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Babesia bigemina enolase binds to plasminogen and contains conserved B-cell epitopes that induce neutralizing antibodies in cattle

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ABSTRACT

Babesia bigemina is an intraerythrocytic parasite that causes bovine babesiosis and is responsible for economic damage to livestock in tropical and subtropical countries. Enolase is a glycolytic enzyme with multiple functions. In many pathogens, enolase contributes to the conversion of plasminogen to plasmin, which helps to degrade the fibrin of blood clots and various elements of the extracellular matrix, supporting the pathogen invasion process. In addition, enolase's antigenic and protective properties in several organisms, including the Apicomplexa parasites *Plasmodium* ssp, have been reported. This protein is considered a vaccine candidate. To determine whether *B. bigemina* enolase is antigenic and has some of the characteristics already described, we isolated the gene, cloned and expressed the recombinant protein, and performed different assays to evaluate its ability to bind to and activate plasminogen and induce neutralizing antibodies. First, we studied the conservation of the *B. bigemina* enolase protein (BbiEno) among Apicomplexa parasites; then, we determined that it is transcribed and expressed in erythrocytic stages and tick stages via RT—PCR. In addition, we expressed recombinant BbiEno (rBbiEno) and studied whether sera from naturally infected cattle recognized it via Western blotting. Besides, the ability of rBbiEno to bind and activate plasminogen was analyzed. Finally, we examined whether specific anti-rBbiEno antibodies could neutralize the merozoite invasion of erythrocytes. The results showed that BbiEno is a conserved and immunogenic protein; it is transcribed and expressed in erythrocytic stages, and rBbiEno is recognized by the sera of naturally infected cattle. In addition, rBbiEno binds and activates plasminogen, and the specific anti-rBbiEno antiserum was able to neutralize the invasion of erythrocytes *in vitro* significantly. These results show that BbiEno could be considered a potential vaccine candidate.

1. Introduction

Parasites of the genus *Babesia* are the causative agents of babesiosis. *Babesia bovis* and *B. bigemina* are the main species that cause this disease in cattle and are transmitted by ticks of the *Rhipicephalus* genus. In Mexico, these ticks are endemic; hence, this disease represents a serious problem for livestock, especially in tropical and subtropical regions, where climatic conditions favor the proliferation of ticks (Schnittger et al., 2012). Although *B. bigemina* is less virulent than *B. bovis,* it is also responsible for economic losses worldwide, so better control methods are needed to manage this parasite (Silva et al., 2018).

The economic impact of babesiosis is significant because of the decrease in meat and milk production, the increase in treatment and control costs, and the losses associated with animal mortality. In addition, the disease compromises animal welfare and affects the sustainability of livestock production. This disease can be partially controlled using ixodicides for vector control and live attenuated parasite vaccines (Alvarez et al., 2020). However, there is still no commercial vaccine since this type of vaccine has disadvantages in terms of safety and long-term immunity (Suarez et al., 2019), hence it is necessary to

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implement new control methods.

The life cycle of *B. bigemina* is complex. In the bovine host, the parasites invade erythrocytes through a well-known invasion mechanism in which *Babesia* infects the bovine red blood cells (RBCs) through interactions between parasite proteins and host cell receptors. Once inside the host's erythrocytes, the parasites divide asexually, exit, and break the erythrocytes to invade new RBCs (Yokoyama et al., 2006), while the mechanism of the invasion of *B. bigemina* into the tick midgut is not known in detail.

In its complex life cycle in the tick vector and bovine host, *Babesia* parasites need to cross different physical barriers, such as the epithelium, the extracellular matrix, and host effector systems, to invade and establish themselves within them. In the literature, it is well documented that a system that favors the invasion and dissemination of parasites is the fibrinolytic system. The fibrinolytic system, also known as the plasmin-plasminogen system, is crucial in maintaining homeostatic balance by regulating intravascular fibrin deposition, wound closure, and thrombosis (Hvas and Larsen, 2023). This process is mediated mainly by plasmin, a serine protease with a key role in fibrin degradation, removing clots, and degrading different extracellular matrix components. Plasminogen activation to plasmin by tissue-type activator (tPA) and the urokinase-type (uPA) has a central role in activating the fibrinolytic system (Alves E Silva et al., 2021). It occurs when plasminogen and its activators bind to the cell surface. On the other hand, it has been reported that the interaction of parasites with the fibrinolytic system is a complex process that stimulates the adherence of parasites to their target cells. The recruitment of plasminogen by parasite receptor proteins helps to immobilize it, facilitating its conversion to plasmin by its activators, resulting in a significant increase in plasmin activity. Most plasminogen receptors in bacteria and parasites show similar patterns of binding through lysine residues and are generally moonlighting proteins within which the protein alpha-enolase has been linked to virulence (Figuera et al., 2013; Miles et al., 2005)

Alpha enolase is a moonlighting protein that has been identified in several microorganisms, where it can perform many functions. Alpha enolase is a 41–50 kDa metalloenzyme that participates in the formation of phosphoenolpyruvate (PEP) from 2-phospho D-glycerate (2PGA) in glycolysis and backward in gluconeogenesis (Pancholi, 2001). This protein is found in diverse organisms, such as archaebacteria and mammals, and has a conserved amino acid sequence and structure (Díaz-Ramos et al., 2012). It has been shown that enolase has multiple functions depending on its cell localization; in addition to its catalytic function in the cytoplasm, alpha-enolase may function as a stress-related protein (Wilkins et al., 2002), whereas in the nucleus, its 37-KDa truncated form can bind to the c-myc transcription factor (Subramanian and Miller, 2000), suppressing its activity. Alpha enolase is also localized in intracellular vacuoles and functions as a regulator (Didiasova et al., 2019). Its expression on the membrane surface is important in the initial cycle of infection of many pathogens (Castaldo et al., 2009; Ghosh and Jacobs-Lorena, 2011; Satala et al., 2020).

Currently, there are multiple reports that enolase, with its multiple functions, can stimulate a protective immune response against diverse pathogenic organisms (Arce-Fonseca et al., 2018; Dutta et al., 2018; Feng et al., 2009; Jiang et al., 2014; Pal-Bhowmick et al., 2007a). Enolase may also be localized extracellularly, mediating host tissue's degradation and allowing parasites to evade the host immune system (Avilán et al., 2011). In different microorganisms, enolase is localized on the cell surface and interacts with plasminogen, promoting microorganisms' dissemination within the host's cells. The binding of enolase to plasminogen increases the activity of the plasminogen system and plasmin generation (López-Alemany et al., 2003a, 2003b), leading to fibrinolysis (Avilán et al., 2011). The relationship between cell surface enolases and plasminogen occurs through the interaction of a lysine located on the C-terminal domain of enolase. However, some studies have revealed that enolase contains a plasminogen-binding motif at the same position (Miles et al., 2005).

To date, no studies have been conducted on the *B. bigemina* enolase protein, which represents a possible virulence factor and a potential candidate for generating vaccines or strategies for the eradication or control of this parasite. Based on existing knowledge of enolase functions in similar organisms, such as apicomplexan parasites, the aim of this study was to characterize the enolase of *B. bigemina* to evaluate its ability to bind plasminogen and induce neutralizing antibodies to generate new strategies for the control of bovine babesiosis.

2. Materials and methods

2.1. Isolation of the B. bigemina enolase (Bbieno) gene from the Chiapas, México strain

B. bigemina-infected blood was acquired as previously described (Camacho-Nuez et al., 2017). Briefly, a calf was splenectomized and inoculated with the Chiapas strain of *B. bigemina*. Nine days later, infected blood was obtained. Infected erythrocytes were washed, and DNA extraction was performed via a Gentra Puregene kit (Gentra Purogene, Germantown, USA) according to the supplier's instructions. The final pellet was resuspended in 100 µl of the hydrating solution and incubated for 5 min at 65 °C before storage at –20 °C until use.

The Bioethics Committee of the School of Natural Sciences, Autonomous University of Queretaro, Mexico (permission number 45FCN2017) approved the protocol for handling and bleeding the cattle.

The *Bbieno* gene was amplified via PCR via the forward primer 5′-ATGGCTTCGATTACTTCGATTC-3′, the reverse primer 5′-TGTAAGCTTTCAGTTTCCGCAAGTGCGG-3′, and Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, USA.). The PCR conditions were as follows: a first step at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 64 °C for 30 s, and 72 °C for 1.30 min, and a final step at 72 °C for 7 min. The amplicons were subsequently ligated to the TOPO-TA vector (Invitrogen, Carlsbad, USA.) following the manufacturer's instructions. Three positive clones were selected for sequencing (Sanger et al., 1977) via an automatic sequencer (AB3130, Applied Biosystems, California, USA). Two internal primers were designed to complete the sequence: internal forward (5′-CGTGGATCCATGAACGAGTGGGGTCACTG-3′) and internal reverse (5′-TGTAAGCTTTCAGTTTCCGCAAGT-3′). The *BbiEno* sequence was uploaded to the GenBank database with the accession number QEG79394.

2.2. Bioinformatic analysis

Multiple sequence alignment of the BbiEno amino acid sequence of the Chiapas, Mexico strain revealed the following sequences: XP_001611923.1 enolase (2-phosphoglycerate dehydratase) [*B. bovis*], XP_012649799.1 enolase [cepa RI de *Babesia microti*], AAA18634.1 enolase [*Plasmodium falciparum*], XP_002365579.1 enolase 1 [*Toxoplasma gondii*], ADU85973.1 enolase [*Theileria annulata*] and XP_028865010.1 enolase [*Babesia ovata*] was carried out via Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/).

The ExPASy-ProtScale tool based on the Kyte J., Doolittle R.F. amino acid hydropathy scale (http://web.expasy.org/protscale/pscale/Hphob.Doolittle.html) was used to search for transmembrane regions, and the SignalP 4.1 server tool (http://www.cbs.dtu.dk/services/Sign+alP/) was used to search for possible signal peptides.

The domains and enolase signature sequences were analyzed using ScanProsite online software (https://prosite.expasy.org/scanprosite/), InterPro (http://www.ebi.ac.uk/interpro/result/InterProScan), and MOTIF Search online software (https://www.genome.jp/tools/motif/).

Multiple alignment of BbiEno with enolase sequences of *R. microplus* and *Bos taurus* was performed using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) with the following sequences: QEG79394.1 enolase [*B. bigemina*], XP_037275137.1 enolase-phosphatase E1-like isoform X2 [*R. microplus*] and NP_776474.2 alpha-enolase [*B. taurus*].

2.3. Transcription analysis by RT—PCR

RNA from ticks was obtained from samples from another study we previously published (Camacho-Nuez et al., 2017). The RNA was purified via TRIzol (Invitrogen, Carlsbad, USA), as described previously (Camacho-Nuez et al., 2017). The samples included *B. bigemina*-infected erythrocytes, a pool of infected ticks at zero h post repletion, and the midgut of infected ticks at 48 and 72 h post repletion. For reverse transcription, 1 μg of total RNA was used with the ThermoScript™ RT—PCR System for first-strand cDNA synthesis (Invitrogen, Carlsbad, USA.). PCR for amplification was performed with Platinum® Taq DNA Polymerase High Fidelity (Invitrogen, Carlsbad, USA.) Two microliters of cDNA from each sample were used. For the amplification of 100 bp of the *Bbieno* gene, the following primers were used: pF (5′-CGCTGCCGTTCAGATTGTG −3′) and pR (5′- CTTCAACAAAA-GAGCGTTGCAA −3′). The PCR program used consisted of a first step at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 60 °C for 30 s and 72 °C for 30 s, and a final extension step at 72 °C for 7 min. The *ama-1* gene was used as a reference, and the primers used were forward (5′- ATTGACCCGAAGTGGGATCAC-3′) and reverse (5′-AAGCAGATTCGAGGGCAACA-3′). The negative controls were: RNA extracted from uninfected erythrocytes, RNA extracted from the midgut of an uninfected tick, and the RNA of each sample with no Reverse Transcriptase (RT) enzyme.

2.4. Obtaining the recombinant protein

To obtain a recombinant product, we cloned a 1290-bp fragment of the *Bbieno* gene from the Chiapas strain, which was amplified via PCR from *B. bigemina* DNA via sense and antisense primers with *Bam*H1 and *Hind*III restriction sites (in bold), respectively: a sense primer (5´-5'CGT**GGATCC**ATGAACGAGTGGGGTCACTG3'-3´) and an antisense primer (5´-TGT**A-AGCTT**TCAGTTTCCGCAAGTGCGG-3´). The amplicons were purified and cloned in-frame into the pCold I expression vector (Takara Bio, Nojihigashi, Japan), after which the ligation reaction was transformed into the *E. coli* strain Rosetta 2. 6xHisBbiEno recombinant protein (rBbiEno) expression was induced with 1 mM isopropyl-beta-D-thiogalactopyranoside (IPTG, Invitrogen, Carlsbad, USA.) After incubation at 15 °C for 24 h, the cells were harvested and sonicated, and the resulting pellet was used to purify the recombinant protein under denaturing conditions via HisTrap HP affinity columns in an ÄKTA Prime Plus FPLC System (both from GE Healthcare, Madrid, Spain). The fractions were analyzed on a 10 % SDS—PAGE polyacrylamide gel, dialyzed and quantified via the Bradford method (Bio-Rad, California, USA) (Bradford, 1976). To confirm the presence of the recombinant protein, a Western blot analysis was performed with anti-His6x antibodies (1:3000) and a secondary anti-mouse IgG-HRP antibody (Santa Cruz Biotechnology, California, USA). The proteins were added at a 1:5000 dilution and incubated for one hour. After three washes, the membranes were incubated with an enhanced chemiluminescence (ECL) Western blotting substrate kit (Promega, Wisconsin, USA). The results were analyzed and photographed with a ChemiDoc™ Imaging System (Bio-Rad, California, USA).

2.5. Determining if sera from naturally infected bovines recognize the protein enolase

A Western blotting technique was used to analyze whether cattle naturally infected with *B. bigemina* developed antibodies against enolase. The sera were previously evaluated via the indirect fluorescence antibody test (IFAT) as described in the manual of the World Organization for Animal Health (WOAH, 2021).

The cold *B. bigemina* antigen for Western blotting was prepared from a culture of infected erythrocytes. The samples were washed several times in cold PBS with the protease inhibitor cOMPLETE Mini, EDTA-free (ROCHE, Basel, Switzerland) to ensure clarity of the supernatant. After freezing and thawing, the final pellet was suspended in a 2X lysis buffer to isolate proteins. The cold antigen extract and rBbiEno were loaded on a 12 % SDS—PAGE preparative gel and transferred to nitrocellulose membranes, which were subsequently incubated with PBS-T (0.5 %) and 5 % nonfat dry milk overnight. The membranes were cut into strips containing 3 μg of cold antigen extract, and field bovine serum samples were added at a 1:20 dilution. After overnight incubation at 4 °C and several washes with PBS-T, the membranes were incubated with a secondary anti-bovine IgG-HRP antibody (Santa Cruz Biotechnology, California, USA) at a 1:3500 dilution for one hour, and further treatment of the nitrocellulose membrane was performed as described previously.

We performed a Western blot assay to ensure no cross-reaction with the bovine enolase. 38 μg of protein extracts from uninfected and infected erythrocytes were separated on 10 % SDS-polyacrylamide gels and then electro-transferred onto nitrocellulose membranes (Bio-Rad, Hercules, USA). After blocking the membranes with 5 % non-fat dried milk in TBS containing 0.05 % Tween-20 % (TBS-T), they were washed and incubated overnight at 4 °C with rabbit antisera anti-rBbEno diluted 1:2000 in TBS-T, containing 1 % non-fat dried milk. After additional washes, the membranes were incubated with an anti-rabbit IgG conjugated with peroxidase (1:500) (Jackson ImmunoResearch) at room temperature for one h. After further washing with TBS-T, the proteins of interest were detected by chemiluminescence by using the ECL-Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, USA). Pre-immune serum diluted at 1:2000 was used as a negative control.

2.6. Generation of specific bovine antibodies against rBbiEno

Anti-enolase antibody generation was performed by immunizing an 8-month-old male steer twice with an emulsion of 1:1 (vol/vol) Montanide ISA™ 201 adjuvant (Seppic, Courbevoie, Francia) with 100 μg of the recombinant protein in PBS. A blood sample was obtained before the first immunization and 15 days after the last immunization to obtain antiserum. Subsequently, Western blotting was performed by loading rBbiEno on a 12 % SDS—PAGE gel and electroblotting it onto a nitrocellulose membrane to test the specificity of the antiserum. The membranes with 1 μg of rBbiEno were incubated with specific antiserum (1:1600) in 1 % nonfat milk powder in PBS-T for 1 hour. Proteins were visualized with peroxidase-conjugated secondary antibodies (1:3000; Invitrogen, Carlsbad, CA, USA) and detected with an ECL-Plus system (Promega, Wisconsin, USA) with a Bio-Rad ChemiDoc™ Imaging System (Bio-Rad, California, USA).

2.7. Enolase/plasminogen ligand blotting

A ligand blotting assay was performed to demonstrate rBbiEno-plasminogen (Plg) binding; two 10 % SDS—PAGE gels were run with 10 μg of rBbiEno and 10 μg of BSA as a negative control and transferred to a nitrocellulose membrane, which was blocked overnight with 5 % PBS-milk and cut into 0.4-cm strips. Afterward, 1 ml of Plg [15 μg/ml], [25 μg/ml], [35 μg/ml], or [45 μg/ml] (Sigma Aldrich, Saint Louis, USA) with 1 % PBS-BSA was added to each strip in duplicate, and the mixture was incubated for 2 h at room temperature. Next, four washes with PBS-Tween (0.5 %) were performed, 1 ml of antibody against Plg [1:1000] (Santa Cruz Biotechnology, California, USA) was added, and the mixture was incubated overnight at 4 °C. The strips were washed with PBS-T and incubated with 1 ml of anti-mouse IgG [1:3500] (Santa Cruz Biotechnology, California, USA) for 1 hour at room temperature. After four washes, the bands were visualized via the Promega ECL Western blotting Substrate Kit (Promega, Wisconsin, USA.) and visualized with a Bio-Rad ChemiDoc™ Imaging System (Bio-Rad, California).

To evaluate the specificity of the binding, we performed an inhibition assay using the lysine analog εACA (Sigma—Aldrich, St. Louis, MO, USA.). Nitrocellulose membrane strips containing 10 μg of rBbiEno were allowed to interact with 1 ml of Plg [35 μg/ml] or εACA at different

concentrations [1.5 M], [0.75 M] and [0.375 M], and the strips were incubated at room temperature for 1 hour. Further procedures were carried out as described in the previous paragraph.

The semiquantitative analysis of binding assays was performed by densitometry of the enolase bands. The intensity of the enolase protein bands was quantified using the Image Lab 4.0.1 program. The means value ± SEM of three quantifications of pixels intensity is represented. A one-way ANOVA was used to analyze the results, and $P < 0.001$ was considered significant; the results were analyzed via the GraphPad Prism 8 program. The three asterisks indicate that the value is statistically significant ($p < 0.0001$).

2.8. Plasminogen activation assay

To evaluate Plg activation in the presence of rBbiEno, an assay was conducted in a Corning Incorporated Costar (Corning Inc., Corning, NY, USA.) 96-well cell culture plate. First, the Plg [20 μg/ml] and rBbiEno [20 μg/ml] mixture was incubated for 1 hour at 37 °C in a 1.5-ml microtube. Afterward, 100 μl/well was dispensed in the microplate in triplicate, and 50 μl of tPA (tissue Plg activator human, Sigma—Aldrich, St. Louis, MO, USA.) was added at a final concentration of 500 ng/μl per well and incubated for 15 minutes at 37 °C. Next, 50 μl of Chromozyme PL [0.3 mM] (ROCHE, Basel, Switzerland) was added per well, and the mixture was incubated at 37 °C. The absorbance was read at 405 nm with a spectrophotometer (SPECTROstar Nano of BMG LABTECH, Ortenberg, Germany) at different times: 0 h, 4 min, 6 min, 8 min, 10 min, 15 min, 20 min, 25 min, 30 min, 35 min, 40 min, 50 min, 60 min, 75 min, 90 min, 105 min, and 120 min. Three conditions were used as controls: reactions without Plg, tPA, or rBbiEno. ANOVA was used for analysis of the results, followed by Tukey's test, and $P < 0.05$ was considered significant; the results were analyzed via the GraphPad Prism 8 program.

2.9. Neutralization assay

To evaluate whether anti-rBbiEno antibodies block the invasion of *B. bigemina* into erythrocytes, a neutralization assay was performed as previously reported (Hernández-Silva et al., 2018) briefly, B. bigemina Nayarit isolate was cultured *in vitro* in bovine erythrocytes in a 96-well cell culture plate with 200 μl/well with 5 % hematocrit and 1 % parasitemia in GIT medium supplemented with 40 % of each serum (pre--immune serum from cattle immunized with rBbiEno, immune serum from bovine immunized with rBbiEno and serum from a bovine immunized with adjuvant), which were decomplemented by heating to 56 °C for 30 minutes. The cells were incubated at 37 °C with 5 % CO_2 for 72 h, and the maintenance medium (150 μl) was changed every 24 h. Pre-immune bovine serum was used as a negative control. The assay was performed in triplicate.

After incubation, two μl of each well was taken, and smear duplicates were made on slides that were stained with Giemsa. For each slide, the percentage of parasitized erythrocytes was determined by counting 2000 erythrocytes. ANOVA was applied to analyze the results, followed by Tukey's test, with a significance of $P < 0.05$, using GraphPad Prism 8.

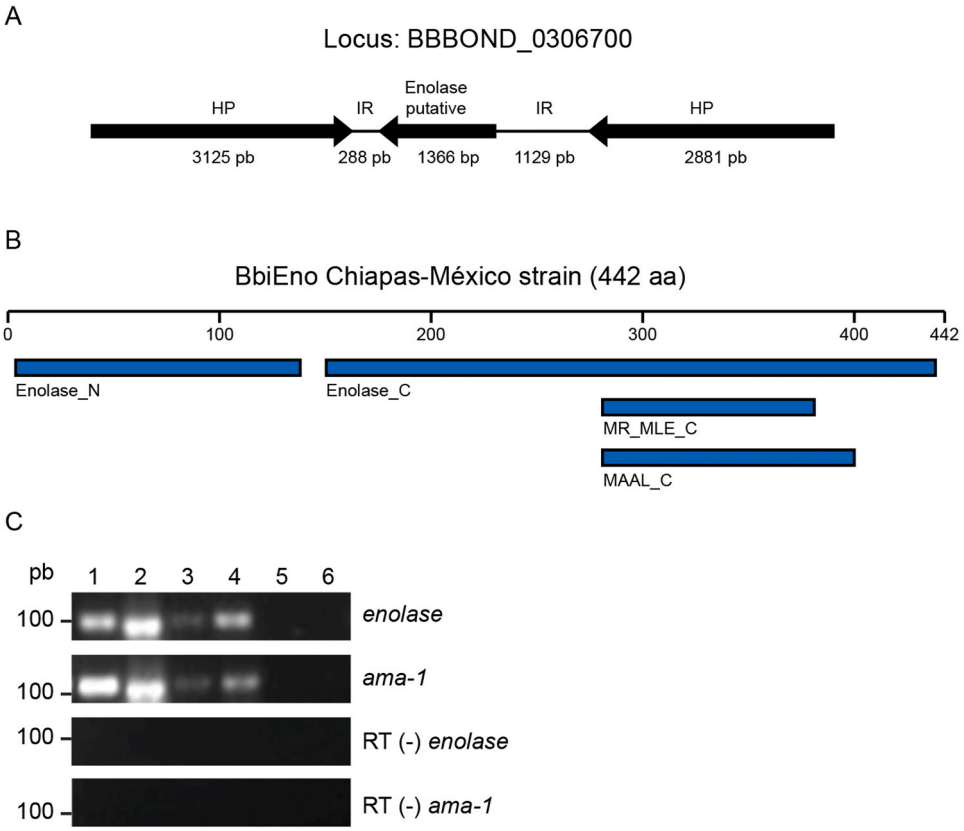

Fig. 1. Localization, analysis, and expression of the *B. bigemina* enolase gene. A. The enolase gene is located on chromosome III. The BLASTp analysis identified a sequence (XP_012768952) as enolase (2-phosphoglycerate dehydratase) putative in the GenBank, in the locus BBBOND_0306700. B. The amino acid sequence of the BbEno Chiapas strain was analyzed using MOTIF Search online software (https://www.genome.jp/tools/motif/). The complete amino acid sequence is represented in black with a ruler. Four characteristic enolase motifs are represented as blue bars. C. Expression of the *Bbieno* gene in the bovine and tick stages. RT-PCR of *Bbieno* and *ama-1* as a positive control: lane 1: infected erythrocytes; lane 2: infected midgut at zero h post-repletion; lane 3: infected midgut at 48 h post-repletion; lane 4: infected midgut at 72 h post-repletion; lane 5: uninfected erythrocyte; lane 6: uninfected midgut. The two lower panels correspond to RT controls for each sample.

3. Results

3.1. The B. bigemina enolase gene encodes a conserved protein that is expressed in the erythrocyte and tick stages of the parasite and contains the enolase signature and domains

The full *Bbieno* gene of the Chiapas, México strain was cloned and sequenced. The BbiEno protein has four characteristic motifs for enolase (Fig. 1), including enolase C (PF00113: C-terminal TIM barrel domain, positions 140–438); enolase_N (PF03952: N-terminal domain, positions 281–381); MR_MLE_C (PF13378: enolase C-terminal domain-like, positions 281–381); and MAAL_C (PF07476: methylaspartate ammonia-lyase C-terminus, positions 282–400). The predicted amino acid sequence of BbiEno showed no transmembrane region or signal peptide.

To examine whether the *Bbieno* gene is expressed in different parasite stages, we performed RT—PCR assays using RNA from infected erythrocytes and infected ticks at three post-repletion times. We found that the *Bbieno* gene is expressed in both infected erythrocytes (Fig. 1C, upper panel, Lane 1) and infected tick samples at 0, 48 and 72 h post repletion (Fig. 1C, upper panel, Lanes 2, 3, and 4, respectively). The *ama-1* gene was used as a positive control for infection in erythrocytes and tick samples, as shown in Fig. 1C (middle panel, Lanes 1–4). No reactions were observed in RNA extracted from uninfected erythrocytes and RNA from the midgut of an uninfected tick used as negative controls (Fig. 1C, upper and middle panel, Lane 5 and Fig. 1C, Lane 6 button two panels, respectively). The RT- controls showed no reactions (Fig. 1 C, bottom two panels).

Multiple sequence alignment revealed that the BbiEno amino acid sequence is highly conserved among enolases from apicomplexan parasites, ranging from 63.72 % with *T. gondii* enolase to 97.74 % with the enolase of *B. ovata* (Fig. 2). BbiEno has the enolase characteristic signature LLLKVNQIGSVTEA (amino acids 349…362, underlined in green), and the probable metal binding sites (S41, D252, E300 D327) are indicated with black asterisks.

Considering the relatively conserved nature of enolase, we conducted multiple alignments with the vertebrate host protein and the enolase of the transmitting vector *R. microplus*. This allowed us to predict the absence of cross-reaction of the sera with these proteins. The results, as shown in Figure S1, indicate that the enolase of *B. bigemina* shares a specific 20.35 % identity with the enolase of *R. microplus* and a significant 65 % with the protein of *B. taurus*; hence, we analyzed the cross-reactivity by Western blotting, results are shown in Fig. 3C.

3.2. Sera from naturally infected cattle specifically recognize rBbiEno

Nine serum samples from naturally infected cattle previously diagnosed as positive for both *B. bigemina* and *B. bovis* were evaluated via Western blotting. The results revealed that seven serum samples (those marked with asterisks) recognized the recombinant protein rBbiEno with a molecular weight of 39.8 kDa (Fig. 3A; Lanes 2, 3, 4, 5, 6, 8 and 9), in addition to the positive control of a serum from a bovine immunized with rBbiEno (Lane 11). To confirm that the positive sera of the bovines recognized the total protein extract of *B. bigemina*, we performed Western blotting using the total protein extract (Panel B). Two of the same positive serum samples barely recognized proteins in the cold extract of proteins from *B. bigemina*-infected erythrocytes (Lanes 4 and 5 of Fig. 3B). The negative control serum from a noninfected bovine did not show any protein recognition against the total protein extract (Fig. 3B, Lane 10). It is important to note that the negative serum displayed a notably low signal against the recombinant protein (Fig. 3A, Lane 10). This signal could be a result of a potential artifact of the technique rather than a cross-reaction with bovine enolase which is supported by Fig. 3C, Lane 3, where a positive antibody to enolase does not recognize any protein from uninfected erythrocytes. Importantly, this anti-rBbiEno serum recognizes the native enolase of approximately 47.5 kDa molecular weight in infected erythrocytes with high specificity (Fig. 3C, Lane 4). The preimmune serum showed no reactivity against uninfected and infected erythrocytes (Fig. 3C, Lanes 1 and 2, respectively).

3.3. rBbiEno binds plasminogen

The binding of enolase and Plg has been tested in other apicomplexan parasites; hence, we decided to evaluate the binding of rBbiEno and Plg via a ligand blotting assay in which different concentrations of Plg were used. The specific anti-Plg antibody recognized the complex rBbiEno/Plg with a molecular weight of 39.8 kDa, which is significant as it corresponds to the molecular weight of rBbiEno. In addition, we demonstrated that the signal of the complex is more intense with statistical significance as the concentration of Plg increased (Fig. 4A, Lanes 2, 3, 4, and 5 in upper and button panels), and no signal was detected when BSA was used as a negative control (Fig. 4A, Lane 6). Moreover, the specificity of this binding was evaluated with increasing concentrations of the lysine analog εACA: 0.375 M, 0.75 M, and 1.5 M, and the results revealed a decrease in the signal as the εACA concentration increased (Fig. 4B, Lanes 2, 3 and 4, respectively, upper panel). The lower panel of Fig. 4B shows that the intensity of the complex decreased with statistical significance by approximately 15 % when 0.375 M of εACA was added, and 35 % with 0.75 M, no statistical difference was displayed between 0.75 M and 1.5 M of εACA. Fig. 4C, Lane 2 shows the electrophoretic pattern of the rBbiEno protein with an apparent molecular weight of 39.8 kDa, and Fig. 4D presents the specificity of the anti-Plg antibody, which recognized Plg with a molecular weight of 92 kDa (Lane 2) and not the rBbiEno protein (Lane 3). Lane 1 in all panels shows the molecular weight marker.

3.4. The binding between rBbiEno and Plg enhances the activation of Plg

To further explore the function of *B. bigemina* enolase, we explored whether the binding of rBbiEno to Plg has any effect on plasminogen activation; hence, Plg was incubated in the presence or absence of rBbiEno and the Plg tissue activator (tPA). The results shown in Fig. 4 indicate that the conversion of plasminogen into plasmin increased when rBbiEno was added in the first hour of the kinetic curve ($P < 0.05$). The curves without tPA or Plg showed no OD at 405 nm.

3.5. Specific anti-rBbiEno antibodies neutralize the invasion of bovine erythrocytes by merozoites in vitro

To further investigate whether *B. bigemina* enolase plays a role in the invasion of erythrocytes by the parasite, we performed a neutralization test *in vitro*. Western blotting was performed to verify the specificity of the bovine serum used in the neutralization assay (Fig. 6A). The immune serum (Lane 2) recognized rBbiEno with a molecular weight of 39.8 kDa, whereas the pre-immune serum did not recognize any protein (Lane 3). The molecular weight marker is shown in the first lane.

Panel B of Fig. 6 shows the neutralization test results in a culture of bovine erythrocytes infected with *B. bigemina.* After 72 h of the test, the bovine anti-rBbiEno antibodies (immune serum) were able to neutralize approximately 65 % of the parasitemia of the red cells with *B. bigemina* (Fig. 6B, gray bar), with $p < 0.05$, compared with the pre-immune serum (Fig. 6B, black bar and the control serum from a bovine immunized with PBS plus adjuvant (Fig. 6B, white bar).

4. Discussion

The lifecycle of *B. bigemina* is complex, with asexual reproduction in the erythrocytes of the vertebrate host and sexual reproduction in the invertebrate vector *R. microplus*. The study of the molecules involved in the host—vector—parasite interface is key to understanding this interaction at the molecular level; the identified proteins could be studied as targets in the development of vaccines against the parasite (Antunes

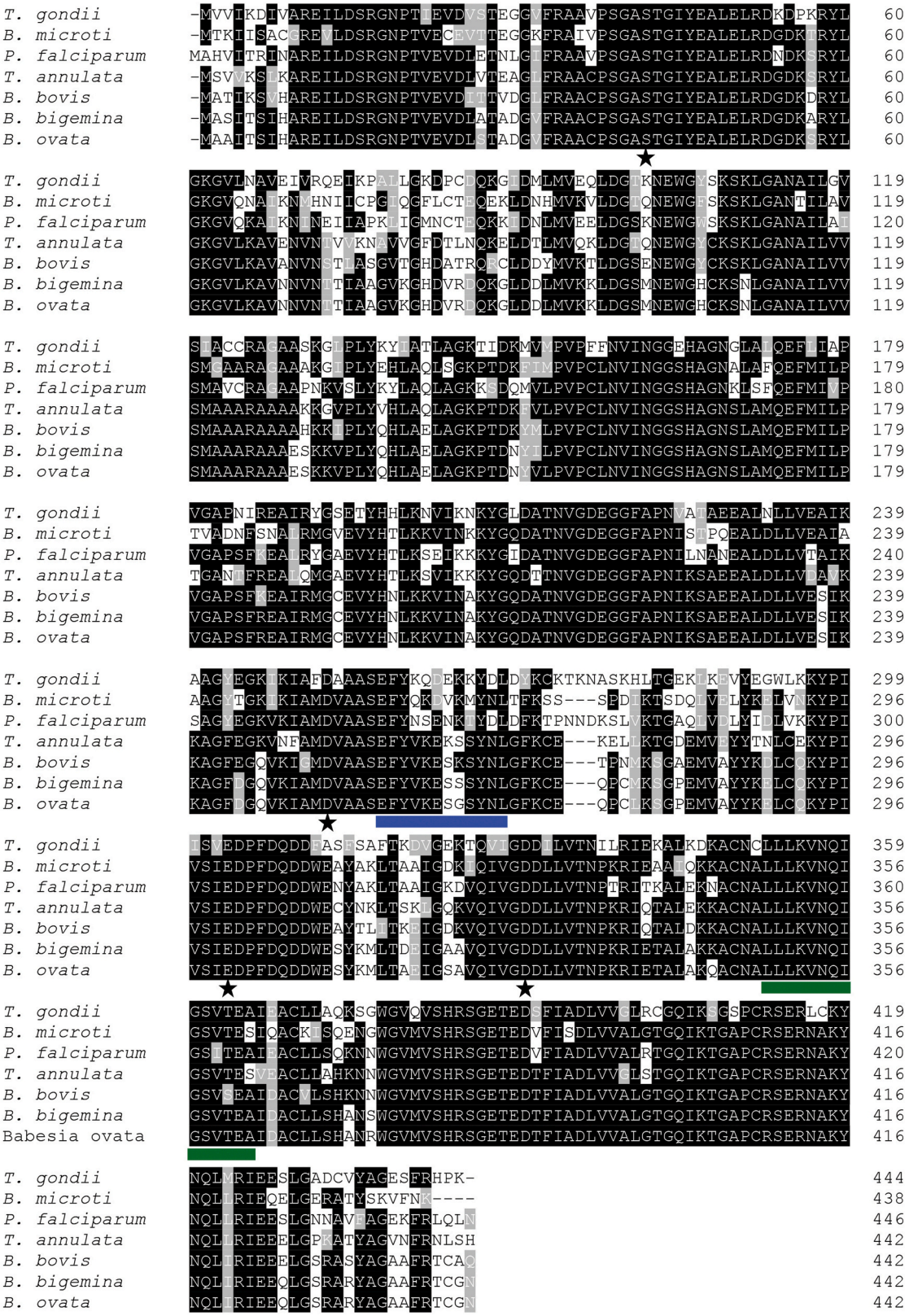

Fig. 2. Multiple alignments of BbiEno with enolase sequences of other apicomplexans. The following sequences were aligned using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/): XP_001611923.1 enolase (2-phosphoglycerate dehydratase) [*B. bovis*], XP_012649799.1 enolase [cepa RI de *B. microti*], AAA18634.1 enolase [*P. falciparum*], XP_002365579.1 enolase 1 [*T. gondii*], ADU85973.1 enolase [*T. annulata*] and XP_028865010.1 enolase [*B. ovata*] Identical amino acids are represented by black letters on a white background, and conserved substitutions have a shaded background. Black asterisks represent the metal binding sites, the enolase signature is underlined in green, and a putative motif for binding plasminogen is underlined in blue. The analysis of the amino acid residues implicated in metal binding was carried out in InterPro (http://www.ebi.ac.uk/interpro/result/InterProScan), and the enolase signature was identified with ScanProsite online software (https://prosite.expasy.org/scanprosite/).

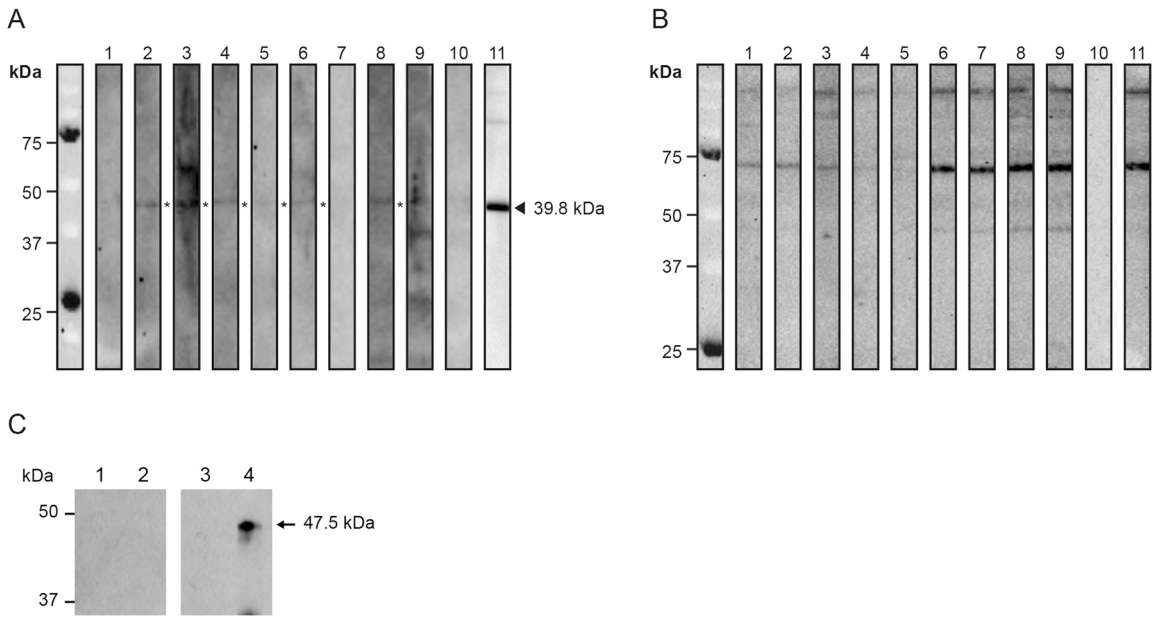

Fig. 3. rBbiEno was recognized by the sera of naturally infected cattle. 3 μg of rBbEno (A) or cold antigen (B) was incubated with 1 ml of sera of naturally infected cattle (1:20) (lane 1–9) and sera of non-infected bovine (10), as positive controls immune serum (11) in interaction with anti-bovine IgG-HRP antibody [1:3500]. The molecular weight of rBbiEno is approximately 39.8 kDa. The asterisks indicate a protein band with a molecular weight similar to the rBbiEno protein. (C) Western blot assay with protein extract of erythrocytes. Lanes 1 and 2: uninfected erythrocytes incubated with pre-immune (1:500) serum and immune serum (1:500), respectively; Lanes 3 and 4: infected erythrocytes incubated with pre-immune serum (1:500) and immune serum (1:500), respectively.

et al., 2014; Mosqueda et al., 2012); Silva et al., 2018; Yokoyama et al., 2006).

In several microorganisms, the enolase protein has been identified as a virulence factor; this protein participates in the invasion process of its target cells (Díaz-Ramos et al., 2012). In parasites such as *Trichomonas vaginalis*, enolase is located on the surface of the parasite, and its binding to plasminogen is related to the rupture of the basement membrane of the urogenital tract, which facilitates infection (Mundodi et al., 2008). In *Leishmania mexicana*, enolase is secreted in vesicles and the parasite membrane and interacts with and activates plasminogen, facilitating invasion (Figuera et al., 2013). In *P. falciparum* and *Plasmodium berghei*, enolase is also located on the surface of the parasite, and its binding to plasminogen results in the formation of plasmin, which facilitates the parasite's passage from the mosquito's intestine to the salivary glands, where it is converted into infective sporozoites (Ghosh et al., 2011). Therefore, the present study focused on analyzing the gene and enolase protein of *B. bigemina*.

The DNA sequence of the *Bbieno* gene of the Chiapas, México strain showed only one change compared with the enolase gene of the BOND strain (BBBOND_0306700) reported in GenBank; this change in nucleotides leads to a change in the amino acid residue at position 31 from a leucine to a valine; however, this change is not considered relevant since both amino acids have a nonpolar side chain.

BbiEno (Chiapas, México strain) has four characteristic enolase domains and a barrel-shaped domain at the carboxy-terminal end, which belongs to the ICL/PEPM_KPHMT protein family. This site contains the active site where the conversion of 2-phosphoglycerate to phosphoenolpyruvate in glycolysis takes place (Keller et al., 2007). Moreover, the amino acid sequence of the BbiEno Chiapas, México strain has no transmembrane regions or signal peptides; the lack of a signal peptide indicates that enolase does not pass through the endoplasmic reticulum, and the secretory pathway of the protein is unknown (López-Villar et al., 2006; Mukherjee et al., 2016). Most proteins in the cell membrane contain an N-terminal signal peptide; however, many moonlighting proteins, which are typically located intracellularly, lack a signal sequence when located on the cell surface (Amblee and Jeffery, 2015). The enolase protein is located extracellularly, in small vesicles outside the cell, and in the cell membrane, where the protein does not have enzymatic activity (Miura et al., 2012); however, there is not much information about the mechanisms used for nonconventional cellular transport pathways. For example, in *Borrelia burgdorferi*, enolase is a plasminogen receptor secreted in membrane vesicles, although the cellular mechanism by which this glycolytic enzyme is transported is unknown (Toledo et al., 2012). In *Plasmodium yoelii,* the enolase protein is encoded by a single gene. However, some isoforms of the protein with different isoelectric points are evident due to posttranslational modifications and fundamental phosphorylation, which seem to be correlated with the different locations of the protein (Pal-Bhowmick et al., 2007b). However, *Saccharomyces cerevisiae*, which is a model organism widely used to study secretory pathways, enolase, and other glycolytic enzymes, may use an unconventional secretion pathway dependent on the SNARE protein TLG2 with unknown machinery (Miura et al., 2012).

The amino acid alignment of the BbiEno Chiapas, México strain, with the enolase sequences of other apicomplexan parasites, revealed that these proteins are highly conserved and all have enolase signatures and metal binding sites. These residues are essential for causing the conformational change of the protein that activates its glycolytic activity (Avilán et al., 2011). The enolase contains a domain of plasminogen-binding lysins (K) at the C-terminal end (FYDKERKVYD) first reported for *Streptococcus pneumoniae* (Bergmann et al., 2003) and then for *T. annulate* (Mutlu et al., 2016); the binding motif of *B. bigemina* enolase (FYVKESSSYN) is similar to that of the previous enolase.

The results presented here demonstrate that the enolase gene *Bbieno* of the Chiapas, México strain is functional and is expressed at the transcriptional level in both the asexual and sexual stages of the parasite; similarly, enolase is expressed in the blood phase in humans and the midgut of the vector in different species of *Plasmodium* (Alvarado-Delgado et al., 2016). Previous reports have shown that enolase is overexpressed in the sexual stages of a great variety of parasites, facilitating the invasion process (Pal Bhowmick et al., 2009). The finding that BbiEno is also expressed in different stages of the parasite's lifecycle represents a promising finding for future studies of this protein as a vaccine candidate.

The recognition of rBbiEno by positive sera to *B. bigemina* from field

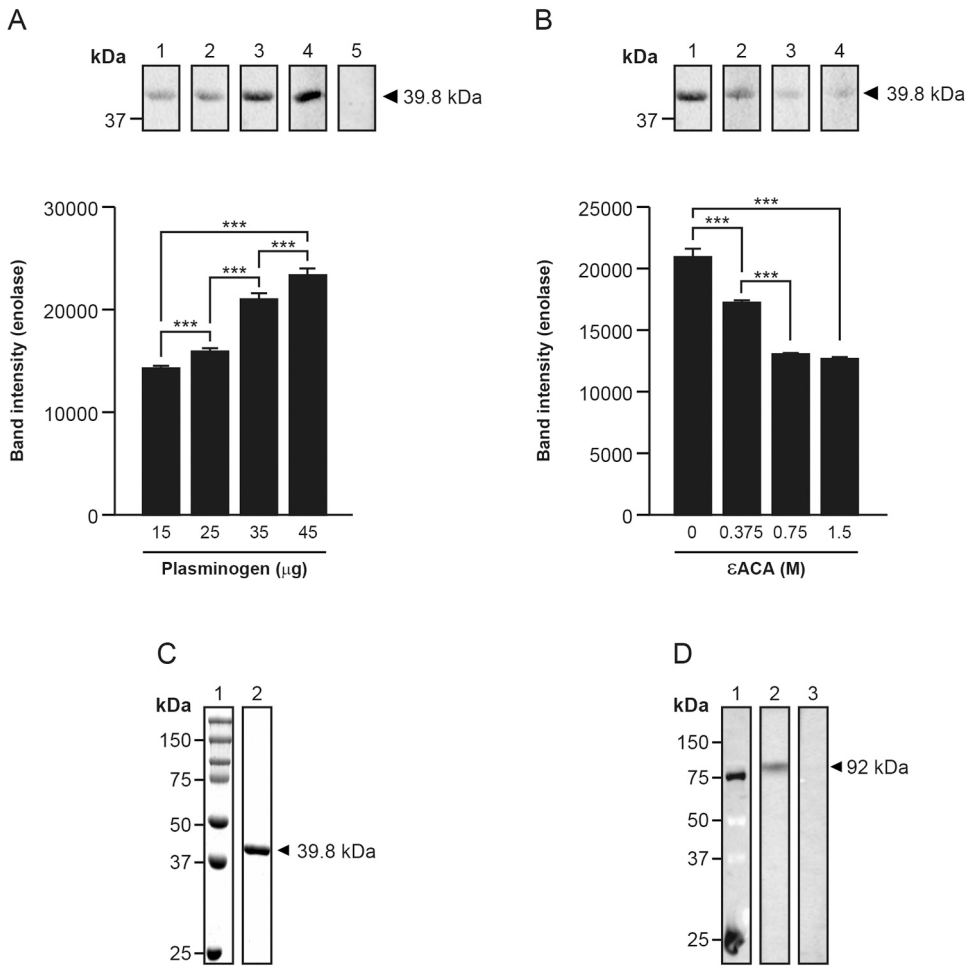

Fig. 4. rBbiEno binds to plasminogen, and its binding is inhibited by ε-aminocaproic acid. (A) Ligand blotting assay: rBbiEno (10 μg) was incubated with: 15 μg of Plg (2), 25 μg of Plg (3), 35 μg of Plg (4), 45 μg of Plg (5), 10 μg of BSA was incubated with 45 μg of Plg as negative control (6) and molecular weight marker (1). (B) Ligand blotting assay in the presence of εACA, Plg (35 μg) was added to enolase rBbiEno (10 μg), incubated with 0.375 mM of εACA (2), 0.75 mM of εACA (3) and 1.5 mM of εACA (4). Bound Plg was detected using mouse anti-Plg and molecular weight marker (1). (C) SDS-PAGE of purified recombinant *B. bigemina* enolase (rBbiEno) with a molecular weight of 39.8 kDa; (1) molecular weight marker, (2) rBbiEno protein purified by FPLC from IPTG-induced E. coli Rosetta™ 2(DE3) transformant clones. (D) Western blot of Plg (2) and rBbiEno (3), in interaction with mouse anti-Plg antibody [1:1000] and HRP-coupled mouse anti-igG [1:3500], molecular weight marker (1). In (A) and (B), the lower panels show the results of semiquantitative analysis of each band of the upper panel. The intensity of the enolase protein bands was quantified using the Image Lab 4.0.1 program. The means value ± SEM of three quantifications of pixel intensity is represented. A one-way ANOVA was used to analyze the results, and $P < 0.001$ was considered significant; the results were analyzed via the GraphPad Prism 8 program. The three asterisks indicate that the value is statistically significant ($p < 0.0001$).

bovines demonstrated that enolase is an immunogenic protein that is in contact with the bovine immune system at some point. It has been reported that even though enolase is a glycolytic metalloenzyme, it can be exposed on the membrane surface. For example, in *P. berghei* and *P. falciparum*, enolase has been found on the surface of the three stages of the parasite (Ghosh et al., 2011; Pal Bhowmick et al., 2009; Pal-Bhowmick et al., 2007a); the enolase protein of *B. microti* is located on the membrane as well (Liu et al., 2017a, 2017b); and in *Leishmania*, enolase is located both on the membrane surface and in vesicles (Avilan et al., 2000; Figuera et al., 2013; Liu et al., 2017a, 2017b).

One important function other than glycolysis that has been reported for enolase in many parasites is the binding and activation of plasminogen; hence, we investigated whether the BbiEno protein has the same ability (Xie et al., 2023). Our results show that the recombinant protein, in addition to binding, activates plasminogen. It has been demonstrated that enolase/plasminogen binding is crucial during the invasion process of parasites through the activation of Plg into plasmin, which degrades the extracellular matrix, allowing for the parasite to cross cells and continue its lifecycle (Avilan et al., 2000; López-Alemany et al., 2003a, 2003b)

The most relevant result of this work was the significant decrease in *B. bigemina* parasite invasion into bovine erythrocytes *in vitro,* which could be a promising result for BbiEno to be considered a candidate for a vaccine against babesiosis. Previous work with *P. falciparum* has demonstrated that the enolase protein has neutralizing epitopes and that specific antibodies against the protein are able to neutralize the invasion of the parasite. The EWGWS epitope is highly conserved, and when it is blocked with monoclonal antibodies, the growth of merozoites in the blood is inhibited (Dutta et al., 2018; Mukherjee et al., 2016). The amino acid sequence of the BbiEno Chiapas, México strain contains the EWGHC (positions 102…107) sequence in a conserved position related to *P. falciparum*; further investigations are needed to determine whether this sequence is a neutralizing epitope in *B. bigemina* enolase.

5. Conclusions

These data show that *Bbieno* is a functional gene of *B. bigemina* that is highly conserved among different apicomplexan parasites. It is expressed in the parasite's asexual stages and within the vector tick. Besides, BbiEno can bind and activate plasminogen, and specific anti-

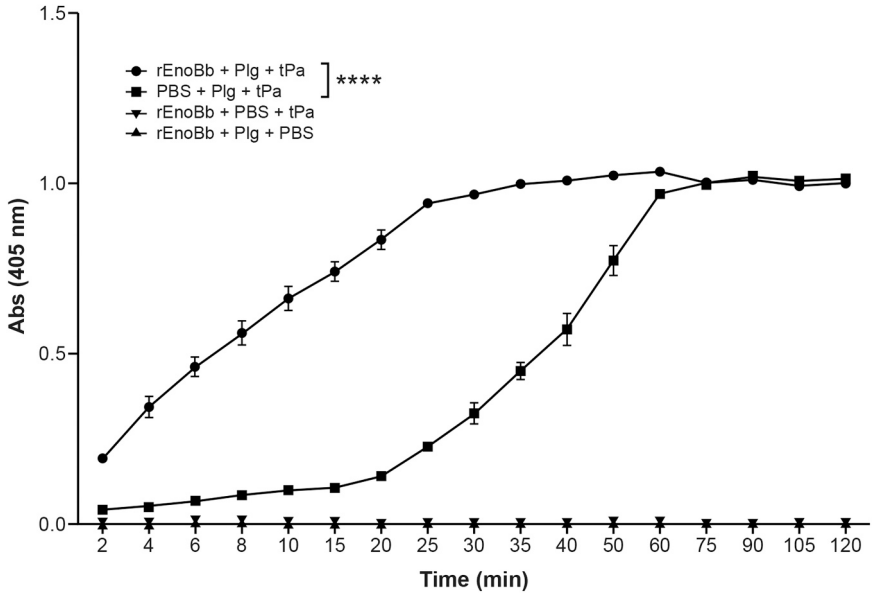

Fig. 5. rBbiEno enhances the activation of Plg in the presence of tPa. Plg was incubated with rBbiEno, and tPa was added subsequently. Plg activation was measured by adding a plasmin-specific substrate (tosyl-glycyl-polyl-lysine-4-nitranilide acetate), and the OD value was measured at 405 nm. As negative controls, wells without Plg or tPA were added. Data represent the means and standard errors from two experiments with three replicates per condition. $P < 0.05$ (****) Two-way ANOVA and Tukey's multiple comparisons test using GraphPad Prism 8 program.

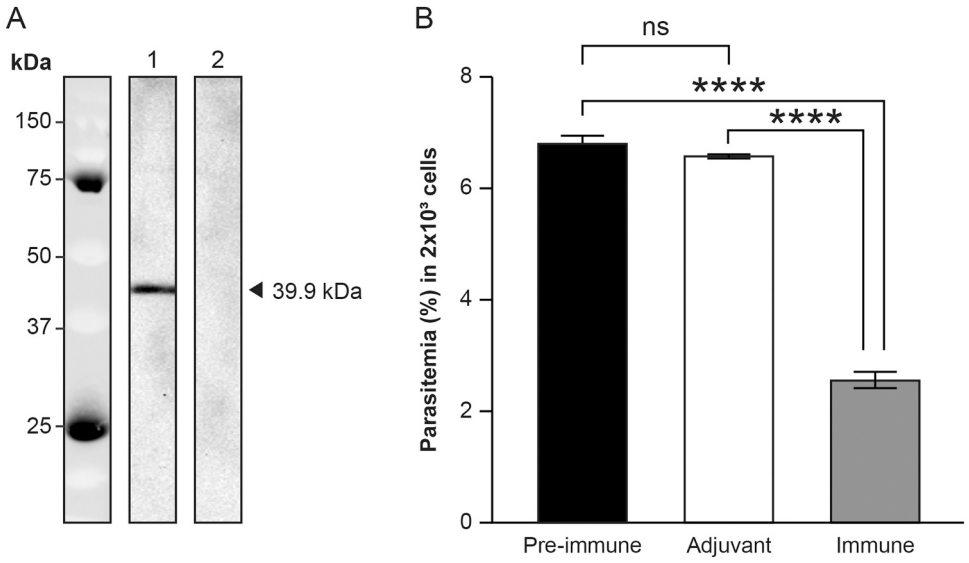

Fig. 6. Specific anti-rBbiEno antibodies decrease the bovine erythrocyte infection *in vitro*. (A) the specificity of the immune and pre-immune sera (tested by using Western blotting against rBbiEno) is shown in lanes 1 and 2, respectively; the molecular weight marker is in the first lane. (B) Culture of *B. bigemina* on erythrocytes maintained with pre-immune bovine serum (black bar), rBbiEno-immunized bovine serum (grey bar), and serum from bovine immunized with PBS plus adjuvant (white bar). The estimation of parasitemia is shown in the y-axis. Statistical analysis was carried out with an ANOVA and Tukey test using a 99 % confidence level using the GraphPad Prism 8 program. The bars indicate the standard deviation; ns, no significant difference, and asterisks indicate statistical difference. $P < 0.05$ (****).

enolase antibodies neutralize the parasite's invasion of erythrocytes *in vitro*. Therefore, it meets the main characteristics that should be studied as a vaccine candidate.

CRediT authorship contribution statement

Castañeda-Ortiz Elizabeth Jacqueline: Validation, Methodology, Investigation. **Mosqueda Juan:** Writing – review & editing, Supervision, Resources, Project administration, Funding acquisition. **Amaro-Ibarra Mariana:** Validation. **Camacho-Nuez Minerva:** Writing – review & editing, Supervision, Resources, Project administration, Methodology, Investigation, Conceptualization. **Álvarez-Sánchez María Elizbeth:** Writing – review & editing. **de La Cruz González Valeria Guadalupe:** Methodology. **Cárdenas-Flores Alma:** Writing – original draft, Validation. **Luna-Rodriguez Ana Laura:** Writing – original draft, Validation, Methodology, Funding acquisition, Formal analysis.

Ethics approval

The "Bioethics Committee" of the School of Natural Sciences, Autonomous University of Queretaro, Mexico (permission number

45FCN2017) approved the protocol for handling and bleeding the cattle and for tick collection.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2025.110503.

Data Availability

Data will be made available on request.

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