

# Identification and characterization of a *Babesia bigemina* thrombospondin-related superfamily member, TRAP-1: a novel antigen containing neutralizing epitopes involved in merozoite invasion

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## Research

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# Abstract

## Background

The thrombospondin-related anonymous protein (TRAP) has been described as a potential vaccine candidate in several apicomplexan parasites. However, this protein and members of this family have not been characterized yet in *Babesia bigemina*, one of the most prevalent species causing bovine babesiosis.

## Methods

The *Babesia bigemina* TRAP-1 (*BbiTRAP-1*) gene of 3186 bp was identified by a bioinformatics search using the *B. bovis* TRAP-1 sequence. Members of the TRAP and TRAP-related protein families (TRP) were identified in *Babesia* and *Theileria* through the search of the TSP-1 adhesive domain, the hallmark motif in both proteins. Structural modelling and phylogenetic analysis were performed with the identified TRAP proteins. A truncated recombinant BbiTRAP-1 that migrates at ~107 kDa and specific antisera were produced and used in Western blot analysis and indirect fluorescent antibody test (IFAT). B-cell epitopes with neutralizing activity in BbiTRAP-1 were defined by ELISA and invasion assays.

## Results

TRAP family has 3 members in *B. bigemina* (BbiTRAP-1-3). All are type 1 transmembrane proteins containing the von Willebrand factor A (vWFA), thrombospondin type 1 (TSP-1) and cytoplasmic C-terminus domains along with transmembrane regions. The BbiTRAP-1 predicted structure also contains a metal ion-dependent adhesion site (MIDAS) for interaction with the host cell. The TRP family in *Babesia* and *Theileria* species contains the canonical TSP-1 domain but lacks the vWFA domain and together with TRAP define a novel gene superfamily. A variable number of tandem repeat units is present in BbiTRAP-1 and could be used for strain genotyping. Western blot and IFAT analysis confirmed expression of BbiTRAP-1 by blood stage parasites. Partial recognition by a panel of sera from *B. bigemina* infected cattle in ELISA using truncated BbiTRAP-1 suggests that this protein is not an immunodominant antigen. Additionally, bovine anti-recombinant BbiTRAP-1 antibodies were capable of neutralizing merozoite invasion *in vitro*.

## Conclusions

We identified the TRAP and TRP gene families in several *Babesia* and *Theileria* species, and characterized BbiTRAP-1 as a novel antigen of *B. bigemina*. The functional relevance and presence of neutralization-sensitive B-cell epitopes suggest that BbiTRAP-1 could be included in future vaccine candidates against *B. bigemina*.

## Background

Parasites of the genus *Babesia* are tick-borne pathogens of human and veterinary importance. In cattle, bovine babesiosis caused by *B. bovis* and *B. bigemina* pose a major constraint to livestock production in tropical and subtropical regions with considerable economic losses (1). A recent meta-analysis of samples from 62 countries representing six continents revealed a pooled global prevalence of bovine babesiosis as 29% (2).

*Babesia bovis* is the most virulent species which can occasionally cause neurological manifestations. However, animals infected with *B. bigemina* may also develop severe symptoms of babesiosis such as high fever, lethargy, anemia, and hemoglobinuria (1). Of both parasites, *B. bigemina* has also the highest prevalence (2).

Animals that survive primary infections become persistently infected for life and are reservoirs for tick transmission (1). The use of acaricides and live attenuated vaccines are currently the only preventive measures used to control outbreaks (3).

In the vertebrate host, *Babesia* parasites are obligate intracellular pathogens that exclusively reside inside the erythrocytes (1). Therefore, studies that aim to decipher the process of erythrocyte invasion by *Babesia* infective stages are crucial to develop novel strategies to control the establishment of infection.

*Babesia* parasites belong to the phylum *Apicomplexa*, which is characterized by the presence of a complex of specialized secretory organelles that include rhoptries, micronemes and spherical bodies. These organelles contain and secrete key proteins implicated in the invasion, establishment, and egress from host cells (4). In general, the mechanism by which *Babesia* merozoites invade red blood cells is similar to other apicomplexan parasites such as *Plasmodium* and *Toxoplasma*. The process involves attachment to the host cell, reorientation, membrane invagination and final internalization of the parasite (4). The initial steps of invasion involve an active specific locomotion movement known as “gliding” which involves the parasite’s actin–myosin motor (5). This motor is connected to host-cell receptors through transmembrane proteins that belong to a family called thrombospondin-related anonymous protein (TRAP) (5). TRAP-1 is a type-1 transmembrane protein that carries two adhesive domains in its extracellular region, a motif similar to the type 1 repeat of thrombospondin (TSP-1) and an A-domain of the von Willebrand factor (vWFA) (6). Even though the overall mechanism of parasite’s actin–myosin motor is widely conserved among *Apicomplexa*, differential expression of distinct and species-specific TRAP proteins was identified among this phylum (7). However, the exact role played by TRAP-1 and their ligands during the process of invasion in apicomplexan parasites remains undefined.

In 2004, a new *B. bovis* merozoite protein with a domain structure resembling the arrangement of TRAP from *Plasmodium* sporozoites was identified and characterized (6). This protein was directly involved in both processes of recognition and invasion of bovine erythrocytes. Recently, other TRAP proteins have also been reported in *Babesia orientalis* and *Babesia gibsoni* (8–10).

The availability of several *Babesia* genomes have facilitated the identification of genes and gene families that are conserved across the phylum and whose presence and function have not been identified in less

studied members.

In this study, we have identified members of the *TRAP* gene family in *B. bigemina* and performed a detailed analysis at the genomic and sequence levels. Besides, we have searched for distantly TRAP related proteins and found a new family of thrombospondin-related proteins (TRP) which share some of the structural domains of TRAP proteins.

Finally, we have analyzed the expression and functional relevance of TRAP-1 in *B. bigemina* merozoites. The role of this protein as a neutralization-sensitive antigen with vaccine potential was also investigated.

## Materials And Methods

### 1. Identification and characterization of BbiTRAP-1

For the identification of the *BbiTRAP-1* coding sequence, a TBLASTN search (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) was performed in the genome of *B. bigemina* (BOND strain). The search was conducted using the predicted amino acid sequence of the annotated *B. bovis TRAP-1* gene as a query (GenBank accession number: EDO06220.1). For *in silico* topology prediction, concatenated amino acid sequences of the vWFA and TSP-1 domains of BbiTRAP proteins were submitted to the Swiss-model server (<http://swissmodel.expasy.org>) and 3D models were constructed. The crystal structure of a fragment containing the vWFA and TSP-1 domains from *Plasmodium vivax* sporozoite surface protein 2 (PDB code: 4hql.2.A, residues 25-283) (11) was used for homology modelling since it was the structure with the highest GMQE score. This score is a quality estimator with values between 0 and 1 and combines properties from the target–template alignment and the template search method.

*BbiTRAP-1* coding sequence of the BOND strain was used to identify the corresponding genes from the other *B. bigemina* genomes (JG29, S3P and Puerto Rico strains) available from the Wellcome Trust Sanger Institute FTP site (<ftp://ftp.sanger.ac.uk/pub/pathogens/Babesia/>) (12). These *BbiTRAP-1* sequences were then translated *in silico* to confirm the presence of the respective open reading frames. All predicted amino acid sequences were further aligned using the Clustal  $\Omega$  algorithm ([www.ebi.ac.uk/Tools/msa/clustalo/](http://www.ebi.ac.uk/Tools/msa/clustalo/)). Percentages of identity and similarity between sequence pairs were calculated using the Sequence Manipulation Suite “Ident and Sim” resource ([https://www.bioinformatics.org/sms2/ident\\_sim.html](https://www.bioinformatics.org/sms2/ident_sim.html)).

### 2. Identification and characterization of members of the TRAP and TRP family in other species

For the identification the TRAP family in species of the *Babesia* and *Theileria* genera, a database search aiming to identify all proteins containing the TSP-1 domain (IPR000884) or vWFA domain (IPR002035) was conducted in PiroplasmaDB (<https://piroplasmadb.org/>) (13). The presence of a signal peptide,

transmembrane domains and other functional domains was recorded. Furthermore, the occurrence of an acidic cytoplasmatic tail domain (CTD) was analyzed by predicting the isoelectric point using <http://isoelectric.org/>. The conservation of a tryptophan residue near the C-terminal end of the protein was also analyzed. To determine that a TSP-1-containing protein belongs to the TRAP family, the presence of the CTD with the conserved tryptophan residue was considered essential (14).

For all the TSP-1-containing proteins, the group of orthology to which they belong was determined using OrthoMCL database (<https://orthomcl.org/orthomcl/>, version 6.1). Synteny of the chromosomal region containing each gene was evaluated in PiroplasmaDB by visualizing the alignment of the genomic region encompassing each gene in different *Babesia* species. The conservation and distribution pattern of genes surrounding the target locus was also analyzed.

### 3. Phylogenetic analysis

Sequence alignment of complete *TRAP* gene family from species of the *Babesia* and *Theileria* genera was performed using the MEGA version X (15). Phylogenetic analysis was performed with Maximum Likelihood and Neighbor Joining methods and tree topologies were compared to have a robust phylogeny. In both cases, TRAP of *Plasmodium falciparum* (Access number: PF13\_0201) was selected as an outgroup and bootstrap values were calculated with 1000 pseudo replicates.

### 4. *B. bigemina* strains and genomic DNA isolation

The following Argentinean strains were used: S1A, S2A and M1A (all attenuated vaccine strains) and the pathogenic B38, S3P and S2P strains. Details of the geographic origin of these strains and the respective multilocus genotypes were already described (16). For *in silico* analysis, the *B. bigemina* genomes of BOND, JG29 and Puerto Rico strains were used (12). We have also included sequences obtained from samples of genomic DNA from Brazil, Mexico and Nayarit (the latter 2 kindly provided by Dr. Juan Mosqueda, Univ. Aut. de Querétaro, Mexico).

Extraction of genomic DNA from blood of experimentally-inoculated bovines or erythrocyte cultures were performed in PBS-washed packed red blood cells by standard phenol-chloroform extraction and ethanol precipitation (17). DNA was quantified using a Nano Drop 1000 spectrophotometer (Thermo Fisher Scientific, USA) and stored in aliquots at -20 °C.

### 5. Identification and analysis of tandem repeats

In order to identify repetitive sequences present in *BbiTRAP-1-3* the Tandem Repeat Finder (TRF) program was used (18) with the amino acid sequence of the S3P strain. A subset of sequences was manually selected from the TRF output of *BbiTRAP-1* according to the period size of the repeat (>176 bp) and copy number (>3). These repeats were further analyzed and documented.

Primers corresponding to the conserved 5' and 3' regions flanking approximately 20-30 bp from the *BbiTRAP-1* repeat region were manually designed using the S3P sequence. Primers (5'-3') were: RepTRAPfw GACTCATCACAGAAAGCGCG and RepTRAPrv TCTTCCCTGCCTAGGTCTGA. These primers flank a region of repeats from positions 1392 to 2326 of the *BbiTRAP-1* nucleotide sequence. PCR amplifications were carried out using the genomic DNA from the *B. bigemina* strains mentioned before. DNA from the *B. bovis* R1A strain was used as a template negative control. Amplifications were performed using the Kit T-Plus DNA polymerase (INBIO HIGHWAY, Argentina), containing 0.4 µmol of each primer, 0.2 mM of each deoxyribonucleotide triphosphate (Promega, Madison, Wi. USA), 0.5 µg/µL BSA and 100 ng of genomic DNA. PCR was performed in a 50 µl reaction mixture in a Bio-Rad MyCycler Thermal Cycler (BioRad, USA). The cycling conditions were: denaturation at 94 °C for 3 min, a touch down step of 9 cycles at 95 °C for 1 min, 65 °C for 1 min and 72 °C for 1 min and 10 s with annealing temperatures decreasing by 1 °C every cycle. This step was followed by 27 cycles at 95 °C for 1 min, 59 °C for 1 min and at 72 °C for 1 min and 10 s. The final extension was at 72 °C for 10 min.

Fifty microliters of each amplified product were electrophoresed in a 1% agarose gel stained with ethidium bromide. DNA bands were visualized on a UV image analyzer (BioRad, USA) and documented. The bands of approximately 1028 bp and 800 bp were excised, purified and cloned into the pGEM-T easy vector (Promega, Madison, Wi. USA) according to standard protocols. The corresponding insert from purified plasmids was sequenced in both strands using vector primers. Sequences were deposited in GenBank under accession numbers MN450376-MN450383.

## 6. Expression and purification of the recombinant BbiTRAP-1

For ease of expression, predicted transmembrane regions of high hydrophobicity of BbiTRAP-1 were identified using the TMHMM Server v.2.0 ([www.cbs.dtu.dk/services/TMHMM](http://www.cbs.dtu.dk/services/TMHMM)) and removed from the final sequence. Therefore, a truncated DNA fragment of 1875bp coding for 625 amino acids (corresponding to amino acids 320 to 944) was commercially synthesized (GenScript, USA) and cloned between the EcoRI and BamHI sites in the pET-28a vector (Sigma-Aldrich, Steinheim, Germany) along with an *in-frame* 6X Histidine tag for further protein purification. The plasmid map can be accessed in <https://www.addgene.org/vector-database/2565/>.

The *BbiTRAP-1*-pET28a construct was used to transform chemically competent *E. coli* Rosetta (DE3) BL21 strain (Invitrogen, Carlsbad, CA, USA). Maximal expression of the protein was obtained after induction with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 20 °C overnight. Protein purification was performed according to the manufacturer's instructions (ProBond™ Purification System, Life Technologies, Carlsbad, CA, USA) for purification of poly Histidine-containing recombinant proteins. The purified BbiTRAP-1 protein was analyzed by SDS-PAGE and the protein identity was confirmed by **Western blot** using an anti-His tag commercial **antibody** (Abcam, Cambridge, USA). The protein concentration was measured using the Pierce BCA Protein Assay Kit (Thermo Scientific, USA).

## 7. Production of anti-BbiTRAP-1 polyclonal sera

Purified BbiTRAP-1 was used to immunize mice and bovines. In all cases, a pre-immunization bleeding was performed. Five male BALB/c mice of seven weeks old were used and housed in cages with food and water provision *ad libitum*. Two female Aberdeen Angus bovines of three years old were housed in plot field facilities at *Instituto Nacional de Tecnología Agropecuaria*, INTA – Castelar (34°35'48.8"S 58°41'01.5"W) and were kept for a week before starting the experiment for adaptation and handling. Trained and experienced animal technical assistants and care workers were in charge of the bovines. In mice, 30 µg of the antigen combined with complete Freund's adjuvant (Sigma) were injected subcutaneously. Immunizations were repeated on days 14 and 21 with 30 µg of antigen combined with incomplete Freund's adjuvant (Sigma). Blood samples were collected by submandibular bleeding at day 28 and sera was stored in aliquots at - 20 °C until use. All mice were anesthetized with isoflurane (Piramal, USA) and further sacrificed by cervical dislocation.

For bovines, 100 µg of recombinant protein plus Montanide ISA-61VG adjuvant (Seppic, Paris, France) were injected 4 times every 21 days and using the same injection via. Blood samples were collected by jugular venipuncture in Vacutainer™ tubes (Becton Dickinson, NJ, USA) at the end of the experiments and sera were stored in aliquots at - 20 °C until use.

All the experiments were carried out under guidelines of the Institutional Committee for the Use and Care of Experimentation Animals (CICUAE – INTA protocols No. 17/2018 and 47/2018).

## 8. BbiTRAP-1 Protein Expression Analysis

The expression of TRAP-1 in *B. bigemina* merozoites was confirmed by Western blot analysis. Parasites were cultured at 37 °C in 5% CO<sub>2</sub> in air in 25 cm<sup>2</sup> flasks (JETBIOFIL, China) containing a 5% (vol/vol) concentration of bovine erythrocytes from a healthy donor in medium 199 (GIBCO, Grand Island, NY, USA) supplemented with 40% autologous bovine serum. For Western blot experiments, 50 ml of this *B. bigemina* culture were centrifuged at 2500 x g at 4 °C for 15 minutes and washed twice with cold PBS (same centrifuge speed, temp.). The pellet was resuspended at 10% packed cell volume in PBS followed by addition of 1.13 ml of ice-cold distilled water per gram of wet pellet under continuous mixing by gentle shaking. The suspension was left for 1 minute on ice, and then 0.11 volumes of 10x PBS were added to bring it back to isotonic condition. The suspension was finally spun down and the pellet was washed 2 times with PBS. This pellet was further resuspended in cracking buffer 2x and used for SDS-PAGE. The gel was then transferred into nitrocellulose membranes (Merck, NJ, USA) using a wet blotting system and the membrane was further blocked with 5% (w/v) skimmed milk in PBS for at least 2 h at room temperature or at 4 °C overnight with continuous shaking. The source of primary antibodies was murine or bovine polyclonal sera respectively. The secondary antibodies used were anti-murine or anti-bovine antibodies (goat anti-mouse IgG H&L (HRP) (Abcam, Cambridge, MA, USA) or rabbit anti-bovine IgG whole molecule (HRP) (Sigma) following standard protocols. After incubation for about 1 h at room temperature and washing the membranes, the bands were developed with a chemiluminescent method (ECL, Pierce™ ECL Western Blotting Substrate, Thermo Scientific).

## 9. BbiTRAP-1 ELISA

An indirect ELISA test using recombinant BbiTRAP-1 was performed following previously developed protocols (19). Different concentrations of antigen (2, 5, 10, 20, 40, 80 and 100 ng/well) and serum dilutions were previously checked in triplicates in order to optimize the assay conditions (data not shown). The optimal concentration of bound antigen was set as 40 ng/well. Serum dilutions were 1:1600 for mice and 1:10 for bovines.

Bovine sera from different geographic locations with reported presence of ticks (Misiones (n = 42) and Santa Fe (n = 27)) were used. All sera were previously selected to be positive to *B. bigemina* infection by an ELISA test based on a merozoite lysate (20). Sera from the tick-free area of the Buenos Aires region (n = 10) were included as negative controls. All these sera were tested as negative for *B. bovis* and *B. bigemina* antibodies by the merozoite-based ELISA. For specificity testing, 5 sera from *B. bovis* positive animals were used. A threshold O.D. value was calculated to discriminate *B. bigemina* positive from negative sera. This value was obtained by calculating averages of O.D. of negative sera of triplicate data sets +2 standard deviation values.

## 10. *In vitro* neutralization assay

Inhibition of *B. bigemina* merozoite invasion of erythrocytes was performed as previously described for *B. bovis* (21). Briefly, the *B. bigemina* Puerto Rico (PR) strain (provided by the ADRU-USDA lab at Washington State University) was cultured in a 96-well plate with 5% hematocrit. To that end, infected erythrocytes with 1% parasitemia were incubated with the sera to be analyzed in a culture medium containing 60% HL-1 medium (pH 7.2) and 40% of the aforementioned sera, that had been previously heat-inactivated for 30 min at 56 °C. The culture was maintained at 37 °C in a 5% CO<sub>2</sub> atmosphere for 72 h with changes of media every 24 h. Pre-immune mice and bovine sera were used as negative controls. Serum from a steer inoculated with *B. bigemina* PR was used as positive control. At the end of the incubation, the percentage of parasitized erythrocytes (PPE) was determined by flow cytometry using a Millipore Guava easyCyte HT flow cytometer. Prior to sorting, samples were stained with hydroethidine (Dihydroethidium, Invitrogen) to stain the parasite's DNA, following the manufacturers' instructions. For the FACS acquisition, cell suspensions were diluted into PBS containing 0.2% sodium azide to obtain a flow rate of 2000-5000 cells/s and each cell suspension was sampled in triplicate for statistical analysis. Measures of hydroethidine (blue fluorescence) were registered for all analyzed samples. The number of gated events was recorded on a fluorescence (BluFL2) versus side scatter (SSC) dot plot, where thresholds were set up based on the infected and non-infected controls (22). The percentages of parasitemia inhibition (% pi) for the anti-BbiTRAP-1 antibodies were calculated with the following formula: % pi = 100 - [(PPE Post-immunization serum) / (PPE Pre-immunization serum)] × 100). P-values < 0.05 of an independent Student's t-test were considered significant.

## 11. Indirect fluorescent antibody test (IFAT)

The IFAT was carried out essentially as described in the OIE Manual (23), with minor modifications. Smears of iRBC from cultured *B. bigemina* parasites were fixed in ice-cold acetone for 30 s and air-dried. Pooled polyclonal bovine sera anti-BbiTRAP-1 were diluted 1:100 in PBS. Pooled pre-immune bovine sera at the same dilution were used as negative controls.

All sera were incubated for 30 min in a humid chamber at 37 °C. After three washes (10 min in PBS and 5 min in bidistilled water), fluorescein isothiocyanate (FITC) conjugated anti-bovine IgG (H+L) (Sigma), (diluted 1:100 in PBS containing Blue Evans (1:1000) and TO-PRO-3 (1:150) stains) was applied as a secondary antibody and incubated for 30 min in a humid chamber at 37 °C. The slides were washed twice for 10 min with PBS-Tween 20 0.05% and once for 5 min with bidistilled water and then mounted with a coverslip in 1:2 glycerol-PBS. Epifluorescence was examined by a confocal microscope (Leica TCS SP5, Mannheim, Germany).

## Results

### 1. Identification and genetic characterization of the *TRAP* family in *Babesia* and *Theileria*

For the identification of the *BbiTRAP-1* coding sequence, a TBLASTN search was performed in the genome of *B. bigemina* (BOND strain) using the predicted amino acid sequence of the *B. bovis TRAP-1* gene as a query.

This search resulted in the identification of an orthologous *TRAP-1* gene of 3186 bp that encodes a 1061 aa protein (BBBOND\_0202740). This gene has 5 exons and is located in chromosome II, as in *B. bovis* (Figure 1.A). At the genome level, *BbiTRAP-1* as well as downstream and upstream surrounding genes are syntenic in comparison with *B. bovis*, *B. divergens*, *B. microti* and *B. ovata* (Figure 2).

The BbiTRAP-1 protein has a signal peptide at the N-terminus, the adhesive domains vWFA (approx. 200 residues) and TSP-1 (approx. 60 residues and also known as TRS) and the characteristic transmembrane domain (Figure 1.A). A metal ion-dependent adhesion site (MIDAS) motif is also present in the vWFA domain. This motif in BbiTRAP-1 is identical to the reported consensus sequence (7) and is composed of five non-contiguous amino acids, Asp-Xaa-Ser-Xaa-Ser (where 'Xaa' can be any amino acid), that are brought together to accommodate a divalent cation.

Even though the amino acid sequence of the TRAP-1 orthologs in *B. bovis* and *B. bigemina* share an amino acid identity of only 49,62%, the modular composition and domain order in both species is the same as in its more distant ortholog, *P. falciparum* (Figure 1.B)

Previous studies in *Plasmodium* sp. show that TRAP proteins belong to a family with at least 6 members (24). As in *Apicomplexa* the genes encoding for these proteins are expanded in a lineage-specific fashion, we searched for the presence of other members of the *TRAP* gene family in the *Babesia* and *Theileria* genera. We first searched on PiroplasmaDB for all the genes that coded for proteins containing the TSP-1 or vWFA domain since these functional domains are present in all TRAP proteins characterized so far (7).

Our criteria to assign a protein to the TRAP family, includes the presence of an acidic CTD with a conserved tryptophan residue near the C-terminal end of the protein, other typical features of canonical apicomplexan TRAP proteins (14).

The results of this search, based on available current information, are shown in supplementary Table 1. All species of *Babesia* and *Theileria* contain at least two TRAP genes in their genome. In general, the percentages of amino acid identity of TRAP proteins were between 18.43-66.25% for TRAP-1 in *Babesia* spp. and 42.66-82.91% for TRAP-1 in *Theileria* spp.

*Babesia bovis* is the species with the largest number of TRAP genes with four members, meanwhile *B. canis* has only one TRAP-2 gene. Particularly, in *B. bigemina*, TRAP-2 has the same domain architecture as TRAP-1 whereas TRAP-3 has two TSP-1 domains and vWA domains respectively (Figure 1.C).

A second search focused on identifying encoded proteins containing just the TSP-1 domain led us to also identify three new members of another group of TRAP-related proteins (Figure 1.C and Supplementary Table 2). These were named TRP after the report of these orthologous proteins in *Plasmodium berghei* (14). Unlike TRAP, these TRP proteins lack the vWFA domain but contain a variable number of TSP-1 domains and a non-acidic CTD where the conserved tryptophan residue is absent.

## 2. Phylogenetic analysis

Phylogenetic analysis of sequences of the TRAP family in *B. bigemina* with paralogs and orthologs of available related species was performed using Maximum Likelihood (Figure 3), Neighbor Joining analysis retrieved a tree with similar topology (data not shown). This analysis, based on TRAP-1, showed that *B. ovata* is the species with the closest genetic relationship with *B. bigemina*. Both parasites are classified as a large-type *Babesia* that infect cattle. A further subclade including *B. bovis* and *B. orientalis* is also recognized with high bootstrap value (100%). In general, all TRAP-1 orthologs cluster together with the exception of *B. microti*, which is considered as a *sensu lato Babesia* sp. In contrast, TRAP-2 and TRAP-3 protein sequences are grouped with their respective paralogs.

## 3. Structural analysis of TRAP family

To further confirm the conservation of BbiTRAP-1-3 proteins with the already reported orthologs, the 3D structure of the 3 proteins was predicted *in silico* (Supplementary Figure S1). Homology modelling was performed upon the structure of a fragment of *Plasmodium vivax* sporozoite surface protein 2 (PDB code: 4hql.2.A) (also referred as TRAP) (25) which was the candidate with the highest GMQE scores (0.60; 0.49 and 0.5 for TRAP-1, TRAP-2 and TRAP-3, respectively).

The predicted 3D structures of the canonical vWFA and TSP-1 domains contain eight  $\alpha$ -helices ( $\alpha$ 1 to  $\alpha$ 8), eight  $\beta$ -strands ( $\beta$ 1 to  $\beta$ 8) and the MIDAS site (Figure 4). While the degree of amino acid conservation between BbiTRAP-1, 2 and 3 and sporozoite surface protein 2 is low (Seq. Similarity 0.34; 0.30 and 0.30

for TRAP-1, TRAP-2 and TRAP-3 respectively) their overall predicted domain structures are well conserved.

#### 4. Sequence analysis of *BiTRAP-1* repeats

The TRF software was used to screen the *BbiTRAP-1* gene for the presence of tandem repeats based on previous findings of these repeats in the central part of the *TRAP-1* gene in *B. bovis* (18).

The *BbiTRAP-1* protein contains four blocks of long amino acid repeats in the central portion of the protein. Two of these blocks have complete repeat modules of 84 amino acids each, while the last two are truncated (comprised of 60 and 73 amino acids, respectively).

The tandem repeat motifs in TRAP-1 proteins were also analyzed in different *B. bigemina* strains obtained either from translated genomic data or from sequenced PCR products. Comparative analysis among strains from Argentina, Australia, Puerto Rico, Brazil and Mexico determined that the tandem repeat modules varied in number and sequence among distinct isolates (Table 1). In order to facilitate the analysis of the variation of the repeat modules, a code number was assigned to each repeat. An alignment of all the repeats identified in this work is shown in Figure 5.

Table 1: Variability of the TRAP-1 protein sequence of different *B. bigemina* strains. A number R I-R IV was assigned to each of the four repeat blocks shown in Figure 1.B and 1.C for the BOND strain.

Strain	R I	R II	R III	R IV	Origin	Source
S3P	1	2	3	4	Argentina	Genome database (12)
S2A	13	14	3	15	Argentina	Own results
S1A	21	14	3	15	Argentina	Own results
M1A	22	14	3	15	Argentina	Own results
38	16	17	3	4	Argentina	Own results
S2P	16	12	23	11	Argentina	Own results
Brazil	1	14	3	18	Brazil	Own results
JG29	10	2	-	11	Mexico	Genome database (12)
Mexico_seed	19	20	-	11	Mexico	Own results
Nayarit	24	-	25	26	Mexico	Own results
PR	9	-	-	4	Puerto Rico	Genome database (12)
BOND	5	6	7	8	Australia	Genome database (12)

The number and sequence of the repeats among strains is rather variable. Strains from Puerto Rico and Mexico have a shorter repeat region with only two and three repeat blocks, respectively. Some of the repeat modules are present in strains from a specific region, such as repeats 1, 3, 14 and 15 that were only found in Argentina and Brazil. Remarkably, all Argentinean attenuated strains analyzed here (S1A, S2A and M1A) show a relatively conserved repeat pattern where the first repeat module is variable but the last three ones (repeats 14, 3, 15) are exactly the same. All the repeats present in the Australian BOND and the Mexican Nayarit strains are unique and are not present in any other strain analyzed here.

Regarding BbiTRAP-2 and 3, four blocks of tandem repeats were found in BiTRAP-2, between amino acids 71-178. Two of these blocks have 27 amino acids each, while the last two have 25 and 28 amino acids, respectively. No repeats were identified in BiTRAP-3.

## 5. Expression of recombinant BbiTRAP-1

For further characterization of TRAP-1 in *B. bigemina*, we generated a recombinant form of this protein containing only predicted extracellular regions and a His tag in order to facilitate expression and purification. The truncated recombinant BbiTRAP-1 protein (rBbiTRAP-1) was obtained at high yield and it could be purified under native conditions. After SDS-PAGE analysis (Figure 6.A, lane 1), a unique band of ~107 kDa, was observed. Even though the recombinant BbiTRAP-1 run with a higher than predicted molecular weight, positive Western blot results with the anti-His antibody confirmed the identity of the purified protein (Figure 6.B, lane 1).

## 6. Evaluation of antibody reactivity against recombinant and native BbiTRAP-1

### 6.1 BbiTRAP-1 is recognized by antibodies from naturally infected cattle

In order to determine if antibodies present in the serum of *B. bigemina* infected cattle would react with rBbiTRAP-1, a Western blot analysis was performed. Sera from these bovines specifically recognized the same band of ~107 kDa protein that was obtained with the anti-His antibody (Figure 7.A, lanes 4, 5 and 6). No reaction was observed with sera from uninfected cattle (Figure 7.A, lanes 7 and 8), or from a bovine infected with *B. bovis* (Figure 7.A, lanes 1, 2 and 3).

To further characterize the expression of the BbiTRAP-1 protein in merozoites, we initially tested the ability of sera from mice and bovines immunized with recombinant BbiTRAP-1 to recognize the native protein in immunoblot assays. In both animal models, antisera reacted with a single band of approximately 107 kDa in parasite lysates derived from *B. bigemina*-infected erythrocytes (Figure 7.B. lane 2 for mice and Figure 7.C. lane 1 for bovine). Accordingly, mice or bovine pre-immune sera did not react with any *B. bigemina* antigen (Figure 7.B. lane 1 and Figure 7.C. lane 2). Altogether, the data confirms expression of BbiTRAP-1 in blood stages of the parasite.

## 6.2 Truncated BbiTRAP-1 is not immunodominant in *B. bigemina* infected bovines

An *in house* indirect ELISA was developed to further assess the immunogenicity of BbiTRAP-1 using sera from *B. bigemina* infected animals. For this purpose, a set of 69 sera from *B. bigemina*-infected herds from 2 different geographic origins (Misiones and Santa Fe, Argentina) was used. The results showed that 29.62% from experimentally-infected and 42.85% of naturally-infected bovines recognized BbiTRAP-1 in this ELISA (Supplementary Figure S2). The specificity of the test was optimal (100%) meanwhile the sensitivity had the same values as above since no false negative results were obtained. This pattern of reactivity suggests that rBbiTRAP-1 is not an immunodominant antigen, and would not be an optimal candidate for developing novel serological diagnostic assays.

## 6.3 BbiTRAP 1 contains neutralization sensitive epitopes

To determine whether BbiTRAP-1 has neutralization-sensitive B-cell epitopes, an *in vitro* invasion assay was performed using anti-recombinant BbiTRAP-1 murine and bovine hyperimmune sera. After 72 h of the initiation of the cultures, antibodies against BbiTRAP-1 present in both sera neutralized invasion by 39.94% in mice (Supplementary Figure S3) and 46.92% in bovine samples. As expected, *B. bigemina* cultures containing pre-immunization serum as a negative control (considered as 100% of infection) exhibited normal parasitemias similar to those observed in control cultures without addition of any serum. Confirmation of the presence of *in vitro* neutralization sensitive epitopes in BbiTRAP-1 suggests that this protein may also be exposed to neutralizing antibodies during infection, and could be targeted as a possible component of a subunit vaccine against *B. bigemina*.

## 6.4 BbiTRAP-1 is expressed in intraerythrocytic merozoites

Finally, we have analyzed the localization of BbiTRAP-1 in intracellular merozoites. The pattern of reactivity of bovine serum raised against recombinant BbiTRAP-1 was tested by IFAT using fixed smears of *B. bigemina* infected erythrocytes. The green fluorescence of the FITC-anti-bovine conjugate localized with a strong focal signal in the wider region of the pear-shaped merozoites compared with the rest of the parasite's body (Figure 8, i). This positive signal was not observed when infected red blood cells were incubated with a *B. bigemina* negative bovine serum (Figure 8, e). Both anti-BbiTRAP-1 and anti *B. bigemina* antisera showed a very similar staining pattern where fluorescence appeared to be concentrated at the bottom side of the parasites.

# Discussion

BbiTRAP-1 is a novel, well conserved, antigen of *Babesia bigemina* with structural and antigenic characteristics similar to several adhesin orthologs already described. These include proteins of diverse origin such as the *Plasmodium* thrombospondin-related anonymous protein and the circumsporozoite

protein as well as *T. gondii* micronemal proteins (7). We propose that both TRAP and TSP are members of a TRAP superfamily sharing a common ancestral origin and non-overlapping functions. The widespread conservation of this multigene TRAP superfamily, including canonical TRAP and the hereby described TRP families, among distinct apicomplexan parasites underscore an important functional role and relevance for parasite survival. Whether TRP proteins are functional equivalents of TRAP, or if they play different roles remains unknown.

Indeed, proteins containing adhesive domains like TRAPs allow the parasite to interact with receptors present at the host cell surface and are linked to the glideosome, a molecular machine necessary for parasite motility and host cell invasion (5). The importance of adhesive domains was demonstrated in recent studies on *Plasmodium berghei* TRAP in which deletion of the vWFA domain abolished gliding motility, mosquito salivary gland invasion and mouse infection (26). For these reasons, these proteins could be important targets for immunological or chemotherapeutic interventions.

Our genomic analysis showed that the gene *BbiTRAP-1* is in fact, part of a family composed of two additional members designated as *BbiTRAP-2* and *BbiTRAP-3*. According to what is widely known in *Apicomplexa*, we confirm here the observation that the TRAP family in Piroplasmida possess 2 or 3 genes according to the genera. Whether the expression of TRAP family proteins is stage-specific as is reported in *Plasmodium* will demand further studies.

Expression of TRAP-2 in merozoites had been already reported only for *B. bovis* and *B. orientalis* (9, 27) and up to our knowledge, no other study has described the presence of these proteins in other members of Piroplasmida. In this sense, our data provides additional information on this gene family for future characterization studies not only in *Babesia* but in *Theileria* sp. as well. The data described here, extends also to the new TRP gene family identified by us in both genera through a domain-driven bioinformatic search. In relation to TRP function, it has been demonstrated that the *P. berghei* TRP ortholog is involved in oocyst egress and salivary gland invasion (14), therefore, further studies in tick stages will shed light on the functional relevance of these proteins in Piroplasmida.

Regarding *BbiTRAP-1*, the chromosomal region that contains this gene is syntenic between *B. bigemina* and the other *Babesia* species analyzed. This observation indicates that this region of the chromosome is well conserved along the *Babesia* genus and that recombination would be infrequent for this region of the genome. At the gene level, the number of exons in *BbiTRAP-1* also coincides with their orthologs in *B. bovis*, *B. orientalis* and *B. ovata*, suggesting a closer genetic relationship among these members of the genus. In this sense, the phylogenetic analysis of *TRAP-1* in *Babesia* sp. supports the hypothesis of a closer association among these species by showing that *B. bigemina*, *B. orientalis*, *B. bovis* and *B. ovata* form a separate clade apart from *B. divergens*. It is interesting to note that the *TRAP-1* tree reflects mostly the taxonomic relationships of members of the order Piroplasmida performed using mitochondrial and 18S genes (28). In both cases, *Babesia* species of the *sensu stricto* group form a separate phylogenetic clade apart from *Theileria* sp. and *B. microti*.

The presence of only one *TRAP-2* member in *B. canis* can be attributed to a sequencing artifact due to the fragmentation of *B. canis* genome in a large number of scaffolds (29). It has been reported that incorrect genome assemblies result in inferring a wrong number of genes belonging to a gene family (30), so the number of *TRAP* genes, TM and acidic CTD in *B. canis* should be further revised.

Even though the overall amino acid identity of the BbiTRAP-1 protein compared with other *Babesia* species is relatively low, the sequence conservation and tertiary structure is very high in key adhesive domains such as vWFA and TSP-1. This conservation exceeds the *Babesia* genus as reflected by the good fitting of the predicted BbiTRAP-1 3D structure with the Sporozoite surface protein 2 of *P. vivax*, confirming the functional importance of this protein in critical processes not only in the *Babesia* genus, but also among other apicomplexan parasites. Conserved domains like the vWFA with similar overall structures in distant phyla were reported to be polyspecific in *Apicomplexa* giving the parasites the versatility to interact with multiple ligands and migrate into diverse organs (26).

Another conserved feature of BbiTRAP-1 and other adhesive proteins is the presence of tandem repeat units in the central part of the protein. The presence of repeats or tandem duplication of adhesive domains have been associated with parasitic evolution strategies to form high avidity complexes for host cell invasion (31) or as a smokescreen to escape from the vertebrate host immune system (32). The presence of these repeats has already been described for *B. bovis TRAP-1* (33) where this high ratio of variation was exploited to be used as a molecular marker. Here, the variation in sequence and order of repeat units in *BbiTRAP-1* could also be applied for high resolution molecular fingerprinting of *B. bigemina* isolates. Further analysis at the genomic level will determine if the high conservation of *BbiTRAP-1* repeat modules in vaccine strains of Argentina is a more widespread phenomenon, and if it could reflect a process of reduced genome diversity after attenuation, similar to what has been reported for *B. bovis* (34).

The truncated recombinant BbiTRAP-1 showed a molecular size higher than expected. This effect has been reported for acidic proteins of eukaryotic origin and was attributed to changes in protein mobility due to low binding of SDS, producing insufficient denaturation and slower migration in the gel (35–36). Consistently, the recombinant BbiTRAP-1 obtained in this work has 17.9% of acidic amino acids and a theoretical pI of 4.72.

Testing the reactivity of anti-rBbiTRAP-1 in Western blot against a *B. bigemina* lysate results in the recognition of native *B. bigemina* merozoite protein with the predicted size of BbiTRAP-1 suggesting that this protein is actually expressed in blood stages of the parasite. This result is fully consistent with the identification of anti rBbiTRAP-1 antibodies in infected cattle and the strong IFAT signal. Furthermore, the immunofluorescence assay using bovine antisera demonstrated that this protein is expressed in intraerythrocytic merozoites, with stronger signal in the region of the apical complex where adhesion proteins for initial attachment are frequently located. In addition, the presence of a signal peptide in BbiTRAP-1 is compatible with its involvement in a possible secretion pathway via association with the apical complex organelles of the parasite.

One of the major thrusts in *Babesia* studies is the identification of immunodominant antigens that can be used in serological diagnostics. At least in Argentina, the only serological test used routinely for *B. bigemina* diagnosis is an indirect ELISA based on a merozoite lysate (20), which requires the maintenance of parasite cultures or infected bovines and is difficult to standardize. The ELISA results with the truncated form of BbiTRAP-1 had values of sensitivity of 29.62 - 42.85%. These values are below optimal for an ELISA test and indicate that at least the selected portion of BbiTRAP-1 expressed by us is not suitable for a diagnostic test and that the epitopes present in this protein are not immunodominant. If, BbiTRAP-1 is in fact a subdominant antigen, it may be a molecule that is essential for parasite survival, and thus, a promising candidate for vaccine development.

The involvement of members of the TRAP family during the erythrocyte invasion by merozoites has been previously demonstrated in *B. bovis* (6, 27) and in other *Babesia* species (8–10). Our results of an important reduction in parasite invasion assays with anti-TRAP-1 antibodies support the hypothesis of the presence of surface exposed neutralizing B-cell epitopes, which is in accordance to the above mentioned reports. As the recombinant BbiTRAP-1 protein obtained in this work lacks hydrophobic portions in the N- and C- terminal regions, it can be assumed that these epitopes are located outside these regions. Further experiments of epitope mapping will be required to determine the exact location of these epitopes.

In summary, this study characterizes a novel TRAP-1 homologue expressed by merozoites of *B. bigemina* and provides evidence that this antigen has B-cell neutralizing epitopes that are exposed during infection and could play a key role in parasite invasion. These functional characteristics together with its possible antigenic subdominance suggest BbiTRAP-1 as a rational candidate for developing a subunit vaccine against *B. bigemina*.

## Conclusions

In the present study, we have identified the *BbiTRAP* and *BbiTRP* gene families in several *Babesia* and *Theileria* species and defined these genes as members of a gene superfamily. We have also characterized BbiTRAP-1 as a novel antigen of *B. bigemina* with similar structural and antigenic characteristics as its orthologs in other *Apicomplexa*. The presence of adhesive domains, perceived functional significance, high level of conservation, added to the identification of surface exposed neutralization-sensitive B-cell epitopes and its possible immunological subdominance, suggests that BbiTRAP-1 should be included as a subunit vaccine candidate against *B. bigemina*.

## Abbreviations

TRAP-1: thrombospondin-related anonymous protein 1; vWFA: von Willebrand factor A; TSP-1: Thrombospondin type-1 repeat; MIDAS: Metal ion-dependent adhesion site; PPE: Percentage of parasitized erythrocyte; iRBC: infected red blood cell.

# Declarations

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## Availability of data and materials

The nucleotide sequences generated during this study were submitted to the GenBank database under the accession numbers MN450376-MN450383.

## Authors' contributions

VM, MP and JJ performed the experiments. VM, MP, CS and SW analyzed the results. SW designed the study and drafted the manuscript. All authors have read and approved the final manuscript.

## Ethics approval and consent to participate

The experiments were carried out under guidelines of the Institutional Committee for the Use and Care of Experimentation Animals (CICUAE – INTA protocols No. 17/2018 and 47/2018). All authors have read and approved the final manuscript.

**Consent for publication:** Not applicable

**Competing interests:** Not applicable

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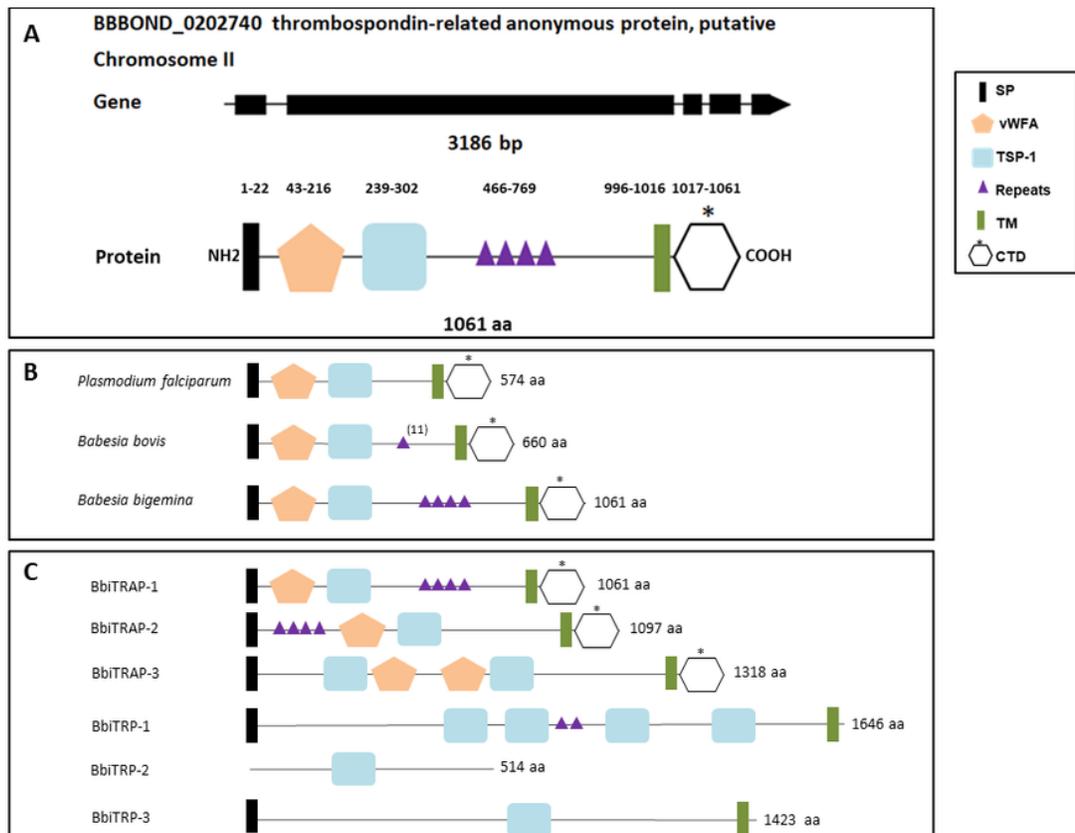
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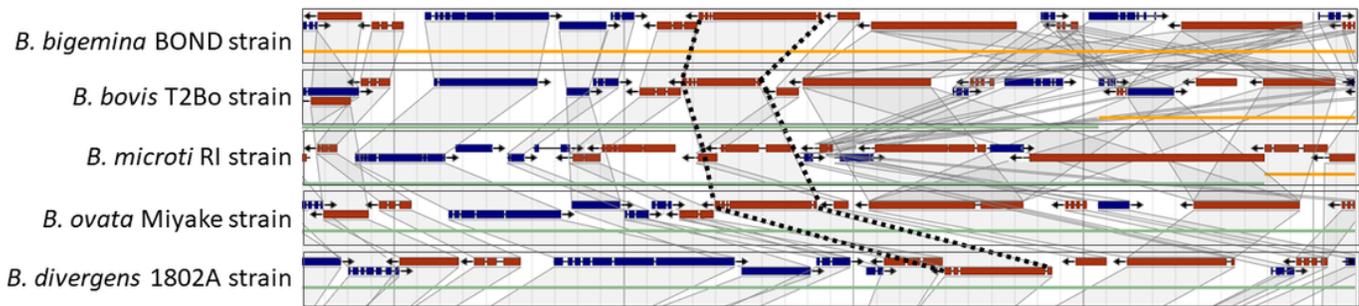
## Figures



**Figure 1**

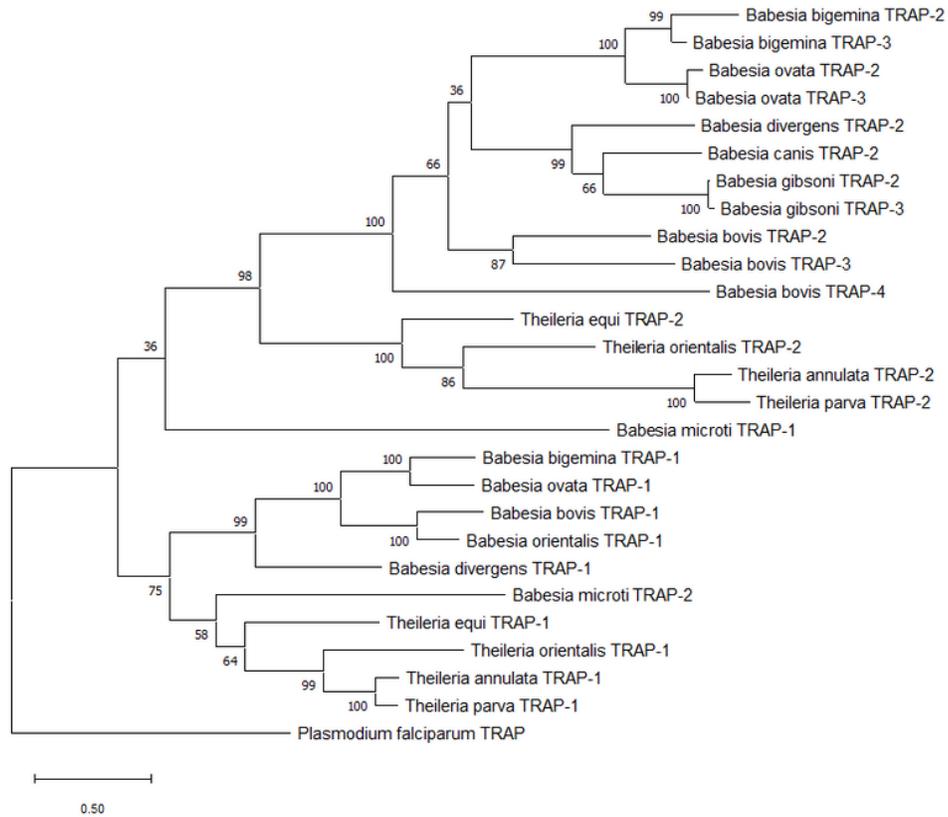
Sequence analysis of BbiTRAP-1. A. Schematic representation of the BbiTRAP-1 gene in the BOND strain with its five exons and the encoded polypeptide containing a signal peptide (SP), a von Willebrand factor A (vWFA), a thrombospondin type-1 repeat domain (TSP-1), tandem repeats and the transmembrane region (TM) as well as a cytoplasmic tail domain (CTD). Asterisk indicate the presence of a tryptophan residue close to the CTD. B. Schematic representation (not to scale) of the domains and repeats of 3 apicomplexan TRAP family proteins. Only long repeats (>7aa) are displayed as purple triangles. The triangles reflect the number of tandem repeat units with the exception of *B. bovis* TRAP-1 which has 11 repeat units in the T2Bo strain. The GenBank accession numbers of the corresponding genes are PF13\_0201 (*Plasmodium falciparum*) and EDO06220.1 (*B. bovis*). The protein sizes in amino acids (aa)

are shown on the right side. C. Schematic representation (not to scale) of the domains and repeats of *B. bigemina* TRAP and TRP family proteins. Only long repeats (>7aa) are displayed as purple triangles.



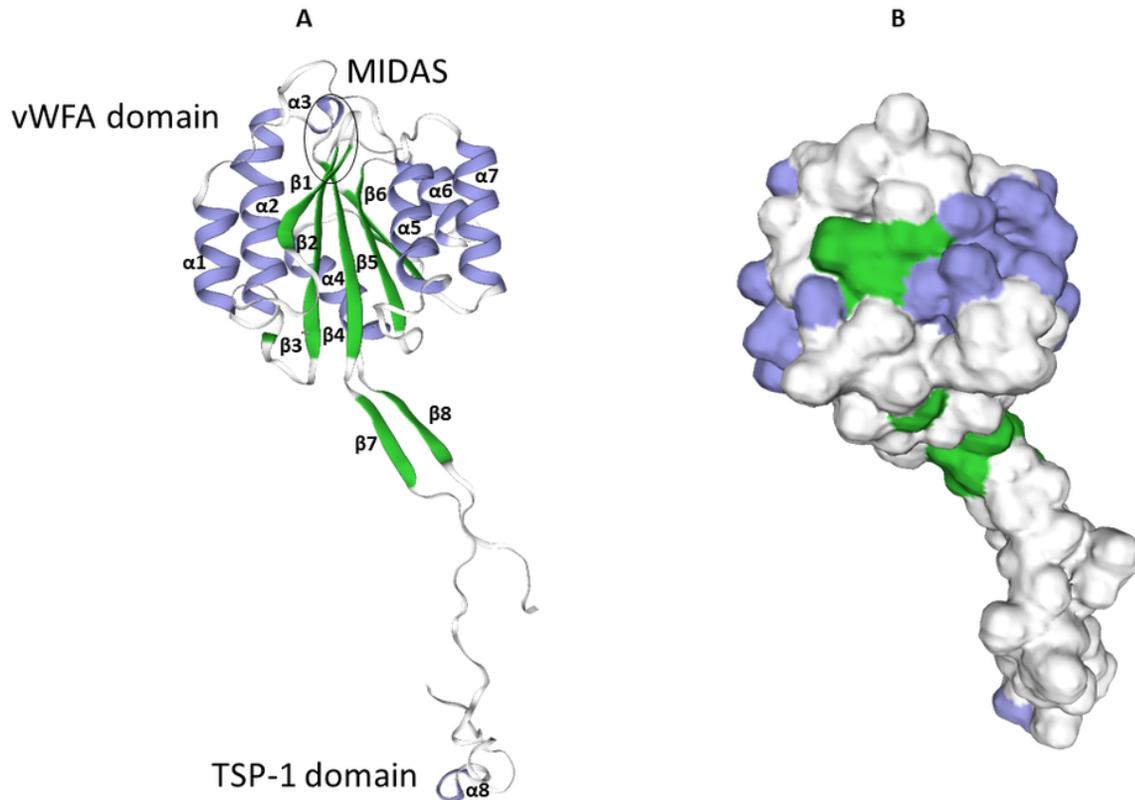
**Figure 2**

Synteny map analysis for BbiTRAP-1. Dotted lines indicate the location of TRAP-1 orthologs in other *Babesia* species. Conservation of surrounding genes is shown as shaded areas.



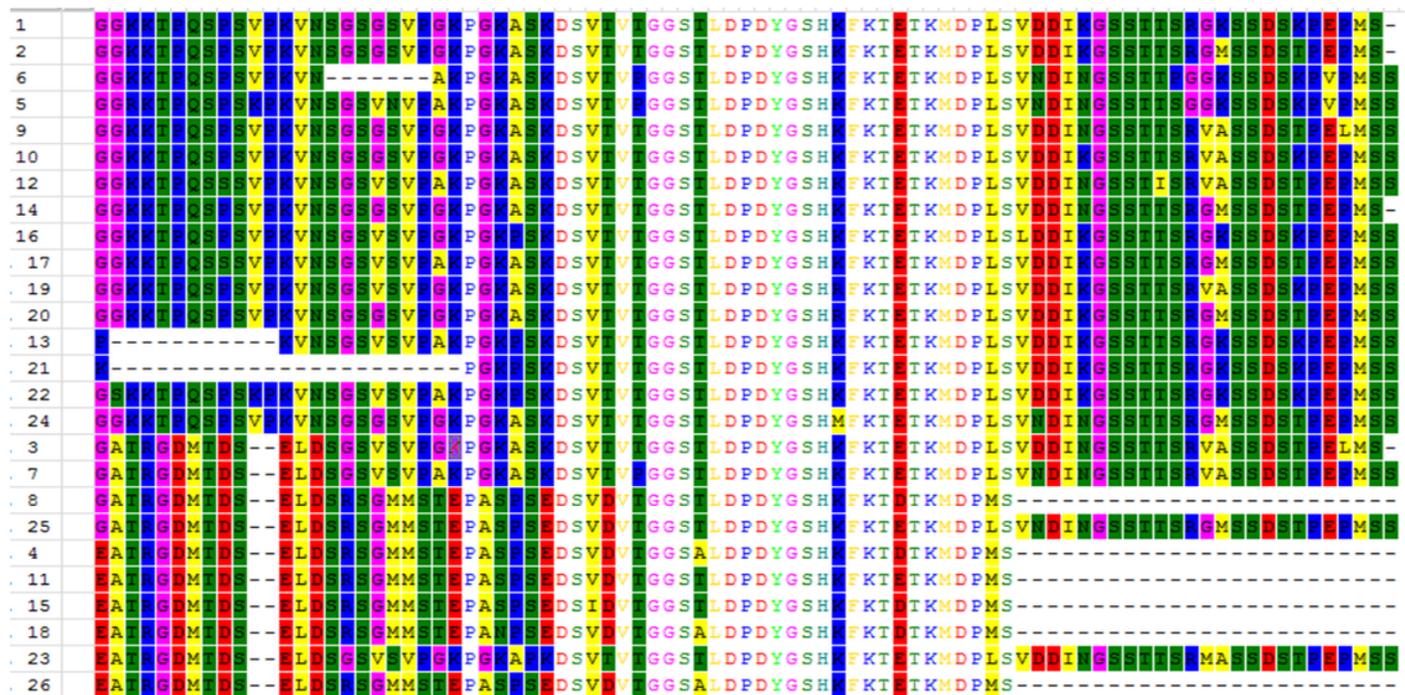
**Figure 3**

Synteny map analysis for BbiTRAP-1. Dotted lines indicate the location of TRAP-1 orthologs in other Babesia species. Conservation of surrounding genes is shown as shaded areas.



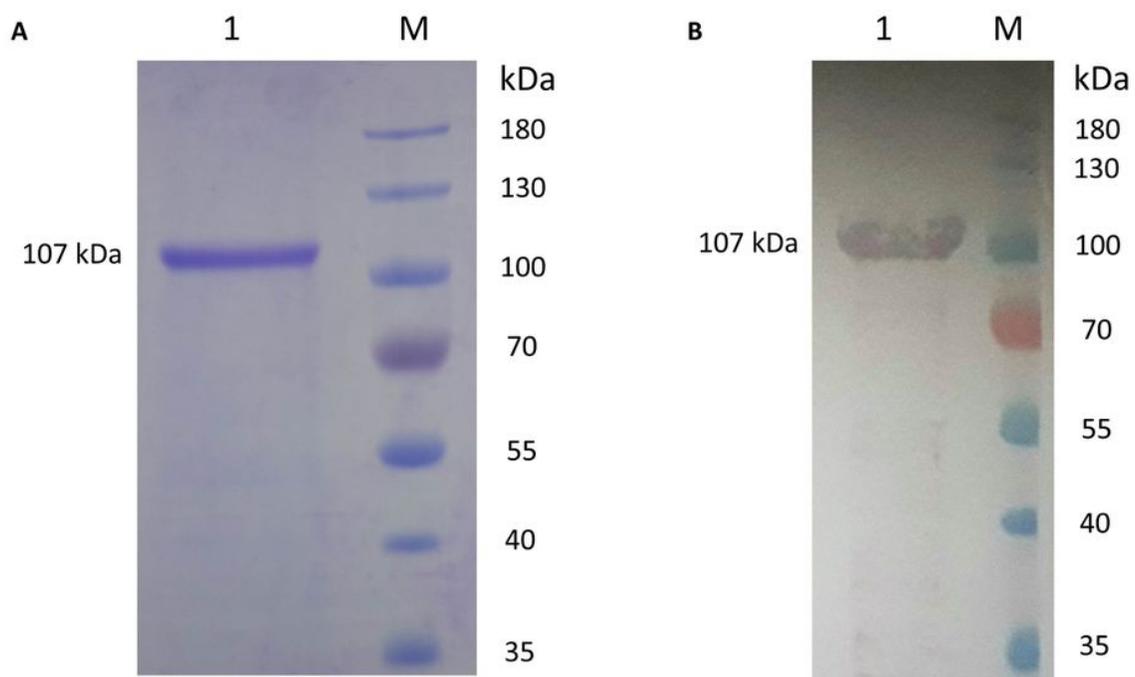
**Figure 4**

Hypothetical 3D structure of BbiTRAP-1. A. Cartoon structure of BbiTRAP-1 (residues 43-302). The  $\alpha$ -helices are shown in violet and  $\beta$ -strands are indicated in green. The MIDAS site for binding ligand is shown in a black circle. B. BbiTRAP-1 surface structure.



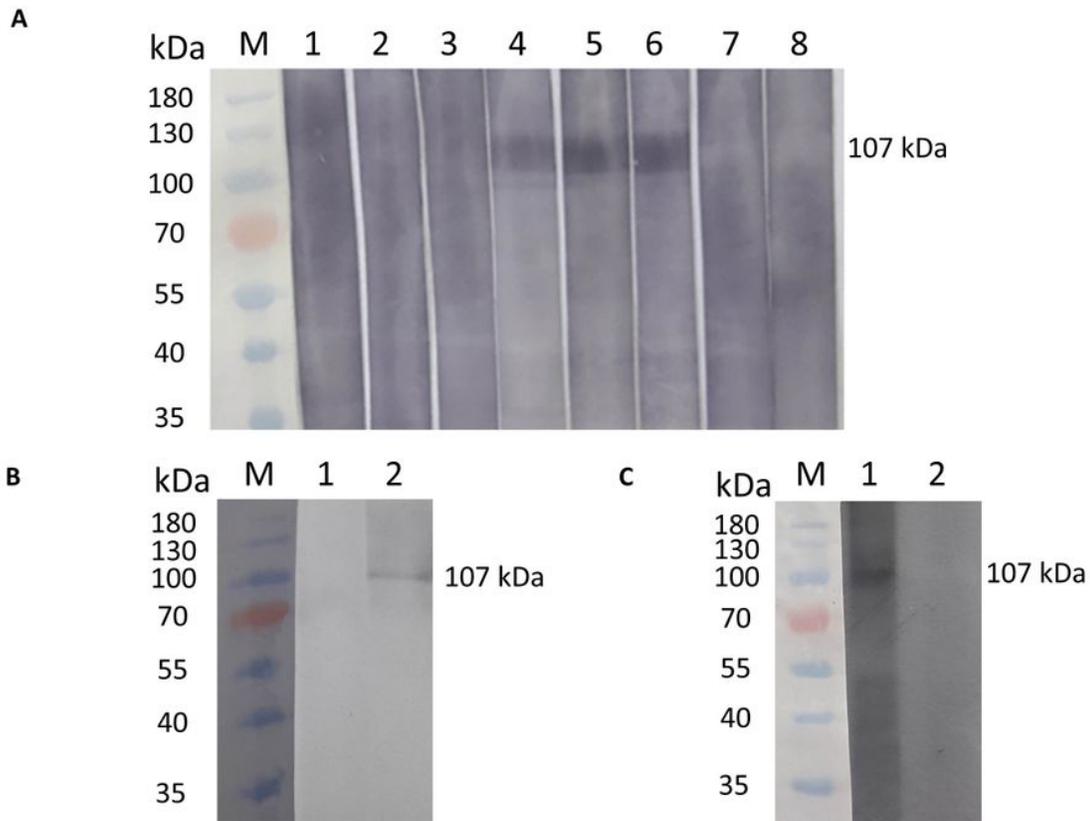
**Figure 5**

Amino acid alignment of the different repeat modules found in BbiTRAP-1. Each number on the left corresponds to a different type of repeat.



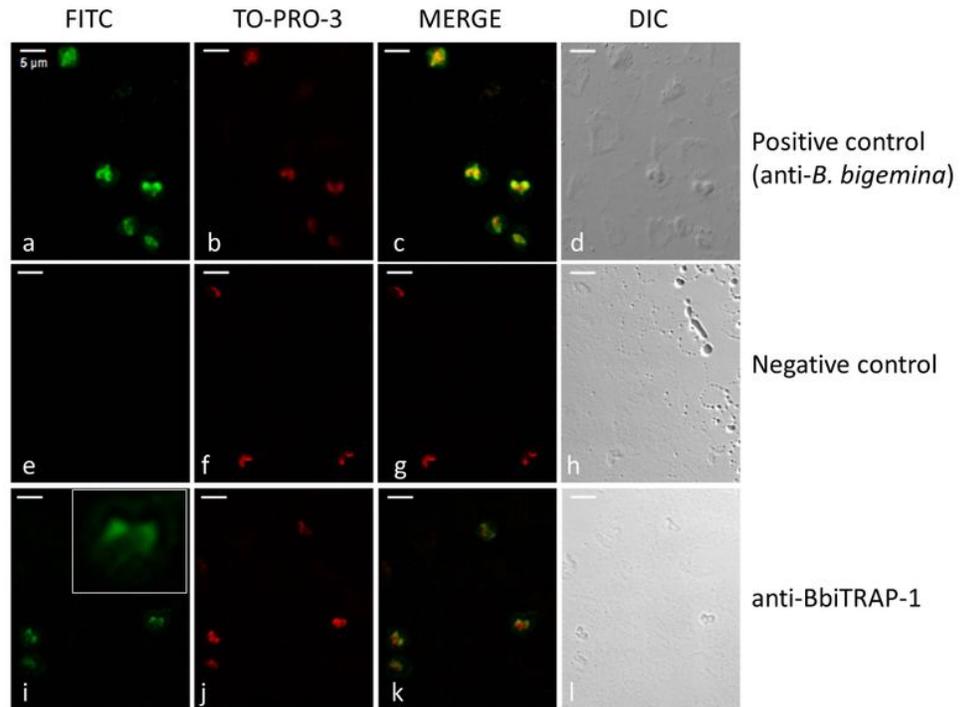
**Figure 6**

Expression of recombinant BbiTRAP-1 as a His-tagged protein. The purified protein fraction is shown in a 12% SDS-PAGE stained with Coomassie blue (A) and in a Western blot (B). Molecular weight marker is indicated as M.



**Figure 7**

Expression of recombinant BbiTRAP-1 as a His-tagged protein. The purified protein fraction is shown in a 12% SDS-PAGE stained with Coomassie blue (A) and in a Western blot (B). Molecular weight marker is indicated as M.



**Figure 8**

Indirect immunofluorescence assay by confocal laser microscopy of *B. bigemina* with antibodies against recombinant BbiTRAP-1. Smears of *B. bigemina*-infected merozoites were incubated with sera from: a *B. bigemina* experimentally-infected bovine (a, b, c and d); a control bovine immunized with adjuvant alone (e, f, g and h); a bovine immunized with recombinant BbiTRAP-1 (i, j, k and l). A FITC-conjugated anti-bovine IgG and TO-PRO-3 DNA stain were used with a merged image (c, g and k). An enlargement of duplicated *B. bigemina* merozoites reacting with anti-BbiTRAP-1 is shown as an inset in panel i. DIC: differential interference contrast microscopy images are located on the far right and were used to confirm the location and number of individual intracellular parasites. Scale-bars 5  $\mu$ m.

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