protein of ~70 kDa co-purified with the fusion protein, it was established that this protein was derived from *E. coli*, since it was detected before the IPTG induction, it was not expressed as a fusion protein and it was not identified in the lysate of *B. bovis* parasitized erythrocytes by anti-rBboRhop68 mice sera.

The early expression of BboRhop68 might be required for the rhoptry biogenesis, as it was proposed for the homologous PfRhop148

[14,15], and it could be an important target to be modified by novel drugs useful for the babesiosis control.

5. Conclusion

A novel protein BboRhop68 was identified in *B. bovis* strains with significant homology with the rhoptry protein of PfRhop148 from *P. falciparum*. The early differential expression of the protein in trophozoites and merozoites observed by three different experimental approaches in this study, suggest that BboRhop68 could be associated to the biogenesis of the rhoptries. However, further studies should be done in order to confirm the precise localization and functional relevance of BboRhop68.

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