Original Article

Serum proteomic profile of subclinical *Babesia bovis* infection in PCR-positive Eld's deer using serum protein electrophoresis coupled with LC-MS/MS

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Abstract

Subclinical babesiosis observed in captive Eld's deer, which act as reservoirs for ticks, poses a risk to healthy herds. However, the knowledge of biomarkers for subclinically infected Eld's deer and host-parasite interactions is limited. This study aimed to investigate the serum proteomic profiles and protein interactions of *Babesia bovis* and subclinically infected Eld's deer, PCR-positive for *B. bovis*, using automated serum protein electrophoresis and liquid chromatography-tandem mass spectrometry (LC-MS/MS). The findings revealed albumin and four globulin fractions: alpha-1, alpha-2, beta, and gamma globulin. Albumin protein (ALB) was consistently detected across all fractions, highlighting its role in maintaining osmotic balance and as a carrier protein. The study also identified scaffold protein involved in DNA repair (SPIDR), a genome stability-maintaining protein, in all globulin fractions. Eld's deer proteins, such as phospholipid transfer protein C2CD2L (C2CD2L), serpin A3 (SERPINA3), and various immune-related proteins were expressed, indicating a host response to *B. bovis* infection. Protein interaction network analysis revealed that the alpha-1 and beta globulin fractions were enriched in pathways associated with the acute phase response and immune defense. Additionally, interactions within the gamma globulin fraction were enriched in telomerase RNA binding, emphasizing cell proliferation. Additionally, Eld's deer C2CD2L interacted with *B. bovis* heat shock protein 70 (HSP70), indicating that *B. bovis* could potentially stimulate the immune response of the infected host. In summary, this study provides insights into the molecular mechanisms of host-pathogen interactions, highlighting the complexity of immune responses during *B. bovis* infection.

Keywords: *Babesia bovis*, Eld's deer, gel electrophoresis, serum proteomic profile, immune response

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Introduction

Siamese (*Rucervus eldii siamensis*) and Burmese Eld's deer (*Rucervus eldii thamin*) are unique and native to Southeast Asia. This species is primarily distributed across the lowland forests and grasslands of Myanmar, Cambodia, Laos, Vietnam, and Thailand (Gray *et al*., 2015). Eld's deer face significant threats to their survival globally. According to the International Union for Conservation of Nature (IUCN), Eld's deer is listed as Endangered (EN) primarily due to habitat loss, hunting, and human encroachment (Gray *et al*., 2015). A warm and humid climate in Thailand provides an ideal environment for the proliferation of ticks and other blood-sucking insects. Reports of Ixodid ticks and deer keds (*Lipoptena* spp.) infesting Eld's deer increase their risk of babesiosis infection (Wattanamethanont *et al*., 2018; Tiawsirisup *et al*., 2023). *Babesia bovis* is one of the most significant species of *Babesia*, capable of causing severe clinical manifestations such as fever, anemia, jaundice, hemoglobinuria, and death (Bock *et al*., 2004). However, many infections in wildlife are often asymptomatic or subclinical, posing a significant risk to herd health. These underdetected infections act as reservoirs, allowing ticks to spread the disease to healthy animals within the population (Kirupananthan *et al*., 2016). Traditional diagnostic methods, such as thin blood smears, often fail to detect the parasite when its load in the blood is low (Nayel *et al*., 2012). Consequently, other serological techniques such as enzyme-linked immunosorbent assay, indirect fluorescent antibody test, and western blotting have been employed (Ryan *et al*., 2001; Thammasirirak *et al*., 2003; Swai *et al*., 2007). Despite these methods, there remains a need for more advanced diagnostic techniques due to their limitations in sensitivity and specificity. To improve disease detection in captive Eld's deer, research on new biomarkers suitable for advanced diagnostic tools is required. The application of PCR assay targeting the conserved spherical body protein 2 (*sbp-2*) gene of *B. bovis* to detect parasitic DNA in blood samples has been reported from both cattle and Eld's deer (Jirapattharasate *et al*., 2016; Srionrod *et al*., 2022; Pumpitakkul *et al*., 2024).

Protein electrophoresis and proteomic profiling are essential tools for health monitoring and diagnosis. For example, plasma protein electrophoresis has been used to analyze the health status of both fawn and adult white-tailed deer (Cray *et al*., 2019). Serum proteomics has also proven effective in revealing alterations in protein expression due to parasitic infections. In African buffalo infected with *Theileria parva*, serum proteomics identified several upregulated acute-phase proteins (Maboko *et al*., 2021). Similarly, in deer infected with *Fascioloides magna,* serum proteomics highlighted changes in proteins related to inflammation and immune responses (Šimonji *et al*., 2022). Additionally, in southern white rhinoceros (*Ceratotherium simum simum*), combining serum protein electrophoresis with mass spectrometry revealed changes in serum protein concentrations and identified candidate proteins associated with serum protein fractions in healthy and various stages of tissue trauma (Hooijberg *et al*., 2018).

The present study aimed to investigate the serum proteomic profiles and predict host-interactions of *B. bovis* and subclinically infected Eld's deer that tested PCR-positive for *B. bovis* across different serum protein fractions, using automated electrophoresis coupled with liquid chromatography-tandem mass spectrometry (LC-MS/MS). The findings provide detailed protein information for each serum protein fraction, which could be instrumental in identifying potential proteins to develop as biomarkers or test kits for investigating subclinical infection in Eld's deer.

Materials and Methods

***Animals*:** Samples were collected from 9 Eld's deer (6 males, 3 females) reared at Nakhon Ratchasima Zoo (Nakhon Ratchasrima province) under the Zoological Park Organization of Thailand under the Royal Patronage of H.M. The King (ZPOT) and Huai Kha Khaeng Wildlife Breeding Center (Uthai Thani province) under the Department of Natural Parks, Wildlife and Plant Conservation (DNP). Samples were taken during routine veterinary examinations, with protocol approval from the Chulalongkorn University Animal Care and Use Committee (CU-ACUC), Thailand (Approval number 2031071). The details of Eld's deer samples used in this study are provided in Supplementary Table 1. All animals appeared clinically normal and showed no signs related to babesiosis. Additionally, these Eld's deer tested positive for nPCR as reported in a previous study (Pumpitakkul *et al*., 2024).

***Sample preparation*:** Microscopic examination of thin blood smears was performed as the gold standard for parasite identification, following established protocols (Böse *et al*., 1995). Additionally, whole blood samples were then centrifuged at 3000 ×*g* for 15 min at 4°C to collect serum. A halt protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) was added to the serum to prevent protein degradation. Samples were then kept at –20°C until they were submitted for automated serum protein electrophoresis and proteomic analysis.

***Determination of serum protein profile using automated serum protein gel electrophoresis*:** Total protein concentration was determined by the Lowry protein method using bovine serum albumin as a protein standard. Serum protein samples were then electrophoresed in SPIFE split beta SPE gel and run on an automated Helena SPIFE 3000 electrophoresis analyzer (Helena Laboratories, Beaumont, TX, USA) in accordance with the manufacturer's protocol. Briefly, 15 µL of each sample was loaded for agarose gel electrophoresis at 650 V for 6 min. The serum protein bands were stained with 0.5% (w/v) acid blue stain and destained in 0.3% (w/v) citric acid. The gel was dried and scanned on the Helena QuickScan 2000 densitometer (Helena Laboratories). The protein fractions were interpreted, including albumin, alpha-1 globulin, alpha-2 globulin, beta globulin and gamma globulin.

***In-gel serum protein digestion and LC-MS/MS analysis*:** To quantify the proteomic profiling within each fraction, we conducted an additional agarose gel electrophoresis, loading 50 µg of protein samples. The gel was stained using 0.1% (w/w) Coomassie brilliant blue R250 for 30 min, followed by destaining in a solution comprising 50% methanol, 10% glacial acetic acid, and 40% Milli-Q water for one hour. Each protein fraction was divided into seven segments corresponding to specific bands on the gel: one segment each for albumin, alpha-1 globulin, alpha-2 globulin, and beta globulin, and three segments for gamma globulin, as the gamma globulin fraction appeared as a broad smeared band. These segments were preserved in 0.1% glacial acetic acid at 4°C until further analysis.

Subsequently, in-gel tryptic digestion was performed according to the in-house protocol developed by the Functional Proteomics Technology Laboratory, National Center for Genetic Engineering and Biotechnology, Thailand. In brief, the gel pieces were homogenized, dehydrated using 100% acetonitrile (ACN), and dried. Disulfide bonds were reduced with 10 mM dithiothreitol in 10 mM ammonium bicarbonate (AMBIC), and sulfhydryl groups were alkylated with 100 mM iodoacetamide in 10 mM AMBIC. The gel pieces were dehydrated with 100% ACN twice for 5 min. For in-gel digestion, the samples were dissolved in 50 ng/µL sequencing-grade modified trypsin (Promega, Madison, WI, USA) overnight at 37°C. The digested samples were dried and protonated with 0.1% formic acid before being subjected to the analysis of LC-MS/MS.

The gradient-eluted peptides were analyzed using an Ultimate 3000 Nano/Capillary LC system (Thermo Fisher Scientific) coupled to a hybrid quadrupole time-of-flight (Q-TOF) impact II (Bruker Daltonics, Bremen, Germany) equipped with a Nano-captive spray ion source. In short, digested peptides were separated using a nanocolumn (PepSwift monolithic column, 100 µm inner diameter × 50 mm). Eluent A consisted of 0.1% formic acid in water, while Eluent B comprised 0.1% formic acid in 80% ACN. Peptides were eluted using a linear gradient from 10% to 70% Eluent B over 13 min at a 300 nL/min flow rate. This process included a regeneration step with 90% Eluent B and an equilibration step with 10% Eluent B. The total run time was 20 min. Peptide fragment mass spectra were obtained in data-dependent AutoMS mode, with a scan range of 300–1,500 m/z, three averages, and up to five precursor ions from the MS scan ranging from 50 to 3,000 m/z. Each sample underwent LC-MS/MS analysis in triplicate.

***Bioinformatics and data analysis*:** Protein quantification was carried out on DeCyder MS Differential Analysis software (GE Healthcare, Chicago, IL, USA). The raw mass spectrometry data were converted, and the PepDetect module was employed for automated peptide identification, charge state assignments, and quantification using peptide ion signal intensities in MS mode. The MS/MS data were analyzed against the annotated protein sequences of Eld's deer in FASTA format (Pumpitakkul *et al.*, 2023), as well as the *B. bovis* protein database (taxon ID: 5865, proteome ID: UP000002173) available in UniProt (http://www.uniprot.org/) using MASCOT software (Matrix Science, London, UK). The parameters set included digesting enzyme (trypsin), fixed modifications (carbamidomethylation of cysteine), variable modifications (oxidation of methionine residues and acetylation of the protein N-terminus), mass values (monoisotopic), peptide mass tolerance (1 Da), peptide mass (unrestricted), fragment mass tolerance (± 0.4 Da), peptide charge state (1+, 2+ and 3+) and maximum missed cleavage (1).

Gene ontology (GO) annotations in terms of biological process and molecular function categories were acquired from UniProtKB/SwissProt. The functional enrichment annotation was analyzed using the Database for Annotation, Visualization, and Integrated Discovery (DAVID) (Huang *et al.*, 2009; Sherman *et al.*, 2022). These identified proteins were then submitted to STRING (v 12.0) to determine the protein-protein interactions and potentially related pathways between Eld's deer and *B. bovis* (Szklarczyk *et al.*, 2023). A significant enriched pathway was considered at FDR < 0.05.

Results

***Serum protein profiles of SED and BED*:** The samples used in this study showed negative results for *B. bovis* in the thin blood smear examination but tested positive with nPCR. Serum protein profiling using electrophoresis revealed separation into albumin and 4 globulin fractions, including alpha-1, alpha-2, beta, and gamma globulin. The concentration levels of these serum protein fractions, along with total protein and A/G ratio were also interpreted (Table 1). A representative electrophoretogram was presented in Fig. 1.

Table 1 The relative concentrations of serum protein fractions and albumin/globulin ratio (A/G) in Eld's deer used in this study.

Parameter	Concentration (mean ± S.E.M.)
Total protein (g/dL)	7.94 ± 0.37
Albumin (g/dL)	3.47 ± 0.12
Alpha-1 globulin (g/dL)	0.65 ± 0.04
Alpha-2 globulin (g/dL)	1.01 ± 0.11
Beta globulin (g/dL)	1.21 ± 0.06
Gamma globulin (g/dL)	1.60 ± 0.13
A/G ratio	0.79 ± 0.03

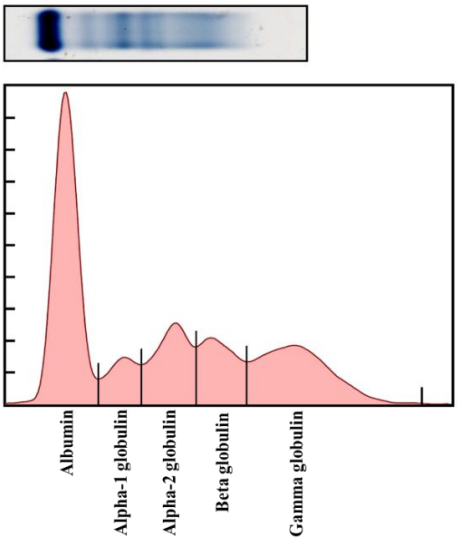


Figure 1 Representative serum protein electrophoresis pattern of subclinically B. *bovis*-infected Eld's deer (ED52MMy)

***Eld's deer and B. bovis proteins found in all serum protein fractions of subclinically infected Eld's deer that tested PCR-positive for B. bovis*:** The proteins of Eld's deer observed in albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin fractions were 2, 6, 3, 20 and 59 proteins, respectively (Table 2). Notably, albumin (ALB) was the only protein detected across all fractions using the Venn diagram. The scaffold protein involved in DNA repair isoform X2 (SPIDR) was presented in all globulin fractions, while palmitoyltransferase ZDHHC12 (ZDHHC12) was observed in three globulin fractions, including alpha-1, alpha-2, and gamma globulin (Fig. 2A).

Focusing on each serum protein fraction, phospholipid transfer protein (C2CD2L) was another identified protein expressed in the albumin fraction. The major proteins in alpha-1 globulin fraction were serpin A3 (SERPINA3) and lipolysis-stimulated lipoprotein receptor isoform X1 (LSR). Interestingly, the beta and gamma globulin fractions included proteins related to the variable domain of immunoglobulins and complements of Eld's deer, such as immunoglobulin gamma-2 chain C region (IGHG2), complement C4-like isoform X3 (C4A) immunoglobulin epsilon heavy chain (IGHG), immunoglobulin lambda variable 2-8 (IGLV2-8). Additionally, several types of keratin proteins were also seen in both fractions. Other major proteins observed in gamma globulin fraction included those involved in metabolisms, such as palmitoyltransferase ZDHHC5 (ZDHHC5), glucose-6-phosphate 1-dehydrogenase isoform X1 (G6PD), and bisphosphoglycerate mutase (BPGM). Proteins regulating the cell cycle included tudor domain-containing protein 1 (TDRD1), endonuclease-reverse transcriptase (POL), PHD finger protein 10 isoform X1 (PHF10), AT-hook DNA-binding motif-containing protein 1 (AHDC1) and zinc finger protein with KRAB and SCAN domains 8-like (ZNF8). Among these, the significant functional enrichment of proteins in the beta globulin fraction was complement activation via classical pathway (GO: 0006958, GO level: biological process, FDR = 2.7E-2) and intermediate filament organization (GO: 0045109, GO level: biological process, FDR = 2.2E-3). Similarly, the gamma globulin fraction showed significant enrichment in complement activation via the classical pathway (GO: 0006958, GO level: biological process, FDR = 4.2E-1).

In the context of *B. bovis* proteins, the proteins observed in albumin, alpha-1 globulin, alpha-2 globulin, beta globulin, and gamma globulin fractions were 17, 6, 3, 9, and 111 proteins, respectively (Table 3). No proteins of *B. bovis* were found across all fractions. However, ubiquitinyl hydrolase 1 (USP1), mitogen-activated protein kinase (MAPK), and SET domain-containing protein (SET) were detected in two globulin fractions: alpha-1 and gamma globulin (Fig. 2B).

In the albumin fraction, the *B. bovis* proteins detected included chaperonin-related proteins such as heat shock protein 70 (HSP70), ATP-dependent protease La family protein (LONP), and chaperonin cpn60 (HSPD1). The alpha-1 globulin fraction contained major proteins related to immune response, including variable erythrocyte surface antigen-1 (VAR1). In the beta globulin fraction, the predominant proteins identified in all samples were involved in cellular bioenergetics, such as ubiquinone biosynthesis O-methyltransferase family protein (COQ3), aconitate hydratase (ACO1), and flavodoxin domain-containing protein (FDX1). Lastly, the gamma globulin fraction revealed proteins associated with vesicle mediated transport proteins, including adaptin N terminal region domain containing protein (AP1G1), rab11b protein (RAB11B), t-SNARE coiled-coil homology domain-containing protein (STX), and FYVE-type domain-containing protein (ZFYVE). Additionally, proteins involved in the central dogma processes were identified, such as splicing factor 3b, subunit 3 (SF3B3), trimethylguanosine synthase (TGS1), pumilio-family RNA binding repeat domain containing protein (PUM), C3H1-type domain-containing protein (ZC3H1) and DNA-directed RNA polymerase subunit (POLR).

Table 2 List of Eld's deer proteins found in all serum protein fractions of subclinically *B. bovis*-infected Eld's deer. Gene Ontology (GO) annotations for biological process and molecular function categories were retrieved from UniProtKB/SwissProt.

Protein name	Peptide	Average log_2 protein intensity	GO annotation in biological process category	GO annotation in molecular function category
Albumin fraction:				
Phospholipid transfer protein (C2CD2L)	LDSPSRSPSK	18.75	phospholipid transport	phospholipid binding
Albumin (ALB)	AACLLPK	10.91	regulation of blood osmotic pressure	metal ion binding
Alpha-1 globulin fraction:				
DNA repair-scaffolding protein isoform X2 (SPIDR)	VVTSPVLR	16.51	DNA repair	DNA binding
Palmitoyltransferase ZDHHC12 (ZDHHC12)	VAVSPVPR	14.09	protein palmitoylation	protein-cysteine S-palmitoyltransferase activity
Albumin (ALB)	AACLLPK	13.35	regulation of blood osmotic pressure	metal ion binding
Serpin A3 (SERPINA3)	DTQSIIFLGK	12.55	negative regulation of peptidase activity	protease binding
Lipolysis-stimulated lipoprotein receptor isoform X1 (LSR)	APALRQR	10.84	lipid metabolic process	lipoprotein receptor activity
Myosin-VIIa isoform X1 (MYO7A)	GKDRLWNHTR	10.27	Cellular component organization	Actin binding
Alpha-2 globulin fraction:				
DNA repair-scaffolding protein isoform X2 (SPIDR)	VVTSPVLR	16.32	DNA repair	DNA binding
Palmitoyltransferase ZDHHC12 (ZDHHC12)	VAVSPVPR	14.08	protein palmitoylation	protein-cysteine S-palmitoyltransferase activity
Albumin (ALB)	AACLLPK	11.95	regulation of blood osmotic pressure	metal ion binding
Beta globulin fraction:				
DNA repair-scaffolding protein isoform X2 (SPIDR)	VVTSPVLR	16.36	DNA repair	DNA binding
Albumin (ALB)	AACLLPK	13.14	regulation of blood osmotic pressure	metal ion binding
Phospholipid transfer protein (C2CD2L)	CDCCDYSCK	12.51	lipid transport	lipid binding
Ig gamma-2 chain C region (IGHG2)	CKVYNEGLPAPIVR	12.40	immune response	antigen binding
Complement C4-like isoform X3 (C4A)	AEFQGVLEK	12.40	complement activation	antigen binding
Immunoglobulin epsilon heavy chain (IGHG)	GSYSCEVTHEGSTVAK	10.61	immune response	antigen binding
Immunoglobulin lambda variable 2-8 (IGLV2-8)	EETQMCVWTWTPGGAR	10.46	immune response	antigen binding
Haptoglobin (HP)	AGDGVYTFNNK	10.31	acute-phase response	hemoglobin binding
Protein C8orf37 homolog (C8orf37)	LGTVELPR	8.34	ciliogenesis	protein binding
Sister chromatid cohesion protein PDS5 homolog A isoform X4 (PDS5A)	NENNSHAFMKK	8.01	sister chromatid cohesion	chromatin binding
Serpin A3-1 (SERPINA3)	DTQSIIFLGK	7.99	negative regulation of peptidase activity	protease binding
Keratin, type I cytoskeletal 10 (KRT10)	DAEAWFNEK	7.53	epidermis development	structural molecule activity
Joining Chain Of Multimeric IgA And IgM (JCHAIN)	DIETCYTYDR	7.25	immune response	immunoglobulin receptor binding
C4b-binding protein alpha chain (C4BPA)	DASLEASER	7.35	immune response	protein binding
Keratin, type I cytoskeletal 14 (KRT14)	ALEEANADLEVK	6.92	keratinocyte differentiation	structural molecule activity
Keratin, type II cytoskeletal 6A (KRT6A)	AQYEEIAQR	6.68	epidermis development	structural molecule activity
Endonuclease-reverse transcriptase (POL)	GYIMRNTGLDEAQAGIK	6.57	DNA integration	nucleotidyltransferase activity
Keratin, type II cytoskeletal 3 (KRT3)	AQYEEIAQR	6.00	keratinization	structural molecule activity

Alpha-adducin isoform X1 (ADD1)	ILIQKNLGPK	5.95	cell junction organization	actin binding
Cationic amino acid transporter 3 isoform X2 (SLC7A3)	CSFCCCSSCCSVAR	4.83	amino acid transport	amino acid transmembrane transporter activity
Gamma globulin fraction:				
DNA repair-scaffolding protein isoform X2 (SPIDR)	VVTSPVLR	18.28	DNA repair	DNA binding
Tudor domain-containing protein 1 (TDRD1)	LQSPSASLSR	18.24	Regulation of transcription, DNA-templated	RNA binding
Voltage-gated potassium channel subunit beta-1-like (KCNAB1)	VALPTVSR	18	potassium ion transport	voltage-gated potassium channel activity
Beta-crystallin A4 (CRYBA4)	SLISAVPR	18	visual perception	structural constituent of eye lens
DNA repair-scaffolding protein isoform X2 (SPIDR)	VVTSPVLR	17.9	DNA repair	DNA binding
Ig gamma-2 chain C region (IGHG2)	CKVYNEGLPAPIVR	17.62	Immune response	Antigen binding
Tudor domain-containing protein 1 (TDRD1)	LQSPSASLSR	17.54	regulation of transcription, DNA-templated	RNA binding
Beta-crystallin A4 (CRYBA4)	SLISAVPR	17.37	Visual perception	Structural constituent of eye lens
Ig gamma-2 chain C region (IGHG2)	CKVYNEGLPAPIVR	16.2	immune response	antigen binding
Palmitoyltransferase ZDHHC12 (ZDHHC12)	VAVSPVPR	16.18	protein palmitoylation	protein-cysteine S-palmitoyltransferase activity
Endonuclease-reverse transcriptase (POL)	IAGGNIK	15.91	DNA integrzation	nucleotidyltransferase activity
Palmitoyltransferase ZDHHC12 (ZDHHC12)	VAVSPVPR	15.79	protein palmitoylation	protein-cysteine S-palmitoyltransferase activity
Craniofacial development protein 2 (CFDP2)	QNQSKKNTQLWMSLVMEVK	15.54	Multicellular organism development	Chromatin binding
Immunoglobulin epsilon heavy chain-like (IGH)	ADGTPITR	15.47	Immune response	Antigen binding
Ig gamma-2 chain C region (IGHG2)	CKVYNEGLPAPIVR	15.16	Immune response	Antigen binding
Craniofacial development protein 2 (CFDP2)	TLMIYVQCISSSTNVQKAQK	15.01	multicellular organism development	chromatin binding
Glucose-6-phosphate 1-dehydrogenase isoform X1 (G6PD)	DGLLPEDTYIVGYAR	14.94	Glucose metabolic process	Glucose-6-phosphate dehydrogenase activity
Complement C4-like isoform X3 (C4A)	AAGLAFSDGDHR	14.83	Immune response	Complement binding
Glucose-6-phosphate 1-dehydrogenase isoform X1 (G6PD)	DGLLPEDTYIVGYAR	14.37	glucose metabolic process	glucose-6-phosphate dehydrogenase activity
Immunoglobulin epsilon heavy chain-like (IGH)	ADGSTITR	14.32	immune response	antigen binding
Immunoglobulin lambda variable 2-8 (IGLV2-8)	AFLQGLPR	14.24	Immune response	Antigen binding
Immunoglobulin epsilon heavy chain-like (IGH)	GSYSCEVTHEGSTVAK	13.65	Immune response	Antigen binding
Immunoglobulin lambda variable 2-8 (IGLV2-8)	ASGVPDR	13.28	immune response	antigen binding
Complement C4-like isoform X3 (C4A)	AEFQGVLEK	13.23	Immune response	Complement binding
PHD finger protein 10 isoform X1 (PHF10)	QRITDHYKQYSQMQQQK	12.89	regulation of transcription, DNA-templated	zinc ion binding
Keratin, type I cytoskeletal 10 (KRT10)	DAEAWFNEK	12.33	keratinocyte differentiation	structural molecule activity
Craniofacial development protein 2 (CFDP2)	GIKIIPK	12.19	Multicellular organism development	Chromatin binding
Phospholipid transfer protein (C2CD2L)	ALLRLRATR	12.11	Regulation of signal transduction	Calcium ion binding
Albumin (ALB)	AACLLPK	12.04	regulation of blood osmotic pressure	metal ion binding

Albumin (ALB)	AACLLPK	11.8	regulation of blood osmotic pressure	metal ion binding
AT-hook DNA-binding motif-containing protein 1 (AHDC1)	GGGHAAK	11.75	Regulation of transcription, DNA-templated	DNA binding
Keratin, type II cytoskeletal 1 (KRT1)	LALDVEIATYR	11.71	intermediate filament organization	structural molecule activity
DALR anticodon-binding domain-containing protein 3 (DALRD3)	APTEALRNR	11.62	tRNA processing	RNA binding
Casein kinase I isoform X1 (CSNK1)	GPKRGPR	11.6	protein phosphorylation	protein serine/threonine kinase activity
Lipolysis-stimulated lipoprotein receptor isoform X1 (LSR)	APALRQR	11.48	lipid metabolic process	lipoprotein receptor activity
Lipolysis-stimulated lipoprotein receptor isoform X1 (LSR)	ALPRGCPPPSR	11.46	lipid metabolic process	lipoprotein receptor activity
Phospholipid transfer protein (C2CD2L)	LDSPSRSPSK	11.25	Lipid transport	Phospholipid binding
Endonuclease-reverse transcriptase (POL)	EMGIADYLTCLLR	11.22	DNA integration	Nucleotidyltransferase activity
Keratin, type II cytoskeletal 1 (KRT1)	DYQELMNTK	11.1	Intermediate filament organization	Structural molecule activity
Zinc finger protein with KRAB and SCAN domains 8-like (ZNF8)	EKPYECNECGKTFR	11.07	regulation of transcription, DNA-templated	DNA binding
Formin-1 isoform X1 (FMN1)	ELKRTPR	11.05	Actin cytoskeleton organization	Actin binding
Membrane-bound immunoglobulin gamma3 heavy chain constant region (IGHG3)	AKGQALEPQVYVLAPPR	11.02	Immune response	Antigen binding
Craniofacial development protein 2 (CFDP2)	VVRGGLQIAENRR	11.01	multicellular organism development	chromatin binding
Reverse transcriptase (RT)	ALFWTLK	11	DNA integration	Nucleotidyltransferase activity
Formin-1 isoform X1 (FMN1)	ELKRTPR	10.97	actin cytoskeleton organization	actin binding
Isthmin-1 (ISM1)	VDVLPWIICK	10.86	Angiogenesis	Heparin binding
Endonuclease-reverse transcriptase (POL)	VGHSFSSKEQK	10.86	DNA integration	Nucleotidyltransferase activity
Formin-1 isoform X1 (FMN1)	ELKRTPR	10.84	Actin cytoskeleton organization	Actin binding
Keratin, type II cytoskeletal 1 (KRT1)	DYQELMNTK	10.78	Intermediate filament organization	Structural molecule activity
Bisphosphoglycerate mutase (BPGM)	GACCGER	10.67	Glycolytic process	Phosphoglycerate mutase activity
Peroxisome biogenesis factor 2 (PEX2)	IWKTQQWPQYWK	10.36	Peroxisome organization	Ubiquitin-protein transferase activity
SUN domain-containing ossification factor isoform X1 (SUN1)	ALEVNMSLSGR	10.26	nucleus organization	protein binding
Receptor-type tyrosine-protein phosphatase gamma isoform X1 (PTPRG)	AVTNLHSTLNGRTITLPTMVR	10.19	Regulation of signal transduction	Protein tyrosine phosphatase activity
Olfactory receptor 8B3-like (OR8B3)	IDAFELWCWGR	10.03	Sensory perception of smell	G-protein coupled receptor activity
Conserved oligomeric Golgi complex subunit 8 (COG8)	VFVNPSLRVLDSR	9.96	Protein transport	Protein binding
Histone-lysine N-methyltransferase 2A isoform X5 (KMT2A)	KRGRPPTFPGVK	9.52	histone H3-K4 methylation	histone-lysine N-methyltransferase activity
Palmitoyltransferase ZDHHC5 (ZDHHC5)	PAESAKR	9.45	Protein palmitoylation	Protein-cysteine S-palmitoyltransferase activity
Prospero homeobox protein 2 isoform X1 (PROX2)	LALVPPVK	9.39	Regulation of transcription, DNA-templated	Sequence-specific DNA binding
Golgi SNAP receptor complex member 1 (GOSR1)	SGSGVNNR	9.22	Vesicle-mediated transport	SNAP receptor activity

Table 3 List of *Babesia bovis* proteins found in all serum protein fractions of subclinically *B. bovis*-infected Eld's deer. Gene Ontology (GO) annotations for biological process and molecular function categories were retrieved from UniProtKB/SwissProt.

Protein name	Peptide	Average log_2 protein intensity	GO annotation in biological process category	GO annotation in molecular function category
Albumin fraction:				
Rab-GAP TBC domain-containing protein (TBC1D)	QLTWLLIK	15.79	regulation of GTPase activity	GTPase activator activity
Heat shock protein 70 (HSP70)	EQMSSEDIDSLK	14.30	protein folding	ATP binding
Ribonucleoprotein (RNP)	AVLAVSHK	14.23	RNA processing	RNA binding
Structural maintenance of chromosomes protein (SMC)	DETKRTLDQLCFK	14.23	chromosome organization	structural molecule activity
Sec63 domain containing protein (SEC63)	DAFGEVMTHLR	14.21	protein targeting to ER	ATPase activity
RNA helicase (DDX)	DIETYTHRIGR	13.90	RNA helicase activity	ATP binding
Tetratricopeptide repeat domain containing protein (TPR)	AARAQQK	13.74	protein transport	protein binding
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	ALEAFMKWAEQDEK	13.47	immune response	antigen binding
MMS19 nucleotide excision repair protein (MMS19)	AAGDHIGR	12.81	nucleotide-excision repair	DNA binding
Suppressor of forked domain-containing protein (SUPT)	ETVSFDLDNELAK	12.70	regulation of transcription	protein binding
Kinase (STK)	KCVVNALVK	12.52	phosphorylation	kinase activity
Putative GTP-ase activating protein for Arf (ARFGAP)	AVQNIKDGIATAIK	12.51	vesicle-mediated transport	GTPase activator activity
Condensin complex subunit 1 C-terminal domain-containing protein (CAP-G)	CKCIELLSK	11.77	chromosome condensation	chromatin binding
ATP-dependent protease La family protein (LONP)	DSFKSSLSR	11.73	protein catabolic process	ATP-dependent peptidase activity
Mitogen-activated protein kinase (MAPK)	DYFMDRNVK	10.99	signal transduction	MAP kinase activity
U5 snRNP-associated subunit (PRPF8)	AVLVPDQSK	10.96	mRNA splicing	RNA binding
Chaperonin cpn60 (HSPD1)	LRYEDAINAVR	10.34	protein refolding	chaperone binding
Alpha-1 globulin fraction:				
Merozoite surface antigen-2b (MSA2)	DYESLVKFCSDFLDGESPFTNMYK	11.53	cell adhesion	structural molecule activity
Variable erythrocyte surface antigen-1 (VAR1)	QLPDYLIEK	11.00	immune response	antigen binding
Ubiquitinyl hydrolase 1 (USP1)	AAGSLGLEGNR	10.97	protein deubiquitination	thiol-dependent ubiquitin-specific protease activity
Chaperonin cpn60 (HSPD1)	VTLGPRGR	10.55	protein refolding	chaperone binding
Mitogen-activated protein kinase (MAPK)	GIDLYKR	10.14	signal transduction	MAP kinase activity
SET domain containing protein (SET)	CKEFNTIIRDIDPVDILR	9.70	RNA helicase activity	ATP binding
Alpha-2 globulin fraction:				
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	CQKDNKK	11.94	immune response	antigen binding
Coatomer subunit beta (Beta-coat protein) (COPB1)	LLLDESDLNVK	11.36	vesicle-mediated transport	structural molecule activity
LCCL domain-containing protein CCP2 (CCP2)	AVRAIRYLEVPK	10.55	signal transduction	protein binding
Beta globulin fraction:				
Phospholipid acyltransferase (LPCAT)	EAILKLNPNVDR	16.32	lipid metabolic process	acyltransferase activity
Sec63 domain containing protein (SEC63)	FDDDVTKKEQK	14.05	protein targeting to ER	ATPase activity
Ubiquinone biosynthesis O-methyltransferase family protein (COQ3)	VTKYRAEIFDEDTVSEGVIK	13.31	ubiquinone biosynthetic process	O-methyltransferase activity
Endonuclease/exonuclease/phosphatase family protein (INPP5A)	DFLMDKR	11.06	phosphatidylinositol dephosphorylation	phosphatase activity
Aconitate hydratase (ACO1)	FIQRVSMVQSR	10.66	tricarboxylic acid cycle	aconitate hydratase activity

t-SNARE coiled-coil homology domain-containing protein (STX)	LSLQLGMR	10.37	vesicle-mediated transport	SNARE binding
BCNT-C domain-containing protein (BCNT)	VDVIPPPR	9.95	regulation of gene expression	protein binding
Flavodoxin domain containing protein (FDX1)	VPKSFFK	9.25	electron transport chain	flavodoxin activity
Integral membrane protein (ITM2A)	QGEDPTEAGSDDLK	8.91	cell differentiation	structural molecule activity
Gamma globulin fraction:				
Adaptin N-terminal region domain containing protein (AP1G1)	DQLKEYLVR	18.63	vesicle-mediated transport	protein binding
Adaptin N terminal region domain containing protein (AP1G1)	FEPYAMQATQMAEYK	18.51	vesicle-mediated transport	protein binding
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	ALFYQLYFLRK	18.39	immune response	antigen binding
Rab11b protein (RAB11B)	GTLPKQAK	18.39	vesicle-mediated transport	GTPase activity
Phospholipid or glycerol acyltransferase (LPCAT)	LNPNVDR	18.37	lipid metabolic process	acyltransferase activity
Phospholipid or glycerol acyltransferase (LPCAT)	LNPNVDR	18.24	lipid metabolic process	acyltransferase activity
t-SNARE coiled-coil homology domain-containing protein (STX)	QLADEVKSR	18.17	vesicle-mediated transport	SNARE binding
Phospholipid or glycerol acyltransferase (LPCAT)	LNPNVDR	18.14	lipid metabolic process	acyltransferase activity
Secreted protein (SEC)	LKAPPLL	17.48	extracellular region	protein secretion
Secreted protein (SEC)	LKAPPLL	17.48	extracellular region	protein secretion
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	ALFYQLYFLRK	17.41	immune response	antigen binding
Secreted protein (SEC)	LKAPPLL	16.91	extracellular region	protein secretion
Ribonucleoprotein (RNP)	AVLAVSHK	16.24	RNA processing	RNA binding
Ribonucleoprotein (RNP)	AVLAVSHK	16.12	RNA processing	RNA binding
WD domain, G-beta repeat containing protein (WDR)	DTREPELEFDRVYNCHTK	16.11	signal transduction	protein binding
Ribonucleoprotein (RNP)	AIGHHTKLGR	16.07	RNA processing	RNA binding
Heat shock protein 70 (HSP70)	ECHKRLQELSWK	16.02	protein folding	ATP binding
WD domain, G-beta repeat containing protein (WDR)	ANGVKYYHK	15.89	signal transduction	protein binding
Heat shock protein 70 (HSP70)	HMQFKLTR	15.84	protein folding	ATP binding
Heat shock protein 70 (HSP70)	DKISEEDKK	15.82	protein folding	ATP binding
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	CSDSGGK	15.46	immune response	antigen binding
Acylphosphatase (ACYP1)	IYVASYRVYGR	15.4	phosphate metabolic process	acylphosphatase activity
Mitochondrial phosphate transporter (SLC25A3)	FFFFEYIQDIFYEHILK	14.69	phosphate transport	phosphate ion transmembrane transporter activity
Acylphosphatase (ACYP1)	IYVASYRVYGR	14.39	phosphate metabolic process	acylphosphatase activity
Formin homology 2 domain containing protein (FHOD1)	AKEEAEAKR	13.41	actin filament organization	actin binding
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	ALEAFMK	13.34	immune response	antigen binding
Splicing factor 3b, subunit 3, 130kD (SF3B3)	DAIVAFKPR	13.3	mRNA splicing	RNA binding
Formin homology 2 domain containing protein (FHOD1)	AKEEAEAKR	13.28	Actin cytoskeleton organization	Actin binding
Mitochondrial phosphate transporter (SLC25A3)	FFFFEYIQDIFYEHILK	13.28	phosphate transport	phosphate ion transmembrane transporter activity
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	ALEAFMKWAEQDEK	13.21	immune response	antigen binding
Merozoite surface antigen-2b (MSA2)	ALNEYDELVK	13.02	cell adhesion	structural molecule activity

Mitogen-activated protein kinase (MAPK)	GIDLYKR	13.01	signal transduction	MAP kinase activity
Mitogen-activated protein kinase) (MAPK)	GIDLYKR	12.95	signal transduction	MAP kinase activity
Mitogen-activated protein kinase (MAPK)	EVEDEMCQVGVR	12.8	signal transduction	MAP kinase activity
General transcription and DNA repair factor IIH helicase subunit XPD (ERCC2)	GALFLSICR	12.79	DNA repair	helicase activity
Splicing factor 3b, subunit 3, 130kD (SF3B3)	IFAGDIREGIQILR	12.73	mRNA splicing	RNA binding
Lysine-specific demethylase-like domain-containing protein (KDM)	AYDNEDRVSFDEVR	12.61	histone demethylation	oxidoreductase activity
Small open reading frame (smORF)	EDSETAGPPRYSVEWYLLPK	12.6	gene expression	structural constituent of ribosome
RNA helicase (DDX)	ASEPLGVLLTTLRR	12.52	RNA helicase activity	ATP binding
SET domain containing protein (SET)	DMAPNGAENSTTPNR	12.42	histone methylation	methyltransferase activity
Chromo-helicase DNA-binding protein (CHD)	EIIRTFELARVHVPGK	12.4	chromatin remodeling	ATP-dependent helicase activity
Rab GDP dissociation inhibitor (GDI1)	ICNKLCPDK	12.38	regulation of GTPase activity	GTPase activator activity
SET domain containing protein (SET)	CKEFNTIIRDIDPVDILR	12.3	RNA helicase activity	ATP binding
Rp16 (RPLP1)	FTYIIDK	12.29	translation	structural constituent of ribosome
Chromo-helicase DNA-binding protein (CHD)	ANQPLGYQQADPKQMYYQQQYMYPR	12.23	chromatin remodeling	ATP-dependent helicase activity
SET domain containing protein (SET)	CKEFNTIIRDIDPVDILR	12.22	RNA helicase activity	ATP binding
Glycerol-3-phosphate-acyltransferase (GPAT)	LAGCIPVHR	12.22	lipid metabolic process	acyltransferase activity
Trimethylguanosine synthase (TGS1)	IGNIVTIYLPR	12.21	RNA cap formation	methyltransferase activity
Trimethylguanosine synthase (TGS1)	EIGVGGIFPPIATI	12.18	RNA cap formation	methyltransferase activity
Rp16 (RPLP1)	FTYIIDK	12.15	translation	structural constituent of ribosome
Condensin complex subunit 1 C-terminal domain-containing protein (CAP-G)	FRLSLNTSTVK	12.12	chromosome condensation	chromatin binding
DEAD/DEAH box helicase and helicase conserved C-terminal domain containing protein (DDX)	FIYSRTEDKVATLCR	12.07	RNA helicase activity	ATP binding
Membrane protein (MEM)	LLKFSKVGCVLPLDK	12.06	cell adhesion	structural molecule activity
DEAD/DEAH box helicase and helicase conserved C-terminal domain containing protein (DDX)	ASYDELMHMFPK	11.99	RNA helicase activity	ATP binding
Ribosomal protein L10 (RPL10)	CYRYCKNKPYPK	11.92	translation	structural constituent of ribosome
Nucleolar GTP-binding protein 2 (GNL2)	EGQYRDK	11.85	GTPase activity	GTP binding
Condensin complex subunit 1 C-terminal domain-containing protein (CAP-G)	FRLSLNTSTVK	11.83	chromosome condensation	chromatin binding
Prefoldin Subunit 6 (PFDN6)	LEKERLR	11.8	protein refolding	chaperone binding
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	ALEGEKTEGIK	11.71	immune response	antigen binding
Chaperonin cpn60 (HSPD1)	NIELEDRK	11.67	protein refolding	chaperone binding
GCC2 and GCC3 domain containing protein (GCC2)	ALYLGTEYK	11.66	vesicle-mediated transport	protein binding
Trimethylguanosine synthase (TGS1)	EIGVGGIFPPIATI	11.65	RNA cap formation	methyltransferase activity
Membrane protein (MEM)	DAKQYTLK	11.63	cell adhesion	structural molecule activity
Ubiquitinyl hydrolase 1 (USP1)	AAGSLGLEGNR	11.63	protein deubiquitination	ubiquitin-specific protease activity

Proline-tRNA ligase (PARS)	DEARPCNSNMRCR	11.56	protein biosynthesis	aminoacyl-tRNA ligase activity
Merozoite surface antigen 2c (MSA2)	AQKDDYR	11.44	cell adhesion	structural molecule activity
Pumilio-family RNA binding repeat domain containing protein (PUM)	EIAENSFGNYVLQAVTRNK	11.36	regulation of translation	RNA binding
Condensin complex subunit 1 C-terminal domain-containing protein (CAP-G)	KSIMESVKLLIIK	11.35	chromosome condensation	chromatin binding
LCCL domain-containing protein CCP2 (CCP2)	ALGSAGYRK	11.33	signal transduction	protein binding
Sec1 family protein (VPS45)	AVSDMLQLSVDDDSDGRSLK	11.2	vesicle-mediated transport	SNARE binding
Merozoite surface antigen 2c (MSA2)	AQKDDYR	11.2	cell adhesion	structural molecule activity
FYVE-type domain-containing protein (ZFYVE)	ARSPPRK	11.19	endocytosis	phosphatidylinositol-3-phosphate binding
Elongation factor G, mitochondrial (GFM1)	CTAYGANSQAFGINRYPYRK	11.17	mitochondrial translation	GTP binding
Snf2-related chromatin remodeling factor SRCAP (SRCAP)	DKSSSRDSGSR	11.16	chromatin remodeling	chromatin binding
Trehalose-6-phosphate synthase domain containing protein (TPS)	AFRKYLESYPVSR	11.15	carbohydrate metabolic process	transferase activity
ClpC (CLPC)	EYLNRFK	11.14	protein folding	chaperone binding
Prefoldin Subunit 6 (PFDN6)	LEKERLR	11.12	protein refolding	chaperone binding
Pumilio-family RNA binding repeat domain containing protein (PUM)	HFIRIAKAQQK	11.11	regulation of translation	RNA binding
Macro domain-containing protein (MACROD)	AIEDMREQFK	11.1	DNA repair	ADP-ribosylhydrolase activity
Chromo-helicase DNA-binding protein (CHD)	DVEKSLPNKVER	11.07	chromatin remodeling	ATP-dependent helicase activity
Nucleolar GTP-binding protein 2 (GNL2)	HFSVGFIGYPNVGK	11.06	GTPase activity	GTP binding
Prefoldin Subunit 6 (PFDN6)	LEKERLR	11.04	protein refolding	chaperone binding
Formin homology 2 domain containing protein (FHOD1)	AKEEAEAKR	11.04	actin cytoskeleton organization	actin binding
Chaperonin cpn60 (HSPD1)	NIELEDRK	11.02	protein folding	chaperone binding
ubiquitinyl hydrolase 1 (USP1)	AAGSLGLEGNR	10.98	protein deubiquitination	thiol-dependent ubiquitin-specific protease activity
Chaperonin cpn60 (HSPD1)	TNSYGDMIQQGVIDPTK	10.93	protein refolding	chaperone binding
Chorein N-terminal domain-containing protein (VPS13)	ELDIGTLLGAYKSYVK	10.85	vesicle-mediated transport	protein binding
RNA recognition motif-containing protein (RRM)	ADSAGQK	10.81	RNA processing	RNA binding
Cell division cycle protein ATPase (CDC)	ARAAAPCILFFDEIDSIAK	10.67	cell cycle	ATPase activity
C3H1-type domain-containing protein (ZC3H1)	GESCVKGNIEKGK	10.65	regulation of gene expression	RNA binding
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	ALFYQLYFLRK	10.65	immune response	antigen binding
ClpC (CLPC)	ILNKLTINFIKLFGDK	10.6	protein folding	chaperone binding
p18 protein (CDKN2C)	EAFEARR	10.6	cell cycle regulation	cyclin-dependent kinase inhibitor activity
Spindle assembly abnormal protein 6 N-terminal domain-containing protein (SAS6)	ANEFRIVRNITYR	10.59	spindle assembly	structural constituent of centrosome
Glycerol-3-phosphate-acyltransferase (GPAT)	GWIDLCGSLYPPERSMVPTNK	10.53	glycerolipid biosynthetic process	acyltransferase activity
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	ALEGEKTEGIK	10.52	immune response	antigen binding
Programmed cell death protein 2 (PDCD2)	DYSLAAK	10.51	glycerol-3-phosphate metabolic process	glycerol-3-phosphate dehydrogenase activity
glycerol-3-phosphate dehydrogenase (GPD1)	EVARMSAEAK	10.44	methylation	methyltransferase activity

Heat shock protein 90 (HSP90)	AIDMFKNLQAA NPDR	10.42	protein folding	ATP binding
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	DELNGHQGALS PK	10.08	immune response	antigen binding
DNA-directed RNA polymerase subunit (POLR)	CAFLLLPENTVK	10.06	transcription	DNA-directed RNA polymerase activity
DNA-directed RNA polymerase subunit (POLR)	DFEYALMVK	9.99	transcription	DNA-directed RNA polymerase activity
Small open reading frame (smORF)	DYDEPIDAALEK RIR	9.93	gene expression	structural constituent of ribosome
Methyltransferase type 11 domain-containing protein (METTL11)	LVVCRPEYK	9.87	dephosphorylation	phosphoprotein phosphatase activity
RNA helicase (DDX)	ARVESLLVAPISQ ASAAQR	9.77	RNA helicase activity	ATP binding
Peptidase C14 caspase domain-containing protein (CASP)	ALADMQQRK	9.62	apoptotic process	cysteine-type endopeptidase activity
Serine/threonine-protein phosphatase (PPP)	IKYPENFFLLR	9.51	lipid metabolic process	acyltransferase activity
Membrane protein (MEM)	DFLKEYFR	9.47	cell adhesion	structural molecule activity
Variant erythrocyte surface antigen-1, alpha subunit (VAR1)	GDYSITER	9.43	immune response	antigen binding
Casein kinase II, alpha chain (CK II) (CSNK2A1)	LGRGKYSEVFEG TNLLTHER	9.25	phosphorylation	protein kinase activity
Long chain fatty acid CoA ligase (ACSL)	AFGGNIK	8.86	lipid metabolic process	Long chain fatty acid CoA ligase activity

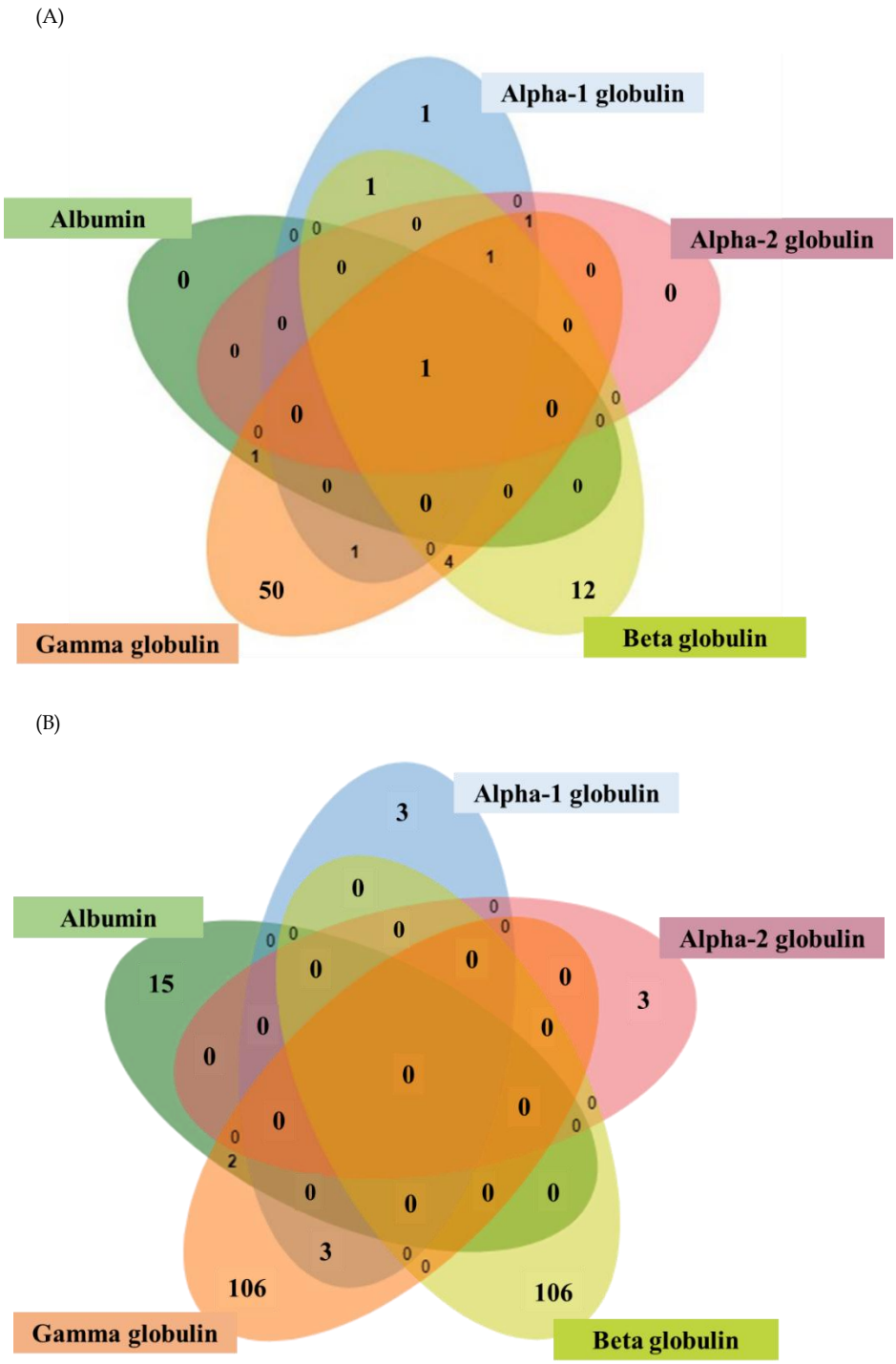


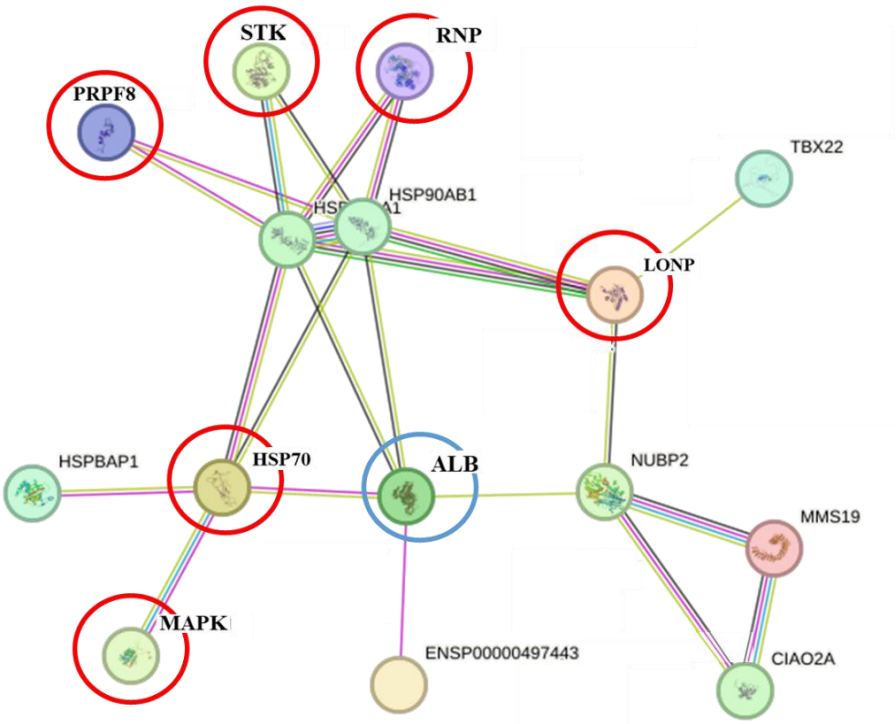
Figure 2 Venn diagram of common protein expression in albumin, alpha-1 globulin, alpha-2 globulin, beta globulin and gamma globulin fractions of (A) Eld's deer proteins and (B) *Babesia bovis* proteins found in PCR-positive Eld's deer serum samples.

***The protein interaction network of Eld's deer and identified B. bovis proteins in subclinically B. bovis-infected Eld's deer*:** We conducted a protein interaction network analysis between the identified Eld's deer proteins and *B. bovis* proteins detected in PCR-positive Eld's deer serum samples, focusing on the interactions within each serum protein fraction (Fig. 3). In albumin fraction, interactions were observed between Eld's deer ALB and several *B. bovis* proteins: HSP70, ribonucleoprotein (RNP), serine/threonine protein kinase (STK), LONP, MAPK, and U5 snRNP-associated subunit (PRPF8). These proteins were functionally enriched in ATP-dependent activity (FDR = 0.042) and anion binding (FDR = 0.042) in terms of the molecular function category of GO (Fig. 3A). For alpha-1 globulin, interactions were noted between Eld's deer proteins, ALB, LSR and SERPINA3, and *B. bovis* variable antigen 1 (VAR1). The significant functional enrichments of this network included the acute-phase response (FDR = 9.6E-4) and defense response (FDR = 0.002) in the biological process category (Fig. 3B). We also conducted the protein interaction network of identified proteins in beta globulin fraction. The interactions were found between

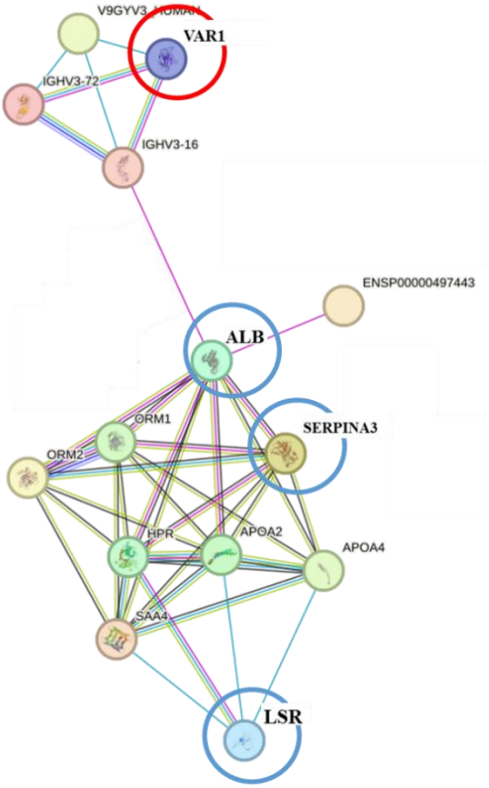
B. bovis phospholipid acyltransferase (LPCAT) and various immunoglobulin-related proteins. The significant functional enrichment pathways of this network related to the immune system, including the acute-phase response (FDR = 0.003), regulatory of immune effector process (FDR = 0.04), and defense response (FDR = 0.001) in the biological process category. This finding was corresponding to the protein network of alpha-1 globulin fraction (Fig. 3C).

Due to the gamma globulin fraction appearing as broad bands, we combined the proteins from three segments and conducted a protein network analysis between Eld's deer and *B. bovis* proteins. This analysis revealed interactions between several proteins from Eld's deer and *B. bovis*, with the GO functional enrichment pathway identified as telomerase RNA binding (FDR = 0.009) (Fig. 3D). However, no interactions were detected between the identified Eld's deer and *B. bovis* proteins within the alpha-2 globulin fraction.

(A)



(B)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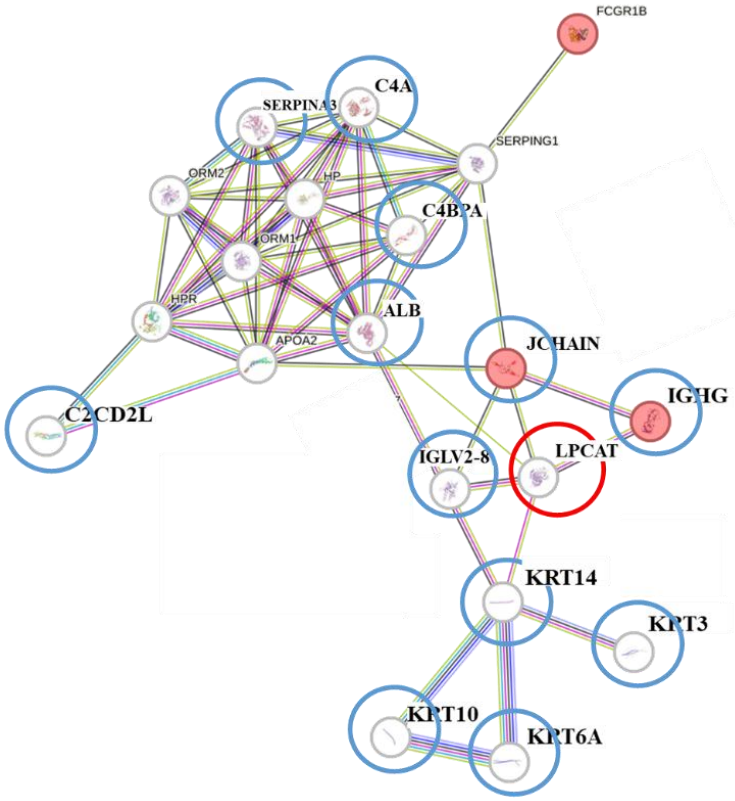
IGHV3-72
IGHV3-16
ENSP00000497443
ALB
ORM1
SERPINA3
ORM2
HPR
APOA2
APOA4
SAA4
LSR

(C)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C4A
SERPINA3
SERPING1
ORM2
HP
C4BPA
ORM1
HPR
ALB
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JCHAIN
IGHG
C2CD2L
IGLV2-8
LPCAT
KRT14
KPT3
KPT10
KPT6A

(D)

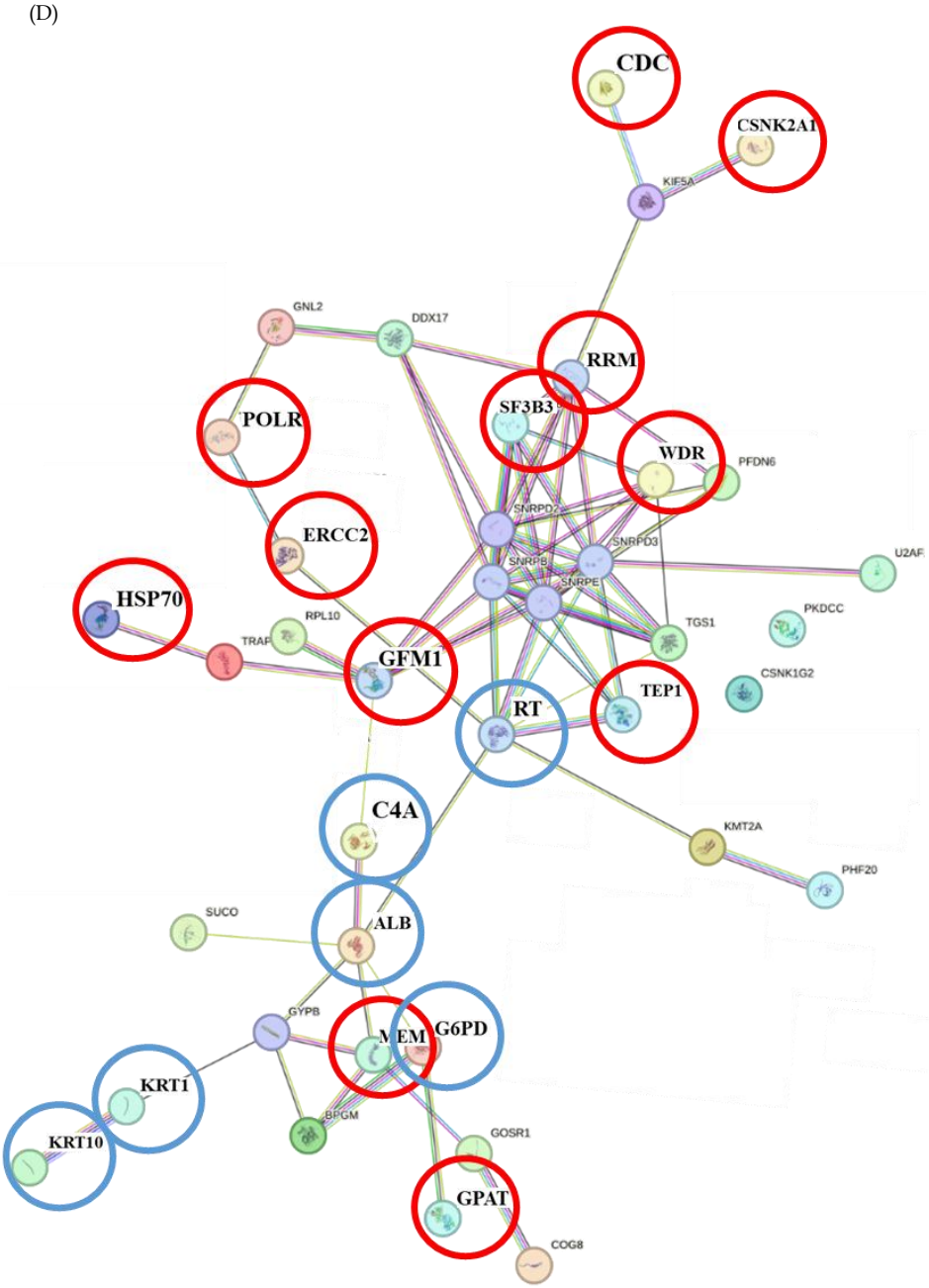

Figure 3 Protein interaction network of Eld's deer proteins and *Babesia bovis* proteins detected in Eld's deer serum showed in each serum protein fraction, including (A) albumin, (B) alpha-1 globulin, (C) beta globulin and (D) gamma globulin. Blue circle indicates the identified protein of Eld's deer, while red circles represent the proteins of *Babesia bovis* presented in the PCR-positive Eld's deer serum samples.

Discussion

The present study provided an in-depth analysis of the Eld's deer and *B. bovis* proteins in each serum protein fraction of these subclinically infected Eld's deer. It also explored the interactions between *B. bovis* and host proteins. ALB was detected in all fractions of Eld's deer protein due to its high abundance in serum. This protein plays a crucial role in maintaining osmotic balance and serves as a carrier for other proteins (Bertholf, 2014). Furthermore, SPIDR was present in all globulin fractions and is involved in maintaining genome stability and acting as a scaffold for DNA repair and chromatin related proteins (Aprosoff *et al.*, 2023).

In terms of protein interactions between Eld's deer and *B. bovis* proteins, C2CD2L of Eld's deer interacted with *B. bovis* proteins involved in ATP activity and anion binding, such as HSP70 and LONP, which appeared in the albumin fraction. Generally, C2CD2L is involved in maintaining cellular integrity and immune function in mammals (Sun *et al.*, 2019). HSP is a chaperone protein activated by a cellular stress response induced by parasitic infection and has been previously reported in humans infected with *B. microti* (Pérez-Morales and Espinoza, 2015; Magni *et al.*, 2019). Interestingly, HSP90 of *B. bovis* has been demonstrated to interact with Eld's deer serum proteins, indicating that the parasite could promote host immune responses (Pumpitakkul *et al.*, 2024). Therefore, the

interactions in this albumin fraction highlighted that *B. bovis* can stimulate the immune response of the infected host. The differences in serum protein digestion and proteomic techniques between in-gel-based and gel-free proteomic techniques possibly led to varied protein expression results as electrophoresis can simplify and separate sample mixtures, while in-solution protein digestion can enhance peptide yields. Despite these methodological variations, our current study identified protein network interaction pathways between Eld's deer and *B. bovis* proteins in each fraction, highlighting associations with immune and inflammatory responses consistent with previous findings. This suggests a host-defense response in Eld's deer during *B. bovis* infection.

We also analyzed the host-parasitic interaction from alpha-1 and alpha-2 globulin fractions. Indeed, the presence of ZDHHC12 and lipolysis-stimulated lipoprotein receptor isoform X1 (LSR) in the *Babesia*-positive Eld's deer serum indicated significant roles in lipid metabolism and immune response regulation (Andersen *et al*., 2003; Liao *et al*., 2024). These proteins in the alpha-1 globulin fraction interacted with variable antigen-1 (VAR1) of *Babesia*, indicating the acute phase response and host defense mechanisms as previously reported in other species (Mohammadi *et al*., 2021; Janjić *et al*., 2022). Nevertheless, no interactions between the Eld's deer and B. bovis proteins were found in the alpha-2 globulin fraction.

Further investigation of enriched functional pathways in the beta globulin fraction of Eld's deer serum revealed mechanisms related to the complement activation classical pathway, such as C4BPA, complement C4-like isoform X3 (C4A), IGHG2, IGLV2-8, and IGHG. The complement system, including proteins C3 and C4, plays a crucial role in the host defense against *Babesia* infections by promoting opsonization and lysis of the parasites (Chapman and Ward, 1976; Jack and Ward, 1980). In our study, IGLV2-8 was also found to interact with *B. bovis* proteins as previously reported (Pumpitakkul *et al*., 2024). Thus, we suggest that this protein possesses the potential to be a reliable biomarker, and further studies should be warranted. Moreover, the presence of proteins associated with intermediate filament organization, including alpha-adducin isoform X1 (ADD1) and keratins, indicated potential involvement in maintaining cellular structure and stability during infection (Friedman, 2006; Moztarzadeh *et al*., 2022). These findings suggested that the complement system and intermediate filament organization are crucial in the host's immune response and cellular integrity during *Babesia* infection. Understanding these interactions provides insight into the molecular mechanisms of the host-pathogen interaction and may inform the development of therapeutic strategies to enhance host defense mechanisms against *Babesia* infections.

The analysis of gamma globulin fractions from *B. bovis*-infected Eld's deer serum revealed significant functional enrichments, notably in the complement activation pathway. Detection of proteins such as immunoglobulins like IGHG2, IGLV2-8, IGHG, and C4A highlighted their role in the adaptive immune response. The interaction network revealed telomerase binding RNA interactions between Eld's deer and *B. bovis*, suggesting a potential mechanism regarding the cellular response to infection and cell proliferation (Belair *et al*., 1997; Nassour *et al*., 2023). Overall, our findings underscore the complex interplay between host and pathogen at the protein level and potentially aid in the detection of subclinical babesiosis. Future research should focus on further elucidating these pathways and their roles in the immune response to improve disease management and treatment outcomes for *Babesia* infections in Eld's deer.

Conflicts of interest: There were no conflicts of interest that may have biased the work reported in this study.

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