RESEARCH

Identification and molecular characterization of a novel *Babesia orientalis* rhoptry neck protein 4 (BoRON4)

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Abstract

Babesia orientalis, a protozoan parasite transmitted by the tick *Rhipicephalus haemaphysaloides*, holds significant economic importance along the Yangtze River. Key factors in the host invasion process include rhoptry neck proteins (RON2, RON4, and RON5) and apical membrane antigen 1 (AMA1). However, the intricacies of the interaction between AMA1 and RONs remain incompletely elucidated in *B. orientalis*. To better understand these crucial invasion components, the RON4 gene of *B. orientalis* (BoRON4) was cloned and sequenced. RON4 is 3468 base pairs long, encodes 1155 amino acids, and has a predicted molecular weight of 130 kDa. Bioinformatics analysis revealed a unique region (amino acid residues 109–452) in BoRON4, which demonstrates higher sensitivity to epitope activity. The BoRON4 gene was strategically truncated, amplified, and cloned into the pGEX-6p-1 vector for fusion expression. We successfully used the mouse polyclonal antibody to identify native BoRON4 in *B. orientalis* lysates. Furthermore, the corresponding BoRON4 protein band was detected in the water buffalo serum infected with *B. orientalis*, while no such band was observed in the control. Additionally, I-TASSER and Discovery Studio software were used to predict the tertiary structures of BoRON4 and its ligands, CH-PKA and CH-complex. These ligands can serve as lead compounds for the development of anti-babesiosis drugs. In conclusion, BoRON4 emerges as a promising candidate antigen for distinguishing water buffalo infected with *B. orientalis* from their normal counterparts. This study positions BoRON4 as a potential diagnostic antigen for babesiosis in water buffalo, contributing valuable insights to the field of parasitology.

Keywords *Babesia orientalis* · Rhoptry neck protein 4 · Babesiosis · Immunogenicity

Introduction

Babesiosis is a globally significant zoonotic disease caused by various species of the genus *Babesia*, parasitic protozoans belonging to the phylum Apicomplexa (He et al. 2014). *Babesia* spp. have the capability to infect a wide range of vertebrates, including humans, significantly impacting global livestock, wildlife conservation, pet health, and public health (Gohil et al. 2013; He et al. 2021, 2017; Liu et al. 2005). The main characteristics of *Babesia* infection are high fever, icterus, anemia, hemoglobinuria,

and respiratory distress syndrome (Guo et al. 2018; He et al. 2009; Schnittger et al. 2012). In 1997, *B. orientalis* was identified as a novel species within the *Babesia* genus, distinguished by variations in host susceptibility, morphology, vector competence, pathogenicity, and in vitro cultivation characteristics (Ma et al. 1989; Zhao et al. 1997, 1994). *B. orientalis* exclusively infects water buffalo, leading to significant economic repercussions for the water buffalo population and industry in central and southern China (He et al. 2017; Zhao et al. 2002).

Apicomplexan parasites, including *Plasmodium*, *Toxoplasma*, *Cryptosporidium*, *Eimeria*, and *Babesia*, utilize proteins derived from parasitic elements to invade host cells through specialized apical complexes. This complex invasion process undergoes four stages: adhesion, invasion, vacuole formation, and the sustained presence of the parasite within the host cell (Liu et al. 2005, 1997). The intricate events during protozoan invasion are mediated by apical membrane antigen 1 (AMA1) and rhoptry neck proteins

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(RONs), which leads to the formation of the moving junction and constitutes a pivotal aspect of the parasite's life cycle (Besteiro et al. 2009; Fernandes et al. 2022; Giovannini et al. 2011). RONs (RON2, RON4, RON5, and RON8) play crucial roles in host cell invasion in other apicomplexan parasites such as *T. gondii* and *P. falciparum* (Najm et al. 2023; Srinivasan et al. 2013). Deficiencies in TgRON2, TgRON4, and TgRON5 result in profound impairments of invasion and replication, with KO-RON4 resulting in a 60% reduction in invasion, underscoring the significance of RON4 in the invasion process (Guérin et al. 2017). RON4 is an exceptionally conserved protein within parasites of the apicomplexan complex (Tokunaga et al. 2019).

However, investigations of the interaction of RON proteins with siderophores are limited. While more studies have focused on RON2 and RON5 (Cuy-Chaparro et al. 2024; He et al. 2014; Richard et al. 2010; Tian et al. 2020), there is less emphasis on RON4. In this study, the RON4 of *B. orientalis* was identified and characterized for the first time, and a bioinformatics analysis was initiated to elucidate biological properties of RON4 during host invasion. This research is aimed at providing candidate antigens for diagnostics and contributing to the development of vaccine studies against babesiosis.

Materials and methods

gDNA and cDNA preparation

Blood infected with *B. orientalis* was extracted from the jugular vein of water buffaloes in Hubei Province, China, and leukocytes were removed. Five volumes of PBS suspension were added, followed by three washes with PBS. Each wash involved resuspending the mixture, centrifuging at 5000 rpm for 5 min, gently removing the white cell layer on the surface of the red cell pellet before resuspending for the third time, and finally discarding the supernatant. This process resulted in white blood cell-depleted red blood cells (Aston et al. 2014). The genomic DNA (gDNA) of *B. orientalis* was extracted from infected erythrocytes. gDNA was extracted according to the instructions of the genomic DNA kit (QIAamp DNA Blood Mini Kit, Qiagen, Germany) and stored at −20 °C. The cDNA was mainly extracted from RNA in infected erythrocytes by removing approximately 450 µL of white blood cells (FastQuant®RT kit, Tiangen Biotechnology, China) and stored at −80 °C.

Identification, amplification, and sequencing of the BoRON4 gene

To obtain the BoRON4 gene, the *B. orientalis* genome (unpublished data) was screened using the Basic Local Alignment Search Tool (BLAST) (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Using the hypothetical *B. microti* RON4 (BmRON4, Gene symbol: BMR1_01G02031) as the query, the *B. orientalis* genome was scanned and BLAST-searched for homologous genes. The RON4 complete open reading frame was compared with other protein homologs and analyzed by nBLAST to determine its sequence. Primers for cloning the BoRON4 gene were designed based on the BLAST search results with the genome sequences of *B. orientalis*. The Lasergene Protein program, part of the DNASTAR Lasergene® package (SeqMan NGen®. Version 17.5. DNASTAR, Inc. Madison, WI), predicted a segment of superior antigenic epitope activity from amino acids 109 to 452 for truncated clone expression.

The following primers were designed for cloning the BoRON4 gene: forward primer (5′-ATGTCATTCTGTGCCAAACTGGGTAC-3′) and reverse primer (5′-ATGTCATTCTGTGCCAAACTGGGTAC-3′). Recombinant plasmids were constructed using homologous recombination methods. Primers for cloning the BoRON4-T gene and the pGEX-6p-1 vector (Tianyi Huiyuan Biological Technology, Wuhan, China) were synthesized: forward primer for cloning the BoRON4 gene (5′-TACTTATATGACGGACAGTGCC-3′) and reverse primer (5′-TTCATATTTTGACAAAATCCTCTGCATATC-3′); forward primer for cloning the pGEX-6p-1 vector (5′-CAT CGT GAC TGA CTG ACG ATC-3′) and reverse primer (5′-CAG GGG CCC CTG GAA CAG AA-3′). Thermal cycling parameters included activation of Phanta Max Super-Fidelity DNA polymerase (Enzyme, Nuoweizan, Nanjing, China) at 95 °C for 5 min, 35 cycles consisting of denaturation for 30 s, annealing at 56 °C for 30 s, extension for 1 min, and final extension at 72 °C for 10 min.

Bioinformatics analysis

The BoRON4 nucleotide sequence was uploaded to the NCBI database (GenBank: QUF61461), and its amino acid sequence was compared with other RON4 sequences in the database using BLASTp. The amino acid sequence of BoRON4 was obtained using the ExPASy online tool (https://web.expasy.org/translate/). Homology to RON4 of other apicomplexan protozoa was determined through multiple sequence alignment with BLASTp on NCBI, and phylogenetic analysis was performed using MEGA 11 software (Koichiro et al. 2021). Signal peptides were predicted using the SignalP 5.0 server (https://services.healthtech.dtu.dk/service.php?SignalP-5.0), and the transmembrane region of BoRON4 was analyzed using the TMHMM-2.0 server (https://services.healthtech.dtu.dk/service.php?TMHMM-2.0). Protein tertiary structures were predicted with the I-TASSER online tool (https://zhanggroup.org/I-TASSER/), revealing structures similar to those of other

protozoa. The secondary structure, hydrophilicity, antigenic index, flexible regions, and surface probability of BoRON4 were predicted and analyzed using the PROTEAN program of DNASTAR Lasergene® software (SeqMan NGen®. Version 17.5. DNASTAR, Inc. Madison, WI).

Molecular docking

The tertiary structure of BoRON4 was predicted using the I-TASSER server (Yang and Zhang 2015; Zhang 2009). The prediction results revealed that BoRON4 can bind to the ligand CH-PKA within the CH complex of the protein structure (PDB: 4YRI). Discovery Studio 2018 Client (v18.1.100.18065) software is licensed to the State Key Laboratory of Agricultural Microbiology at Huazhong Agricultural University, Wuhan, Hubei, and ensures a comprehensive optimization and docking strategy. Discovery Studio 2018 was used to predict the receptor binding site of BoRON4 and dock the CDOCKER molecule with the ligand CH-PKA within the CH complex. The Site Finder tool in Discovery Studio was utilized to identify potential binding sites or cavities within the protein. The receptor binding site of BoRON4 was also evaluated based on characteristics such as cavity size, shape, and charge distribution. The tools available in Discovery Studio (such as Prepare Ligands) were used to optimize the ligand structure, including adding hydrogen atoms and calculating charges. The docking analysis was performed using the CDOCKER module in Discovery Studio, opening the module and setting the relevant parameters, such as docking precision and energy threshold. The previously predicted receptor binding site was specified as the docking target for initiating the CDOCKER docking calculation. The visualization tools in Discovery Studio were utilized to inspect the docking conformations and analyze the interactions between the ligand and receptor, focusing on hydrogen bonds and hydrophobic interactions. To model the three-dimensional structure of BoAMA1, three-dimensional structures of homologous proteins available in the PDB database (https://www.rcsb.org/) were used as templates for further analysis. The amino acid sequence of AMA1 was compared with these homologous proteins to construct the three-dimensional model of BoAMA1. The protein structure files of RON4 (receptor) and AMA1 (ligand) were imported into Discovery Studio. Then, preprocessing steps were performed, including removing water molecules, adding hydrogen atoms, and correcting conformational issues. The protein structure files of RON4 (receptor) and AMA1 (ligand) were import into Discovery Studio. In the ZDOCK module, RON4 and AMA1 were set as receptor and ligand, respectively. The Angular Step Size was adjusted to a recommended sampling of 15°. Distance thresholds and required binding site amino acids were set to filter out inappropriate docking poses. For smaller systems,

it is recommended to set RMSD Cutoff and Interface Cutoff values. After setting all parameters, the ZDOCK docking calculation was run. Upon completion of docking, analyze the docking results. Discovery Studio's visualization tools were utilized to view and analyze the docking results.

Expression and purification of recombinant protein

The constructed plasmids were transformed into *E. coli* BL21 (*Escherichia coli*, TransGEN, Beijing, China) and cultured at 37 °C. Induction was performed with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) at 37 °C for approximately 4.5 h. After induction, BL21 cells were collected by centrifugation at 7000 rpm for 10 min using a high-speed refrigerated centrifuge (Hitachi, Tokyo, Japan). Subsequently, the cells were re-suspended in 30 ml of PBS binding buffer (8 g NaCl, 0.2 g KCl, 3.63 g $Na_2HPO_4 \cdot 12H_2O$, and 0.24 g KH_2PO_4) and lysed using a high-pressure homogenizer at 1000 bar. Following lysing, the cell lysate was centrifuged at 10,000 rpm for 10 min at 4 °C, and the supernatant was collected and filtered through a 0.45 µm filter. The filtered supernatant was then loaded onto a GSH Purpose 4 Fast Flow column (Sangon Biotech, Jiangsu, China) and eluted with 2 mM reduced glutathione. Subsequently, the purified protein was concentrated and stored at 4 °C.

Polyclonal antibody preparation

The Freund's complete adjuvant (MilliporeSigma, Burlington, MA, USA) was thoroughly mixed with recombinant protein rBoRON4 in appropriate proportions to achieve a final concentration of 500 µg/ml. This mixture was then injected into three Kunming mice (without specific pathogens) in four separate doses. Immunizations were administered on days 0, 14, 21, and 28. One week after the fourth immunization, whole blood samples were collected from Kunming mice. The collected blood was incubated at 37 °C for 1 h, followed by overnight incubation at 4 °C. Serum was then separated by centrifugation at 600 g for 10 min and stored at −20 °C.

Western blot analysis

To evaluate the immunoreactivity of rBoRON4, SDS-PAGE was performed, and the separated proteins were subsequently transferred onto polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Kenilworth, NJ, USA) for Western blotting. The membranes were blocked with 5% skim milk (TBST) and probed with water buffalo serum infected with *B. orientalis* (1:200) and control water buffalo serum separately (37 °C, 4 h). Binding proteins were identified via Western blotting using horseradish peroxidase (HRP)-conjugated anti-bovine IgG secondary antibody (1:5000, 37

°C, 1 h, Bioss, Beijing, China). Additionally, infected erythrocyte lysates were incubated with anti-rBoRON4 mouse serum for identifying the native form of RON4 in *B. orientalis* lysates. Infected and uninfected erythrocytes lysates were separated on 12% SDS-PAGE gels, transferred onto PVDF membranes (Merck Millipore, Kenny, NJ, USA), and probed with anti-rBoRON4 mouse serum (1:200, 37 °C, 4 h) or negative mouse serum as a control for probe detection. The membranes were washed with TBST and then incubated with goat anti-mouse IgG/HRP (1:2000, Bioss) as a secondary antibody (37 °C, 1 h). These reactions were detected using enhanced chemiluminescence (ECL) detection from WesternBright™ (Advansta, San Jose, CA, United States).

Results

Molecular characterization and sequence analysis of BoRON4 gene

PCR successfully achieved specific amplification of the BoRON4 gene in genomic DNA (gDNA), cDNA samples, and negative controls (water, Fig. 1a). The results indicate that BoRON4 is consisted of 3468 base pairs with devoid of introns and encodes a protein of 1155 amino acids with a molecular weight of 130 kDa. Further analysis revealed three specific low-complexity regions in BoRON4, located at positions 179–192, 391–401, and 1070–1084 (Fig. 2a). Due to the significance of antigenic epitopes, we intentionally selected a target fragment spanning amino acids 109 to 452 (Fig. 1b) for the creation of a truncated clone. This aspect of the study laid the foundation for subsequent investigations.

### Phylogenetic and bioinformatics analyses

The amino acid sequence of BoRON4 was aligned with the sequences of two closely related species, *B. bovis* (T2Bo) (XP 001612212) and *B. ovis* (GFE55147). The comparison revealed that the sequences of BoRON4 and *B. bovis* shared 64% identity and 79% positive match, suggesting the presence of highly conserved regions in BoRON4 with potential functional significance. Comparative analysis with the sequences of *Babesia* sp. Xinjiang (XP 028872845) and *B. bigemina* (XP 028872845) showed 55% and 58% identity, respectively (Fig. 2b).

To explore the evolutionary relationship between RON4 proteins, various related species were analyzed, including *B. bigemina*, *B. ovata*, *Babesia* sp. Xinjiang, *B. bovis* (T2Bo), *T. equi* strain WA, *T. orientalis*, *T. annulata*, *B. microti* strain RI, *P. yoelii*, *P. vivax*, *P. falciparum* 3D7, and *T. gondii* (Fig. 2b and Fig. 3). The resulting phylogenetic tree grouped the RON4 proteins of *B. orientalis* and *B. bovis* (T2Bo) into one clade, while *B. ovata* and *B. bigemina* formed another. This observation suggests a close evolutionary affinity between *B. orientalis* and other *Babesia* spp., particularly with *B. ovata*. Additionally, the genus *Babesia* protozoa exhibited a clear affinity with the genus *Theileria*. The branching pattern of the phylogenetic tree, with values indicated for each branch, provided insight into the evolutionary relationships among these parasitic species.

The three-dimensional structure of BoRON4 was predicted using the I-TASSER software (Fig. 4a). Subsequently, the protein model with the highest similarity to the predicted structure of BoRON4 was selected from the PDB library, with the PDB code 5IJO (Fig. 4b). To further investigate the specific features of the predicted BoRON4 three-dimensional structure, including helix, strand, coil, and normalized B-factor, the structure was analyzed. Interestingly, the predicted structure bears a striking resemblance to that of the human nuclear pore complex (NPC). The 3D structure of BoAMA1 was derived from a homology model created by Discovery Studio software (DS, Fig. 4c). To identify the active site of RON4, the 3D structure of the BoRON4 receptor was predicted using the DS software (Fig. 4d). Subsequently, protein–protein docking (ZDOCK) was performed

Fig. 1 PCR amplification of BoRON4 gene from gDNA and cDNA of *B. orientalis*. **a** Lane M: molecular marker; lane 1: amplicon from gDNA; lane 2: negative control (water); lane 3: amplicon from *B. orientalis* cDNA. **b** Lane 1: truncated BoRON4-T gene amplification (gDNA); lane 2: truncated BoRON4-T gene amplification (cDNA); lane 3: negative control (water)
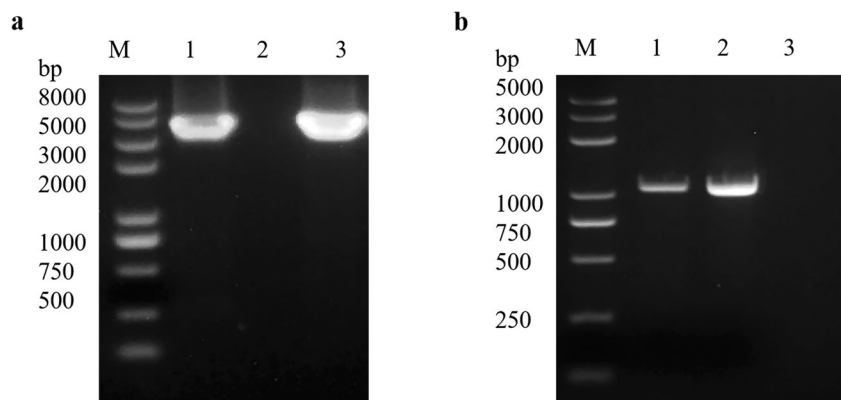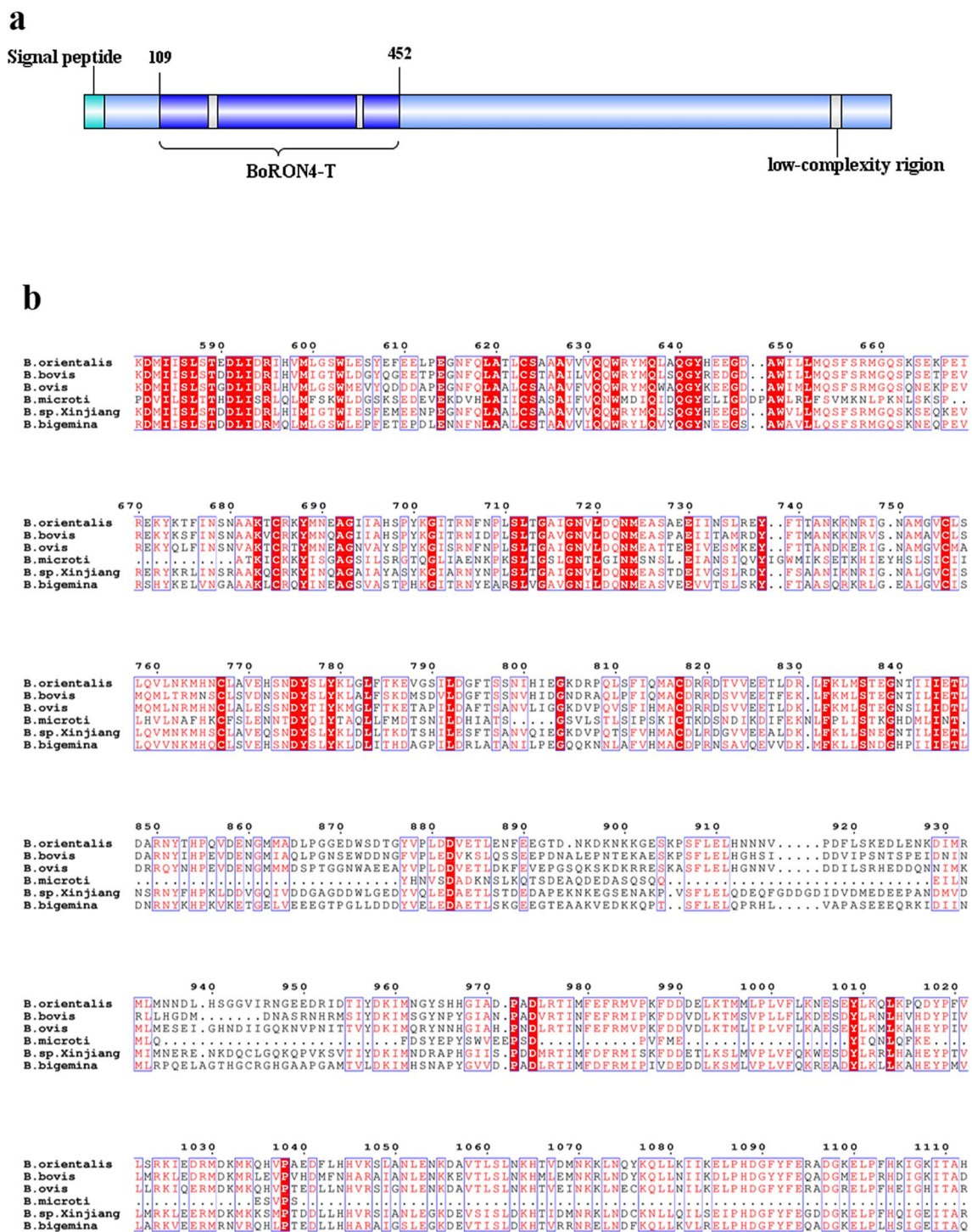
Fig. 2 Analysis of BoRON4 gene information and multi-species amino acid sequence alignment. **a** Analysis of BoRON4 gene information. Gray indicates low-complexity regions, blue denotes the BoRON4-T position, and green represents the signal peptide. **b** BoRON4 sequence alignment across various strains of apicomplexan parasites, and the sequences were aligned using ClustalW

using DS software to generate a structural map of the interaction between AMA1 and RON4 (Fig. 4e). This simulated interaction network diagram further validates the interaction between BoAMA1 and RON4, highlighting the role of RON4 in the invasion of erythrocytes by *B. orientalis*. These findings lay the groundwork for subsequent experimental work.

In addition, the interaction of RON4 with two ligands (CH-PKA and CH-complex) was further investigated. CH-PKA

Fig. 3 Molecular phylogenetic analysis of BoRON4 amino acid sequences using the neighbor-joining method. Trees were drawn to scale and clade length was measured as the number of substitutions per site. The analysis involved 13 amino acid sequences. All positions containing gaps and missing data have been eliminated. Sequences newly identified in this study are presented in bold font. Show GenBank accession numbers
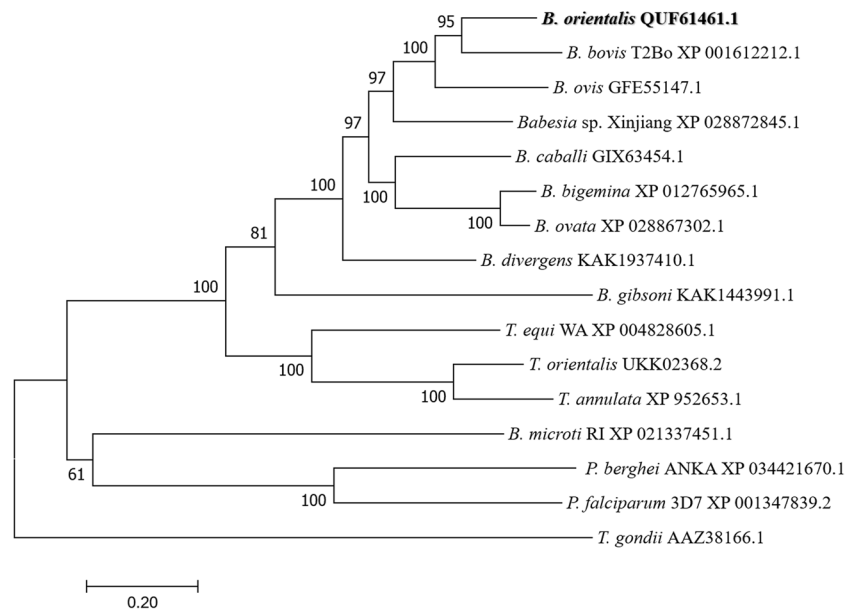
can form hydrogen bonds with ASP333, VAL366, ALA367, and LYS370 of BoRON4. Similarly, CH-complex can form hydrogen bonds with ASP333, GLN334, VAL366 of BoRON4, ALA367, LYS370, and ILE593 (Fig. 4f-g). Comprehensive bioinformatics analysis indicated that BoRON4 exhibits high similarity to other *Babesia* protozoa, suggesting conservation of RON4 among parasitic protozoa. The prediction results indicate that there are no transmembrane regions, and the probability of a signal peptide is 7.725%, located within the first 30 amino acids of BoRON4 (Fig. S1). The structural analysis predicting the interaction of RON4 with AMA1 provides a solid foundation for future studies.

Truncated BoRON4 cloning and expression of BoRON4-T

Following an extensive analysis of antigenic epitopes and low-complexity regions, a sequence spanning amino acids 109–452 of truncated BoRON4 was identified, resulting in a 344 amino-acid sequence (1032 bp). To produce this truncated protein, a recombinant plasmid with GST-tagged fusion expression was constructed and subsequently purified using Glutathione Sepharose HP.

The predicted molecular size of the truncated BoRON4 was 66 kDa; however, the actual molecular weight observed was 85 kDa (Fig. 5a). This disparity can be elucidated by previous studies on non-repeat regions in the heavy chain of Bombyx mori silk fibroin proteins. These studies have demonstrated that an increase in the number of copies of non-repeat regions leads to a larger-than-expected molecular weight for GST fusion proteins (Wu et al. 2017). The predicted molecular size of the

truncated BoRON4 was 66 kDa, but the actual molecular weight observed was 85 kDa (Fig. 5a). This disparity can be elucidated by previous studies on non-repeat regions in the heavy chain of Bombyx mori silk fibroin proteins. These studies have demonstrated that an increase in the number of copies of non-repeat regions leads to a larger-than-expected molecular weight for GST fusion proteins.

Identification of the immunoreactivity and native form of BoRON4-T

To systematically evaluate the immunoreactivity of rBo-RON4-T, immunoblot analysis was conducted in this study by reacting rBoRON4-T with serum from water buffaloes infected and uninfected with *B. orientalis*. In the serum samples from infected water buffaloes, a specific band was observed, corresponding to the molecular weight of rBo-RON4-T (85 kDa, Fig. 5b), while no corresponding band was observed in the control serum samples from uninfected water buffaloes. These findings suggest that rBoRON4-T can effectively bind to antibodies present in the serum of infected water buffaloes, demonstrating a high immunoreactivity of rBoRON4-T.

Furthermore, this study utilized co-incubation experiments with anti-rBoRON4-T serum and lysates from infected erythrocytes to identify the presence of native BoRON4. Experimental results revealed a band of approximately 130 kDa in the co-incubation samples, consistent with the expected molecular weight of native BoRON4 (Fig. 5c), while no such band was observed in the control. This result not only validates the immunological characteristics of the rBoRON4 protein but also confirms the presence of native BoRON4 protein

Fig. 4 Schematic diagram of the predicted tertiary structure of BoRON4 and BoAMA1. **a** Predicted tertiary structure of BoRON4, showing the alpha helix (red) and coils (green). **b** Tertiary structure of human nuclear pore complex (NPC) protein (PDB code: 5ioj), showing the alpha helix (red) and coils (green). **c** Discovery studio software homology modeling predicted the tertiary structure of BoAMA1. **d** Predicted receptor binding site of BoRON4, red is the helix, and green is the binding site. **e** Protein interaction structure of BoAMA1 and BoRON4, green is AMA1, and yellow is RON4. **f** The two-dimensional visual structure diagram combined with ligand CH-PKA and BoRON4. **g** The two-dimensional visual structure diagram combined with ligand CH-complex and BoRON4
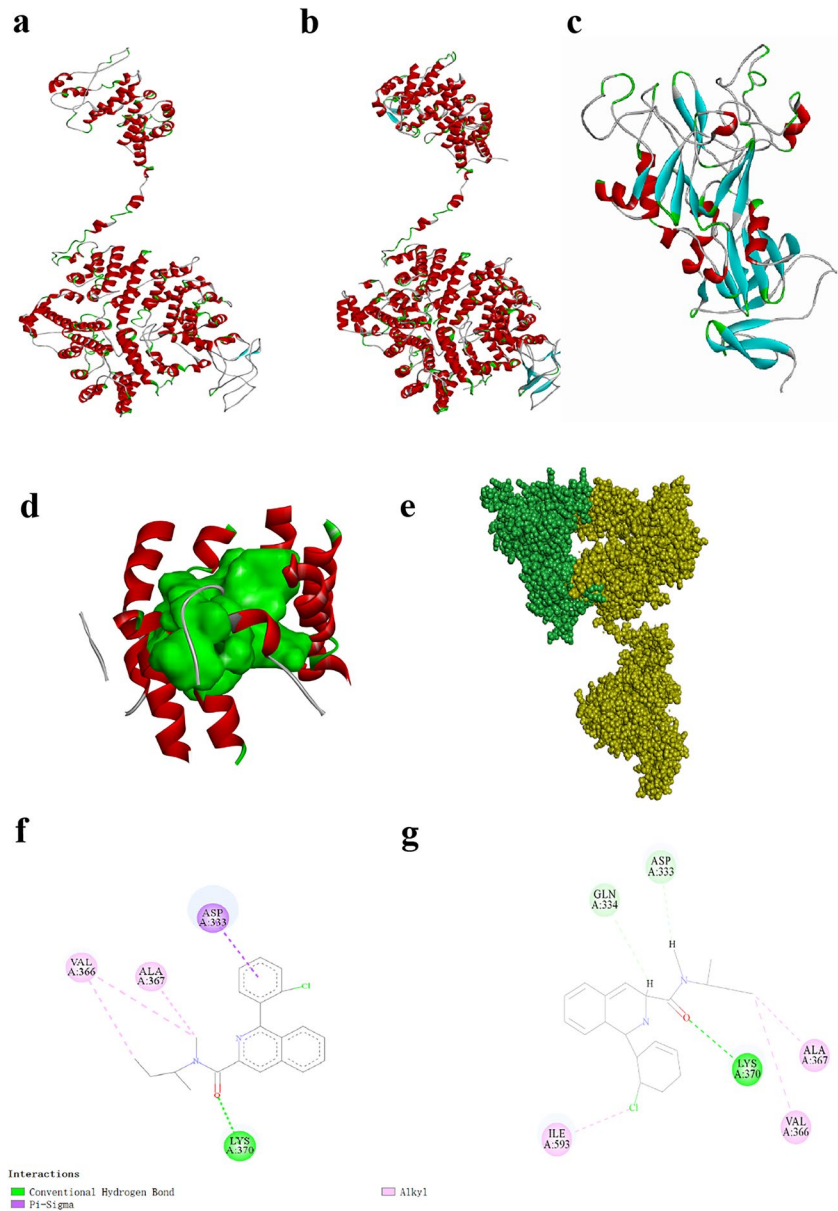
in the RBC lysates of *B. orientalis*, with a molecular weight of approximately 130 kDa. The full-length size of BoRON4 protein is indeed 130 kDa, which corresponds to the natural protein size of RON4 detected in Western blot analysis. The rBoRON4 protein indeed holds potential diagnostic value in the detection of *B. orientalis* infection.

Discussion

The Western blot (WB) results from our study provide crucial insights into the expression of the BoRON4 protein in *B. orientalis*. The BoRON4 gene was successfully cloned, purified, and characterized. Bioinformatics analysis revealed that the BoRON4 gene shares significant homology with other *Babesia* species, showing 64% similarity with *B. bovis* (T2Bo), 55% with *Babesia* sp. Xinjiang, and 58% with *B. bigemina*, indicating the relatively conserved nature of the RON4 gene among *Babesia* species. This study reports the RON4 in *B. orientalis* for the first time, addressing a gap in previous research. Our experimental results demonstrated the presence of a band around 130 kDa in water buffalo erythrocyte lysates from animals infected with *B. orientalis* when reacted with mouse anti-rBoRON4 serum. This band corresponds to the predicted molecular weight of the BoRON4 protein, confirming its expression in infected animals. The specificity of the detected band was corroborated by the absence of a similar band in the lysates of uninfected water buffalo erythrocytes, which were used as negative controls. This demonstrates that the observed band is specifically
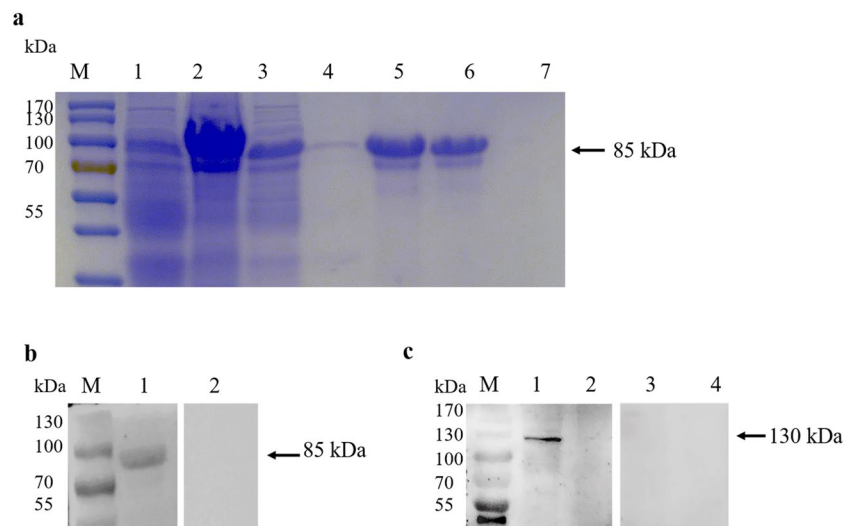
Fig. 5 SDS-PAGE analysis of expressed and purified rBoRON4, Western blot analysis of BoRON4-T immunoreactivity and native form. **a** lane M: molecular weight marker; lane 1: uninduced lysate of rBoRON4; lane 2: IPTG induced lysate of rBoRON4; lane 3: supernatant; lane 4: inclusion bodies; lane 5–6: purified rBoRON4; lane 7: negative control (water). The corresponding bands are indicated by arrows. **b** Lane M: molecular weight marker; lane 1: rBoRON4-T reaction with *B. orientalis*-infected water buffalo serum; lane 2: rBoRON4-T reaction with uninfected buffalo serum. **c** Identification of natural BoRON4 in *B. orientalis* merozoite lysates. Lane 1: lysate of *B. orientalis*-infected water buffalo erythrocytes probed with rBoRON4-T immunized mouse serum; lane 2: lysate of uninfected buffalo erythrocytes probed with rBoRON4-T immunized mouse serum; lane 3: lysate of *B. orientalis*-infected buffalo erythrocytes probed with normal mouse serum; lane 4: lysate of *B. orientalis*-infected buffalo erythrocytes probed with lysates of uninfected buffalo erythrocytes probed with serum from normal mice. The corresponding lanes are indicated by arrows

associated with *B. orientalis* infection. The use of mouse anti-rBoRON4 serum ensured that the detected protein was indeed BoRON4, and the consistency of the band size with the predicted molecular weight further validated the specificity of our antibody. Furthermore, the intensity of the band suggests a significant expression of BoRON4 in the merozoite stage of *B. orientalis*. The successful detection of BoRON4 in infected erythrocyte lysates not only confirms the presence of this protein but also suggests its potential role in the parasite's invasion mechanism, given the conserved nature of RON4 proteins among *Babesia* species. In conclusion, the Western blot analysis effectively verified the expression of BoRON4 in *B. orientalis*-infected water buffalo erythrocytes, supporting its potential as a diagnostic marker for detecting *B. orientalis* infections. This study thereby contributes valuable new information to the field of parasitology and the ongoing efforts to understand and diagnose babesiosis in water buffalo.

The N-terminus of the encoding protein comprises these low-complexity regions, with previous research indicating that tandem repeats might serve as a crucial mechanism for evading the host immune response (Ferreira et al. 2004; Hisaeda et al. 2005; Ramasamy 1998; Verra and Hughes 1999). Examination of solvent availability and hydrophobicity results highlights that the N-terminal region of PvRON4 in *Plasmodium vivax* emerges as the most exposed protein segment. This region presents itself as a promising antigenic target, hosting the highest number of potential B-cell linear epitopes. The identified repeats contribute to the expansion of solvent-exposed areas and enhance the antigenic potential of the proteins. Consequently, the N-terminal region of BoRON4 may serve as the segment exposed to the host immune system, while the repeat region may act as a mechanism for immune evasion. To substantiate this hypothesis, further studies on immunoreactivity are warranted.

Apicomplexan parasites such as *B. orientalis*, *P. falciparum*, and *T. gondii* exhibit remarkable similarities in their invasion mechanisms. Sporozoites play a crucial role in the invasion of *Plasmodium* into mammalian hosts, containing proteins such as RONs (RON2, RON4, and RON5) at their tips. Studies in *Plasmodium* have demonstrated the essential roles of PfRON4 and PfRON5 in sporozoite invasion of salivary glands (Baba et al. 2023; Pulido-Quevedo et al. 2023). The interaction between RON2 and RON4 during transport within the developing sporozoite is crucial for stability and attachment during sporozoite invasion. Combined with microneme proteins associated with sporozoite sliding, further analysis of RONs protein secretion mechanisms and interactions contributes to elucidate the comprehensive mechanism of sporozoite infection (Nozaki et al. 2020). Similarly, RON4 may represent a new target for the development of vaccines capable of recognizing the stages of *Babesia* infection. Relevant reports indicate that the anti-RON4 antibody titer is positively correlated with malaria protection (Kanoi et al. 2017; Richards et al. 2013). In the case of *B. orientalis*, the introduction of the sporophyte into buffalo occurs through the bite of *Rhipicephalus haemaphysaloides*.

This unique situation suggests the potential adaptability of BoRON4 in response to tick-borne diseases. Considering this similarity provides a basis for basic research aimed at developing treatments against such tickborne diseases.

As far as protein structure regulation is concerned, the structural analysis of BoRON4 remains uncharted territory to date. Predictions suggest that certain components of the Histidyl-tRNA synthetase in complex with 1-(3-bromophenyl) methanamine (Chem 166) of *T. cruzi* could serve as ligands for the RON4 structure (PDB number 4YRI). This ligand binds to the pocket site of HisRS in the trypanosome group and targets trypanosome drugs due to its ATP-binding properties. Based on this research, high-throughput virtual screening (HTVS), small molecule calculations, and trypanosome free energy dynamics and molecular dynamics simulations have led to the successful identification of five potential drugs against trypanosomes (Koh et al. 2015; Vetrivel and Nagarajan 2018). This suggests that the design modification of the BoRON4 ligand, 4YRI, may lead to the development of an effective inhibitor of babesiosis, thus paving the way for the development of lead compounds against babesiosis.

The 2D interaction between BoRON4-CH complex and BoRON4-PKA was successfully visualized using a combination of molecular dynamics simulations and molecular docking methods. While CH-PKA (PubChem CID: 1345) has been commercialized as PK11195, the Median IC50 values for PK11195 were 14.2 μM for *L. amazonensis*, 8.2 μM for *L. major*, and 3.5 μM for *L. braziliensis* (Guedes et al. 2018). This indicates that CH-PKA has the potential to be a lead compound.

It is important to note that the ligands considered are quinoline molecules, which are effective against both *Plasmodium* and *Babesia* parasites. Quinolines, such as chloroquine, amodiaquine, and tafenoquine, strongly inhibit purine nucleotide activity and hemoglobin metabolism in *Plasmodium*. Tafenoquine (TAF) is an 8-aminoquinoline that has shown efficacy in erasing acute *Plasmodium* infections (Liu et al. 2021). Based on these findings, tafenoquine is a promising candidate for the treatment of babesiosis. The BoRON4 receptor binding site is expected to be the drug target, designing compounds that inhibit invasion by impeding the RON4 receptor binding site. These findings highlight the great promise of BoRON4 as a potential candidate for vaccine development and disease diagnosis.

Conclusions

In summary, this study identified and characterized BoRON4, a novel protein of *B. orientalis*. BoRON4 exhibits good immunoreactivity, making it a potential candidate for the diagnosis of *B. orientalis*. Furthermore, RON4-related ligands, such as CH-PKA and CH-complexes, have been regarded as drug targets for the prevention and control of *B. orientalis* and other tick-borne diseases.

Declarations

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