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Innovative approaches for precise identification and control of ticks and tick-borne pathogens

Edited by
Mourad Ben Said and Rosa Estela Quiroz Castañeda

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Innovative approaches for precise identification and control of ticks and tick-borne pathogens

Topic editors

Mourad Ben Said — University of Manouba, Tunisia

Rosa Estela Quiroz Castañeda — Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Mexico

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EDITED AND REVIEWED BY
Antoinette Marsh,
The Ohio State University, United States

*CORRESPONDENCE
Mourad Ben Said
✉ bensaidmourad83@yahoo.fr
Rosa Estela Quiroz-Castañeda
✉ requiroz79@yahoo.com.mx

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Editorial: Innovative approaches for precise identification and control of ticks and tick-borne pathogens

Mourad Ben Said[1,2]* and Rosa Estela Quiroz-Castañeda[3]*

[1]Higher Institute of Biotechnology of Sidi Thabet, University of Manouba, Manouba, Tunisia, [2]Laboratory of Microbiology at the National School of Veterinary Medicine of Sidi Thabet, University of Manouba, Manouba, Tunisia, [3]Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP, Jiutepec, Morelos, Mexico

KEYWORDS

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Editorial on the Research Topic
Innovative approaches for precise identification and control of ticks and tick-borne pathogens

This Research Topic presents a collection of original and review articles that explore the diverse aspects of tick and tick-borne disease management. It addresses (i) innovative tick control methods using natural products, (ii) advancements in tick diagnostics and pathogen detection, (iii) novel vaccine strategies for combating vector-borne diseases, and (iv) in-depth research on tick proteins and their roles in pathogen interactions. Together, these contributions enhance our understanding of ticks and their impact on animal and public health, paving the way for effective management strategies (Figure 1).

Innovative and sustainable solutions are crucial in the ongoing fight against tick infestations and the diseases they transmit. The study by Abd-Elrahman et al. explore the effectiveness of natural extracts, specifically Chrysanthemum extract and neem oil emulsion, for controlling tick populations and cattle-associated infections. Their findings indicate a significant relationship between tick infestations and the presence of pathogens such as *Theileria annulata* and *Babesia bigemina*, highlighting the critical role ticks play in disease transmission. The research also demonstrates the potential of these natural products as effective alternatives to conventional acaricides. Complementing this work, Phaahla et al. investigate the traditional knowledge and practices used by local communities in Sekhukhune District, South Africa, for managing cattle ticks. Their study emphasizes the value of integrating traditional, plant-based methods into livestock health care, especially in resource-limited settings. By engaging with community members, they identified numerous plant species with potential acaricidal properties, advocating for eco-friendly farming practices and preserving indigenous knowledge. These insights support the conservation of traditional practices and suggest ways to incorporate ethnoveterinary methods into modern tick control strategies.

In diagnostics and pathogen detection, Kamau et al. focus on identifying *Coxiella burnetii* DNA in ticks, emphasizing the zoonotic risks of Q fever linked to infected livestock and wildlife. Their study evaluates three diagnostic methods:

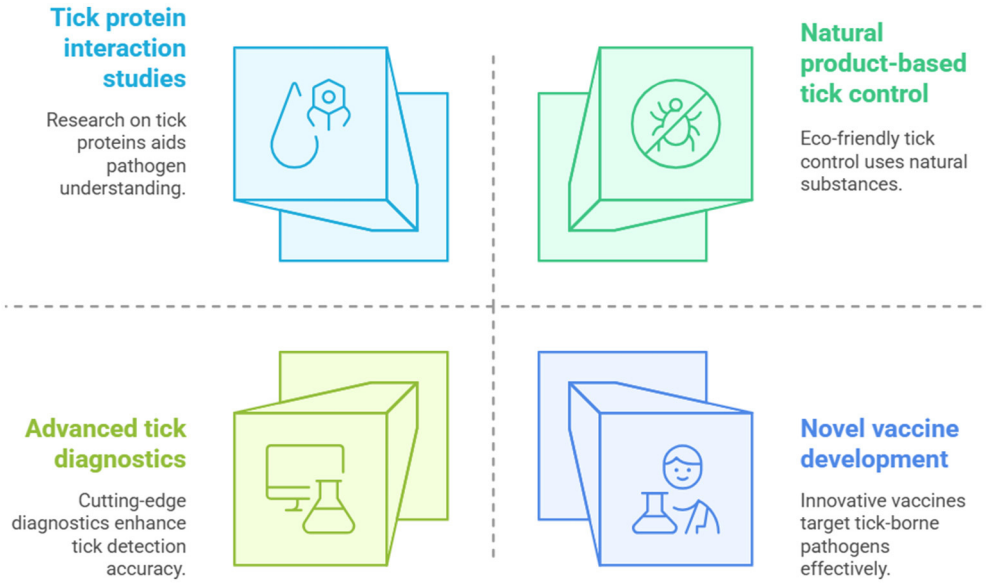

FIGURE 1
Comprehensive strategies for managing ticks and tick-borne diseases presented in this Research Topic.

conventional PCR (cPCR), Biomeme's *C. burnetii* qPCR Go-strips, and a novel PCR high-resolution melt (PCR-HRM) assay. Analyzing ticks collected from wildlife and cattle in northern Kenya, the researchers used Bayesian latent class analysis (BLCA) to assess the sensitivity and specificity of these tests without a gold standard. The results showed that the PCR-HRM assay had the highest sensitivity at 86%, followed by the Biomeme test at 57% and cPCR at only 24%, while all methods maintained high specificity (94% to 98%). Notably, about 16% of the ticks tested positive for *C. burnetii* DNA, indicating the endemic presence of this pathogen in the area. This suggests that PCR-HRM could serve as a reliable tool for *C. burnetii* surveillance in tick populations, offering valuable insights for future epidemiological research and public health initiatives. Additionally, the study by Sri-in et al. examine tick diversity and their role as vectors for various pathogens, including *Anaplasma*, *Babesia*, and *Theileria*. Conducted at Khao Kheow Open Zoo in Thailand, this research involved the collection of over 10,000 ticks over a year, revealing a high pathogen infection rate of 66.37%, with *Anaplasma* spp. being the most prevalent at 55.23%. This work highlights the importance of understanding tick diversity and pathogen prevalence as indicators of wildlife health and ecosystem dynamics.

Regarding immunization against vector-borne diseases, Alzan et al. offer a comprehensive overview of current vaccines, experimental trials, and innovative strategies to control veterinary-relevant blood parasites. These parasites, transmitted by ticks and other blood-sucking arthropods, pose substantial threats to human and animal health, exacerbated by environmental changes. The infections they cause can lead to a range of clinical symptoms, including fever, anemia, jaundice, and neurological disorders, with varying mortality rates. The review emphasizes the need for effective control measures primarily involving vector control, drug treatments, and vaccination strategies. However, many existing approaches face limitations, such as environmental concerns linked to the use of parasiticides and the practicality of vaccine deployment in non-endemic areas. The authors highlight recent advances in vaccine development, particularly those utilizing recombinant antigens, vectored vaccines, and live attenuated or genetically modified parasites. Despite ongoing research, the challenge of creating effective subunit vaccines against blood-stage parasites persists. The authors advocate for leveraging insights from previous vaccine development efforts and employing emerging technologies to refine immune mechanisms of protection, identify appropriate adjuvants, and discover protective antigens, thus enhancing the arsenal against these significant veterinary pathogens. In a complementary study, Quiroz-Castañeda et al. explore an alternative vaccine target for bovine anaplasmosis, focusing on enolase, a moonlighting protein. This research shifts away from traditional vaccine targets, such as Major Surface Proteins and Outer Membrane Proteins, toward multifunctional proteins that can perform various biological roles. The study identifies three enolase proteins in Mexican strains of *Anaplasma marginale* and employs bioinformatics to predict their catalytic domains and binding capabilities. Importantly, the molecular docking analysis indicates that one of these enolases, AmEno01, may interact with erythrocyte proteins, which is crucial for the pathogen's adhesion and invasion.

Focusing on tick biology and diagnostics, Li et al. examine the proteins in the hemolymph of *Haemaphysalis flava* ticks, providing important insights into the biological mechanisms that affect these ectoparasites. Their study emphasizes the role of hemolymph as a circulating fluid that transports nutrients, immune factors, and waste, with proteins being its main soluble components. Using blue native polyacrylamide gel electrophoresis (BN-PAGE) and sodium dodecyl sulfate PAGE (SDS-PAGE), the researchers identified critical proteins, particularly from the vitellogenin (Vg)

family and α-macroglobulin. These protein identifications are vital, as they may play significant roles in tick physiology and immunity, presenting potential targets for tick management strategies. In another study, Tila et al. report the first detection of *Hepatozoon ayorgbor* in *Rhipicephalus haemaphysaloides* and *Hepatozoon colubri* in *Haemaphysalis sulcata* and *Hyalomma anatolicum*. This research highlights the risks of pathogen spillover from wildlife to domestic animals, stressing the importance of vigilance in monitoring tick-borne diseases. Their study involved extensive tick collection and molecular analysis, revealing significant infection rates and identifying environmental risk factors for spillover events. Additionally, Zamiti et al. contribute to improving tick diagnostics by developing PCR assays that utilize minimal *Bm*86 cDNA fragments to identify *Rhipicephalus* and *Hyalomma* tick species. This research addresses the limitations of traditional morphological identification methods, providing a sensitive and specific molecular tool for tick species delineation. By concentrating on shorter, targeted sequences, the study enhances our ability to effectively classify ticks, which is crucial for managing the spread of tick-borne diseases.

As Editors of this Research Topic, we would like to extend our gratitude to all authors for their valuable contributions of original research and insightful reviews. We believe that readers will discover this Research Topic to be an essential reference on recent advancements in the field, covering (i) cutting-edge tick control strategies utilizing natural compounds, (ii) progress in tick diagnostics and pathogen identification, (iii) innovative vaccine approaches for addressing vector-borne diseases, and (iv) detailed investigations into tick proteins and their functions in pathogen interactions.

Author contributions

MB: Conceptualization, Validation, Visualization, Writing – original draft, Writing – review & editing. RQ-C: Conceptualization, Validation, Visualization, Writing – original draft, Writing – review & editing.

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We would like to acknowledge all authors who have contributed with high-quality original articles and very interesting reviews.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Nicola Pugliese,
University of Bari Aldo Moro, Italy

REVIEWED BY
Alberto Moraga-Fernández,
Spanish National Research Council (CSIC), Spain
Reginaldo G. Bastos,
Washington State University, United States

*CORRESPONDENCE
Mohamed Aziz Darghouth
✉ damaziz@yahoo.fr
Mourad Ben Said
✉ bensaidmourad83@yahoo.fr

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Development and field evaluation of PCR assays based on minimum length *Bm86* cDNA fragments required for *Rhipicephalus* and *Hyalomma* tick species delineation

Sayed Zamiti[1], Moez Mhadhbi[1], Mokhtar Dhibi[1], Mohamed Aziz Darghouth[1]* and Mourad Ben Said[1,2]*

[1]Laboratory of Parasitology, National School of Veterinary Medicine of Sidi Thabet, University of Manouba, Manouba, Tunisia, [2]Department of Basic Sciences, Higher Institute of Biotechnology of Sidi Thabet, University of Manouba, Manouba, Tunisia

Introduction: *Hyalomma* and *Rhipicephalus* ticks are important genera that can transmit diseases to both animals and humans, including Crimean-Congo hemorrhagic fever, tick-borne encephalitis, and several types of spotted fever. The accurate identification of tick species is essential for the effective control and prevention of tick-borne diseases. However, traditional identification methods based on morphology can be challenging and subjective, leading to errors. The development of DNA markers has provided more precise and efficient methods for tick species identification, but the currently available markers have limitations in their discriminatory power and sensitivity. To address this need for more sensitive and specific markers, this study aimed to identify two minimum sequence fragments required for tick *Hyalomma* and *Rhipicephalus* species identification using the *Bm86* cDNA marker, which has previously been shown to be in perfect agreement with the current taxonomy of hard ticks based on its complete sequence.

Methods: Based on our *in silico* determination that a minimum sequence of 398 bp for *Rhipicephalus* spp. (from 1487 to 1884) and 559 bp for *Hyalomma* species (from 539 to 1097) was necessary for species delineation, two distinct PCR assays were developed to apply these sequences in practice.

Results and discussion: Discrimination between species within each genus was achieved through sequence homology and phylogenetic analysis following the sequencing of the two PCR products. Subsequently, their performance was evaluated by testing them on the field-collected ticks of the *Hyalomma* and *Rhipicephalus* genera obtained from various host animals in different geographic regions of Tunisia. The use of shorter partial sequences specific to the tick genera *Rhipicephalus* and *Hyalomma*, which target the tick's RNA banks, could represent a significant advance in the field of tick species identification, providing a sensitive and discriminatory tool for interspecific and intraspecific diversity analysis.

KEYWORDS

***in silico* selection, minimum length partial sequences, *Bm86* cDNA, *Hyalomma and Rhipicephalus* spp., species delineation**

1. Introduction

Rhipicephalus is a genus of ticks that includes several species known to transmit a range of pathogens to both animals and humans (1). Some of the most common species of *Rhipicephalus* ticks include *R. sanguineus* sensu lato, *R. microplus*, and *R. annulatus* (2). These ticks are distributed in different regions worldwide, with *R. sanguineus* s.l. found in the temperate and tropical regions and *R. microplus* and *R. annulatus* being more commonly found in tropical and subtropical regions (3). *Rhipicephalus* ticks are known to transmit various pathogens, including bacteria, viruses, and protozoans, such as Crimean-Congo hemorrhagic fever virus, *Babesia*, *Anaplasma*, *Ehrlichia*, and *Rickettsia* species, which can cause diseases such as Crimean-Congo hemorrhagic fever, Lyme disease, Rocky Mountain spotted fever, and other tick-borne illnesses (3, 4).

The genus *Hyalomma* comprises several species of hard ticks that are widely distributed in Africa, Asia, and Europe (5). These ticks are known to transmit a number of pathogens, including viruses, bacteria, and protozoa, some of which can cause severe diseases in humans and animals (3). *Hyalomma* ticks are important vectors of the Crimean-Congo hemorrhagic fever virus, a highly pathogenic virus that can cause severe hemorrhagic fever in humans (6, 7). They are also known to transmit several other viral pathogens, such as the Nairobi sheep disease virus and the Alkhumra virus (8). In addition, *Hyalomma* ticks can transmit bacterial pathogens such as *Rickettsia*, *Ehrlichia*, and *Anaplasma*, as well as protozoan pathogens such as *Theileria* and *Babesia* (9–12). Some *Hyalomma* species are also associated with the transmission of tick-borne encephalitis virus, a flavivirus that causes a range of neurological symptoms in humans (13).

The identification of *Rhipicephalus* and *Hyalomma* species using morphological diagnosis keys might be constrained by several factors (14). For instance, species from the *R. sanguineus* group have very similar morphologies, making their distinction quite laborious (15) and requiring therefore advanced taxonomic knowledge (i.e., male sclerites) for reliable species identification. In addition, tick species morphological diagnosis could be rendered problematic due to the alteration of some key morphological traits during the course of engorgement, in particular, for female ticks. Moreover, some species may exhibit significant variations in morphology depending on their geographic location or the host they are feeding on (16). Finally, in comparison to adult ticks, species identification is more problematic with juvenile stages of *Rhipicephalus* and *Hyalomma* due to the absence of expression of some key morphological features present in adults. Accordingly, morphological diagnosis can be time-consuming, requiring specialized knowledge and training, and these taxonomic competences may not be readily available in some regions (17). These challenges underscore the importance of incorporating additional approaches to morphological diagnosis, including molecular and genetic techniques, to identify and classify tick species more accurately (17).

The use of DNA markers has significantly improved the reliability of the identification of tick species, including those of the genera *Hyalomma* and *Rhipicephalus* (18). Compared to morphological diagnosis, DNA-based identification was shown, for several tick species, to be highly accurate and able to differentiate between closely related species (19). Some of the DNA markers used for species identification in these genera include mitochondrial DNA (mtDNA) genes, such as cytochrome c oxidase subunit 1 (CO1) and 16S rRNA, as well as nuclear DNA markers like the internal transcribed spacer (ITS) region (20–22). These markers were successfully applied to species identification for instance with *Hyalomma dromedarii* and *Rhipicephalus microplus* (23–25).

However, and despite their usefulness, there are limitations to the use of existing DNA markers for tick species identification (26, 27). Indeed, there are still gaps in the available DNA sequence databases for some tick species, emphasizing accordingly, the need to design more reliable molecular markers for their identification (28). Moreover, the existence of hybridization and introgression events between different tick species can further complicate the identification process. Therefore, the development of more comprehensive DNA sequence databases and the use of multiple molecular markers can help to increase the accuracy and reliability of tick species identification (29).

In this context, the present study targeted an mRNA-based marker as a complementary tool to morphological diagnosis and to genomic and/or mitochondrial DNA markers. Unlike genomic DNA markers, which have several limitations, mRNA markers provide more specific and sensitive information for species identification. mRNA is a complementary copy of a gene that is used as a template for protein synthesis (30). mRNA-based markers are highly conserved among species and are often more species-specific than genomic markers (31, 32). These markers can be used in combination with traditional morphological methods and other molecular markers to improve the accuracy of tick species identification.

Our study focused on identifying and validating mRNA-based markers for the diagnosis of *Hyalomma* and *Rhipicephalus* species. The rational base of our study was to determine the minimum length of partial *Bm86* cDNA fragments required for species identification in two tick genera, namely *Rhipicephalus* and *Hyalomma*. The decision to use *Bm86* cDNA encoding the vaccine target gut protein is justified by the fact that the complete cDNA sequence perfectly aligns with the recent classification of hard ticks (31). This strategy offers several expected benefits, including the potential to detect several tick species using a single molecular marker (33). Although changes in climate and in the length of the different seasons will directly affect tick survival, activity, and development, our mRNA-based markers can be applied to both engorged and non-engorged ticks, making it useful for analyzing ticks in various developmental stages and environmental settings (34, 35).

Therefore, the aims of this study were to conduct an *in silico* analysis to identify the minimum partial *Bm86* cDNA sequence required for the identification of species within the *Hyalomma* and *Rhipicephalus* genera, to develop a molecular method based on the amplification and sequencing of these two minimal sequences, and to apply this technique for the identification of tick field specimens belonging to the two genera collected from several hosts located in different Tunisian regions.

2. Materials and methods

2.1. Sequence and data acquisition from GenBank

In this study, a selection of minimum length partial *Bm86* cDNA fragments required for species delineation within *Rhipicephalus* and *Hyalomma* tick genera was performed. This selection was based on all complete or nearly complete *Bm86* cDNA sequences available in GenBank on 1 January 2023. All sequences were screened and downloaded from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/) through a BLAST analysis (maximum and minimum recovery of 100 and 68%, respectively). In particular, 175 isolates or strains belonging to the six classified *Rhipicephalus* species and 19 isolates or strains belonging to the five classified *Hyalomma* species, which is available in the GenBank® database of NCBI (http://www.ncbi.nlm.nih.gov/nuccore/), were analyzed in this study (36–45) (Supplementary Tables 1–3).

2.2. Multiple sequence alignments

Multiple sequence alignments and sequence similarities were calculated using the CLUSTAL W 1.81. The DNAMAN software (Version 5.2.2; Lynnon Biosoft, Que., Canada) was also used to create two *Bm86* sequences' alignment profiles. Partial *Bm86* sequences that ensure effective discrimination between species of *Rhipicephalus* and *Hyalomma* genera were selected to investigate interspecific diversity positions with the analysis of sequence alignments (Figures 1, 2).

2.3. Collection and species identification of tick samples

Between June and August 2020, adult tick specimens were collected from four different bioclimatic areas and five governorates (Table 1). Specimens were hand-picked from live domestic animals (e.g., cattle, camels, goats, sheep, and dogs). Tick specimens were identified based on morphological characteristics according to the identification keys of Walker et al. (46). Tick samples were preserved in 1 ml of TRIzol reagent (Invitrogen life technologies, Invitrogen Corporation, California, USA) and stored at −80°C for subsequent uses.

2.4. Total RNA and genomic DNA co-extraction

A total of 14 field tick specimens belonging to *Rhipicephalus* and *Hyalomma* genera were selected for the field evaluation of minimum length *Bm86* cDNA fragments. DNA and RNA co-extraction from a single adult tick specimen were performed using TRIzol reagent (Invitrogen, CA, USA). Each whole tick was crashed and homogenized in TRIzol reagent (Invitrogen, CA, USA) (1 mL/100 mg tissue) using a pestle and liquid nitrogen. Phases' separation was performed by adding chloroform in a TRIzol to chloroform ratio of 3:1 according to the manufacturer's instructions. For each sample, the aqueous phase was separated from the interphase/organic phase and subjected to RNA isolation according to the TRIzol reagent protocol. The final resuspension of total RNA was performed in 50 µl of RNase-free water, followed by incubation at 56°C for 10 min for complete resolubilization of the nucleic acid. RNA samples were immediately stored at −80°C until further use. The DNA samples present in the interphase/organic phase were purified according to the TRIzol reagent according to the manufacturer's instructions. DNA isolation was carried out with a final resuspension in 0.1–0.2 ml of 8 mM NaOH. Insoluble materials were pelleted by centrifugation for 10 min at 12,000 × g at 4°C. The supernatant was transferred to a new 1.5 ml tube and stored at −20°C until use.

2.5. Amplification of mitochondrial 16s rRNA partial sequences

To confirm the results of the morphological diagnosis, a partial mitochondrial 16S rRNA sequence (273 bp) was amplified from all analyzed tick specimens by using primers TQ16S+1F (5′-CTGCTCAATGATTTTTTAAATTGCTGTGG-3′) and TQ16S-2R (5′-ACGCTGTTATCCCTAGAG−3′), as previously described by Black and Piesman (47).

2.6. cDNA synthesis and amplification of *Bm86* partial sequences

The first-strand synthesis reaction was carried out using the SuperScript First-strand Synthesis System for the RT-PCR kit (Invitrogen USA) following the manufacturer's instructions. Two primer sets employed to amplify the selected minimum length partial *Bm86* cDNA fragments needed for species delineation within *Rhipicephalus* and *Hyalomma* tick genera were designed, and the position of each primer was obtained with respect to the *Bm86* cDNA sequence isolated from the *R. (B.) australis* (formerly *R. microplus*) Yeerongpilly strain (GenBank Accession number M29321) and the *Ha98* cDNA sequence isolated from the *H. anatolicum* India strain (GenBank accession number AF347079), for *Rhipicephalus* and *Hyalomma* tick genera, respectively (Table 2).

All PCRs were performed in a final volume of 50 µl containing 0.125 U/µl of Taq DNA polymerase, 1× PCR buffer 10×, 2.5 mM of MgCl2, 0.2 mM of dNTPs, 4 µl (50 to 150 ng) cDNA, and 0.5 µM of primers. PCR reactions were performed in an automated DNA thermal cycler. The selected minimum length partial *Bm86* cDNA fragment (603 bp) for *Hyalomma* species delineation was amplified using the following conditions: an initial denaturation step of 5 min at 94°C followed by 40 cycles of 30 s at 94°C, 30 s at 54°C, 50 s at 72°C, and a final extension step of 72°C for 10 min. However, the thermal profile used for the amplification of partial *Bm86* cDNA

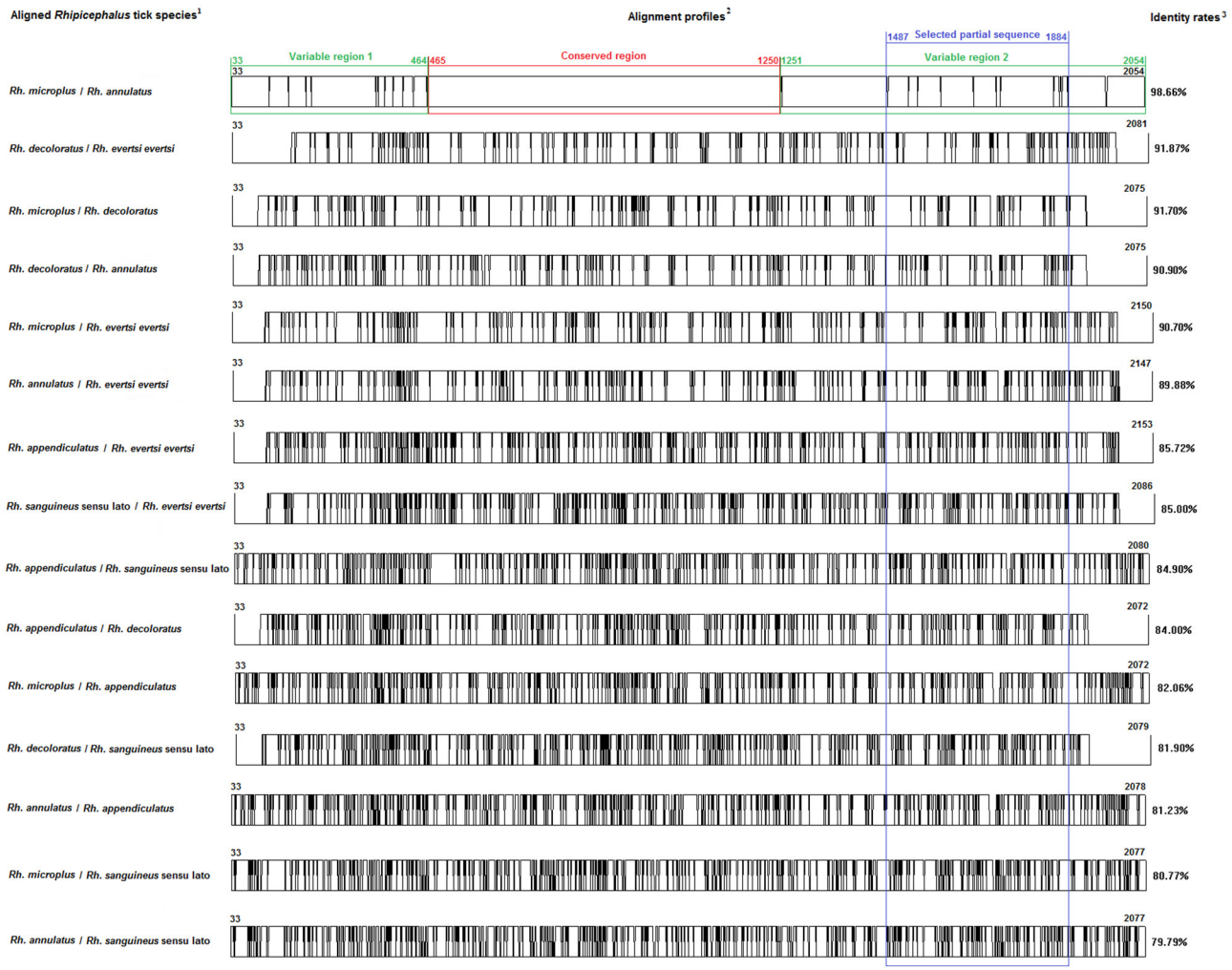

FIGURE 1
Multiple alignment profiles allowing the selection of minimum length *Bm86* cDNA fragments required for species delineation within *Rhipicephalus* tick genera. [1]*Rhipicephalus* species analyzed in each alignment profile. [2]Alignment profiles of all available genetic variants isolated from each *Rhipicephalus* species. [3]Average percent identity between genetic variants of each *Rhipicephalus* species.

fragment required for the *Rhipicephalus* spp. discrimination was as follows: an initial denaturation step of 5 min at 94°C followed by 35 cycles of 30 s at 94°C, 30 s at 57°C, 1 min at 72°C, and a final extension step of 72°C for 15 min. PCR products were observed after electrophoretic migration on 1% agarose gels stained with ethidium bromide and under UV transillumination (Figure 3).

2.7. Specificity and sensitivity of PCR assays

To determine the specificity of the PCR reactions, we utilized two sets of primers—AD-Bm86-Hyl/AR-Bm86-Hyl and AD-Bm86-Rhip/AR-Bm86-Rhip—to analyze samples of *Rhipicephalus* and *Hyalomma* spp. through PCR. In addition, to evaluate the sensitivity of the assay, we conducted serial dilutions of a quantified cDNA and calculated the PCR sensitivity in terms of the amount of cDNA per µl (Figure 4).

2.8. DNA sequencing, sequence analysis, and phylogenetic trees' construction

PCR products generated from mitochondrial 16S rRNA and *Bm86* partial sequences were purified and sequenced on both forward and reverse strands by using the same primers employed in PCR to obtain maximal data accuracy. The Big Dye Terminator cycle sequencing ready reaction kit (Applied Biosystems, Foster City, USA) and an ABI3730XL automated DNA sequencer (Macrogen Europe, Amsterdam, The Netherlands) were employed.

The chromatograms were evaluated with Chromas Lite v 2.01 (http://www.technelysium.com.au/chromas_lite.html). Obtained raw sequences were edited, primer region sequences were manually removed, and the overlapping parts were selected. BLAST analysis was performed to assess the level of similarity with previously reported sequences (http://blast.ncbi.nlm.nih.gov/).

Nucleotide sequences of the partial *Bm86* orthologs were compared with previously reported sequences isolated from

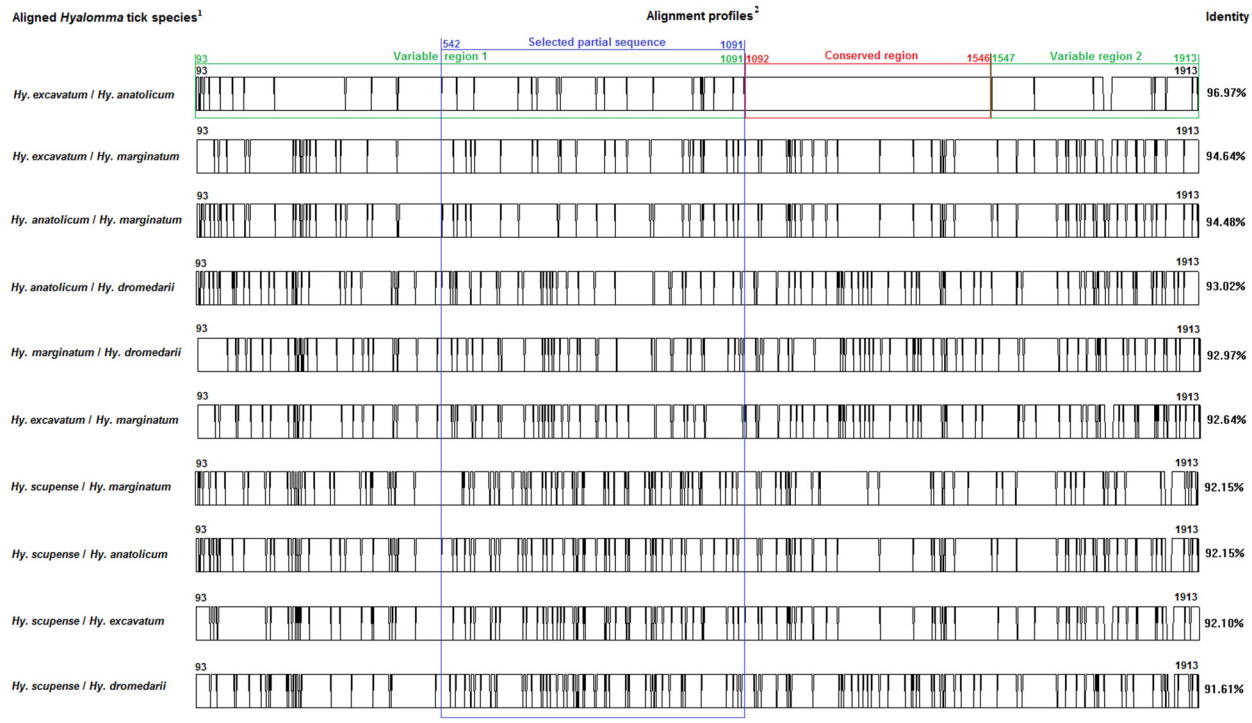

FIGURE 2
Multiple alignment profiles allowing the selection of minimum length *Bm86* cDNA fragments required for species delineation within *Hyalomma* tick genera. [1]*Hyalomma* species analyzed in each alignment profile. [2]Alignment profiles of all available genetic variants isolated from each *Hyalomma* species. [3]Average percent identity between genetic variants of each *Hyalomma* species.

Hyalomma and *Rhipicephalus* spp. ticks using the DNAMAN program (Version 5.2.2; Lynnon Biosoft, Quebec, Canada). By using the same software, genetic distances were computed by the maximum composite likelihood method (50) and were used to construct neighbor-joining trees (50). Statistical support for the internal branches of trees was established by bootstrap analysis with 1,000 reiterations (51).

The mitochondrial 16S rRNA and *Bm86* partial sequences of *Hyalomma* and *Rhipicephalus* spp. isolates were deposited in the GenBank under the accession numbers OP749949-OP749962 and OP762544-OP762557, respectively.

3. Results

3.1. Selection of minimum length partial *Bm86* cDNA fragments needed for species delineation

3.1.1. Identification of genetic variants

Based on all available complete and nearly complete sequences of *Bm86* cDNA isolated from *Rhipicephalus* and *Hyalomma* species, we precisely selected the minimal length partial sequences that will allow us to discriminate between all classified species within each tick genus. All genetic variants of *Bm86* cDNA belonging to each *Rhipicephalus* and *Hyalomma* species were included in our data set. These genetic variants differ from each other by at least one mutation and represent complete or nearly complete sequences of all strains and isolates submitted in GenBank (Supplementary Tables 1–3). For all available *Rhipicephalus* and *Hyalomma* species, the two genetic variants (each representing one classified species) that showed the lowest diversity were selected, and the two sequences' alignment profiles are shown in Figures 1, 2.

3.1.2. Identification of conserved and variable sequences

First, we identified the *Bm86* regions that are conserved at least between the two closest genetic variants belonging to the two closest classified species within *Rhipicephalus* and *Hyalomma* genera to eliminate them when selecting the minimal partial sequences required for species delineation (Figures 1, 2).

Analysis of all possible combinations of two sequence alignments for all selected variants belonging to the *Rhipicephalus* species revealed that only the alignment profile resulting from *Rh. microplus* and *Rh. annulatus* variants showed one conserved region of 786 bp (from 465 to 1,250) and two variable regions. The first variable region is located from position 33 to position 464 by having a size of 432 pb, while the second variable region begins just after the conserved region and continues until the end of the *Bm86* cDNA sequence (from position 1,251 to position 2,054, 804 bp).

Moreover, the same analysis was performed for the selected genetic variants of each *Hyalomma* species. The two sequences' alignments showed that only the alignment between the two selected genetic variants of *Hy. excavatum* and *Hy. anatolicum* showed a conserved region having a size of 455 bp (from 1,092 to

TABLE 1 Information about origins and hosts of studied field tick specimens belonging to *Hyalomma* and *Rhipicephalus* genera from Tunisia and blast analysis of sequenced mitochondrial 16S rRNA and *Bm86* cDNA fragments.

Tick specimen	Host species	District (governorate, bioclimatic area)	GenBank Acc. Nb. (mito16SrRNA/*Bm86*)	Morphological diagnosis	Blast analysis	
					Mito 16S rRNA partial sequence	*Bm86* cDNA minimal sequence
Hs1	*B. taurus*	Raoued (A, SA)	OP749949/OP762544	*Hy. scupense*	100% to *Hy. scupense* (MK601705)	100% to *Hy. scupense* (HQ872022)
Ha1	*B. taurus*	Amdoun (B, H)	OP749950/OP762545	*Hy. anatolicum*	100% to *Hy. anatolicum* (MT509435)	99.28% to *Hy. anatolicum* (EU665682)
He1	*B. taurus*	Nefza (B, H)	OP749951/OP762546	*Hy. excavatum*	100% to *Hy. excavatum* (MK601704)	99.10% to *Hy. excavatum* (JF298786)
He2	*B. taurus*	Kalâa Kebira (S, SA)	OP749952/OP762547	*Hy. excavatum*	100% to *Hy. excavatum* MK601704	99.11% to *Hy. excavatum* (JF298786)
Hm1	*B. taurus*	Raoued (A, SA)	OP749953/OP762548	*Hy. marginatum*	100% to *Hy. marginatum* (MT229186)	100% to *Hy. marginatum* (KF527438)
Hm2	*B. taurus*	Nefza (B, H)	OP749954/OP762549	*Hy. marginatum*	100% to *Hy. marginatum* (MT229186)	100% to *Hy. marginatum* (KF527438)
Hm3	*B. taurus*	El Fahs (Z, SA)	OP749955/OP762550	*Hy. marginatum*	100% to *Hy. marginatum* (MT229186)	100% to *Hy. marginatum* (KF527438)
Hi1	*C. dromedarius*	Remada (T, A)	OP749956/OP762551	*Hy. impeltatum*	100% to *Hy. impeltatum* (MN960583)	97.67% to *Hy. anatolicum*[1] (MH325952)
Hd1	*C. dromedarius*	Remada (T, A)	OP749957/OP762552	*Hy. dromedarii*	100% to *Hy. dromedarii* (MN960589)	99.82% to *Hy. dromedarii* (JF298785)
Rb1	*O. aries*	Raoued (A, SA)	OP749958/OP762553	*Rh. bursa*	100% to *Rh. bursa* (MT302761)	96.61% to *Rh. evertsi evertsi*[2] (GU144600)
Rs1	*B. taurus*	Amdoun (B, H)	OP749959/OP762554	*Rh. sanguineus* s.l.	100% to *Rh. sanguineus* s.l. (MK732012)	97.34% to *Rh. sanguineus* s.l. (EF222203)
Rs2	*B. taurus*	Raoued (A, SA)	OP749960/OP762555	*Rh. sanguineus* s.l.	100% to *Rh. sanguineus* s.l. (KY413785)	97.34% to *Rh. sanguineus* s.l. (EF222203)
Rs3	*B. taurus*	Amdoun (B, H)	OP749961/OP762556	*Rh. sanguineus* s.l.	99.27% to *Rh. sanguineus* s.l. (MH630344)	96.85% to *Rh. sanguineus* s.l. (EF222203)
Rs4	*C. l. familiaris*	Sidi Thabet (A, SA)	OP749962/OP762557	*Rh. sanguineus* s.l.	100% to *Rh. sanguineus* s.l. (KY413785)	96.85% to *Rh. sanguineus* s.l. (EF222203)

B. taurus , *Bos taurus*; *C. dromedarius* , *Camelus dromedarius*; *O. aries* , *Ovis aries*; *C. l. familiaris* , *Canis lupus familiaris*; A , Ariana; B , Beja; S , Sousse; Z , Zaghouan; T , Tataouine; SA , Semi-arid; H , Humid; A , Arid; *Hy. scupense*, *Hyalomma scupense*; *Hy. anatolicum*, *Hyalomma anatolicum*; *Hy. excavatum*, *Hyalomma excavatum*; *Hy. marginatum*, *Hyalomma marginatum*; *Hy. impeltatum*, *Hyalomma impeltatum*; *Hy. dromedarii*, *Hyalomma dromedarii*; *Rh. bursa*, *Rhipicephalus bursa*; *Rh. sanguineus*; s.l., *Rhipicephalus sanguineus* sensu lato.

[1] Since this is the first time that the *Bm86* ortholog has been sequenced in *Hyalomma impeltatum*, blast analysis indicated that the closest sequence was that of *Hyalomma anatolicum* from Iran (MH325952); [2] Since this is the first time that the *Bm86* ortholog has been sequenced in *Rhipicephalus bursa*, blast analysis indicated that the closest sequence was that of *Rhipicephalus evertsi evertsi* from South Africa (GU144600).

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TABLE 2 Primers employed to amplify and sequence *Bm86* cDNA minimal sequence used for species delineation within *Hyalomma* and *Rhipicephalus* genera.

Primer name	Nucleotide sequence (5'-3')	Primer position[1]	Amplified (sequenced)[2] fragments		*Bm86* cDNA minimal sequence		Annealing temperature (°C)	Reference
			Position	Size (bp)	Position	Size (bp)		
AD_Bm86_Hyl	GCGAGAAAAACYTGCTTGGAAA	517-538	517-1119	603 (559)	539-1097	559	54	Present study
AR_Bm86_Hyl	TCTCRTACCACTCGCAATGGTC	1098-1119	(539-1097)					
AD_Bm86_Rhip	TTCTGGTTCCAGTGCGCTGA	1416-1435	1416-1917	502 (460)	1487-1884	398	57	Present study
AR_Bm86_Rhip	CAGCACTYGACTTYCCASGAT	1897-1917	(1436-1896)					

[1]Numbers represent the nucleotide position of AD_Bm86_Hyl/AR_Bm86_Hyl and AD_Bm86_Rhip/AR_Bm86_Rhip primer sets with respect to the India strain of *Hyalomma anatolicum* tick from India (GenBank accession number AF347079) (48) and the Yeerongpilly strain of *Rhipicephalus (Boophilus) microplus* tick from Australia (GenBank accession number M29321) (49). [2]Represent the amplified sequences without the primers' sequences.

1,546) and two variable regions of 999 bp (from 93 to 1,091) and 367 bp (from 1,547 to 1,913).

3.1.3. Selection of partial sequences required for species delineation

For the two genera and only within these two variable regions, successive phylogenetic analyses were performed using different window lengths by transferring approximately 50 nucleotides from one assay to another. After evaluating several fragment sizes, we found that the minimum size to delineate species in each genus was located for *Rhipicephalus* spp. in the second variable region from the position 1,487 to 1,884 with a size of 398 bp (Figure 1) and in the first variable region from the position 539 to 1,097 with a size of 559 bp for *Hyalomma* species (Figure 2).

3.2. Morphological and molecular identification of tick species

Based on the morphological characteristics, collected tick specimens were classified into two genera and eight species: *Hyalomma scupense*, *Hy. anatolicum*, *Hy. excavatum*, *Hy. marginatum*, *Hy. impeltatum*, *Hy. dromedarii*, *Rhipicephalus sanguineus* sensu lato, and *Rh. bursa.* It is relevant to note that this is the first time that *H. anatolicum* has been recorded in Northern Tunisia in the humid zone. Tick species identification was also confirmed by the analysis of mitochondrial 16S rRNA partial sequences (272 bp, GenBank accession numbers OP749949-OP749962). Blast analysis showed 99–100% identity of our sequences isolated from *Hyalomma* and *Rhipicephalus* spp. with those genetically closest published in GenBank, which thus confirms the morphological diagnosis (Table 1).

3.3. Amplification of minimal length *Bm86* cDNA partial sequences

Selected tick field samples, nine from *Hyalomma* spp. and five from *Rhipicephalus*, were amplified using the specific set of primers AD-Bm86-Hyl and AR-Bm86-Hyl as well as AD-Bm86-Rhip and AR-Bm86-Rhip with the optimized PCR conditions. All *Hyalomma* samples gave an amplicon of the expected size (603 bp) when amplified with primers specific for *Hyalomma* spp. and reacted negative when amplified with primers specific for *Rhipicephalus* species (Figure 3A). Similarly, all *Rhipicephalus* samples gave an amplicon of the expected size (502 bp) only when amplified using the set of primers specific for *Rhipicephalus* species (Figure 3B). Using the newly designed primers, the cDNA *Bm86* was not amplified for any of the *Rhipicephalus* spp. tick samples using the specific primers for the *Hyalomma* genus, and similarly, cDNA *Bm86* was not amplified for any of the *Hyalomma* spp. tick samples using the specific primers for the *Rhipicephalus* genus. Our results clearly indicate that the newly developed PCR assays were highly specific and capable of accurately distinguishing between closely related tick genera, such as *Hyalomma* and *Rhipicephalus*. Additionally, the sensitivity of the assays was found to be 1.4 pg/µl

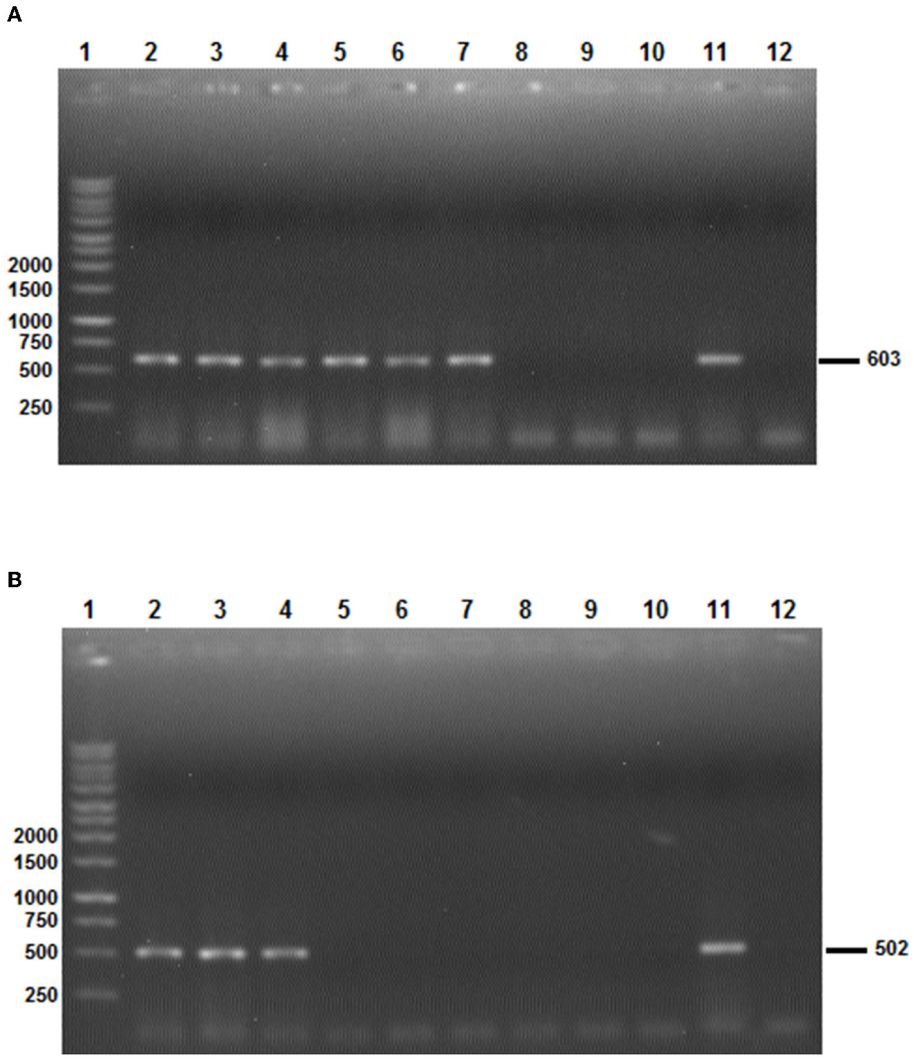

FIGURE 3
The results of the two *Bm86* minimal partial fragments' amplification specific to *Hyalomma* **(A)** and *Rhipicephalus* **(B)** species. **(A)** Line 1: 1 Kb ladder; lines 2–7: PCR products after the amplification of the *Hyalomma* spp. partial fragment based on *Bm86* cDNA orthologs isolated from *Hy. scupense*, *Hy. marginatum*, *Hy. excavatum*, *Hy. anatolicum*, *Hy. Dromedarii*, and *Hy. impeltatum*, respectively; lines 8–10: PCR products showing the absence of amplification of the *Hyalomma* spp. partial fragment based on *Bm86* cDNA orthologs isolated from *Rh. bursa*, *Rh. sanguineus* s.l (Rs1 from cattle), and *Rh. sanguineus* s.l (Rs4 from a dog), respectively; and lines 11 and 12: positive and negative controls, respectively. **(B)** Line 1: 1 Kb ladder; lines 2–4: PCR products after the amplification of *Rhipicephalus* spp. partial fragment based on *Bm86* cDNA orthologs isolated from *Rh. bursa*, *Rh. sanguineus* s.l (Rs1 from cattle), and *Rh. sanguineus* s.l (Rs4 from a dog); lines 5–10: PCR products showing the absence of amplification of the *Rhipicephalus* spp. partial fragment based on *Bm86* cDNA orthologs isolated from *Hy. scupense*, *Hy. marginatum*, *Hy. excavatum*, *Hy. anatolicum*, *Hy. Dromedarii*, and *Hy. impeltatum*, respectively; and lines 11 and 12: positive and negative controls, respectively.

and 1.9 pg/μl for the genus-specific PCRs for *Hyalomma* and *Rhipicephalus*, respectively (Figure 4).

3.4. Genetic analysis of minimal length *Bm86* cDNA partial sequences

To assess the taxonomic interest of *Bm86* orthologs, the amplified fragments of 559 bp and 460 bp containing partial targeted sequences of the *Bm86* transcript were sequenced for *Hyalomma* and *Rhipicephalus* spp., respectively. All sequences were subjected to a Basic Local Alignment Search Tool (BLAST) search to confirm tick species and estimate the homology rate of each sequence with the closest sequence already published in GenBank.

Blast analysis performed on the sequenced *Hy. scupense* ortholog showed 100% sequence homology with the previously published *Hy. scupense* strain Beja (GenBank accession number HQ872022) recovered from cattle in Tunisia. Only one genotype HmBm86G1 was revealed from three *Hy. marginatum* isolates which also shared 100% homology with the *Hy. marginatum* strain Kutahya from Turkey (GenBank accession number KF527438). Two distinct and novel genotypes (HeBm86G1 and HeBm86G1) were identified (GenBank accession numbers OP762546 and OP762546) from two *Hy. excavatum* isolates which shared 99% homology with *Hy. excavatum* strain Sousse recovered from

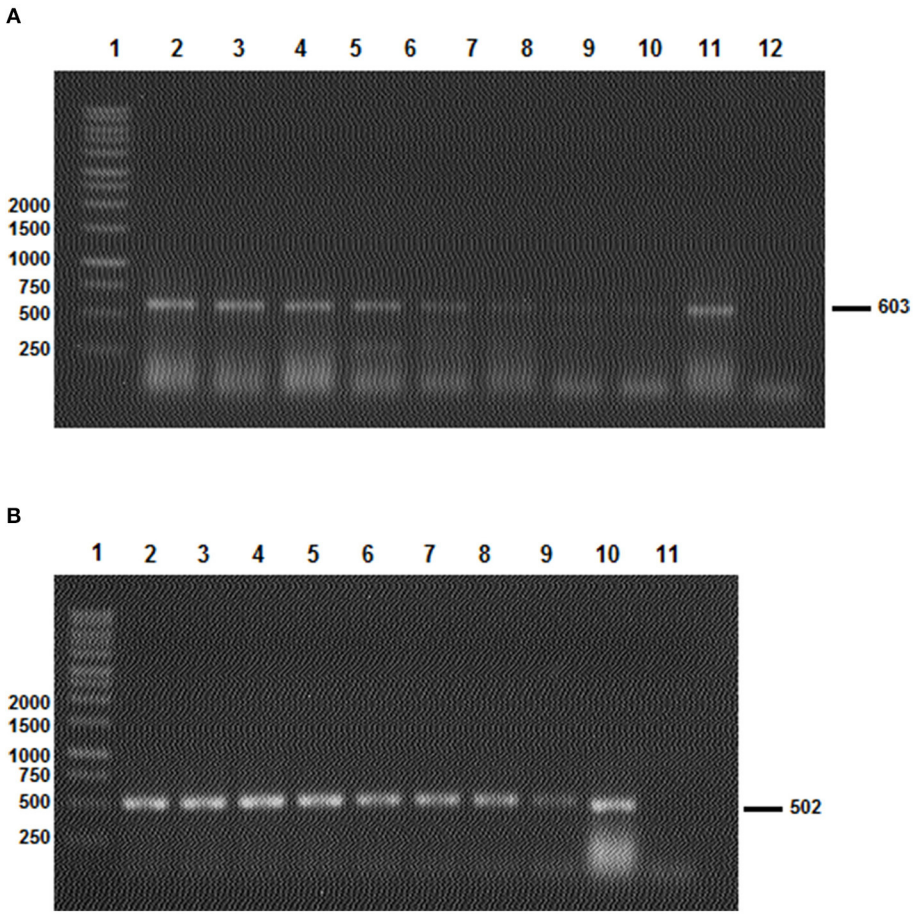

FIGURE 4
The results of the two *Bm86* minimal partial fragments' amplification sensibility test to *Hyalomma* **(A)** and *Rhipicephalus* **(B)** species. **(A)** Line 1: 1 Kb ladder; lines 2–10: PCR products after the amplification of the *Hyalomma* spp. partial fragment based on a serial dilution of a quantified *Bm86* cDNA (from 1.4 $\mu g/\mu l$ to 1.4×10^{-8} $\mu g/\mu l$) isolated from *Hy. scupense* and lines 11 and 12: positive and negative controls, respectively. **(B)** Line 1: 1 Kb ladder; lines 2–4: PCR products after the amplification of *Rhipicephalus* spp. partial fragment based on a serial dilution of a quantified *Bm86* cDNA (from 1.9 $\mu g/\mu l$ to 1.9×10^{-8} $\mu g/\mu l$) isolated from *Rh. sanguineus* s.l (Rs1 from cattle) and lines 10 and 11: positive and negative controls, respectively.

cattle in Tunisia (GenBank accession number JF298786). For *Hy. anatolicum* and *Hy. dromedarii* orthologs, nucleotide sequence identities were superior to 99% in comparison with other previously published *Bm86 Hyalomma* species, resulting in one novel genotype each (HaBm86G1 and HdBm86G1, respectively; Table 1). Since there is no published sequence in GenBank of the *Bm86* gene from *Hy. impeltatum*, one genotype HiBm86G1 was revealed as novel, and blast analysis performed on the *Bm86* ortholog isolated from *Hy. impeltatum* (GenBank accession number OP762551) showed that this sequence has 97.6% sequence homology to the closest sequence that was isolated from the *Hy. anatolicum* isolate Alborz found in an Iranian sheep (GenBank accession number MH325952).

Based on nucleotide alignments of *Bm86* partial nucleotidic sequences (413 bp) of four Tunisian *Rhipicephalus sanguineus sensu lato* isolates, a total of three distinct and novel genotypes RsBm86G1-3 were identified (GenBank accession numbers OP762554 to OP762557). The revealed genotypes were 96.8% to 97.3% similar to *Rh. sanguineus* s.l. (GenBank accession number EF222203; Table 1). Since this is the first time that the *Bm86* ortholog has been sequenced in *Rhipicephalus bursa*, one genotype RbBm86G1 was revealed as novel, and the blast analysis indicated that the closest sequence was that of *Rhipicephalus evertsi evertsi* from South Africa (GU144600) with 96.6% homology (Table 1).

3.5. Comparative analysis of phylogenetic trees built for species delineation

Phylogenetic trees based on the alignment of studied minimum length *Bm86* fragments belonging to both *Hyalomma* and *Rhipicephalus* species were compared with those generated from all complete or nearly complete *Bm86* gene sequences of all *Hyalomma* and *Rhipicephalus* species found in GenBank.

Phylogenetic analysis based on the alignment of Tunisian genotypes with different minimal partial sequences of the *Bm86* gene of all classified species of the genus *Hyalomma* obtained from the GenBank generated various clades (Figures 5A, B). The phylogenetic tree demonstrated the discriminatory power of the minimal *Bm86* fragment between *Hyalomma* species. Both revealed

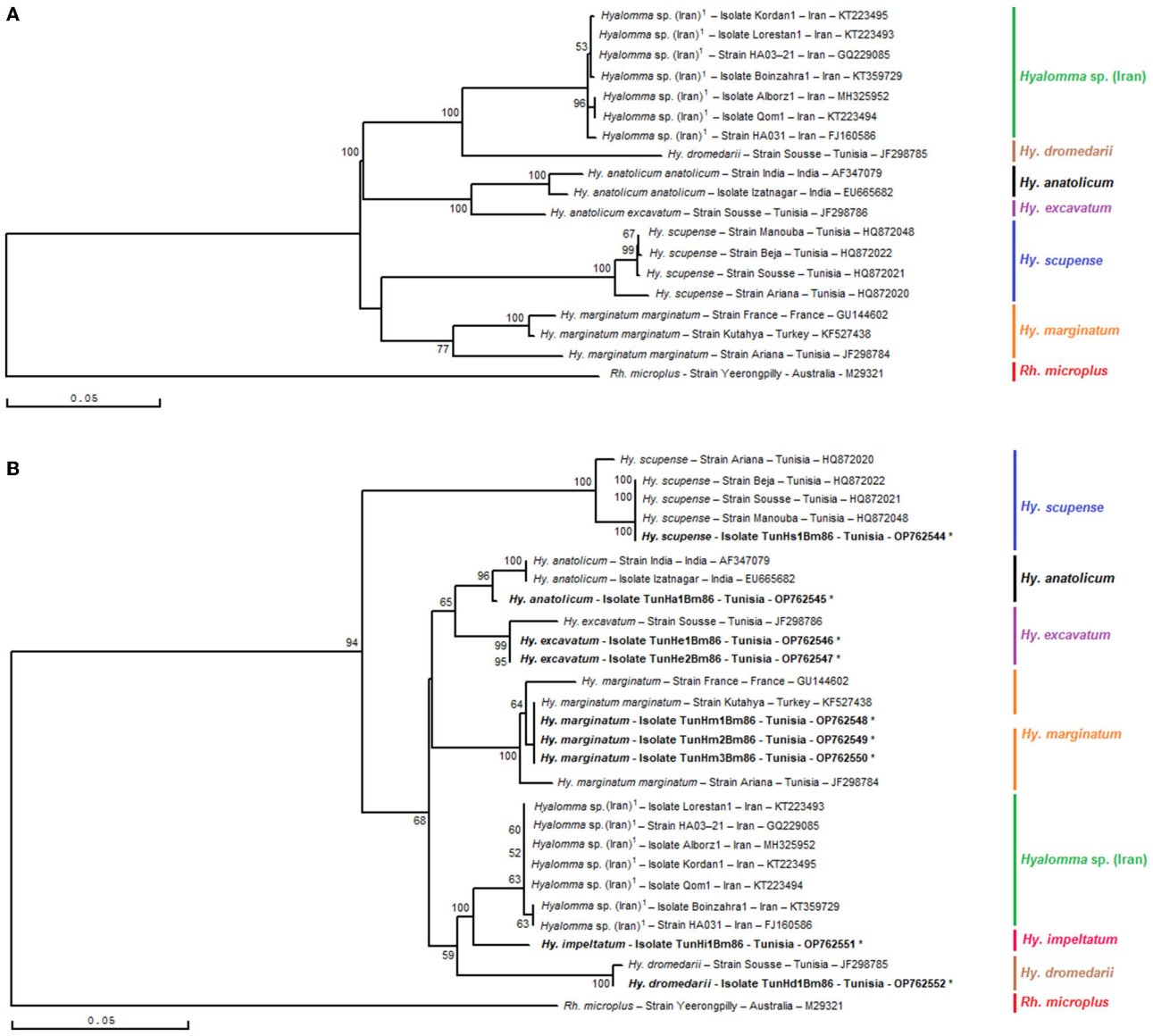

FIGURE 5
Phylogenetic tree inferred with complete or nearly complete *Bm86* gene sequences **(A)** and selected minimal partial *Bm86* sequences (559 bp) **(B)** required for species delineation within *Hyalomma* tick genera using the neighbor-joining method. Numbers associated with nodes represent the percentage of 1,000 bootstrap iterations supporting the nodes (only percentages >50% are show). The host or vector, strain or isolate name, the country of origin, and the GenBank accession number are indicated. The Yeerongpilly strain of *Rhipicephalus* (*Boophilus*) *microplus* tick from Australia (M29321) was added as an out-group.

Hy. excavatum Tunisian strains were classified separately with the Tunisian ortholog strain Sousse isolated from cattle (GenBank accession number JF298786) within *Hy. excavatum* cluster. This latter strain was closely related to the Tunisian *Hy. anatolicum* isolate TunHa1Bm86 found on cattle (GenBank accession number OP762545), which was clustered within the *Hy. anatolicum* cluster containing both orthologs, strain India and isolate Izatnagar, isolated from *Hyalomma anatolicum* in India (GenBank accession numbers AF347079 and EU665682, respectively).

Moreover, the studied minimal length *Bm86* fragment allowed the same isolates' discrimination within each one of the *Hyalomma* species than that obtained with the complete or nearly complete sequences. In particular, as shown by the *Hyalomma* tree generated by complete or nearly complete sequences, all *Hy. marginatum* Tunisian strains were grouped into one *Hy. marginatum* sub-cluster with the Kutahya strain isolated from Turkey and closely related to *Hy. marginatum* strain France and strain Ariana isolated from Tunisian cattle (GenBank accession numbers GU144602 and JF298784, respectively) in the tree based on our minimal length *Bm86* fragment. Furthermore, the studied minimal length *Bm86* fragment allowed the discrimination within *Hy. scupense* isolates resembling that made by the complete or nearly complete sequences. In fact, the Tunisian *Hy. scupense* isolate TunHs1Bm86 found on cattle (GenBank accession number OP762544) was grouped into one of two *Hy. scupense* sub-clusters containing three ortholog strains, namely, Beja, Sousse, and Manouba from Tunisia

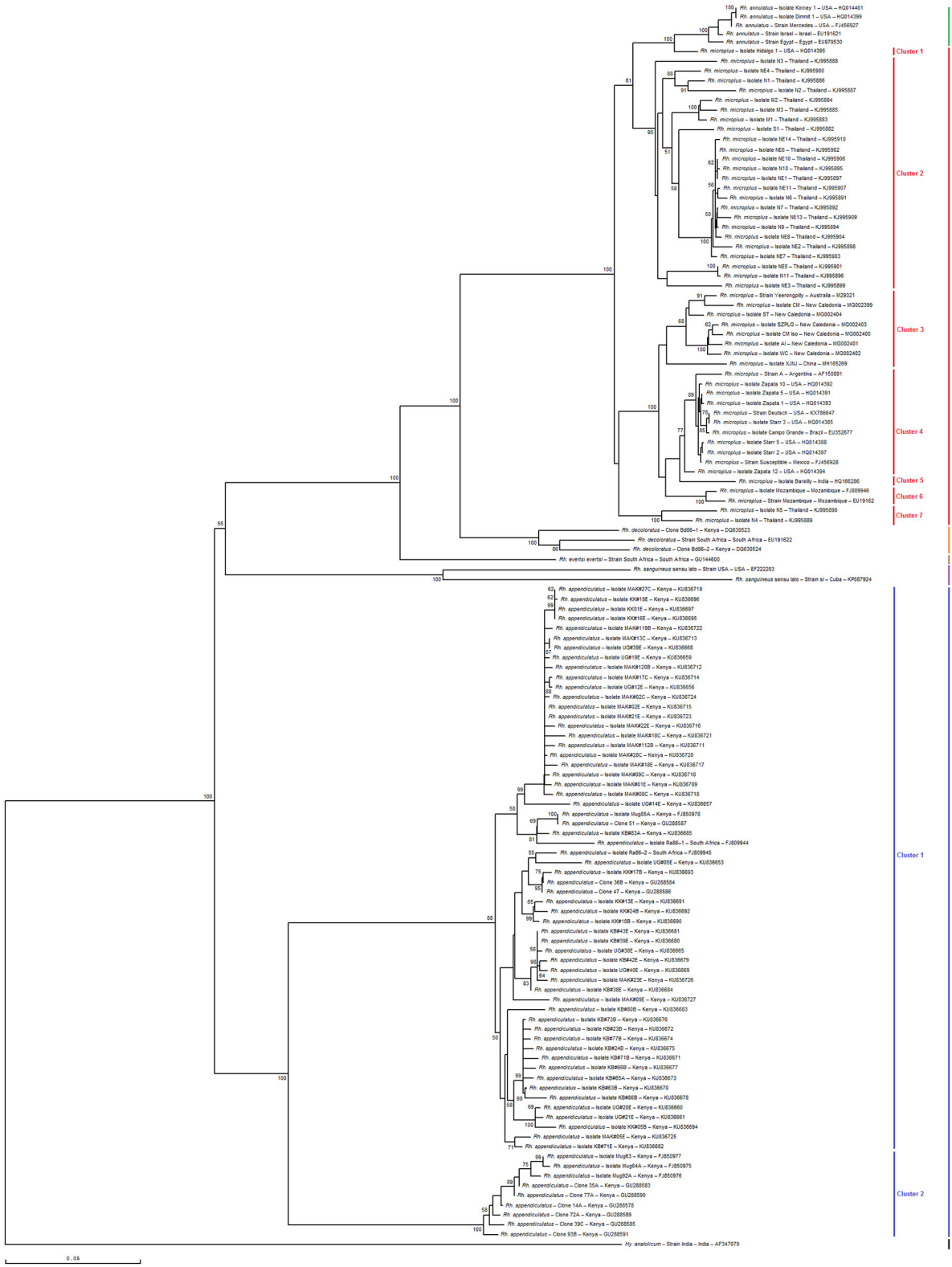

FIGURE 6
Phylogenetic tree inferred with complete or nearly complete *Bm86* gene sequences required for species delineation within *Rhipicephalus* tick genera using the neighbor-joining method. Numbers associated with nodes represent the percentage of 1,000 bootstrap iterations supporting the nodes (only percentages >50% are show). The host or vector, strain or isolate name, the country of origin, and the GenBank accession number are indicated. The India strain of *Hyalomma anatolicum* tick from India (AF347079) was added as an out-group.

FIGURE 7
Phylogenetic tree inferred with selected minimal partial *Bm86* gene sequences (398 bp) required for species delineation within *Rhipicephalus* tick genera using the neighbor-joining method. Numbers associated with nodes represent the percentage of 1,000 bootstrap iterations supporting the nodes (only percentages >50% are shown). The host or vector, strain or isolate name, the country of origin, and the GenBank accession number are indicated. The India strain of *Hyalomma anatolicum* tick from India (AF347079) was added as an out-group.

and thereby leaving the *Hy. scupense* strain Ariana from Tunisia (GenBank accession number HQ872020) in a different separate *Hy. scupense* sub-cluster (Figures 5A, B).

As shown in Figures 6, 7, the phylogenetic tree based on selected minimal partial *Bm86 Rhipicephalus* spp. fragments exhibited almost the same topology found by the analysis of complete or nearly complete *Bm86* gene sequences from all available *Rhipicephalus* species available in GenBank. Almost no changes in phylogenetic relationships were observed. All revealed Tunisian *Rhipicephalus* spp. isolates were classified into the *Rh. sanguineus* s.l. cluster which was closely related to the USA strain (GenBank accession number EF222203) except for the novel Tunisian *Rh. bursa* isolate which was closely related to *Rh. evertsi evertsi* strain from South Africa (GenBank accession number GU144600).

The results also revealed that the minimal length *Bm86* transcript sequence specific to the *Rhipicephalus* genus was effective in discriminating between different groups of *Rh. microplus* and *Rh. appendiculatus* isolates. Specifically, phylogenetic analysis based on the minimal length *Bm86* transcript sequence generated groups of *Rh. microplus* isolates based on the geographic regions similar to those found by the phylogenetic analysis based on the complete or nearly complete sequence. In fact, the groups of isolates from the North and Latin America group (USA, Mexico, and Brazil), Asia-Pacific group (New Caledonia, Australia, China, and India), Thailand, and Mozambique were revealed in both phylogenetic trees based on the two types of sequences (Figures 6, 7). In addition, our partial sequence was able to distinguish between two exceptionally divergent homologs (Ra86) of *Rh. appendiculatus* isolates in a manner similar to the complete or nearly complete transcript *Bm86* sequence, as indicated by the two types of generated trees (Figures 6, 7).

Both phylogenetic trees, constructed by aligning the studied minimum length *Bm86* fragments with complete or nearly complete *Bm86* gene sequences of all *Rhipicephalus* species available in GenBank, clearly showcased the remarkable discriminatory ability of the minimal *Bm86* fragment in distinguishing *Rhipicephalus* species at a phylogeographic level. In particular, as shown by the *Rhipicephalus* tree generated by minimum length *Bm86* fragments, all *Rh. microplus* sequences isolated from Thailand were grouped into *Rh. microplus* clusters 1, 4, and 6, which were also grouped into *Rh. microplus* clusters 2 and 7 regarding the *Rhipicephalus* tree generated by complete or nearly complete *Bm86* gene sequences. Nonetheless, the studied minimal length *Bm86* fragment allowed the discrimination within *Rh. microplus* sequences isolated from countries of the American continent (USA, Mexico, Brazil, and Argentina) which were grouped into *Rh. microplus* cluster 2 and *Rh. microplus* sequences isolated from countries of the African continent, specifically from Mozambique, which were grouped into *Rh. microplus* cluster 5. A consistent discrimination profile was created similar to that made by the *Rhipicephalus* phylogenetic tree generated by complete or nearly complete *Bm86* gene sequences, in which *Rh. microplus* sequences isolated from countries of the American continent were grouped into *Rh. microplus* clusters 1 and 4, and *Rh. microplus* sequences isolated from Mozambique were grouped into *Rh. microplus* cluster 6 (Figures 6, 7).

Furthermore, the studied minimal length *Bm86* fragment allowed the discrimination within *Rh. annulatus* isolates similar to that made by the complete or nearly complete sequences. In particular, five isolates were grouped into *Rh. annulatus* cluster that differ by the country of origin, a total of three *Rh. annulatus* isolates from the USA, one *Rh. annulatus* strain from Palestine (GenBank accession number EU191621), and one *Rh. annulatus* strain from Egypt (GenBank accession number EU979530) (Figures 6, 7).

4. Discussion

The identification of tick species has traditionally relied on morphological methods, but these methods can be difficult and subjective. Difficulties may arise during the morphological diagnosis of tick species due to the presence of significant morphological variations related to the stages of development, the state of engorgement of ticks, and the possible presence of hybrid specimens resulting from crossovers between two closely related species (52–54). In recent years, the use of DNA markers for determining tick species has gained widespread popularity due to its precision and efficiency compared to traditional morphological methods (17) and its ability to provide more detailed information about the evolutionary relationships between different species (55).

In recent years, several DNA markers such as the internal transcribed spacer (ITS) region, the cytochrome c oxidase subunit I (COI) gene, and the 16S rRNA gene have been used for tick species identification (15, 52, 56, 57). However, despite their wide use, these markers have been found to have limitations in terms of their discriminatory power and sensitivity (15, 27, 58, 59). For example, the ITS region has been shown to have limited resolution in differentiating between closely related species (27), while the COI gene has been found to have low sensitivity in detecting intraspecific genetic variations (27). Similarly, the 16S rRNA gene has limitations in its ability to differentiate between tick species that belong to different genera (60). In light of these limitations, there is a need for more effective DNA markers that can provide better tick species identification. These limitations are the reason where the proposed study comes in, as it aims to identify the minimum length of partial *Bm86* cDNA fragments needed for species delineation in the tick genera *Rhipicephalus* and *Hyalomma*.

The *Bm86* cDNA marker represents a new and innovative way of species delineation in ticks as it targets the tick's RNA banks that have been previously utilized in various research studies. In many research laboratories, these RNA libraries are for the most part widely studied for their potential in the development of anti-tick vaccines mainly based on proteins from the intestine and salivary glands of ticks (61–65) and in the search for RNA viruses that infect ticks such as tick-borne encephalitis virus (TBEV) and Kyasanur forest disease virus (KFDV) (66–71). In fact, the *Bm86* cDNA marker could represent a significant advance in the field of delineating tick species that make up these banks, providing a sensitive and discriminatory tool that researchers can use in interspecific and intraspecific diversity analyses. Its ability to target the tick's RNA banks, which have already been utilized in previous research, offers new avenues for a more accurate and efficient method for tick species and isolate identification, which will have

important implications for ticks and tick-borne disease research and control.

The selection of the *Bm86* transcript as a species identification marker for ticks in the genera *Hyalomma* and *Rhipicephalus* was motivated by several factors. First, this transcript had been previously used in commercial and experimental anti-tick vaccines (48, 49, 72–75), making it a readily available target in tick RNA banks (64, 76). Second, research showed that the phylogeny based on the complete or almost complete sequence of this transcript was in perfect agreement with the recent taxonomy of hard ticks (31, 77). This made it an ideal candidate for species identification as it had already demonstrated its utility in representing the genetic diversity of these ticks (78–81). Furthermore, using this transcript as a marker for species identification would help to better understand the evolution and ecology of these ticks, which are important vectors of various diseases affecting livestock and humans.

However, the purpose of our study was to identify the minimum length partial *Bm86* cDNA fragments required to differentiate between species within the tick genera *Rhipicephalus* and *Hyalomma*. Using a shorter sequence has practical benefits as it only requires one primer pair for amplification and a single primer for sequencing. This reduces the cost of the test compared to analyzing the complete or nearly complete sequence, which requires multiple primer pairs.

Through a combination of genetic analysis and phylogenetic analysis, the study identified the conserved and variable regions within the *Bm86* cDNA of these tick species. The crucial factor in choosing the partial sequences specific to *Hyalomma* and *Rhipicephalus* genera was the alignment of two genetically closest species *Hy. excavatum* and *Hy. anatolicum* (96.9% identity rate) and *Rh. microplus* and *Rh. annulatus* (98.6%), respectively. As a result of these alignments, one conserved and two variable regions were identified in each genus, as shown in Figure 2 for *Hyalomma* and Figure 1 for *Rhipicephalus*. Therefore, to ensure accurate results in future molecular diagnosis studies of *Hyalomma* and *Rhipicephalus* spp., we suggest avoiding the use of partial sequences within these conserved regions and instead focusing on amplifying and sequencing fragments found in the two variable regions of each genus (Figures 1, 2). By evaluating different fragment sizes within the variable regions, we determined that the minimum size for species delineation was 398 bp for *Rhipicephalus* spp. (from 1,487 to 1,884) and 559 bp for *Hyalomma* species (from 539 to 1,097).

The study also confirmed tick species identification through morphological characteristics and mitochondrial 16S rRNA partial sequences, with a 99.27–100% identity with the closest published *Hyalomma* and *Rhipicephalus* sequences in GenBank. Interestingly, we identified a *Hyalomma anatolicum* tick specimen based on its morphological characteristics, and its identity was validated by mitochondrial 16S rRNA partial sequences. The validation revealed a 100% match with the closest published *Hyalomma anatolicum* sequence in GenBank. Notably, this finding marks the second report of this tick species since the discovery of a single *Hyalomma anatolicum* specimen in 1970 by Van Den Ende in Southern Tunisia (82). This discovery highlights the risk that this tick becomes a vector of the apicomplexan hemoparasite *Theileria annulata* in Tunisia as already advanced by Gharbi et al. (83).

Furthermore, the field evaluation study successfully amplified and genetically analyzed the minimal length *Bm86* cDNA partial sequences for the identified tick species, with 99.10–100% and 96.85–97.34% homology rates with previously published *Hyalomma* and *Rhipicephalus* sequences, respectively. These findings have important implications for the accurate identification and classification of tick species, which is crucial for understanding the epidemiology and transmission of tick-borne diseases.

In addition, our results also support the utility of *Bm86* orthologs as a potential marker for tick isolate or strain identification. In particular, the results suggest that the studied minimal length *Bm86* fragment is just as effective at discriminating between isolates and strains within each *Hyalomma* and *Rhipicephalus* species as the complete or nearly complete sequences. The phylogenetic inference based on the two partial sequences that were chosen showed the same pattern as previously observed when using the complete or nearly complete sequence (Figures 5, 6). No changes in the relationships between the isolates and strains of each species were detected, which suggests that there is no incongruity in the phylogeny. The trees generated using the neighbor-joining method had high support values at the terminal nodes and most of the deeper branches. There were also no instances of polytomies, which is likely because the selected fragments contained the most informative sites, resulting in a conservation of the phylogenetic signal. These findings were consistent with previous studies (84, 85). This is interesting because it suggests that this shorter fragment could be a more efficient and cost-effective reliable method for identifying and characterizing *Hyalomma* isolates and strains. In fact, our findings indicate that the two *Hyalomma* trees generated using the complete or nearly complete sequences and the minimal sequence showed similar results. Specifically, the genetic analysis revealed that the *Hy. marginatum* strain from Turkey (Kutahya) (86) is more closely related to the *Hy. marginatum* strain from France (80) than to all the *Hy. marginatum* Tunisian strains (31). Additionally, the isolate Ariana from *Hy. scupense* was relatively more distant from the isolates from Sousse, Manouba, and Beja (31).

Additionally, our result suggests that the minimal length *Bm86* transcript sequence selected in this study can be as effective as the complete or nearly complete sequence in discriminating between isolates and strains within *Rh. microplus* and *Rh. appendiculatus* species. First, the phylogenetic analysis based on the minimal length sequence was able to generate groups of isolates based on their geographic regions, which were similar to the groups generated by the analysis based on the complete or nearly complete sequence. Second, the results showed that the minimal length *Bm86* transcript sequence specific to the *Rhipicephalus* genus was also effective in discriminating between the two genospecies groups of *Rh. appendiculatus* isolates (87) in a similar way than with the complete or nearly complete transcript *Bm86* sequence (Figures 6, 7). This finding indicates that the shorter fragment could also be a useful tool for identifying and characterizing *Rhipicephalus* strains. Overall, these results highlight the potential benefits of using shorter sequence fragments to identify and distinguish between different strains and isolates of tick species belonging to these two genera, which could have important implications for tick control and disease prevention strategies. However, it is important to note that further research may be necessary to confirm these findings and to determine whether they are applicable to other tick species.

However, the primers we designed for the amplification of each *Bm86* minimum partial sequence specific to *Hyalomma* and *Rhipicephalus* were found to be sensitive and specific for each targeted tick genus. We were able to confirm this by successfully obtaining an amplicon of the expected size for all samples when amplified with primers specific for their respective genus. This indicates that the two sets of primers we created are suitable for amplifying and sequencing our cDNA marker and are capable of differentiating between the two closely related tick genera.

The use of a minimal *Bm86* cDNA sequence for tick species differentiation offers advantages such as the use of a single pair of genus-specific primers for amplification and only a single primer for sequencing, followed by a simple blast analysis to determine the species within each genus. However, it is important to acknowledge the limitations associated with cDNA, as researchers need to extract total RNA and obtain cDNA through reverse transcription. Nonetheless, the decision to exclusively use cDNA for both the *Hyalomma* and *Rhipicephalus* genera was made due to the presence of a large 2,888 bp intron at position 1,752 bp in *Bm86* cDNA (88), which is located within the minimal sequence used to discriminate *Rhipicephalus* species. Consequently, amplification of gDNA using specific primers for the *Rhipicephalus* genus is not feasible. To maintain consistency, we chose to utilize cDNA exclusively for both *Hyalomma* and *Rhipicephalus* genera.

Furthermore, this cDNA marker is expected to be particularly useful in identifying tick specimens that are difficult to recognize morphologically, for instance, with fully engorged tick immature females, in case of occurrence of malformations, or in the occurrence of hybrids of two species of ticks that are taxonomically closely related (31, 89, 90). Our marker, which is able to accurately identify tick species that may be otherwise difficult to differentiate, could be used as an additional taxonomic tool for morphological diagnosis for improving the reliability of tick species identification, particularly when considering the increased risks for changes in tick fauna and the emergence of new tick species driven by the combination of climate change with anthropogenic factors.

5. Conclusion

Genomic and mitochondrial DNA markers have been shown to be effective in identifying tick species compared to traditional morphological methods. In addition to these existing markers, we have identified here a novel *Bm86* cDNA marker that provides a new approach to tick species identification and can target tick RNA banks. In this study, we developed a molecular method based on the minimum length of partial *Bm86* cDNA fragments needed for identifying accurately tick species within the *Rhipicephalus* and *Hyalomma* genera, increasing, therefore, the range of taxonomic tools applied to identify reliably hard tick species.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary material.

Ethics statement

The collection of ticks from cattle was done in accordance with the Tunisian National School of Veterinary Medicine's guidelines for animal care and handling and required no ethical approval. Consent was sought from the farmers before sampling. The animals were gently restrained by their owners in the same way as during routine clinical examinations, and invasive sampling or tranquilizers were not used. Moreover, the sampling was overseen by veterinarians and veterinary technicians from the National School of Veterinary Medicine in Sidi Thabet.

Author contributions

MBS conceived the idea, conducted *in silico* analysis, and designed the experiments. SZ, MM, and MD performed the experiments. SZ and MBS wrote the manuscript. MAD collaborated with MBS in developing the study concept and in editing and finalizing the manuscript. All authors have reviewed and approved the current version of the manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1209210/full#supplementary-material

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EDITED BY
Münir Aktaş,
Firat University, Türkiye

REVIEWED BY
Sezayi Özübek,
Firat University, Türkiye
Osama Mohammed,
King Saud University, Saudi Arabia

*CORRESPONDENCE
Abid Ali
✉ uop_ali@yahoo.com
Kun-Hsien Tsai
✉ kunhtsai@ntu.edu.tw

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First report on detection of *Hepatozoon ayorgbor* in *Rhipicephalus haemaphysaloides* and *Hepatozoon colubri* in *Haemaphysalis sulcata* and *Hyalomma anatolicum*: risks of spillover of *Hepatozoon* spp. from wildlife to domestic animals

Hadia Tila[1], Mehran Khan[1], Mashal M. Almutairi[2], Abdulaziz Alouffi[3], Haroon Ahmed[4], Tetsuya Tanaka[5], Kun-Hsien Tsai[6]* and Abid Ali[1]*

[1]Department of Zoology, Abdul Wali Khan University Mardan, Mardan, Khyber Pakhtunkhwa, Pakistan, [2]Department of Pharmacology and Toxicology, College of Pharmacy, King Saud University, Riyadh, Saudi Arabia, [3]Department of Biotechnology, King Abdulaziz City for Science and Technology, Riyadh, Saudi Arabia, [4]Department of Biosciences, COMSATS University Islamabad (CUI), Islamabad, Pakistan, [5]Laboratory of Infectious Diseases, Joint Faculty of Veterinary Medicine, Kagoshima University, Kagoshima, Japan, [6]Department of Public Health, Institute of Environmental and Occupational Health Sciences, College of Public Health, National Taiwan University, Taipei, Taiwan

This study aimed to detect *Hepatozoon* spp. in ticks infesting asymptomatic domestic animals and to provide insight into their potential spillover from wild to domestic animals. In total, 537 tick specimens were collected in Khyber Pakhtunkhwa, Pakistan, and morphologically identified. The most prevalent tick species was *Haemaphysalis cornupunctata* (69; 12.8%), followed by *Haemaphysalis kashmirensis* (62; 11.5%), *Rhipicephalus microplus* (58; 10.8%), *Haemaphysalis montgomeryi* (51; 9.5%), *Rhipicephalus sanguineus* (49; 9.1%), each *Haemaphysalis bispinosa* and *Haemaphysalis sulcata* (43; 8.0%), each *Hyalomma anatolicum* and *Rhipicephalus turanicus* (37; 6.9%), *Rhipicephalus haemaphysaloides* (33; 6.1%) *Hyalomma scupense* (30; 5.6%), and *Hyalomma isaaci* (25; 4.7%). The extracted DNA from a subset of each tick species was subjected to PCR to amplify 18S rRNA fragments of *Hepatozoon* spp. By BLAST analysis, the *Hepatozoon* sp. detected in *Hy. anatolicum* infesting cows and in *Ha. sulcata* infesting sheep showed 99.7% maximum identity with *Hepatozoon colubri*. Similarly, the *Hepatozoon* sp. detected in *R. haemaphysaloides* infesting goats shared 99.49% maximum identity with *Hepatozoon ayorgbor*, and the *Hepatozoon* sp. detected in *R. sanguineus* infesting dogs exhibited 99.7% identity with *Hepatozoon canis*. Having an overall infection rate (9.3%; 16/172), the highest infection rate was recorded for each *H. canis*, and *H. colubri* (3.5%; 6/172), followed by *H. ayorgbor* (2.3%; 4/172). In the phylogenetic tree, *H. colubri* clustered with corresponding species from Iran, *H. ayorgbor* clustered with the same species from Croatia, Ghana, and Portugal, and *H. canis* clustered with the conspecifics from Iran, Israel, Romania, and Zambia. Regarding the potential spillover of *Hepatozoon* spp. from wildlife through ticks, free ranging animals was at higher risk compared to confined animals (RR = 3.05), animals consuming food from

wildlife habitats were at higher risk compared to those consuming domestic food (RR = 3.06), and animals residing in farm buildings located in wildlife habitats were at higher risk compared to those residing in farm buildings located in villages (RR = 3.28). In addition to the first report on *H. canis* in *R. sanguineus* in Pakistan, this is the earliest data showing *H. ayorgbor* in *R. haemaphysaloides* and *H. colubri* in *Ha. sulcata* and *Hy. anatolicum*. These preliminary findings suggest a potential spillover of *Hepatozoon* spp. from wild to domestic animals via ticks under certain risk factors.

KEYWORDS

ticks, *Hepatozoon ayorgbor*, *Hepatozoon colubri*, *Hepatozoon canis*, spillover

Introduction

Hepatozoon is a diverse genus of apicomplexan, primarily comprised of haemoparasites transmitted by arthropods, especially by ticks to all classes of terrestrial vertebrates, including wild and domestic animals (1). *Hepatozoon* is comprised of more than 300 apicomplexan haemoparasites, with ∼50 species in mammals and 130 species in snakes (1–3). This genus was assigned to Haemogregarinidae prior to allocating to the family Hepatozoidae (4). Due to encompassing some distinct lineages, certain studies have proposed that *Hepatozoon* spp. may not belong to a single genus (5, 6). *Hepatozoon* spp. are globally distributed depending on multiple factors, including the abundance of vertebrate and arthropod hosts (1). With a heteroxenous life cycle, *Hepatozoon* spp. exhibit sporogonic development and oocyst generation occurs in arthropods, while merogony and gametogony in their vertebrate hosts (1, 7, 8).

As intermediate hosts, *Hepatozoon* spp. infect all classes of terrestrial vertebrates, including wild and domestic animals. With low host specificity, depending on the species, some *Hepatozoon* spp. are more prevalent in a particular group of hosts (9). For instance, *Hepatozoon ayorgbor* largely infects wild snakes and wild rodents, whereas *Hepatozoon colubri* mainly infects wild snakes, while *Hepatozoon canis* primarily infects domestic and wild canids. However, *Hepatozoon* spp. can cross-over from their specific wild hosts to unnatural wild and domestic hosts through different agents such as ticks (10–13). In the case of spillover, *Hepatozoon* spp. are considered more pathogenic in unnatural hosts compared to their specific hosts (14–16).

Hematophagous arthropods get infected with *Hepatozoon* spp. by feeding on vertebrate hosts and subsequently act as the definitive host and vectors (17). For instance, mosquitoes serve as principal vectors for *H. ayorgbor* (1, 18), while *Rhipicephalus sanguineus* is the main vector for *H. canis* (1, 19, 20). The principal vectors of many *Hepatozoon* spp., such as *H. colubri*, are not well-established yet. Unlike other tick-borne pathogens which are mainly transmitted during tick bite, *Hepatozoon* spp. are transmitted when vertebrate hosts ingest infected ticks (21, 22). Other transmission routes include via predation of prey, such as *H. ayorgbor*, transmission to snakes through feeding on rodents (18), and vertical transmission such as *H. canis* in *R. sanguineus* (23, 24).

The morphometrics of the peripheral blood gamonts seen in the hosts' blood smears were previously the most widely employed techniques for the identification of *Hepatozoon* spp. However, the limited number of apparent structures and few morphological features make it difficult and unreliable to differentiate *Hepatozoon* spp. (25). A sensitive method for detecting *Hepatozoon* spp. is molecular analysis based on the amplification and sequencing of 18S rRNA fragments (26, 27). Furthermore, ticks have been largely neglected as vectors of *Hepatozoon* spp. and little data is available on *Hepatozoon* spp. of wildlife origin in ticks infesting domestic animals. Therefore, the purpose of this study was to detect *Hepatozoon* spp. in ticks infesting domestic animals and to provide insight into their possible spillover.

Materials and methods

Ethical approval

The Advanced Studies and Research Board of Abdul Wali Khan University, Mardan (AWKUM) granted ethical permission (Dir/A&R/AWKUM/2018/1410) for the conduction of this study. Additionally, the owners of the animals also gave their oral permission to collect ticks from their animals.

Study area

This study was conducted in four districts of Khyber Pakhtunkhwa, including Bajaur (34.7865° N, 71.5249° E), Malakand (34.5030° N, 71.9046° E), Mohmand (34.5356° N, 71.2874° E) in the north-western and Lakki Marwat (32.6135° N, 70.9012° E) in the south-eastern. Global Positioning System was used for finding the actual coordinates of collection points in the studied area and subsequently used in designing the map using ArcGIS v 10.3.1 (ESRI, Redlands, CA, USA; Figure 1).

Tick collection, preservation, and identification

Ticks were randomly collected using forceps from different asymptomatic domestic animals, including cows, livestock guardian dogs, goats, and sheep from March 2022 to September 2022. The appropriate information was recorded during tick collection, including the host type, collection date, and collection site, as well as general observations about the field.

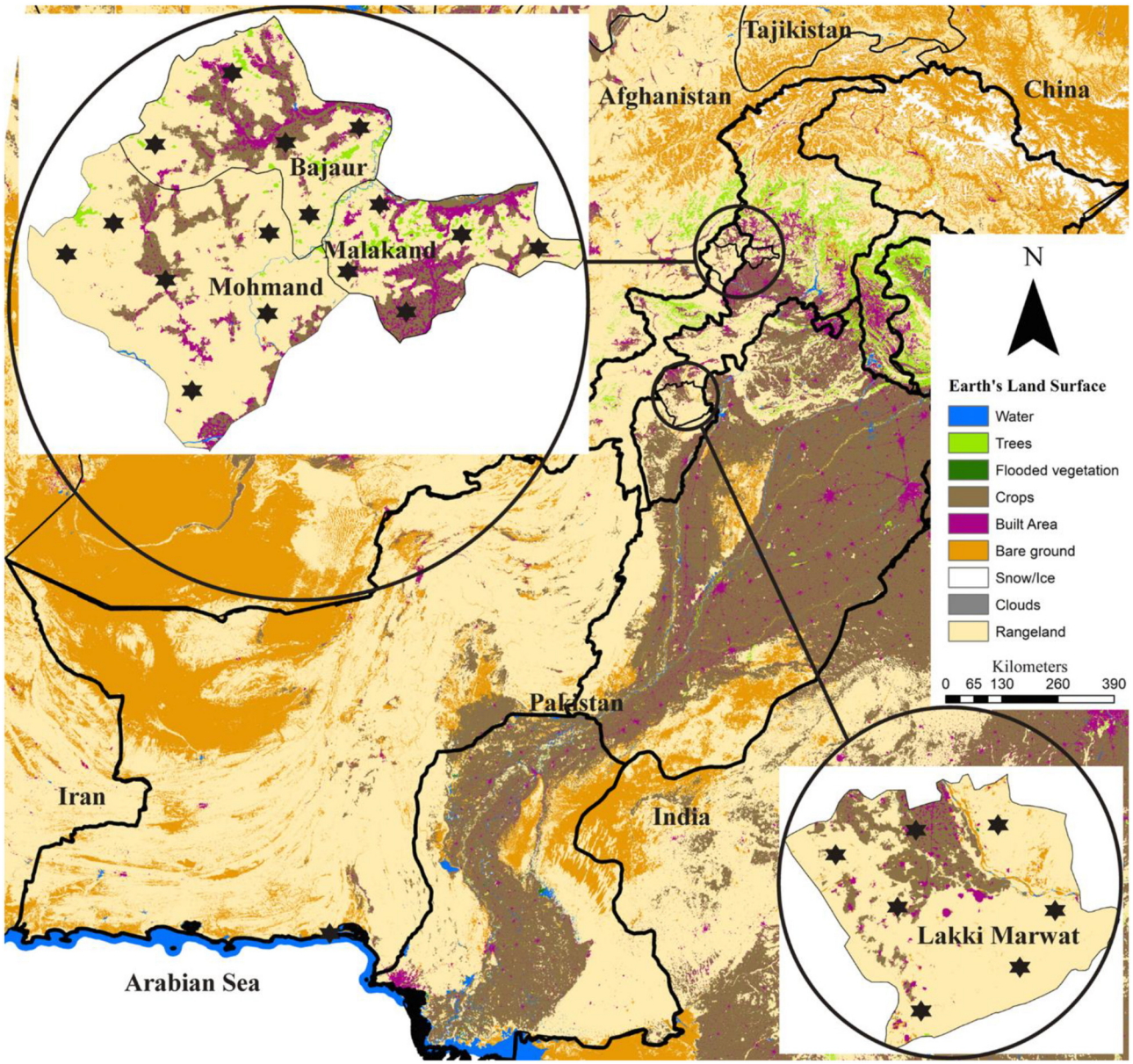

FIGURE 1
Locations (marked by an asterisk) where domestic hosts were examined for ticks are shown on the land-use and land-cover based map.

Furthermore, the owners of domestic animals were given a questionnaire to complete in order to gather possible risk factors for *Hepatozoon* spp. spillover. The questionnaire consisted of closed-ended questions about host-related details such as gender, age, living status, farming type, food supply, and farm building location, nature, and its altitude. It also contained open-ended questions regarding wildlife and anthropogenic activities such as land use and land cover changes, and human-wildlife conflict. Ticks were stored in a mixture of 95% ethanol, 4% distilled water, and 1% glycerol in properly labeled tubes after cleaning with distilled water followed by 70% ethanol. Using a stereomicroscope (Stemi 508, Zeiss, Germany), tick specimens were morphologically identified based on various morphological features, and validated up to the species level using the standard taxonomic identification keys (28–32).

DNA extraction and polymerase chain reaction

A sum of 172 tick specimens (one adult female, one male, and two nymphs per species per district) were selected and subjected to DNA extraction individually. Prior to DNA extraction using the phenol-chloroform method with minor modifications (33), the homogenization of ticks was individually carried out using a hygienic scissor coupled with a sterile pestle in a 1.5 ml Eppendorf

tube. The extracted DNA pellet was hydrated with 20–30 μl (depending on the tick size) of "nuclease-free" water and subjected to NanoDrop spectrophotometer (Nano-Q, Optizen, South Korea) to measure its quality and quantity.

For the amplification of 18S rRNA fragments of *Hepatozoon* spp., the extracted genomic DNA samples were subjected to conventional PCR (BIOER, China) using specific primers: HEP2 144-169-F (5′-GGTAATTCTAGAGCTAATACATGAGC-3′) and HEP2 743-718-R (5′-ACAATAA AGTA AAAAACAYTTCAAAG-3′), and PCR experimental conditions were set as previously described (34). Each PCR reaction of 25 μL volume contained the following components: 8.5 μL of PCR water "nuclease free," 1 μL of each primer with a concentration of 10 pmol/μL, 2 μL of extracted DNA (50–100 ng/μL), and 12.5 μL *DreamTaq* green MasterMix (2X). All PCR procedures employed PCR water as a negative control and *H. canis* DNA as a positive control. The obtained amplicons were run on a 2% agarose gel followed by observing via Gel Documentation (BioDoc-It^{TM} Imaging Systems, Upland, CA, USA) and purified using the GeneClean II Kit (Qbiogene, Illkirch, France).

DNA sequencing and phylogenetic analysis

The purified amplicons were submitted for DNA sequencing (Macrogen, Inc., Seoul, South Korea) by Sanger sequencing method using an ABI 373XL system. Low-quality nucleotide sequences and contaminated reads were eliminated during trimming and assembling of raw sequences through SeqMan version 5.0 (DNASTAR; DNASTAR, Inc., Madison, WI, USA). With the aim to determine the closest identities with other sequences previously deposited in the GenBank, the cleaned sequences were subjected to BLAST (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information). For the subsequent phylogenetic tree using Molecular Evolutionary Genetics Analysis (MEGA-X) (35), the homologous sequences found in the BLAST results were downloaded in FASTA format. These sequences along with a suitable outgroup were aligned in BioEdit alignment editor v 7.0.5 (36) using ClustalW Multiple alignment (37). The alignment was used for constructing a phylogenetic tree with 1,000 bootstrap replicates under Maximum Likelihood method.

Statistical analysis

The questionnaire data along with PCR data were collected and assembled in spreadsheets using Excel (Microsoft V, 2016). Using GraphPad Prism version 5.0 (GraphPad Software Inc., San Diego, CA, USA), the assembled data was subjected to chi-square test (χ^2), selecting significance at $P < 0.05$, and relative risk (RR), with the 95% confidence interval.

Results

Tick and host description

Altogether, 537 ticks were morphologically identified, which were belonging to three distinct genera of hard ticks, including *Haemaphysalis*, *Hyalomma*, and *Rhipicephalus*. Herein, majority of the ticks were *Haemaphysalis cornupunctata* (69, 12.8%), followed by *Haemaphysalis kashmirensis* (62, 11.5%), *Rhipicephalus microplus* (58, 10.8%), *Haemaphysalis montgomeryi* (51, 9.5%), *R. sanguineus* (49, 9.1%), each *Haemaphysalis bispinosa* and *Haemaphysalis sulcata* (43, 8.0%), each *Hyalomma anatolicum* and *Rhipicephalus turanicus* (37, 6.9%), *Rhipicephalus haemaphysaloides* (33, 6.1%) *Hyalomma scupense* (30, 5.6%), and *Hyalomma isaaci* (25, 4.7%). The highest prevalence of tick infestation was recorded on dogs (68.4%: 13/19), followed by cows (64.7%: 22/34), goats (58%: 29/50), and sheep (56.3%: 27/48), resulting in overall prevalence (60.9%; 92/151). The details about each tick species, including life stage, host association, and collection site, are provided in Table 1.

Detection of *Hepatozoon* spp. in ticks

According to BLAST analysis, *Hepatozoon* sp. detected in *Hy. anatolicum* from cows and *Ha. sulcata* from sheep shared a maximum identity of 99.66% with *Hepatozoon colubri*, whereas *Hepatozoon* sp. detected in *R. haemaphysaloides* from goats revealed a maximum identity of 99.49% with *Hepatozoon ayorgbor*, and *Hepatozoon* sp. detected in *R. sanguineus* from dogs showed a maximum identity of 99.66% with *Hepatozoon canis*. With a total infection rate (9.3%, 16/172), the highest rate was recorded for each *H. canis*, and *H. colubri* (3.5%, 6/172), followed by *H. ayorgbor* (2.3%, 4/172). Table 1 provides additional information regarding *Hepatozoon* spp., including the associated vertebrate hosts and the corresponding locality.

The obtained sequences for 18S rRNA were submitted to GenBank under the following accession numbers; OR241141 (*H. ayorgbor*), OR241161 (*H. colubri*), and OR241147 (*H. canis*).

Sequence and phylogenetic analysis

A sum of 64 sequences (four sequences, two being forward and two reverse per detection) were obtained from all amplified fragments. All sequences of *Hepatozoon* sp. amplified from the genomic DNA of *Hy. anatolicum* and *Ha. sulcata* were identical to each other, resulting in a consensus sequence of 590 bp. Similarly, all sequences of *Hepatozoon* sp. amplified from the genomic DNA of *R. haemaphysaloides* were identical, while all sequences amplified from the genomic DNA of *R. sanguineus* were identical, yielding a consensus sequence of 588 and 596 bp, respectively. The *Ha. sulcata* and *Hy. anatolicum* based sequences showed the highest identity of 99.66% with *H. colubri* (MN723844), while the *R. haemaphysaloides* based sequence displayed the highest identity of 99.49% with *H. ayorgbor* (EF157822.1), as well as with two undetermined *Hepatozoon* spp. (MT919387 and MT919388), and *R. sanguineus* based sequence revealed maximum identity of 99.66% with *H. canis* (KX712126 and KX880505). The details regarding the infection rate and the association of *Hepatozoon* spp. with tick species in each district are shown in Table 1.

Based on 18S rRNA, a phylogenetic tree was obtained encompassing the three *Hepatozoon* spp. detected in the present survey. *Hepatozoon ayorgbor* clustered with the same species

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TABLE 1 Data about tick species, associated *Hepatozoon* spp., corresponding vertebrate hosts, locality, and molecular analysis.

Tick species	Tick life stages			Total ticks	Tick hosts	Tick collection sites	PCR details		
	Female	Male	Nymph				Ticks subjected to PCR	Positive ticks	*Hepatozoon* spp. detected
Ha. cornupunctata	35	20	14	69	Goats, Sheep	Bajaur, Malakand, Mohmand	3F, 3M, 6N	0	
Ha. bispinosa	17	15	11	43	Goats, Sheep	Bajaur, Malakand, Mohmand	3F, 3M, 6N	0	
Ha. kashmirensis	28	21	13	62	Goats, Sheep	Bajaur, Malakand, Mohmand	3F, 3M, 6N	0	
Ha. montgomeryi	22	18	11	51	Goats, Sheep	Bajaur, Malakand, Mohmand	3F, 3M, 6N	0	
Ha. sulcata	16	15	12	43	Goats, Sheep*	Bajaur*, Malakand, Mohmand	3F, 3M, 6N	3N	*H. colubri*
Hy. anatolicum	18	9	10	37	Cows*, Goats, Sheep	Bajaur*, Malakand, Mohmand, Lakki Marwat*	4F, 4M, 8N	1F, 2N	*H. colubri*
Hy. scupense	11	10	9	30	Cows	Bajaur, Malakand, Mohmand, Lakki Marwat	4F, 4M, 8N	0	
Hy. isaaci	10	7	8	25	Cows, Goats, Sheep	Bajaur, Malakand, Mohmand, Lakki Marwat	4F, 4M, 8N	0	
R. haemaphysaloides	14	10	9	33	Dogs, Goats*, Sheep	Bajaur, Malakand*, Mohmand*, Lakki Marwat	4F, 4M, 8N	1F, 3N	*H. ayorgbor*
R. microplus	22	18	18	58	Cows, Goats, Sheep	Bajaur, Malakand, Mohmand, Lakki Marwat	4F, 4M, 8N	0	
R. sanguineus	21	15	13	49	Dogs*, Goats, Sheep	Bajaur*, Malakand*, Mohmand, Lakki Marwat*	4F, 4M, 8N	2F, 4N	*H. canis*
R. turanicus	15	12	10	37	Dogs, Goats, Sheep	Bajaur, Malakand, Mohmand, Lakki Marwat	4F, 4M, 8N	0	
Total	229	170	138	537			172	16	

*Associated with Hepatozoon spp. positive ticks.

previously detected in *Apodemus flavicollis*, *Python regius*, and an unknown tick species from Croatia, Ghana, and Portugal, respectively. *Hepatozoon colubri* clustered with the corresponding species found in *Zamenis lineatus* from Iran, and *H. canis* clustered with conspecifics detected in *Canis lupus*, *Canis aureus*, and *Canis familiaris* from Iran, Romania, and Israel and Zambia, respectively (Figure 2).

Risk factors associated with *Hepatozoon* spp. spillover

Different factors were noted which could lead to the spillover of *Hepatozoon* spp. from wild to domestic hosts via ticks. For instance, locals were found handling, poaching, and hunting of wild animals using dogs. Moreover, dogs were found freely wandering between the habitats of wild and domestic animals, and occasionally feeding on dead wild canids. The overlapped area between domestic and wild animals was found to be increasing as a result of expansion of human settlements and deforestation. Deforestation, which is associated with climate change, was observed to create space for agriculture land and pastures for animal grazing. Under these circumstances, alongside an increase in the population of small generalist hosts like rodents, there was a noticeable decline in overall biodiversity. The invasion of the territory of domestic animals by wild animals were noted in the studied area. Furthermore, the habitat, food sources, and climate were found suitable for both wild and domestic animals, as well as ticks. Using a questionnaire, several other risk factors were gathered (Table 2). Herein, with statistical significance ($P < 0.05$), free ranging animals were at greater risk compared to those confined in shelters (RR = 3.05), animals getting food from wildlife habitat were at greater risk compared to those getting domestic food (RR = 3.06), and animals living in farm buildings located in wildlife habitats were at greater risk compared to those living in farm buildings located in villages (RR = 3.28).

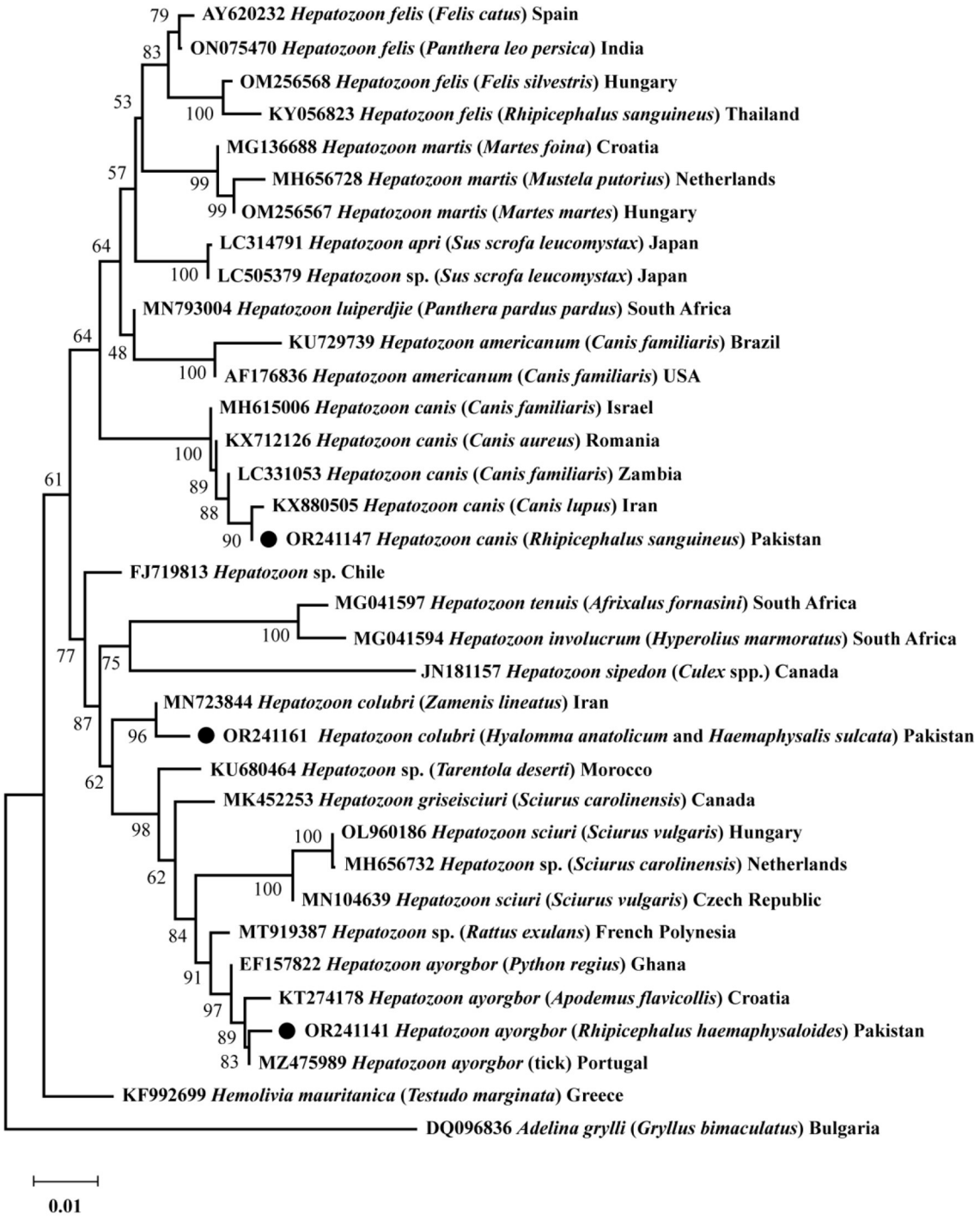

FIGURE 2
The phylogenetic tree was constructed for *Hepatozoon* spp. based on 18S rRNA sequences. The Neighbor-Joining method was used with 1,000 replicates for bootstrap analysis. The tree was rooted and subrooted using the 18S rRNA sequence of *Adelina grylli* and *Hemolivia mauritanica*, respectively. The GenBank accession numbers, species names, sources, and geographic locations were assigned to all sequences. The *Hepatozoon* spp. detected in this study are marked with a black circle.

Discussion

Despite potential agents of spillover of *Hepatozoon* spp. from wildlife to domestic animals, ticks have been widely overlooked as vectors of *Hepatozoon* spp., and there is a scarcity of knowledge regarding *Hepatozoon* spp. of wildlife origin in ticks infesting domestic animals. However, it is crucial to address this knowledge as these parasites pose a potential zoonotic threat, which, in turn could negatively impact food security within ecosystem (38). In addition to highlighting the possible spillover of *Hepatozoon* spp.,

TABLE 2 Details on coupling questionnaire-based risk factors with molecular survey for potential spillover of *Hepatozoon* spp.

Variable associated with hosts	Condition	No. of ticks subjected to PCR	No. of infected ticks	Mean (standard deviation)	Relative risk (95% confidence interval)	*P*-value (χ^2)
Gender	Female	117	10	58.5 (68.59)	0.78 (0.30–2.05)	0.62 (0.25)
	Male	55	6	27.5 (30.41)		
Age	3≤	46	5	23 (25.46)	1.25 (0.46–3.39)	0.67 (0.18)
	3>	126	11	63 (73.54)		
Farming type	Free-ranging	51	9	25.5 (23.33)	3.05 (1.20–7.54)	0.01 (5.98)
	Confined	121	7	60.5 (75.66)		
Food supply	Wildlife habitat*	72	11	36 (35.36)	3.06 (1.11–8.41)	0.02 (5.24)
	Domesticated•	100	5	50 (63.64)		
Farm building location	Wildlife zone	58	10	29 (26.87)	3.28 (1.25–8.56)	0.01 (6.54)
	Village	114	6	57 (72.12)		
Farm building nature	Mud	95	8	47.5 (55.86)	0.81 (0.32–2.06)	0.66 (0.20)
	Concrete	77	8	38.5 (43.13)		
Living status	Herd	105	9	52.5 (61.52)	0.82 (0.32–2.10)	0.68 (0.17)
	Single	67	7	33.5 (37.48)		
Altitude	Hilly	56	6	28 (31.11)	1.24 (0.48–3.25)	0.66 (0.20)
	Plain	116	10	58 (67.88)		

*Encompassed both free ranging and those confined animals which were provided with fresh forage from wildlife habitat.
•Included chaff and straw, cooked and raw food, hay, and mustard cake.

to the best of our knowledge, it is the first report on the occurrence of *H. canis* in *R. sanguineus* in Pakistan, *H. ayorgbor* in *R. haemaphysaloides* and *H. colubri* in *Ha. sulcata* and *Hy. anatolicum*.

Having a large number of small ruminants, the northern part of Pakistan is considered endemic to *Haemaphysalis* ticks compared to *Hyalomma* and *Rhipicephalus* ticks (39–48). *Haemaphysalis* ticks being the most abundant in this study could be attributed to two main factors. Firstly, a larger portion of the study focused on the north-western KP, and secondly, the inclusion of its two common domestic hosts, goats and sheep. This study revealed that dogs exhibited the highest prevalence of tick infestation, highlighting their status as one of the most overlooked hosts regarding tick control practices. Examined dogs were in close interaction with other domestic animals, including cows, goats, and sheep for their role in protecting animals from predators. Furthermore, knowing the scarcity of DNA based information of *Hepatozoon* and the effectiveness of 18S rRNA in their accurate detection and differentiation (26, 27, 49), the collected ticks in the current endeavor were subjected to PCR targeting 18S rRNA of *Hepatozoon*. On comparison of the obtained sequences, like (26, 50), the differences were considerable to distinguish them as three distinct *Hepatozoon* spp. By clustering of query sequences of the *Hepatozoon* spp. with their corresponding homologous sequences, the phylogenetic analysis confirmed the evolutionary relationship suggested by previous reports (26, 27, 49, 51).

Looking at the details of the vertebrate hosts of the corresponding three *Hepatozoon* spp.; *H. colubri* in snakes (*Zamenis lineatus* and *Zamenis longissimus*), *H. ayorgbor* in snakes (*Python regius*) and the closely related *Hepatozoon* spp. in rats and gerbils (*Rattus exulans*, *Rattus rattus*, and *Rhombomys opimus*), and *H. canis* in jackals (*Canis aureus*) and dogs (*Canis lupus familiaris*) (20, 52–57), their wildlife origin is prominent. With *H. ayorgbor* detection for the first time in *R. haemaphysaloides* in the present study, previous studies have molecularly detected *H. ayorgbor*-like sequences in ticks, including *Amblyomma rotundatum* infesting snakes (*Chironius multiventris*, *Corallus hortulanus*, *Oxyrhopus melanogenys*, and *Philodryas viridissima*) in Brazil, and *Amblyomma fimbriatum* infesting monitor lizard (*Varanus panoptes*) and snake (*Liasis fuscus*) in Australia (58, 59), *Ixodes tasmani* infesting Tasmanian devils (*Sarcophilus harrisii*) in Australia and *Ha. sulcata* (host-seeking) in Turkey (60, 61). Some sequences in GenBank such as MZ475989 belonging to *H. ayorgbor* have been attributed to unknown tick species. Besides the detection of *H. colubri* for the first time in *Hy. anatolicum* and *Ha. sulcata* in the current study, previously *H. colubri*-like species has been detected in *Amblyomma nitidum* infesting reptiles in Japan (62). Unlike previous studies on the detection of *H. ayorgbor*-like sequences and *H. colubri*-like sequences in ticks infesting wild reptiles, herein, *H. ayorgbor* and *H. colubri* were detected in ticks infesting domestic animals. Such preliminary findings require further investigation because the presence of pathogen DNA in a tick species does not guarantee its role as a biological vector. Like previous studies that described *R. sanguineus* as the main vector of *H. canis* in many regions of the world (1, 19, 20, 63), *H. canis* was detected in *R. sanguineus* infesting domestic dogs. Furthermore, the absence of infection in male ticks compared to females and nymphs could indicate that males

may not play a role as reservoirs of these parasites due to the fact that they may not feed on wild animals and they die soon after mating with females. Based on the documented evidence of tick infestation on wildlife in the region (44, 64–66), it could be anticipated that the ticks of the current study have acquired the corresponding three *Hepatozoon* spp. from wildlife somewhere during their lifecycle.

A pathogen spillover could be influenced by several factors associated with the pathogen itself, its different types of hosts and climate changes (67, 68), and human behavior. *Hepatozoon* spp. spillover could be determined by their abilities such as infecting a wide host range (1, 69), viability in the environment, adaptability and vector-borne transmission. An increase in the population of generalist small hosts, such as rodents, which are capable of hosting a broad spectrum of pathogens, may contribute to pathogen spillover by different routes, including ticks (68, 70). Additionally, decline in the diversity and abundance of wildlife, and their close proximity to public and domestic animals, may also amplify the spillover events of tick-borne pathogens (68, 71). The decline in biodiversity could be associated with rapid transformations in land use and land cover, as well as their conflict with human due to fears, attacks on crops and livestock, and financial benefits. The factors associated with domestic animals, including grazing in wildlife habitat (free-ranging), feeding on fresh forage from wildlife habitat, and their staying in farm buildings located in the wildlife habitat were noted as considerable risk factors associated with potential spillover. *Hepatozoon* spp. spillover may be facilitated by domestic dogs moving back and forth between wildlife habitats and human settlement (68), which was noted in the current study. Pakistan is the seventh most vulnerable country to climate change (72), which may influence vector dynamics, including ticks, potentially leading to cross-species transmission of *Hepatozoon* spp. Like previous studies, which provided evidences for the spillover of *Hepatozoon* species, including *Hepatozoon americanum*, *H. canis*, and *Hepatozoon silvestris* (10–12, 73–76), the current study proposes the spillover of *Hepatozoon* spp. from wild to domestic hosts via ticks. *Hepatozoon* spp. may have pathogenicity in unnatural hosts compared to their natural hosts (14–16), potentially posing a significant threat to domestic animals.

Conclusion

Besides the scarcity of knowledge on ticks as vectors of *Hepatozoon* spp., there is little information on *Hepatozoon* spp. of wildlife origin in ticks infesting domestic animals. Besides detecting *H. canis* in *R. sanguineus*, this is the earliest report on the detection of *H. ayorgbor* in *R. haemaphysaloides* and *H. colubri* in *Ha. sulcata* and *Hy. anatolicum*. By detecting *Hepatozoon* spp. of wildlife origin in ticks infesting domestic animals, this study proposed a possible spillover of *Hepatozoon* spp. from wild to domestic animals through ticks. The current study could assist in understanding the epidemiological surveillance and adopting preventive measures against tick-borne *Hepatozoon* spp. by minimizing the exposure of domestic animals to key risk factors. Further studies are needed in order to uncover ticks' role in the life cycle of *Hepatozoon* spp.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The animal studies were approved by the owners of the animals also gave their oral permission to collect ticks from their animals. The studies were conducted in accordance with the local legislation and institutional requirements. Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

AAli: Conceptualization, Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Writing—original draft, Writing—review and editing. HT: Formal analysis, Investigation, Methodology, Visualization, Writing—original draft, Writing—review and editing. MK: Data curation, Investigation, Methodology, Software, Validation, Visualization, Writing—original draft, Writing—review and editing. MA: Data curation, Funding acquisition, Investigation, Methodology, Project administration, Writing—original draft, Writing—review and editing. AAlo: Data curation, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Writing—original draft, Writing—review and editing. HA: Data curation, Software, Writing—original draft, Writing—review and editing. TT: Data curation, Formal analysis, Methodology, Project administration, Software, Writing—review and editing. K-HT: Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Software, Visualization, Writing—original draft, Writing—review and editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Yadong Zheng,
Zhejiang Agriculture and Forestry University, China

REVIEWED BY
Panat Anuracpreeda,
Mahidol University, Thailand
Eliane Esteves,
University of South Alabama, United States

*CORRESPONDENCE
Rosa Estela Quiroz-Castañeda
✉ requiroz79@yahoo.com.mx;
✉ quiroz.rosa@inifap.gob.mx

†These authors have contributed equally to this work

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An alternative vaccine target for bovine Anaplasmosis based on enolase, a moonlighting protein

Rosa Estela Quiroz-Castañeda*†, Hugo Aguilar-Díaz† and Itzel Amaro-Estrada

Centro Nacional de Investigación Disciplinaria en Salud Animal e Inocuidad, INIFAP. Carretera Federal Cuernavaca-Cuautla, Col. Progreso, Jiutepec, Morelos, Mexico

The discovery of new targets for preventing bovine anaplasmosis has moved away from focusing on proteins that have already been extensively studied in *Anaplasma marginale*, including the Major Surface Proteins, Outer Membrane Proteins, and Type IV Secretion System proteins. An alternative is moonlighting or multifunctional proteins, capable of performing various biological functions within various cellular compartments. There are several reports on the role of moonlighting proteins as virulence factors in various microorganisms. Moreover, it is known that about 25% of all moonlighting is involved in the virulence of pathogens. In this work, for the first time, we present the identification of three enolase proteins (AmEno01, AmEno15, and AmEno31) in the genome of Mexican strains of *A. marginale*. Using bioinformatics tools, we predicted the catalytic domains, enolase signature, and amino acids binding magnesium ion of the catalytic domain and performed a phylogenetic reconstruction. In addition, by molecular docking analysis, we found that AmEno01 would bind to erythrocyte proteins spectrin, ankyrin, and stomatin. This adhesion function has been reported for enolases from other pathogens. It is considered a promising target since blocking this function would impede the fundamental adhesion process that facilitates the infection of erythrocytes. Additionally, molecular docking predicts that AmEno01 could bind to extracellular matrix protein fibronectin, which would be significant if we consider that some proteins with fibronectin domains are localized in tick gut cells and used as an adhesion strategy to gather bacteria before traveling to salivary glands. Derived from the molecular docking analysis of AmEno01, we hypothesized that enolases could be proteins driven by the pathogen and redirected at the expense of the pathogen's needs.

KEYWORDS

multifunctional proteins, enolase, erythrocytes, ticks, extracellular matrix, veterinary diseases

1. Introduction

The performance of more than one function (moonlighting) by a single protein has been recognized as a common phenomenon with significant implications in metabolic processes and other functions in bacteria, plants, yeasts, fungi, parasites, and vertebrates (1–8). Moonlighting proteins were described in the late 1980s as structural proteins in the lens of the eye (crystallins) with a second and even a third function (9–11). Most of the functions of moonlighting proteins are related to physiologically relevant biochemical or biophysical functions (12–14). The repertoire functions of moonlighting proteins reported in the last years include their

participation as enzymes of the TCA cycle (aconitase, *Homo sapiens, Saccharomyces cerevisiae, Mycobacterium tuberculosis*) (15, 16); glucose metabolism (aldolase, *Arabidopsis thaliana*, hexokinase; *Plasmodium vivax*) (17, 18); chaperones (GroEL, *S. cerevisiae*, HSP60; *Enterobacter aerogenes*) anti-oxidant proteins (thioredoxin, *Escherichia coli*) (19); virulence-associated functions (elongation factor Tu and enolase, *Mycoplasma pneumoniae*, *Pseudomonas aeruginosa, Streptococcus pneumoniae, Plasmodium* spp.) (20, 21); among other roles in different organisms that have been widely reported (6, 22–24) In the last years, due to sequencing technologies and metagenomics advances, more moonlighting proteins and their functions are being discovered in diverse organisms (25).

Currently, two large groups have been proposed to classify moonlighting proteins: 1) "trigger enzymes" and 2) intracellular/secreted moonlighting proteins (4, 26). The first subset comprises enzymes that regulate transcription or translation by directly binding to DNA or RNA or by binding to other proteinaceous translation or transcription factors (27). The second subset includes the most extensive known moonlighting proteins, with activities as housekeeping enzymes, chaperones, translation factors, adhesion, DNA-binding proteins, and many others that are secreted and either reside attached to the cell surface, acting as receptors for soluble proteins or small molecules, or function in the fluid phase, often for intracellular signaling (4). In this regard, as moonlighting proteins perform their canonical and moonlighting functions in separate cell compartments (cytoplasm and the cell surface), this dual cellular localization of the protein strongly suggests a multifunctional activity (28).

The presence of moonlighting proteins is relevant in bacteria, and their study focuses on elucidating their alternative functions since they are present in both pathogenic and commensal bacteria (23).

In this regard, *Anaplasma marginale* is a Gram-negative intracellular pathogen known as the causal agent of bovine Anaplasmosis, an infectious, non-contagious disease characterized by progressive hemolytic anemia, abortions, loss of condition, milk production, and even death (6, 29, 30). Up to now, seven genomes (~1.2 Mbp) of Mexican strains of *A. marginale* have been reported and annotated (31–33). Due to their reduced genome size, moonlighting proteins in this pathogen could be a strategy to efficiently maximize their proteins' use (31, 34).

Currently, our interest focuses on moonlighting proteins of this vector-borne pathogen since they could participate in its pathogenicity or evasion of the host immune system, as it has been reported for many pathogens, which employ moonlighting/multitasking proteins as virulence factors to interfere with multiple cellular processes, in different compartments at different times during infection, augmenting their virulence (6). Thus, we performed a deep and sharp genomic analysis that allowed us to identify potential moonlighting proteins in *A. marginale*, including enolase (AmEno), which have not been reported before in this pathogen. Enolase (2-phospho-D-glycerate hydrolase, EC 4.2.1.11) is an intensely studied moonlighting protein that converts 2-phosphoglycerate to phosphoenolpyruvate in glycolysis. Besides participating in this metabolic pathway, the enolase facilitates binding to host cells, as reported in *Anaplasma phagocytophilum*, in which enolase binds to the host plasminogen. In 2018, Gao et al. (35) demonstrated that recombinant enolase from *A. phagocytophilum* can bind and activate plasminogen and promote conversion to plasmin, thus being crucial to pathogen infection. In addition, this enolase was considered a potential target to control anaplasmosis infection. In *Borrelia burgdorferi,* the surface-expressed enolase plays an essential role during pathogen invasion by binding mammalian plasminogen (36, 37). Recently, Xie et al., (38) confirmed that *Mycoplasma hyopneumoniae* enolase is localized on its surface and is capable of adhesion to swine tracheal epithelial cells.

In this work, we performed bioinformatic analysis, three-dimensional (3D) modeling, and docking of the *A. marginale* enolase, AmEno. This study aimed to identify *in silico* their potential to interact with different proteins from the extracellular matrix (ECM), erythrocyte membrane (EM), and the zymogen plasminogen that circulates in the mammals' blood. In addition, it could guide the development of a rational and sharp strategy to understand the interaction and functions of enolase and some ligands, which are essential for the success of the pathogen establishment.

2. Materials and methods

2.1. Identification of moonlighting enolases in *Anaplasma marginale* genomes

All seven *A. marginale* draft genomes reported (MEX-01-001-01, MEX-14-010-01, MEX-15-099-01, MEX-17-017-01, MEX-30-184-02, MEX-30-193-01, and MEX-31-096-01) had been previously annotated automatically using the RAST (version 2.0) server (39). Derived from this annotation, we identified one enolase gene in each of the seven genomes. Besides, sequences of enolases from different organisms were retrieved from a Blastp search at NCBI. Additionally, a search in AlphaFold Protein Structure Database (40) and MoonProt 2.0 (41) allowed a comparison of *A. marginale* enolase with those reported as pathogen virulence proteins. The selected sequences were used in the phylogenetic reconstruction.

2.2. Phylogenetic reconstruction

Enolase sequences were selected from the Domains Eukarya and Bacteria (Table S1). All multiple alignments were performed with Clustal Omega (42) and visualized with Jalview (43). A neighbor-joining method was used to reconstruct a phylogeny using Mega 11 software (44) with a Poisson substitution model and a bootstrap value of 1,000 replicates.

2.3. Bioinformatics analyses of *Anaplasma marginale* enolases

The magnesium (Mg^{2+}) binding sites, which are essential for the catalytic activity of the enolase and the conserved domains, were predicted in the Conserved Domains database (CDD-NCBI) (44) and ScanProsite (45). Transmembrane regions were predicted with DeepTMHMM (46). The subcellular localization of the proteins was predicted in PSORTb 3.0 (46), and the secondary structure and function were predicted in PSIPRED Workbench (47). The topology of the proteins was predicted in CATH (48).

2.4. Three-dimensional (3D) modeling

SwissModel is a protein structure homology-modeling server widely used to predict the 3D structure of proteins (49). We used this server to predict the 3D structures of the *A. marginale* enolases from strains MEX-01-001-01 (AmEno01), MEX-15-099-01 (AmEno15), and MEX-31-096-01 (AmEno31). The rest of the enolases from strains MEX-30-193-01, MEX-30-184-02, MEX-14-010-01, and MEX-17-017-01 are essentially identical to MEX-01-001-01; consequently, these structures were not modeled.

Homology modeling is currently an accurate method to generate reliable 3D protein structure models, using experimental protein structures from PDB ("templates") to build models for evolutionary-related proteins ("targets"). All generated models in SwissModel are based on the GMQE (Global Model Quality Estimate) and QMEAN model quality.

2.5. Molecular docking and interaction analysis

The docking of the modeled AmEno01 and five possible ligands was performed in ClusPro (50) to analyze their binding affinity. The PDB ID numbers of the ligands are plasminogen (4DUR), Fibronectin (3M7P), Spectrin (3LBX), Ankyrin (4RLV), and Stomatin (4FVF).

ClusPro is a server that uses a fast Fourier transform (FFT) method called Piper, where one of the proteins is placed at the origin of the coordinate system on a fixed grid, the second protein is placed on a movable grid, and the interaction energy is written as a sum of a few correlation functions. The algorithm rotates the ligand with 70,000 rotations. The 1,000 rotations/translation combinations out of the 70,000 rotations with the lowest score are chosen, and these 1,000 ligand positions are clustered with a 9 Å C-alpha RMSD radius (51). In ClusPro, we generated docking models using AmEno01 and the five potential ligands. For each molecular docking, ten models were generated that were downloaded in PDB format and visualized in UCSF ChimeraX (52). The model with the highest score of the ten docking models was selected to visualize the contact surface model in HDOCK (53).

The analysis of the interactions between amino acids of the five AmEno01-ligand complexes and the visualization were performed in PDBsum (54). For this, we first used the option PDBsum Generate to upload each docking model and generate a PDB code. Once we retrieved the PDB code of the five docking models, we used it as an entry in PDBsum to analyze the AmEno01-ligand interactions.

3. Results

3.1. Identification of moonlighting enolases in Mexican *Anaplasma marginale* genomes

We identified one enolase per genome in the seven *A. marginale* Mexican strains. The enolases of strains MEX-30-184-02 (GenBank KAB0450913.1), MEX-17-017-01 (KAB0451331.1), MEX-30-193-01 (KAB0450361.1), MEX-14-010-01 (TZF77690.1), and MEX-01-001-01 (RCL19410.1) had 425 amino acids of length. The enolase of strain MEX-15-099-01 (KAA8472002.1) had 450 amino acids, including 26 additional amino acids (MLYLSLLCLLFRKDCLF CPPLGVRAV) in the N-terminal end, and finally, the enolase of strain MEX-31-096-01 (KAA8473352.1) had 431 amino acids, considering six additional amino acids (MGVRAV) in the N-terminal end. Only eleven differences in amino acid sequences were observed in the seven enolase sequences (Figure 1). We performed bioinformatics predictions to all the enolases of the three Groups; however, the modeling and docking analyses we present here were performed only with the strain MEX-01-001-01, AmEno01 sequence. This model was representative of the three *A. marginale* enolases. Nevertheless, to confirm that 3D modeling and molecular docking of the enolases of Groups 2 and 3 did not vary substantially due to the differences at the sequence level, we also performed a docking of these proteins with the ligands. However, we found no significant variation at the structure or interaction level (data not shown).

3.2. Phylogenetic reconstruction

We reconstructed a phylogenetic tree to determine the phylogenetic relationship of AmEno01 and other enolases reported. It is known that enolases are well-defined in alpha, beta, and gamma groups in mammals. In the phylogenetic reconstruction, we found this classification in Chordata. The enolases of ticks *Rhipicephalus* spp. and *Ixodes scapularis* were clustered in a clade belonging to Arthropoda, and those from Protist organisms were separated from Animalia. In Bacteria, enolases MEX-01-001-01 (AmEno01), MEX-14-010-01 (AmEno14), MEX-17-017-01 (AmEno17), MEX-30-184-02 [(AmEno30-02), and MEX-30-193-01 (AmEno30-01)] were clustered in a unique clade that we named Group 1. The enolase MEX-15-099-01 (AmEno15) and Brazilian strains Jaboticabal and Palmeira clustered in Group 2. Finally, the enolase MEX-31-096-01 (AmEno31) was clustered with the reference strain *A. marginale* St. Maries in Group 3 (Figure 2). Additionally, the sequence identity of Mexican strains ranged from 97.65 to 99.53% in Group 1; the sequence identity between AmEno15 and Brazilian strains was 100%; and the identity between AmEno31 and the reference strain St. Maries was 98.38%.

3.3. Bioinformatics analyses of *Anaplasma marginale* enolases

The analysis in the CDD database showed four Mg^{2+} binding sites identified in AmEno01, AmEno14, AmEno17, AmEno30-02, AmEno30-01, AmEno15, and AmEno31, which were S, D, E, and D, varying in position along the sequences (Table 1). These residues are significant because of their role in the enolase catalytic activity. The enolase signature was identified in the analysis of ScanProsite. Additionally, DeepTMHMM predicted a localization inside the cell for all enolases since no transmembrane regions were identified, and no signal peptide was predicted with PSORT. Beta strands, alpha helixes, and coils were also identified (Supplementary Figure S1). To identify and compare the sequences of enolase signature, the amino acids of the catalytic site, and the loops of the active site, we contrasted enolases AmEno01, AmEno14, AmEno17, AmEno30-02, AmEno30-01, AmEno15, and AmEno31with information previously reported for *bona fide* enolases of *H. sapiens, T. cruzi, S. pneumoniae, A. phagocytophilum* and, *R. microplus* (Table 1). We found that the

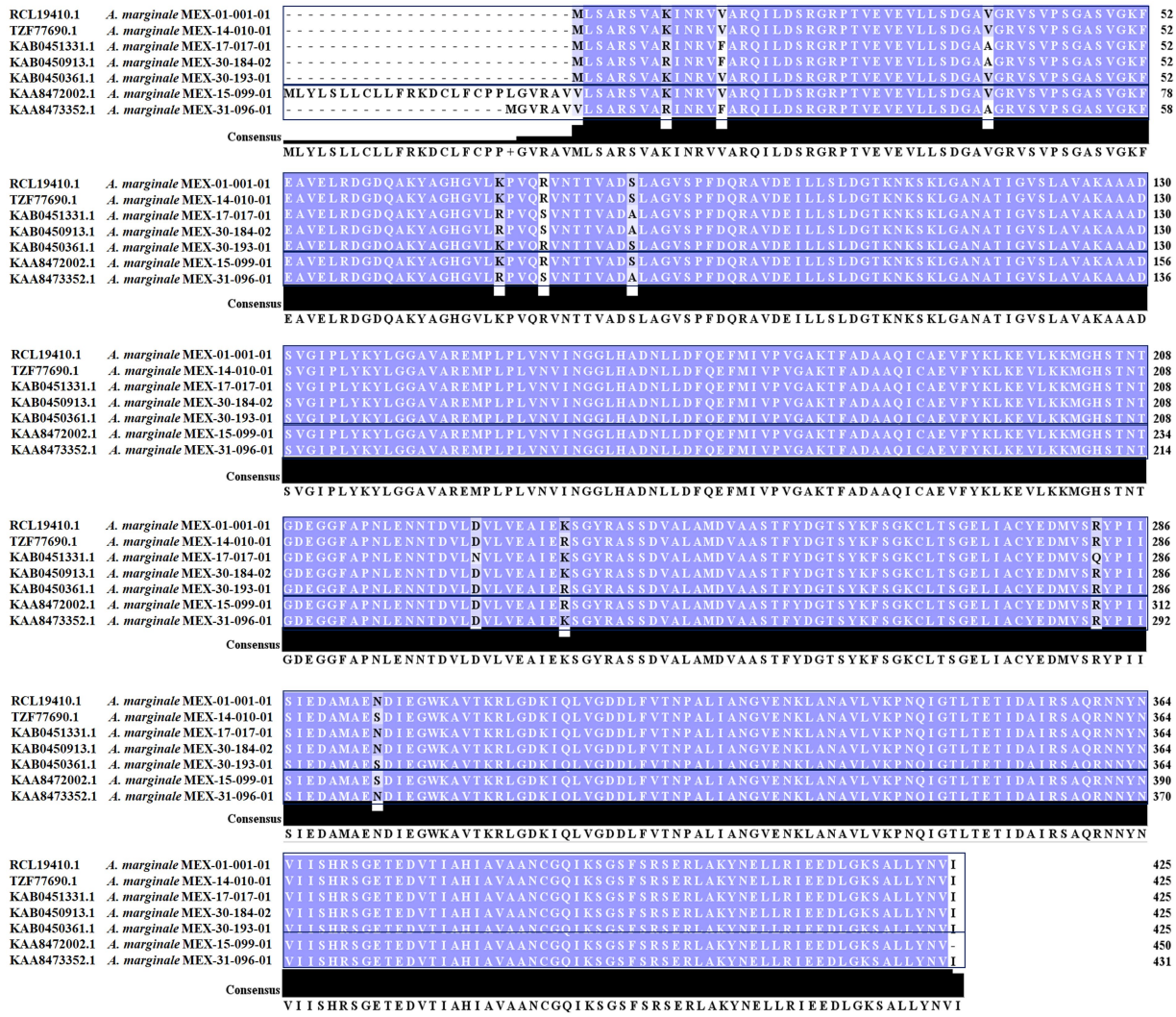

FIGURE 1
Multiple alignments of *A. marginale* Mexican strains enolases using Clustal Omega. Enolases are classified into three Groups. Group 1 comprises strains MEX-01-001-01, MEX-14-010-01, MEX-17-017-01, MEX-30-184-02, and MEX-30-193-01. These sequences share the same length of 425 aa. Group 2 comprises strain MEX-15-099-01 with 450 aa. Notice the 26 additional amino acids in the N-terminal end. Group 3 comprises MEX-31-096-01 with 431 aa, including six additional amino acids in the N-terminal end. The percentage of identity ranges from 97.41–99.76%.

amino acids with a significant role in the catalytic site of the enolases were essentially H, E, E, E, D, K, R, S, and K, which varied in the sequence position. Additionally, we found variants of the enolase signature and plasminogen-binding site in Mexican strains (Table 1 and Figure 3).

3.4. Three-dimensional (3D) modeling and molecular docking

We selected the three representative enolases AmEno01, AmEno15, and AmEno31, as input target sequences in the SwissModel server. The results in SwissModel for templates matching with the target sequence were sorted by higher GMQE value, and the enolase from *Enterococcus hirae* (PDB 1IYX) with a value of GMQE of 0.88 was selected as a template for modeling the three enolases. The identity and coverage percentages between template 1IYX and the modeled AmEno01 were 56.83 and 98%, respectively; for modeled AmEno15, were 56.49 and 92%, respectively; and for modeled AmEno31 were 56.83 and 97%, respectively (Figures 4A–C).

The GMQE value for the modeled enolases AmEno01, AmEno15, and AmEno31 were 0.82, 0.80, and 0.82, respectively, which is a significant accuracy value; hence, these were reliable models. QMEAN value for modeled AmEno01, AmEno15, and AmEno31 was 0.82 ± 0.05.

Since AmEno01, AmEno15, and AmEno31 presented a very similar 3D structure (Figure 4D), with a topology of an alpha-beta barrel (TIM barrel) in the C-terminal end and a two-layer sandwich in the N-terminal end (Figure 4E), we performed the docking with only AmEno01 as a representative model of Mexican strains enolases.

Therefore, the model of AmEno01 was downloaded in PDB format to be used in molecular docking in ClusPro. Five protein–protein dockings were performed in ClusPro, where AmEno01 was considered as a receptor, and the proteins plasminogen (4DUR),

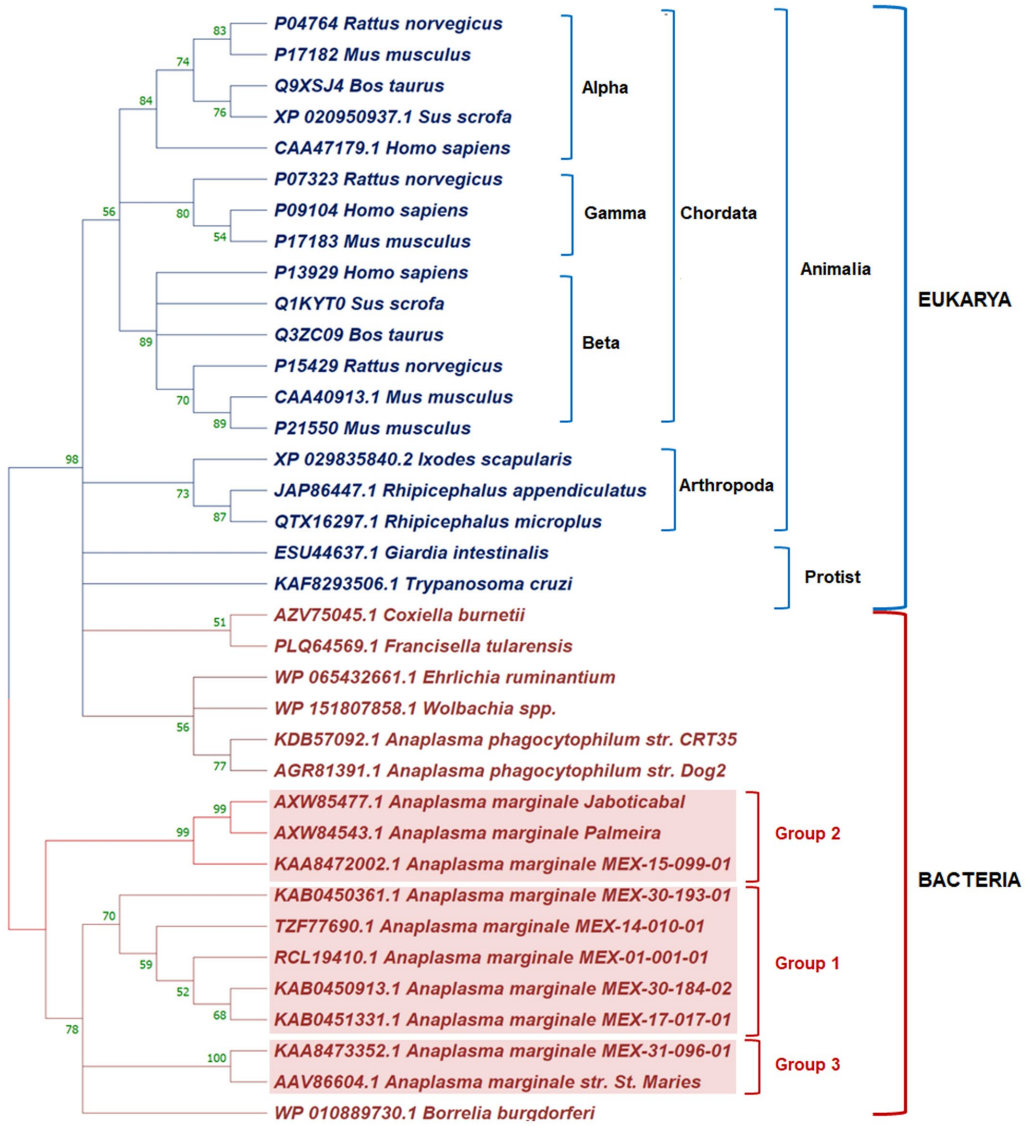

FIGURE 2
Phylogenetic reconstruction of enolases from Domains Eukarya and Bacteria in Mega 11. In Eukarya, Chordata' enolases are organized into alpha, beta, and gamma groups. Tick enolases form a clade separated from Animalia and Protist. In Bacteria, the Mexican strains of *A. marginale* grouped with Brazilian strains and North American strains (red boxes).

fibronectin (3M7P), spectrin (3LBX), ankyrin (4RLV), and stomatin (4FVF), as ligands.

The ten generated docking models by ClusPro were retrieved as "models with balanced coefficients since we do not know what forces dominate the complex protein–protein," as recommended in the ClusPro manual. One of the ten docking models for each AmEno01-ligand was visualized by UCSF ChimeraX. The molecular dockings are shown in Figure 5, and contact surface models for the five interactions of AmEno01-ligands are shown in Supplementary Figure 2.

In addition, the five molecular dockings were evaluated using the Ramachandran plots to validate the quality of the docking by PDBsum. According to this, the obtained values for each docking were 86.6% (AmEno01-3M7P), 87.3% (AmEno01-3LBX), 86.6% (AmEno01-4FVF), 83.9% (AmEno01-4RLV), and 76.3% (AmEno01-4DUR).

Lastly, the analysis of protein–protein (AmEno01-ligand) performed in PDBsum for the five docking models allowed the identification of the residue interactions across the interface. Thus, the salt bridges, disulfide bonds, hydrogen bonds, and non-bonded contacts were identified for each docking model (Supplementary Figure 3).

4. Discussion

The functions reported for enolases as moonlighting proteins in the last years extend their potential applications in the little-explored area of veterinary diseases, such as bovine Anaplasmosis. *A. marginale* is the causal agent of this disease, and seven genomes from Mexican strains have been reported without any moonlighting protein reported in this pathogen up to now (5, 55, 57). However,

TABLE 1 Features of some enolases reported for animals, protists, and bacteria, including *A. marginale* enolases.

Species (accession number and length)	Enolase signature	Mg^{2+} binding sites	aa catalytic sites	Loops of active site	Plasminogen binding sequence	Ref.
H. sapiens (Alpha enolase, P06733; 434 bp)	^{340}LLLKVNQIGSVTES^{353}	S^{40} D^{245} E^{293} D^{318}	H^{158} E^{167} E^{210} E^{293} D^{318} K^{343} R^{372} S^{373} K^{394}	^{37}SGASTGIY^{44} ^{157}SHAGNKLA^{164} ^{248}ASEFFRSGKYDLD FKSPDDPSRYI^{271}	^{251}FFRSGKY^{257}	(55)
R. microplus (QTX16297.1; 434 bp)	^{341}LLLKVNQIGSITEA^{354}	S^{40} D^{245} E^{292} D^{319}	H^{158} E^{167} E^{210} E^{294} D^{319} K^{344} R^{373} S^{373} K^{395}	^{37}SGASTGIH^{44} ^{157}CHAGNKLA^{164} ^{248}ASEFCKDGKYDLDFKNQTSDPSKH^{272}	^{251}FCKDGKY^{257}	This work
Bos taurus (Alpha and beta enolase Q9XSJ4, Q3ZC09; 434 bp)	^{340}LLLKVNQIGSTES^{353}	S^{40} D^{243} E^{289} D^{318}	H^{158} E^{167} E^{210} E^{293} D^{318} K^{343} R^{372} S^{373} K^{394}	Alpha enolase ^{37}SGASTGIY^{44} ^{157}SHAGNKLA^{164} ^{248}ASEFYRSGKYDLDFKSPDDPSRYIT^{272} Beta enolase ^{248}ASEFYRSGKYDLDFKSPDDPSRYIT^{272}	^{251}FYRSGKY^{257}	This work
T. cruzi (KAF8293506.1; 429 bp)	^{340}LLLKINQIGTITEA^{353}	S^{40} D^{243} E^{289} D^{318}	H^{156} E^{165} E^{205} E^{291} D^{319} K^{343} R^{372} S^{373} K^{394}	^{37}SGASTGIH^{44} ^{155}KHAGNALP^{162} ^{245}ASETYDENKKQYNLTFKSPEATWVT^{270}	^{251}FFRSGKY^{257}	(56)
S. pneumoniae (Q97As2.1; 434 bp)	^{340}ILIKVNQIGTLTET^{353}	D^{242} E^{291} D^{318}	H^{155} E^{164} E^{205} E^{291} D^{318} K^{343} R^{372} S^{373} K^{394}	^{39}SGASTGEH^{46} ^{154}SHSDAPIA^{161} ^{245}SSEFYDKERKVYD YTKFEGEGAAVR^{269}	^{248}FYDKERKVY^{256}	(57)
A. phagocytophilum (KDB57092.1; 429 bp)	^{338}VLIKPNQIGTLSET^{351}	S^{48} D^{248} E^{289} D^{316}	H^{161} E^{170} E^{211} E^{289} D^{316} K^{341} R^{370} S^{371} K^{392}	^{45}SGASVGKN^{52} ^{160}LHADNGLD^{167} ^{250}ASTFYDGKIYKFSG^{264}	^{254}FYDGKIYK^{261}	This work

(Continued)

TABLE 1 (Continued)

Species (accession number and length)	Enolase signature	Mg^{2+} binding sites	aa catalytic sites	Loops of active site	Plasminogen binding sequence	Ref.
A. marginale MEX-30-184-02 MEX-17-017-01 MEX-30-193-01 MEX-14-010-01 MEX-01-001-01 (RCL19410.1 TZF77690.1 KAB0451331.1 KAB0450913.1 KAB0450361.1; 425 bp)	^{338}VLVKPNQIGTLTET^{351}	S^{48} D^{248} E^{289} D^{316}	H^{161} E^{170} E^{211} E^{289} D^{316} K^{341} R^{370} S^{371} K^{392}	^{37}SGASVGKF^{44} ^{159} LHADNLLD ^{167} ^{250}ASTFYDGKIYKFSG^{264} ^{250} ASTFYDGTSYKFSGK ^{264}	^{254}FYDGTSYK^{261}	This work
A. marginale MEX-15-099-01 (KAA8472002.1;450 bp)	^{364}VLVKPNQIGTLTET^{377}	S^{74} D^{274} E^{315} D^{342}	H^{187} E^{196} E^{237} E^{291} D^{315} K^{367} R^{396} S^{397} K^{418}	^{37}SGASVGKF^{44} ^{185}LHADNLLD^{193} ^{276}ASTFYDGTSYKFSGK^{291}	^{37}SGASVGKF^{44} ^{165}LHADNLLD^{173} ^{256}ASTFYDGTSYKFSGK^{271}	This work
A. marginale MEX-31-096-01 (KAA8473352.1; 431 bp)	^{338}VLIKPNQIGTLSET^{351}	S^{54} D^{254} E^{295} D^{322}	H^{167} E^{176} E^{217} E^{295} D^{322} K^{347} R^{376} S^{377} K^{398}	^{279}FYDGTSYK^{287}	^{259}FYDGTSYK^{267}	This work

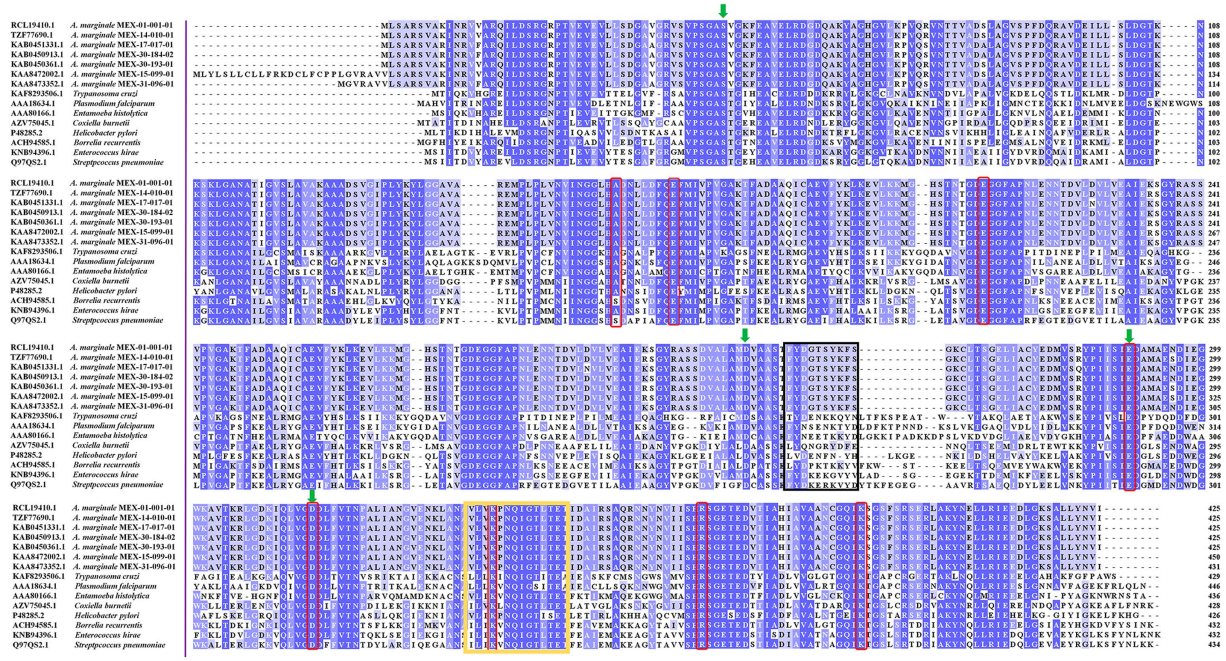

FIGURE 3
Enolases of parasites and bacteria aligned with Clustal Omega. The plasminogen binding sequence is shown in the black box. The sequence FYDGTSYKFS in *A. marginale* strains varies by 70% from the reported in *S. pneumoniae*, which binds to plasminogen by the sequence FYDKERKVYD. The enolase signature in *A. marginale* strains has seven amino acid changes compared to the sequences shown in the yellow box. The amino acids binding magnesium atoms are shown in green arrows and the amino acids of the catalytic site are shown in red boxes.

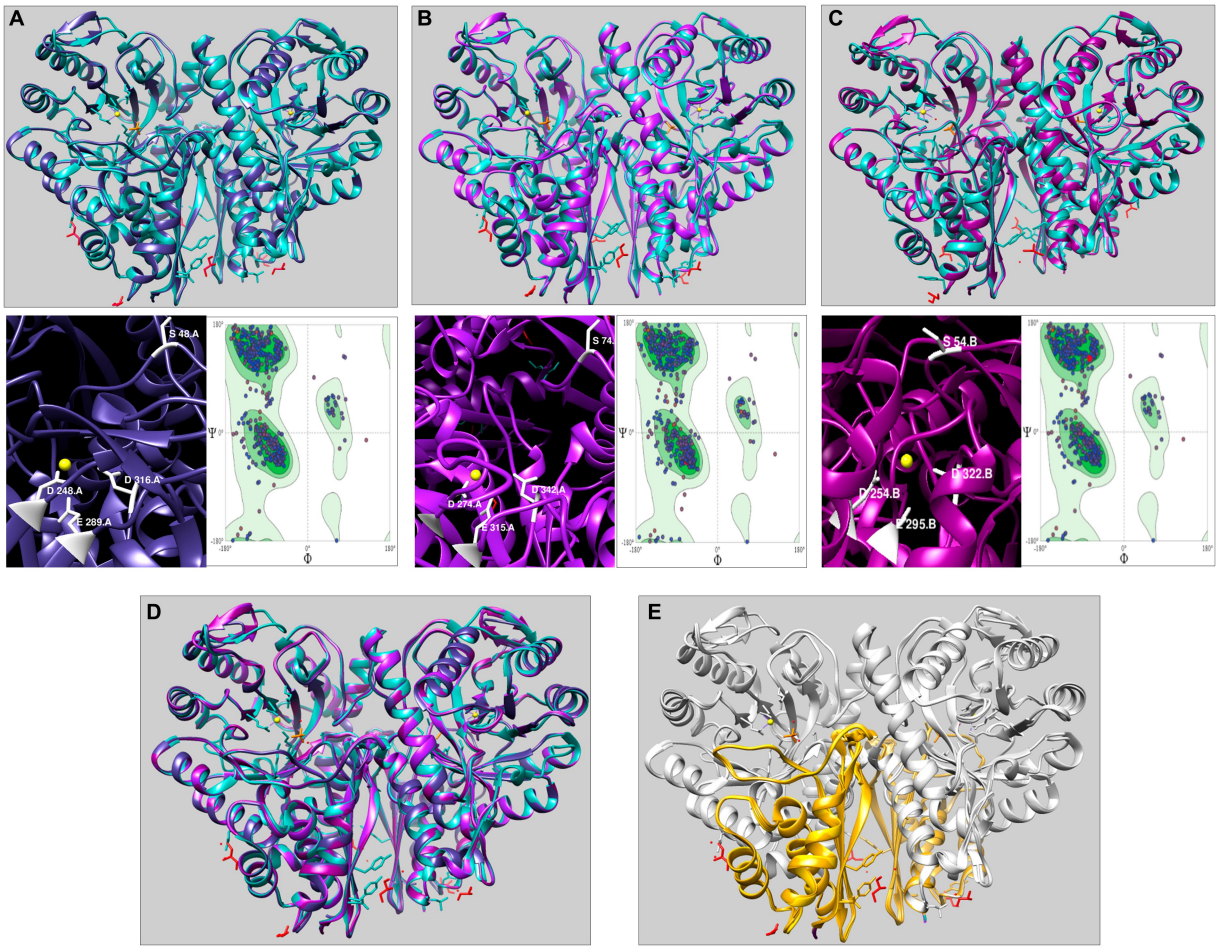

FIGURE 4
Three-dimensional modeling of enolases from Mexican strains of *A. marginale* using *Enterococcus hirae* enolase as the template (PDB 1IYX, cyan). In the three modeled enolases obtained in Swiss Model **(A)** AmEno01 (purple), **(B)** AmEno15 (magenta), and **(C)** AmEno31, the magnesium binding atoms S, D, E, and D (which vary in position) are shown. Models were considered feasible according to the Ramachandran plots. **(D)** Superposition of AmEno01, AmEno15, AmEno31, and 1IYX. **(E)** Enolases have the topology of a two-layer sandwich in the N-terminal end (golden) and an alpha-beta barrel (TIM barrel) in the C-terminal end (silver). For all models, magnesium atoms are shown in yellow circles; the structure of glycerol molecules is in red, and sulfates in orange. All 3D models were visualized and colored in ChimeraX.

their presence in genomes of Mexican strains of *A. marginale* suggests they could have similar functions reported elsewhere for this type of protein. The *A. marginale* enolase (AmEno), is of great interest since this protein could have a significant role during the erythrocyte invasion of the pathogen, as it has been reported for *Mycoplasma suis* where the enolase would participate in the adhesion of the pathogen to porcine erythrocytes at early stages of the invasion process (58).

Using bioinformatics approaches allowed us to identify only one enolase sequence per genome in Mexican strains of *A. marginale*, seven enolase sequences. As the phylogenetic reconstruction showed, these sequences grouped with other enolases into three different subclades: Group 1 (five Mexican strains enolases of 425 aa), Group 2 (one Mexican strain enolase of 450 aa and Brazilian strains enolases Palmeira and Jaboticabal), and Group 3 (one Mexican strain enolase of 431 aa and North American strain St. Maries). This suggested that some enolases of Mexican *A. marginale* strains could be related to Brazilian and North American strains, while others could be exclusive to Mexico.

Although the main difference between enolases of Groups 1 to 3 are additional amino acids at the N-terminal end, they have similar secondary structures of beta strands, alpha helix, and coil structures. Additionally, these differences at the sequence level and length observed in Mexican *A. marginale* strains' enolases did not cause significant modifications in the 3D structural arrangements, as we elucidated when we performed 3D modeling of representative members of each group of enolases: AmEno01, AmEno15, and AmEno31. After predicting 3D structures for these three enolases and realizing their structure was practically identical, we selected AmEno01 for further analysis.

Concerning the AmEno01 3D modeling, our results revealed a dimeric structure of the protein as it has been observed in other enolase structures, including *Helicobacter pylori* (59), *Aeromonas hydrophyla* (60), *Candida albicans* (61), and *Mycoplasma pneumoniae* (62). Additionally, an enolase octameric structure has been reported in *Bacillus subtilis, Streptococcus* spp., and *Thermotoga maritima* (63–65), and a monomeric structure has also been identified *in vitro* conditions (66).

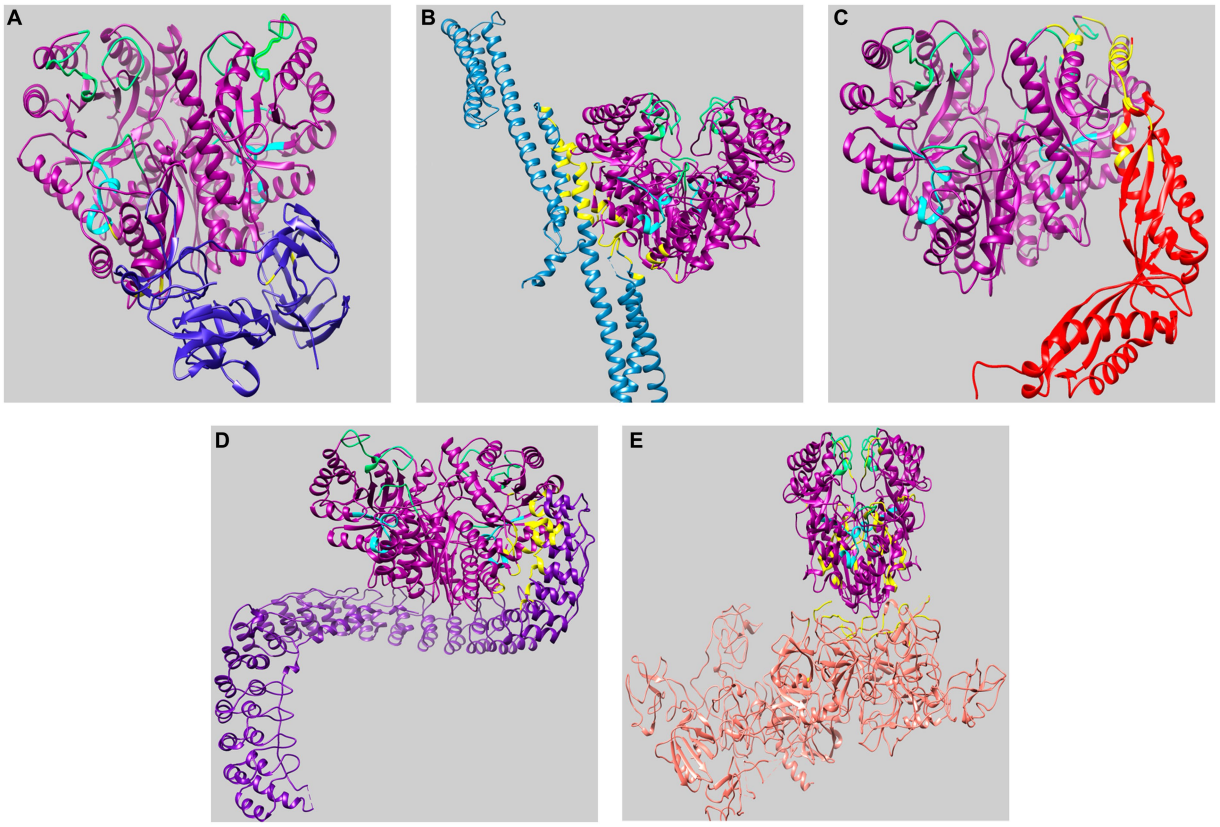

FIGURE 5
Visualization of molecular docking of AmEno01 with ligands retrieved in ClusPro. **(A)** fibronectin (3M7P); **(B)** spectrin (3LBX); **(C)** stomatin (4FVF); **(D)** ankyrin (4RLV); and **(E)** plasminogen (4DUR). The predicted amino acids participating in the interaction between AmEno01 and each ligand are shown in yellow, the enolase signature is shown in cyan, and the loops of the active site are shown in green. The docked structures were visualized and colored in ChimeraX.

Functionally, in the AmEno01 3D structure, we identified the sequences of the loops of the active site (^{37}SGASVGKF^{44}, ^{159}LHADNLLD^{167}, ^{250}ASTFYDGKIYKFSG^{264}, and ^{250}ASTFYDGTSY KFSGK 264) and the enolase signature (^{338}VLVKPNQIGTLTET^{351}), which were similar to those sequences of the rest of the Mexican enolases´strains.

On the other hand, it has been reported that two Mg^{2+} ions bind to the amino acids S, D, E, and D in the classical enolase active site, facilitating its catalytic reaction (67). In AmEno01, we found that amino acid S^{48} has a different spatial location than the one observed in other enolases; however we cannot discard its possible binding to the Mg^{2+} ion. In addition, in the predicted 3D models of AmEno01, AmEno15, and AmEno31, the Ramachandran plots, which predict the possible conformation of a protein, revealed that these models with a different spatial location of the S^{48} were feasible.

Subsequently, to analyze the potential of AmEno01 to bind ligands, we explored its interactions with proteins from the extracellular matrix or ECM (fibronectin), erythrocyte membrane (spectrin, ankyrin, and stomatin), and plasminogen by *in silico* approaches.

In this regard, our analysis of the AmEno01-fibronectin interaction showed that the Ramachandran plot contains 86.6% of residues in the most favored regions [A, B, L], suggesting the occurrence possibility of this interaction. The significance of this interaction relies on the ability to bind to fibronectin as a characteristic reported for many pathogens in the early steps of infection of host tissues (68). Interestingly, as *A. marginale* is a pathogen that does not infect tissues, we proposed that the potential of AmEno01 to bind fibronectin is highly relevant to infect ticks if we consider the possibility to adhere to tick gut cells. Like in spirochaete *B. burgdorferi,* whose membrane protein extract interacts with a protein with fibronectin III domains (Ixofin3D) identified in the gut of *Ixodes* spp., facilitating spirochaete congregation to the gut, and providing a molecular exit to the salivary glands before transmission to the human host (69, 70).

On the other hand, we decided to perform a docking analysis to elucidate if AmEno01 could adhere to erythrocyte proteins spectrin and ankyrin as part of the initial invasion process. According to the Ramachandran plot with 87.3 and 83.9% residues, respectively, in the most favored regions, our docking results suggested the binding of AmEno01 with spectrin and ankyrin. Therefore, we hypothesized that AmEno01 adhere to these proteins and probably mediate the initial erythrocyte invasion process. We must highlight that in *A. marginale*, the entry process to the erythrocyte is not well known; however, the ability to attach to host cells is essential for infection (71).

Some examples of enolase as an adhesion molecule to erythrocytes have been reported. Like *M. suis* enolase, that could act as an adhesion factor to porcine erythrocytes. Schreiner et al. (58)

found that *M. suis* recombinant enolase bound to erythrocytes lysates in a dose-dependent manner, and even transformants *E. coli* acquired the ability to bind to erythrocytes due to the expression of the enolase on their surface.

In addition to spectrin and ankyrin, another erythrocyte protein is stomatin, an integral protein that plays a role as a membrane-bound scaffolding protein modulating transport protein (72). According to this, the docking results of AmEno01 with stomatin showed 86.6% residues in the most favored regions in the Ramachandran plot. Therefore, we proposed that AmEno01 could recognize stomatin in the erythrocyte membrane as an essential step to adhesion and further internalization. In summary, spectrin, ankyrin, and stomatin could be potential targets to be experimentally assessed to avoid the invasion of *A. marginale* to erythrocytes.

As it is known, *A. marginale* infects erythrocytes but not tissues. Therefore, its binding to plasminogen, a zymogen that facilitates migration and invasion of pathogens to host tissues when converted to plasmin by its proteolytic activity, must be analyzed. Interestingly, we found that 76.3% of residues in the docking AmEno01-plasminogen are in favored regions of the Ramachandran plot, a percentage below the expected values to be considered a good quality model. Additionally, the plasminogen binding sequence identified in Mexican strains of *A. marginale* is different to the sequence of well-known enolases that bind plasminogen (73, 74).

In this regard, we theorized that AmEno01 could be binding to erythrocyte membrane proteins instead of binding to plasminogen; this last one is a strategy that parasites use to invade tissues such as *Leishmania mexicana* (cysticercus), *Taenia solium* and bacteria *S. pneumoniae, Bacillus antracis*, and *B. burgdorferi*, among others (37, 73, 75–77).

Finally, the role of enolases in many organisms is still under study; however, many advances in their functions as moonlighting proteins have been achieved (78). Thus, the study of moonlighting activities attributed to enolases in pathogenic bacteria such as *A. marginale* is relevant since it could be a candidate to control bovine Anaplasmosis. This proposal is based on the approaches performed with moonlighting proteins as potential vaccine candidates against several animal diseases. For example, a robust immune response in mice and piglets was observed when an enolase subunit of *M. suis* was used as antigen (20), or the protective immune response obtained when recombinant enolase of *P. vivax* was expressed in *E. coli* and used as an antigen against malaria (79).

In this regard, elucidating the functions of the enolases from Mexican strains of *A. marginale* could be the basis for developing strategies such as an anti-enolase antibody that interferes, avoids the invasion of erythrocytes, or blocks some other vital processes for the pathogenesis. In addition, we neither exclude that enolases could be proteins driven by the pathogen according to its needs nor discard their possible interaction with tick proteins that contribute to the pathogen's survival inside the vector.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

HA-D and RQ-C conducted the experiments. HA-D, RQ-C, and IA-E analyzed the data. RQ-C and HA-D envisioned and designed the study, and RQ-C and HA-D wrote the manuscript. RQ-C, IA-E, and HA-D edited the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2023.1225873/full#supplementary-material

SUPPLEMENTARY FIGURE 1
(A) fibronectin (3M7P); **(B)** spectrin (3LBX); **(C)** stomatin (4FVF) **(D)** ankyrin (4RLV), and **(E)** plasminogen (4DUR).

SUPPLEMENTARY FIGURE 2
(1) fibonectin (3M7P); **(2)** spectrin (3LBX); **(3)** stomatin (4FVF) **(4)** ankyrin (4RLV), and **(5)** plasminogen (4DUR).

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EDITED BY
Sonja Hartnack,
University of Zurich, Switzerland

REVIEWED BY
Bersissa Kumsa,
Addis Ababa University, Ethiopia
Abbey Olsen,
University of Copenhagen, Denmark

*CORRESPONDENCE
Maureen W. Kamau
✉ maureen.wanjiku.k@gmail.com

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Comparison of test performance of a conventional PCR and two field-friendly tests to detect *Coxiella burnetii* DNA in ticks using Bayesian latent class analysis

Maureen W. Kamau[1,2,3]*, Carmel Witte[2,4], Wynand Goosen[2], Mathew Mutinda[5], Jandouwe Villinger[6], Dennis Getange[6], Rua Khogali[6], Michael E. von Fricken[7], Eric Maurice Fèvre[8,9], Dawn Zimmerman[10], Yvonne-Marie Linton[11,12,13] and Michele Miller[2]

[1]Mpala Research Centre, Nanyuki, Kenya, [2]Division of Molecular Biology and Human Genetics, Department of Science, and Innovation – National Research Foundation Centre of Excellence for Biomedical Tuberculosis Research, Faculty of Medicine and Health Sciences, South African Medical Research Council Centre for Tuberculosis Research, Stellenbosch University, Stellenbosch, South Africa, [3]Global Health Program, Smithsonian National Zoo Conservation Biology Institute, Washington, DC, United States, [4]The Center for Wildlife Studies, South Freeport, ME, United States, [5]Kenya Wildlife Service, Nairobi, Kenya, [6]International Centre of Insect Physiology and Ecology (ICIPE), Nairobi, Kenya, [7]College of Public Health and Health Professionals, Department of Environmental and Global Health University of Florida, Gainesville, FL, United States, [8]International Livestock Research Institute, Nairobi, Kenya, [9]Institute of Infection, Veterinary and Ecological Sciences, University of Liverpool, Liverpool, United Kingdom, [10]Veterinary Initiative for Endangered Wildlife, Bozeman, MT, United States, [11]Walter Reed Biosystematics Unit (WRBU) Smithsonian Institution Museum Support Center, Suitland, MD, United States, [12]One Health Branch, Walter Reed Army Institute of Research (WRAIR), Silver Spring, MD, United States, [13]Department of Entomology, Smithsonian Institution, National Museum of Natural History (NMNH), Washington, DC, United States

Introduction: *Coxiella burnetii* (*C. burnetii*)-infected livestock and wildlife have been epidemiologically linked to human Q fever outbreaks. Despite this growing zoonotic threat, knowledge of coxiellosis in wild animals remains limited, and studies to understand their epidemiologic role are needed. In *C. burnetii*-endemic areas, ticks have been reported to harbor and spread *C. burnetii* and may serve as indicators of risk of infection in wild animal habitats. Therefore, the aim of this study was to compare molecular techniques for detecting *C. burnetii* DNA in ticks.

Methods: In total, 169 ticks from wild animals and cattle in wildlife conservancies in northern Kenya were screened for *C. burnetii* DNA using a conventional PCR (cPCR) and two field-friendly techniques: Biomeme's *C. burnetii* qPCR Go-strips (Biomeme) and a new *C. burnetii* PCR high-resolution melt (PCR-HRM) analysis assay. Results were evaluated, in the absence of a gold standard test, using Bayesian latent class analysis (BLCA) to characterize the proportion of *C. burnetii* positive ticks and estimate sensitivity (Se) and specificity (Sp) of the three tests.

Results: The final BLCA model included main effects and estimated that PCR-HRM had the highest Se (86%; 95% credible interval: 56–99%), followed by the Biomeme (Se = 57%; 95% credible interval: 34–90%), with the estimated Se of the cPCR being the lowest (24%, 95% credible interval: 10–47%). Specificity

estimates for all three assays ranged from 94 to 98%. Based on the model, an estimated 16% of ticks had *C. burnetii* DNA present.

Discussion: These results reflect the endemicity of *C. burnetii* in northern Kenya and show the promise of the PCR-HRM assay for *C. burnetii* surveillance in ticks. Further studies using ticks and wild animal samples will enhance understanding of the epidemiological role of ticks in Q fever.

KEYWORDS

***Coxiella burnetii*, diagnostics, Q fever, sensitivity, specificity, ticks, wildlife**

1 Introduction

Coxiella burnetii (*C. burnetii*) is a small gram-negative bacterium and the etiological agent of Q fever, a zoonotic infection with distribution throughout most of the world (1). Although rarely fatal in humans, the disease may lead to substantial morbidity and can be highly debilitating, even with treatment (2). Infections also result in significant economic losses associated with control and treatment of the disease in domestic animals as well as production losses resulting from poor reproductive performance (3). Human Q fever cases are commonly associated with livestock contact, such as the notable Q fever epidemic of more than 4,000 cases in The Netherlands during 2007–2010 (1) that originated from a *C. burnetii* strain in goats (4, 5). However, an increasing number of global reports link human Q fever cases to contact with wild animals (6). For instance, a capybara (7), and three-toed sloth (*Bradypus tridactylus*) (8) were epidemiologically linked to community-acquired pneumonia caused by *C. burnetii* infection in French Guiana (7–10). In another study, two cases of Q fever in South Wales, Australia, were linked to handling kangaroo joeys, and mowing lawns contaminated with kangaroo feces (11). Clinical disease associated with *C. burnetii* infection has also been reported in wild animals, including critically endangered species such as the dama gazelle (*Nanger dama mhorr*) and saiga antelope (*Saiga tatarica tatarica*) (12, 13). Knowledge of the clinical outcome of coxiellosis in wildlife is still limited, however placentitis and reproductive failure have been reported, raising concerns regarding the impact of *C. burnetii* on conservation breeding programs (14). Despite the increasing importance of coxiellosis in wild animals and the recognized risk to human health, study of the disease in wild animals and their environment has been largely neglected (6).

In Kenya, Q fever is ranked among the top 10 priority zoonotic diseases (15). A recent systematic review suggested that both human and animal *C. burnetii* infections are largely unrecognized and therefore underestimated due to limited diagnostic capacity at remote satellite laboratories (16). The review also highlighted that epidemiologic studies in high-risk regions, where there is increased livestock-wildlife interactions, are required to improve our understanding of the role that wild animals play in Q fever outbreaks (16). Previously, studies on Q fever in Kenya have focused on livestock (17–22), humans (23–25), or both (26–28), while only a few studies addressed *C. burnetii* infections in wild animals and their environment (29, 30). Active surveillance programs for wild animal diseases are expensive, logistically difficult, and may result in limited sample sizes, which could underestimate the abundance of *C. burnetii* (14, 31).

An alternative approach to surveillance is to screen disease vectors, such as ticks, to estimate *C. burnetii* abundance in an ecosystem (32). Ticks are ecological bridges for *C. burnetii* spread between wild animals and domestic animals (2). Ticks can get infected with *C. burnetii* when feeding on a bacteremic host and transmit the bacterium to a different host during subsequent feeding (33). Even if the ticks only acquire the bacterium without transmitting it, pathogen surveillance using ticks could provide insights into the presence and abundance of *C. burnetii* in wild animal ecosystems (34). Ticks are easy to collect and can be sampled to represent widespread geographical coverage. In known *C. burnetii* endemic areas, such as northern Kenya (35), tick species collected from wild animals and their environments have been found to harbor *C. burnetii* (20, 29, 36, 37). Furthermore, in these same regions, there was a strong correlation between *C. burnetii* seropositivity in livestock and their infestation with *C. burnetii* infected ticks (20, 21, 26). Therefore, detection of *C. burnetii* in ticks offers a promising approach to further *Coxiella* surveillance in wild animals and their ecosystems.

Therefore, the aim of this study was to compare the performance of three tests for screening tick vectors for *C. burnetii*. We compared (1) conventional PCR assay (cPCR), against two field-friendly techniques: (2) the *C. burnetii* qPCR Go-strips test (Biomeme, Philadelphia, Pennsylvania, United States) on the Biomeme Franklin™ three 9 platform (referred to here as "the Biomeme") and (3) a *C. burnetii* PCR high-resolution melt (PCR-HRM) assay. Additionally, the proportion of ticks with *C. burnetii* DNA was used as an indicator of pathogen abundance in the ecosystem. Because a gold standard test is not available, the performance of the three assays were compared using a Bayesian latent class analysis (BLCA) to assess the feasibility of using ticks for surveillance of *C. burnetii* in an ecosystem.

2 Materials and methods

2.1 Tick collection, identification, and DNA extraction

This study utilized DNA from ticks whose collection and processing has been previously described (38); our study design was retrospective and data collection was opportunistic and occurred before *C. burnetti* DNA testing was performed. Ticks were opportunistically collected (convenience sampling) from 179 wild animals and cattle during routine veterinary interventions in wildlife conservancies within Nyeri, Meru, Laikipia, Isiolo, and Marsabit counties (northern Kenya), between May 2011 and April 2019. The ticks were collected from 13 different wild animal species, as well as cattle present in the conservancies. Wildlife species included: black rhinoceros (*Diceros bicornis*), African buffalo (*Syncerus caffer*), African elephant (*Loxodonta africana*), giraffe (*Giraffa camelopardalis*),

Grévy's zebra (*Equus grevyi*), hartebeest (*Alcelaphus buselaphus*), impala (*Aepyceros melampus*), leopard (*Panthera pardus*), lion (*Panthera leo*), plains zebra (*Equus quagga*), spotted hyena (*Crocuta crocuta*), white rhinoceros (*Ceratotherium simum*), and African wild dog (*Lycaon pictus*), as well as farmed Boran and zebu cattle (*Bos indicus*). Cattle and wild animals were visually examined for ticks during clinical interventions and, if detected, several ticks were collected from each animal and placed in a tube containing 70% ethanol for preservation of the tick DNA. Collection location, date, and host species were recorded on each tube. Tick samples were stored in a −80°C freezer until analyzed.

Field immobilizations and wild animal sample collections were conducted according to Kenya Wildlife Service standard operating procedures. No animals were specifically immobilized or handled for this study. This study was approved by the Stellenbosch University Animal Use and Care (ACU-2023-27912) and Kenya's National Commission for Science, Technology, and Innovation (NACOSTI/P/21/8054).

For *C. burnetii* testing, one tick per host animal (i.e., 179 ticks total, representing 179 different individual animals) was selected. A subset of these were identified to the genus level based on metagenomics, as previously described (38). Each tick ($n = 179$) was placed into liquid nitrogen then homogenized in 1.5 mL microfuge tubes, containing 200 µL of 1x phosphate-buffered saline (PBS), and 150 mg of 0.1 mm and 750 mg of 2.0 mm yttria-stabilized zirconium (YSZ) oxide beads (Glen Mills, Clifton, New Jersey, United States), using a Mini-Beadbeater-16 (BioSpec, Bartlesville, Oklahoma, United States) for 1 min. Tick DNA was extracted with the Qiagen DNeasy Blood and Tissue Kit (Qiagen, Germantown, Maryland, United States) using 100 µL of the tick homogenate, according to manufacturer's instructions. Extracted DNA was stored at −80°C prior to PCR testing. Prior to performing the PCRs, DNA quantity and quality were measured using the Qubit dsDNA High Sensitivity Assay kit (Thermofisher Scientific, Waltham, Massachusetts, United States) and the Qubit 4 fluorometer (Thermofisher Scientific), as described by the manufacturer.

2.2 *Coxiella* PCR detection

Each of the tick DNA samples were screened for presence of *C. burnetii* DNA using three separate methods: cPCR, PCR-HRM, and the Biomeme's *C. burnetii* qPCR Go-strips (Biomeme). The PCR-HRM and cPCR assays were performed at the Molecular Biology and Bioinformatics Unit at the International Centre for Insect Physiology and Ecology (ICIPE, Nairobi, Kenya). Tests using the Biomeme were conducted at Mpala Research Centre (Laikipia, Kenya), following manufacturers' instructions. Primers utilized in all PCRs targeted the IS*1111* transposase elements of the *C. burnetii* genome (Table 1).

2.2.1 *Coxiella burnetii* conventional PCR

This PCR assay included a touch-down PCR amplification, followed by 1.5% agarose gel electrophoresis to visualize the PCR product. Primers used were trans 1 and trans 2 (Table 1), which amplified a 687 bp fragment of the repetitive, transposon-like element, as described previously (40).

The 11 µL PCR mixture consisted of 0.5 µL of each forward and reverse primer at a final reaction concentration of 0.5 µM, 2 µL of template DNA, 2 µL of HOT FIREPol® Blend Master Mix (Solis BioDyne, Tartu, Estonia), and 6 µL of nuclease free water. Known *C. burnetii* positive tick DNA samples and nuclease free water were included on each plate as a positive control and no template control, respectively. The Supercycler Trinity thermocycler (Kyratec, Queensland, Australia) was used for the PCR. The cycling conditions included an initial enzyme activation step at 95°C for 12 min. This was followed by 5 cycles of denaturation at 95°C for 30 s, an annealing step where the temperature was lowered by 1°C between successive steps from 66°C to 61°C for 45 s, and extension at 72°C for 1 min. An additional 35 cycles consisting of denaturation at 95°C for 30 s, annealing at 61°C for 30 s, extension at 72°C for 45 s, and a final extension step at 72°C for 7 min were added. The PCR products were stained with ethidium bromide and loaded onto a 1.5% agarose gel submerged in a TAE (40 mM Tris-acetate, 1 mM EDTA) buffer-filled electrophoresis chamber set at 97 volts for 42 min. The sizes of the PCR products were determined using an O'rangeRuler 100 bp DNA ladder (Thermofisher Scientific). Images of the gels were recorded.

Positive samples had a clear 687 bp band and were reamplified using trans 1 and trans 2 primers with cycling conditions as described in the previous paragraph (40). The amplicons were then cleaned with the ExoSAP-IT PCR Product Cleanup kit (Affymetrix, Santa Clara, California, United States), according to manufacturer's instructions. The purified amplicons were submitted for Sanger sequencing at Macrogen (Amsterdam, The Netherlands) to confirm the identity of the amplified sequences.

2.2.2 Biomeme *Coxiella burnetii* Go-strip qPCR assay

The *C. burnetii* Go-strips (Biomeme) were supplied as individually packaged three-well qPCR test strips. Each three-well qPCR test strip contained lyophilized master mix, primers, and probes. The primers used for this assay target a 290 bp fragment of the IS*1111* transposase elements in the genome of *C. burnetii* (Sarah Senula, pers. comm., February 8, 2022).

The Biomeme *C. burnetii* Go-strip qPCR assays were performed according to the manufacturer's instructions. In brief, 20 µL of tick DNA eluent was transferred into a single well of the Biomeme Go-Strip and a positive control included for each batch of samples. Biomeme provided a synthetic *C. burnetii* positive control on Whatman paper punches. Each punch was resuspended by adding 400 µL of TAE buffer to the punch and incubating overnight at room temperature. A 20 µL aliquot of the positive control eluent represented 5 copies per reaction. The Go-Strips were loaded into the Biomeme Franklin™ three9 Real-Time PCR Thermocycler and the Biomeme Go application was launched on the connected Android phone (Samsung s8+, Samsung, Ridgefield Park, New Jersey, United States). Using the Biomeme Go application, each sample was individually labeled with a sample identifier, and the QR code on the *C. burnetii* Go strips pouch scanned to launch the Biomeme *C. burnetii* qPCR cycling parameters. The PCR cycle consisted of an initial denaturation step for 1 min at 95°C, followed by 45 cycles for 1 s at 95°C, and annealing for 20 s at 60°C. The Biomeme Go mobile application provided views of amplification plots of raw data in real time. Samples positive for *C. burnetii* DNA showed fluorescence on the green channel and the Cq value per sample was also recorded. Samples with a Cq value ≤ 40 were categorized as positive.

TABLE 1 Test type, primer sequence and criteria for positive results for the three evaluated *Coxiella burnetii* PCR tests targeting the IS*1111* transposase elements in the *C. burnetii* genome, using DNA from ticks collected from wildlife and cattle in Kenya between May 2011 and 2019.

Test	Primer name	Primer sequence (5′-3′)	Amplicon length (bp)	Positive result	References
Conventional PCR	Trans 1	TATGTATCCACCGTAGCCAGTC	687	Samples that have a clear band at the expected amplicon size of 687 bp	Hoover et al. (39)
	Trans 2	CCCAACAACACCTCCTTATTC			
Biomeme qPCR	Proprietary	Proprietary *C. burnetii* qPCR Go-strips test (Biomeme, Philadelphia, Pennsylvania, United States)	290	Samples that had a Cq* value ≤40	N/A
PCR-HRM	*C_burnetii*_HRM-F	GGAACTTGTCAGAGATGATTTGGT	150	Samples whose melt profiles' peaks and shape was similar to the positive control	This study
	*C_burnetii*_ HRM-R	AGAGTTCCCGACTTGACTCG			

*Cq, quantification cycle value.

2.2.3 *Coxiella burnetii* PCR-HRM analysis

The PCR-HRM assay involved a PCR step followed by HRM analysis, using *C_burnetii*_HRM-F and *C_burnetii*_HRM-R primers, designed using Geneious Prime software (Biomatters, Inc., San Diego, California, United States) to cross sequences in which *Coxiella*-endosymbiont sequences have insertions and deletions, thus preventing amplification of *Coxiella*-endosymbionts. The amplification regions were identified through alignments of nucleotide sequences available in the GenBank database, ensuring that the 3′ regions of the primers were specific to *C. burnetii* and not the closely related *Coxiella* endosymbionts (Supplementary Figure S1). The specificity of each primer set was evaluated using the Basic Local Alignment Search Tool (BLAST) from the National Center for Biotechnology Information (NCBI) database. The primer sets were adjusted to achieve optimal annealing temperatures and amplification efficiency. Specificity of the designed primers were tested using samples confirmed to be positive for *C. burnetii, Coxiella* endosymbionts, and nuclease free water, as a negative control.

The PCRs were conducted in 11 µL reaction volumes consisting of 2 µL of HOTFIREPol EvaGreen HRM mix (Solis BioDyne), 0.5 µL of each forward and reverse primer at a final reaction concentration of 0.5 µM, 2 µL of template DNA, and 6 µL of nuclease free water. Known *C. burnetii* positive tick DNA samples and nuclease free water were included in each test batch as a positive control and no template control, respectively. A HRM-capable MIC-4 thermocycler (Bio Molecular Systems, Upper Coomera, Queensland, Australia) was used for DNA amplification. The PCR cycling conditions included an initial enzyme activation step at 95°C for 12 min, followed by 40 cycles of denaturation at 95°C for 30 s, annealing at 62°C for 25 s, extension at 72°C for 20 s, and a final extension at 72°C for 7 min. The PCR was immediately followed by the melt curve analysis during which amplicons were gradually melted at 0.1°C increments from 75°C to 95°C, with fluorescence acquisition every 0.5 s. The Mic qPCR Software automatically generated raw graphs of fluorescence against temperature (°C) from the fluorescence acquisition data. The samples' melt profiles were compared to that of the positive control, based on their melt-curve peaks and curve shapes. Positive samples showed melt profiles with peaks and shapes that aligned with the positive control. To confirm HRM based species identification, the positive samples were reamplified by conventional PCR using COX-F (GTCTTAAGGTGGGCTGCGTG) and COX-R (CCCCGAATCTCATTGATCAGC) primers, targeting a 295 bp fragment of the IS*1111* transposase elements in the *C. burnetii* genome (41). The cycling parameters were programed as previously described in the conventional PCR section. The PCR products were visualized as described above. All samples with 295 bp bands were considered positive, then purified with the ExoSAP-IT PCR Product Cleanup kit (Affymetrix) and submitted for Sanger sequencing at Macrogen to confirm the identity of the amplified sequences.

2.3 Data and statistical analyses

Chromatogram files were imported into Geneious Prime software version 2022 (Biomatters Inc.) for trimming, editing, and alignment. To generate consensus sequences, the study sequences were queried against known sequences in GenBank using the Basic Local Alignment Search Tool to reveal their identity and relation to currently deposited sequences (42). The study sequences were then aligned to all available *C. burnetii* sequences in GenBank using the MAFFT plugin (Biomatters Inc.) in Geneious Prime software (Biomatters Inc., version 2022).

The numbers of *C. burnetii* DNA positive and negative ticks were summarized for each method and positive results were reported as proportions of the total number of samples tested. The number of positive and negative results were also summarized by tick genus and host species. Agreement between the three possible pairs of PCR tests was calculated with Cohen's Kappa coefficient using the psych package (43) in R statistical software (44). A Kappa coefficient close to 1 indicated very good agreement, while 0 indicated poor agreement between tests (45).

2.3.1 Bayesian latent class analysis

A Bayesian latent class analysis (BLCA) was used to estimate the proportion of ticks with *C. burnetii* DNA and evaluate the performance of the three tests in the absence of a gold standard reference test. This analytic approach is well-established in human medicine and veterinary science (46–48) and the World Organization for Animal Health has accepted this method for diagnostic test validation (49). A checklist for BLCA reporting standards (47) is included in Supplementary Appendix S2.

For the present study, a three-test, one population model was used with the latent class defined as whether a tick is positive or negative for

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TABLE 2 Results for 169 tick DNA samples tested for *Coxiella burnetii* by conventional PCR, Biomeme's commercially available *C. burnetii* Go-Strip assay, and a *C. burnetii* PCR-high resolution melt (HRM) technique.

Conventional PCR	Biomeme	PCR-HRM	Total samples with test result combination (%)
Positive	Positive	Positive	3 (1.8%)
Positive	Positive	Negative	0
Positive	Negative	Positive	3 (1.8%)
Negative	Positive	Positive	11 (6.5%)
Positive	Negative	Negative	3 (1.8%)
Negative	Positive	Negative	7 (4.1%)
Negative	Negative	Positive	14 (8.3%)
Negative	Negative	Negative	128 (75.7%)
Total			169

C. burnetii DNA, as an indicator of *C. burnetii* presence and abundance in the ecosystem. This is a similar approach as previous studies that have shown density estimates of infected ticks are better predictors of the likelihood of Lyme disease occurrence than pathogen or tick abundance alone (50). The model used in the current study included binary test outcomes from the cPCR, Biomeme Go-Strips, and PCR-HRM assays. Observations from each tick were included in the analysis if results were available for all three tests. The model was structured assuming that the different combinations of positive and negative test results followed a multinomial distribution. The analysis was implemented in OpenBugs within the R statistical software (51) and RStudio environments (52) using packages r2OpenBugs (53). Programming code for the model implementation was adapted from Cheung et al. (48) and Salgadu et al. (54) and is provided in Supplementary Appendix S3. The model was fit using non-informative priors for Bayesian estimates of Se and Sp due to either limited data (cPCR) or no published data (Biomeme and PCR-HRM) on *C. burnetii* detection in African ticks.

Bayesian estimates of the proportion of ticks with *C. burnetii* DNA, Se, and Sp were generated using a Markov Chain Monte Carlo algorithm. A model was fit to the data that assumed all tests were independent. While all three PCR tests are based on the detection of the IS*1111* transposase elements in the *C. burnetii* genome, the primers used anneal and amplify different segments and lengths of the IS*1111* transposase elements. Therefore, these were considered independent detection methods. Three separate chains were run for 30,000 iterations, with the first 10,000 discarded as "burn-in" based on examination of trace plots. Model convergence and unimodality were assessed by visual inspection of the trace plots, autocorrelation plots, and posterior median distributions of each parameter with the R packages "coda" (55) and "mcmplots" (56). Posterior median estimates of each parameter and their corresponding 95% credible intervals were reported.

3 Results

Ticks were identified to the genus level in 67 out of 179 samples; 112 ticks were not assigned a genus since metagenomic data were unavailable. The majority of identified ticks were *Rhipicephalus* spp. (64/67), and 3/67 of the ticks belonged to the *Amblyomma* genus. Extracted DNA quantity from ticks ranged between 0.03 and 116 ng/μL. Samples from 179 ticks were tested by conventional PCR and the PCR-HRM techniques, while 169 samples were tested on the Biomeme (due to limited availability of kits). Summaries of test results by source host species and tick genera are presented in Supplementary Table S1. In total, 9/179 (5.0%) ticks tested positive for *C. burnetii* DNA by cPCR, 21/169 (12.4%) tested positive with the Biomeme PCR, and 32/179 (17.9%) tested positive by PCR-HRM. Frequency distributions of positive test combinations are shown in Table 2. A figure showing the melt profiles of samples used to test the specificity of the *C_burnetii*_HRM-F and *C_burnetii*_HRM-R primers is included in Supplementary Figure S2.

TABLE 3 Results from the Bayesian latent class model estimating the sensitivity and specificity of three assays (conventional PCR, Biomeme's commercially available *C. burnetii* Go-Strip Assay, and a *C. burnetii* PCR-HRM technique) and the estimated proportion of ticks carrying *C. burnetii*.

Parameter	Posterior median estimate (%)*	95% credibility interval
Sensitivity		
Conventional PCR	24%	10–47%
Biomeme	57%	34–90%
PCRHRM	86%	56–99%
Specificity		
Conventional PCR	98%	94–100%
Biomeme	96%	91–100%
PCRHRM	94%	86–100%
Proportion with *C. burnetti*	16%	0.08–28%

*The model assumes independence across assays. Model estimates were generated from 169 tick samples collected from wildlife and cattle in Kenya between May 2011 and 2019. The Deviance Information Criterion (DIC) of the final model was 27.91 (95% credibility interval: 23.41–37.26%).

There was moderate agreement beyond what would be expected by chance between the PCR-HRM and the Biomeme test results (Kappa = 0.46; 95% CI: 0.28–0.64), while agreement was fair between the cPCR and PCR-HRM results (Kappa = 0.23; 95% CI: 0.053–0.41) and only slight agreement between the cPCR and Biomeme results (Kappa = 0.14; 95% CI: 0.061–0.33).

The BLCA model was fit to the 169 data points for tick samples with a test result for all three assays. Posterior median estimates and 95% credibility intervals of Se, Sp, and the proportion of ticks with *C. burnetii* DNA are shown in Table 3. The model estimated that the PCR-HRM assay had the highest Se (86%; 95% credible interval: 56–99%) followed by the Biomeme (Se =57%; 95% credible interval: 34–90%), and the lowest Se for the cPCR (24, 95% credible interval: 10–47%). Specificity estimates ranged from 94 to 98% for all three assays. The estimated proportion of ticks with *C. burnetii* DNA was 16% (95% credible interval: 8–28%).

4 Discussion

The pathogen *C. burnetii* remains endemic in rural regions of northern Kenya, posing a risk to both human and animal health (35). Although ticks have been shown to play a role in *C. burnetii* transmission, their use for non-invasive pathogen surveillance in these ecosystems, shared by livestock, wildlife, and humans, has been largely

neglected due to limited diagnostic capability at remote satellite laboratories (2, 16, 57). Therefore, the goal in the present study was to fill this gap by using BLCA to compare cPCR, the Biomeme, and PCR-HRM assays, using tick DNA, to provide information on detection methods, presence and abundance of *C. burnetii* in wild animal conservancies in northern Kenya. The study found that all three assays detected *C. burnetii* DNA in whole tick DNA extracts, although a higher Se was found with the PCR-HRM and Biomeme assays at 86, and 57%, respectively, compared to the cPCR (24%). All three assays had high Sp (94–98%), therefore, the assays had a high probability of identifying truly negative ticks. Using BLCA, the estimated proportion of ticks that were positive was 16%, which demonstrated the feasibility of detecting *C. burnetii* using non-invasively collected samples for ecosystem surveillance and infection risk evaluation.

All three PCR methods detected *C. burnetii* positive ticks, however, the Se of the assays varied. The PCR-HRM assay had the highest Se, estimated at 86%, which supports its selection as a screening test for identifying *C. burnetii* positive ticks. Although the Biomeme assay had a lower Se of 57%, it might be a more convenient method for screening in remote areas. The currently used cPCR assay had a low Se (24%) when used with tick samples, which could be attributed to differences between cPCR and qPCR assays (58). In general, cPCR assays use longer amplicons and are qualitative, whereas qPCR assays are quantitative and use shorter amplicons, which ensure higher PCR efficiency and improved sensitivity (58, 59).

While PCR-HRM was found to be more sensitive, both the Biomeme and PCR-HRM had sensitivities and specificities higher than the currently used cPCR. Both the Biomeme and PCR-HRM platforms use portable, battery charged thermocyclers, making them field deployable and more reliable for remote satellite laboratories where power supply may be unreliable. At the time of this report, the Biomeme Franklin thermocycler and the MIC-4 thermocycler were similarly priced; however, the running cost for the Biomeme was approximately tenfold higher in our setting, compared to running the PCR-HRM using the MIC-4 thermocycler. Costs and availability will vary depending on setting and likely change over time. Besides cost, both assays offer advantages in different settings. The PCR-HRM approach is better suited for high throughput because the MIC-4 thermocycler can accommodate batches of 48 samples, compared to the Biomeme Franklin three 9 limit of 3 samples with 3 targets (maximum of nine tests per run). Biomeme's lyophilized master mixes and probes are advantageous in truly field-forward situations, where cold storage facilities are unavailable, and where the operator may have limited technical experience. Based on findings from the present study, the cPCR assay using trans 1 and trans 2 primers for *C. burnetii* screening, was not an ideal platform to detect *C. burnetii* DNA in whole tick extracts. The differences in primer lengths across the PCR tests could influence the detection of *C. burnetii* DNA. The longer primer length in the cPCR compared to the shorter lengths in Biomeme qPCR and PCR-HRM might impact the sensitivity and specificity of each test. This variation is intrinsic to the nature of the primers and was a significant factor in our decision to compare these methods. Our results should be interpreted with this consideration in mind, and further studies could explore the impact of primer length on detection performance more comprehensively. Additional studies should evaluate the performance of cPCR using shorter primers such as those reported by Klee et al. (41). Ultimately, the choice of diagnostic test should be based on practical considerations that include the goals of the surveillance program as well as available resources (60).

Estimated specificity was high for all three tests, with the Sp central estimates ranging from 94% (PCR-HRM) to 98% (cPCR). Other reports of Sp for *C. burnetii* detection by Biomeme or PCR-HRM were not available for comparison. Test specificity describes the proportion of individuals without infection who are correctly identified as negative by a screening test. A high test specificity is especially important when there is low pathogen prevalence. Therefore, the *C. burnetii* PCR-HRM assay could be useful for surveillance in low prevalence or negative populations.

In this study, *C. burnetii* DNA was successfully detected in ticks collected from both wild animals and cattle, emphasizing the value of using tick samples for disease surveillance in areas where *C. burnetii* is endemic. Our model estimated that 16% of ticks collected tested positive for *C. burnetii* DNA, which is consistent with the endemic status of the pathogen in this area. Serological surveys in northern Kenya have reported seroprevalence rates of 10.3% in wild animals (30), 12.8–83.7% in livestock (19, 20, 28, 35) and 16.2–24.4% in humans (23, 35). It is unknown how accurately these estimates of *C. burnetii* in ticks translate into true infection prevalence due to the (inherently biased) opportunistic nature of sampling. In addition, positive tick samples may cluster by host species. Therefore, we exercise caution in interpreting our findings of *C. burnetii* in ticks as a true prevalence. It does, however, offer insight into the endemicity of *C. burnetti* in the ecosystem and the usefulness of ticks in revealing this burden.

Ticks can serve as indicators of *C. burnetii* presence in an ecosystem due to their involvement in the sylvatic transmission cycle (2, 14), however, their utility for disease surveillance is not well-established. Ndeereh et al. (30) reported the prevalence of *C. burnetii* in ticks collected from wild animals in Laikipia, Kenya, to be 0.54% (4/137 tick pools). In contrast, a study of ticks collected from ruminants in South Africa detected varying levels of zoonotic pathogens, including 7% positivity for *C. burnetii* DNA (61). This finding suggests that ticks may offer an advantageous and alternative sample type for surveillance of *C. burnetii*. They are widely distributed and can provide a non-invasive means of detecting pathogens, as shown in other studies (19, 29, 62–64). Validated methods to collect ticks from the environment already exist (65, 66) and they can facilitate widespread geographic coverage. This approach may provide insight on the *C. burnetii* status in a region without the need to handle individual animals, restrict sampling activities to culling/hunting seasons, or rely solely on opportunistic post-mortem examinations of wild animals.

Detection of *C. burnetii* in ticks may vary due infection prevalence in different hosts, sampled populations or environments, tick collection procedures, laboratory processing, and screening methods. Previously, studies have relied on cPCR for *C. burnetti* DNA detection in ticks, which the current study found lacking in sensitivity. Therefore, previous studies may have led to an underestimation and under recognition of the role of ticks in *C. burnetii* epidemiology. Further studies using larger numbers of ticks that represent multiple animal populations and incorporate more sensitive detection methods, such as the PCR-HRM assay, would improve estimates of true *C. burnetii* burden and could elucidate drivers of variability in *C. burnetii* DNA detection in ticks.

Since the primary goal of this study was to compare available detection platforms, Bayesian latent class analysis was used to estimate test performance of three *C. burnetii* PCR methods for detection in ticks. This analytic approach was selected since it does not depend on gold standard test results and has been increasingly used to evaluate and compare diagnostic tests (48). In the present study, a single population model was applied (48) and we assumed conditional independence of tests. This assumption was based on the three diagnostic assays using different primers and amplifying different segments of the IS*1111* transposase elements in the *C. burnetii* genome. Models that incorporate covariance (non-independence of assays) could be considered; however, in the present study, we did not have sufficient sample size or additional populations to fit covariance models based on recommended guidelines (48). Repeated studies with larger sample sizes, and multiple comparison populations, could address this limitation as well as reduce the variability in model estimates (67). As knowledge on these assays advance, the inclusion of prior knowledge (including the estimates published herein) into the Bayesian modeling approach will improve inferences.

We acknowledge that there were limitations in this study. First, the estimates of test performance did not incorporate screening of tick pools (usually 3–5 adult ticks or 10 nymphs per pool) (68), which could be a more economic option for screening (48, 69). Therefore, future studies should evaluate test performance using a pooled testing approach. Secondly, the primer target (IS*1111* insertion sequence) and length (290 bp) used in the Biomeme assay were known; however, the specific primer sequence was unavailable since it is proprietary. Therefore, confirmatory sequencing for *C. burnetii* could only be conducted using amplicons re-amplified by cPCR. A notable limitation was the variation in primer lengths among the PCR tests. While each test targeted different segments of the IS1111 transposase elements in the *C. burnetii* genome and was thus considered an independent detection method, the differences in primer lengths (687 bp for cPCR, 290 bp for Biomeme qPCR, and 150 bp for PCR-HRM) could introduce variability in test performance. Future research should aim to standardize primer lengths to minimize this variability and enhance the comparability of different PCR methods. Finally, the three assays were assessed using tick DNA samples, which are distinct from clinical samples in that they may contain *Coxiella*-like endosymbionts (2). These endosymbionts are solely found in ticks, where they enhance fitness, and are genetically related to *C. burnetii* (2). Consequently, they may be detected by certain PCR assays (based on primers) and could lead to biased estimates of *C. burnetii* abundance in an ecosystem (70). Future studies should determine whether the three assays perform differently when applied to clinical samples, where endosymbionts would not affect assay interpretation and infection burden can be more directly estimated.

5 Conclusion

The findings from this study demonstrate the value of using ticks from wildlife and cattle to detect the presence of *C. burnetii* in an ecosystem. A comparison of PCR assays showed that the PCR-HRM is a suitable screening test for *C. burnetii* using ticks; this test has good performance, is field-friendly, and delivers rapid results. While the PCR-HRM had higher Se compared to the Biomeme and cPCR, the Biomeme assay offers some advantages in certain settings. Findings from this study provide a first step to improve feasibility of noninvasive *C. burnetii* surveillance in ecosystems with human-wildlife-livestock interfaces. Future studies should evaluate these newer assays using multiple populations, different sample types, and similar BLCA methods.

Data availability statement

All relevant data are contained within the article. The original contributions presented in the study are included in the article/Supplementary material, further inquiries can be directed to the corresponding author. The sequence data have been deposited in the European Nucleotide Archive (ENA) at EMBL-EBI under accession number PRJEB67399 (https://www.ebi.ac.uk/ena/browser/view/PRJEB67399).

Ethics statement

The animal study was approved by Stellenbosch University Animal Use and Care (ACU-2023-27912) and Kenya's National Commission for Science, Technology, and Innovation (NACOSTI/P/21/8054). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

MK: Software, Project administration, Writing – review & editing, Writing – original draft, Visualization, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. CW: Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Methodology, Formal analysis, Data curation. WG: Validation, Writing – review & editing, Writing – original draft, Visualization, Supervision, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. MMu: Visualization, Validation, Investigation, Writing – review & editing, Resources, Methodology, Data curation. JV: Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation. DG: Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. RK: Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation. MF: Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Methodology, Investigation, Formal analysis, Data curation. EF: Supervision, Writing – review & editing, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Data curation. DZ: Writing – review & editing, Visualization, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Conceptualization. Y-ML: Writing – review & editing, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Data curation, Conceptualization. MMi: Writing – review & editing, Writing – original draft, Visualization, Validation, Supervision, Software, Resources, Project administration, Methodology, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The author(s) declared that they were an editorial board member of Frontiers, at the time of submission. This had no impact on the peer review process and the final decision.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1396714/full#supplementary-material

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EDITED BY
Calin Mircea Gherman,
University of Agricultural Sciences and Veterinary Medicine of Cluj-Napoca, Romania

REVIEWED BY
ThankGod Emmanuel Onyiche,
University of Maiduguri, Nigeria
Aaron Edmond Ringo,
Obihiro University of Agriculture and Veterinary Medicine, Japan

*CORRESPONDENCE
Sonthaya Tiawsirisup
✉ sonthaya.t@chula.ac.th

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Tick diversity and molecular detection of *Anaplasma*, *Babesia*, and *Theileria* from Khao Kheow open zoo, Chonburi Province, Thailand

Chalida Sri-in[1], Kritsada Thongmeesee[1,2], Wittawat Wechtaisong[1], Nichapat Yurayart[1], Ganyawee Rittisornthanoo[3], Chatlada Akarapas[3], Natcha Bunphungbaramee[3], Natthanicha Sipraya[3], Elizabeth Riana[1,4], Thuong Thi Huyen Bui[1,4], Patchana Kamkong[5], Umaporn Maikaew[6], Piyaporn Kongmakee[7], Arpussara Saedan[7], Lyric C. Bartholomay[8] and Sonthaya Tiawsirisup[1]*

[1]Center of Excellence in Animal Vector-Borne Diseases, Veterinary Parasitology Unit, Department of Veterinary Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, [2]Veterinary Pathobiology Graduate Program, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, [3]6th Year Veterinary Student, Academic Year 2022, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, [4]International Graduate Program of Veterinary Science and Technology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, [5]Parasitology Unit, Department of Pathology, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand, [6]Khao Kheow Open Zoo, Zoological Park Organization, Bangpra, Sriracha, Chonburi, Thailand, [7]Animal Conservation and Research Institute, The Zoological Park Organization of Thailand, Bangkok, Thailand, [8]Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI, United States

Ticks are obligate blood-feeding ectoparasites notorious for their role as vectors for various pathogens, posing health risks to pets, livestock, wildlife, and humans. Wildlife also notably serves as reservoir hosts for tick-borne pathogens and plays a pivotal role in the maintenance and dissemination of these pathogenic agents within ecosystems. This study investigated the diversity of ticks and pathogens in wildlife and their habitat by examining ticks collected at Khao Kheow Open Zoo, Chonburi Province, Thailand. Tick samples were collected for 1 year from March 2021 to March 2022 by vegetation dragging and direct sampling from wildlife. A total of 10,436 ticks or 449 tick pools (1–50 ticks per pool) underwent screening for pathogen presence through conventional PCR and DNA sequencing. Out of the 298 samples (66.37%) where bacteria and protozoa were detected, encompassing 8,144 ticks at all stages, 114 positive samples from the PCR screenings were specifically chosen for detailed nucleotide sequencing and comprehensive analysis. Four species of ticks were conclusively identified through the application of PCR, namely, *Rhipicephalus microplus*, *Dermacentor auratus, Haemaphysalis lagrangei*, and *Haemaphysalis wellingtoni*. The highest infection rate recorded was for *Anaplasma* spp. at 55.23% (248/449), followed by *Babesia* spp. and *Theileria* spp. at 29.62% (133/449) and 16.26% (73/449), respectively. Among bacteria identified, three *Anaplasma* genotypes were closely related to an unidentified *Anaplasma* spp., *A. phagocytophilum*, and *A. bovis*. Among protozoa, only an unidentified *Babesia* spp. was found, whereas two *Theileria* genotypes found were closely related to unidentified *Theileria*

spp. and *T. equi*. Significantly, our findings revealed coinfection with *Anaplasma* spp., *Theileria* spp., and *Babesia* spp. While blood samples from wildlife were not specifically collected to assess infection in this study, the data on the presence of various pathogens in ticks observed can serve as valuable indicators to assess the health status of wildlife populations and to monitor disease dynamics. The findings could be valuable in developing programs for the treatment, prevention, and control of tick-borne illnesses in this area. However, additional research is required to determine the ticks' ability to transmit these pathogens and enhance the current understanding of the relationship among pathogens, ticks, and hosts.

KEYWORDS

***Anaplasma*, *Babesia*, *Theileria*, Thailand, tick, zoo wildlife**

1 Introduction

Ticks belong to the class Arachnida, subclass Acari, and are classified into three families: (i) Ixodidae (hard ticks); (ii) Argasidae (soft ticks); and (iii) Nuttalliellidae. There are approximately 899 hard tick species, and 185 soft tick species are known (1), some of which act as vectors for a broad range of pathogens in domestic animals, wildlife, and humans. These pathogens affect the health of animals and humans, consequently causing significant economic losses worldwide (2). Additionally, to this consequence, tick bites may result in paralysis and toxicoses (3). Furthermore, ticks of all developmental stages can induce allergic reactions in the host's skin (4). Their feeding behavior also makes ticks responsible for direct skin damage to the host (2).

In humans, tick-borne infectious diseases include babesiosis, caused by the protozoa *Babesia microti* (5); Lyme disease, caused by *Borrelia burgdorferi* (6); and human granulocytic anaplasmosis, caused by *Anaplasma phagocytophilum* (7). In animals, tick-borne infectious diseases include Crimean–Congo hemorrhagic fever and tick-borne encephalitis virus caused by viruses; Q fever, borreliosis, and relapsing fever, anaplasmosis, and ehrlichiosis caused by bacteria; and theileriosis and babesiosis caused by protozoa (8).

Wildlife is considered an important reservoir of tick-borne protozoal and bacterial pathogens, including *Theileria*, *Babesia,* and *Anaplasma* (9). Wildlife infected by tick-borne pathogens may show only mild symptoms, which makes the diseases difficult to diagnose (10). Thus, these pathogens can circulate among wildlife and tick populations for extended periods before being identified (11). Since disease transmission occurs between wildlife and other hosts via ticks, pathogen identification in ticks can help in disease surveillance. Conventional polymerase chain reaction (PCR) is a comprehensive, specific, and rapid technique for tick species identification and pathogen diagnosis in ticks (12). We performed PCR and DNA sequencing to investigate the diversity of and identify pathogens in tick specimens collected from the various wildlife species at Khao Kheow Open Zoo, Chonburi Province, Thailand.

Khao Kheow Open Zoo is the largest open zoo in Thailand. It covers an area of about 2,000 acres and contains more than 8,000 animals from more than 300 species of wildlife. The zoo is situated within the boundaries of the Khao Kheow Wildlife Sanctuary and was established to rescue injured wildlife and support research about the diversity of the environment and ecosystem. Consequently, certain species of wildlife can move between these two areas and in doing so, transmit pathogens. Given the variety of host species and the possible modes of disease transmission among them, the Khao Kheow Open Zoo is a great resource for studying tick diversity and therefore the possible role of ticks as vectors for important pathogens. In this present study, ticks were collected from tapir, deer, Eld's deer, spotted deer, barking deer, and hybrid cow, and by vegetation dragging in tapir and Eld's deer habitat. We offer insights that can inform programs for the prevention and control of ticks and tick-borne diseases in wildlife at the Khao Kheow Open Zoo. Furthermore, the study findings can be applied to reducing disease transmission between zoo wildlife and livestock, thereby reducing the economic loss from such diseases, improving wildlife conservation, and monitoring the progression of tick-borne diseases in Thailand.

2 Materials and methods

2.1 Tick collection and identification

Two methods were used to collect tick specimens. First, ticks were directly collected from resident wildlife, including tapirs (*Tapirus indicus*), Eld's deer (*Rucervus eldii*), spotted deer (*Axis axis*), red deer (*Cervus elaphus*), barasingha (*Rucervus duvaucelii*), and hybrid cows—a mix of domestic cattle, red cattle, and bulls (*Bos gaurus hubbacki*) in their lineage—during routine health checks at the zoo following anesthesia. Second, ticks were obtained via vegetation dragging. Researchers randomly traversed animal trails within the Eld's deer and tapir habitats for 1 h at each site and collected ticks from the vegetation every month. Three tick collection sites were established for vegetation dragging, namely, the female Eld's deer (13.209146, 101.052077), male Eld's deer (13.21328, 101.07010), and tapir (13.21603, 101.05880) habitats (Figure 1). All collected tick specimens were stored in microcentrifuge tubes with RNA stabilization solution (RNA Later™ Soln, Invitrogen, United States) and transported to the Parasitology Unit, Faculty of Veterinary Science, Chulalongkorn University, Bangkok, Thailand. The samples were stored at −40°C until further examination. Morphological identification was employed to categorize the collected ticks according to morphology, sex, and life stage, following established protocols (13). Subsequently, tick species were identified through the polymerase chain reaction technique and DNA sequencing.

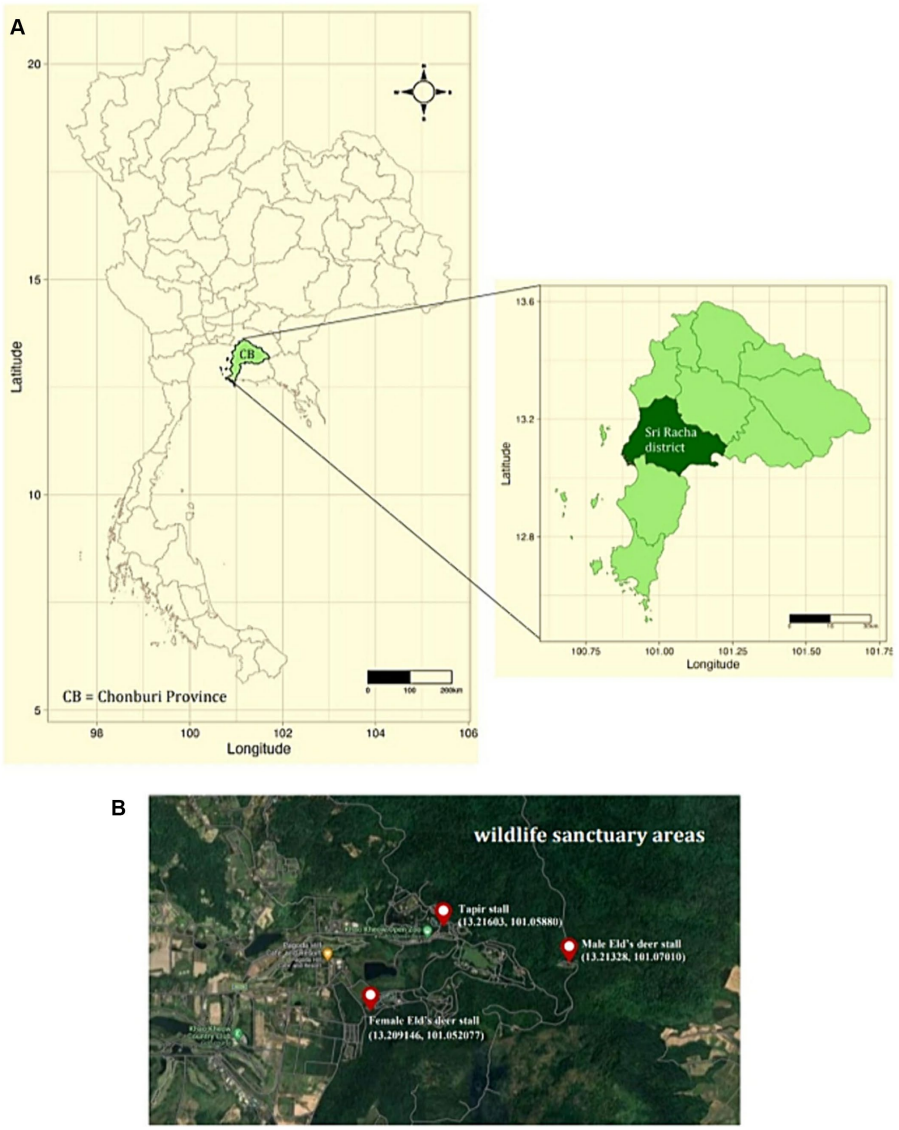

FIGURE 1
(A) Map of Thailand depicting sampling sites in Sri Racha District, Chonburi Province. **(B)** Study sites at Khao Kheow Open Zoo, Chonburi Province of Thailand. The three sampling locations were the tapir, male, and female Eld's deer habitats.

2.2 DNA extraction and conventional polymerase chain reaction

Conventional polymerase chain reaction (PCR) was used for tick species identification and pathogen detection. Due to the substantial quantity, the collected larvae and nymphs were subdivided into pools containing 1 to 50 larvae, and 1 to 10 nymphs, categorized based on morphology, location, and collection time (14, 15). For adult ticks, females and males were examined individually. Tick samples, encompassing males, females, nymph pools, and larval pools, underwent screening for pathogen presence, including *Anaplasma, Babesia*, and *Theileria,* utilizing PCR and DNA sequencing techniques (Supplementary Table 1).

For DNA extraction, the tick samples were homogenized, and genomic DNA was extracted using an IndiSpin Pathogen Kit (Indical Bioscience, Germany) according to the manufacturer's instructions. Nucleic acid samples were stored at −80°C. PCR was performed using KOD One™ PCR Master Mix Blue (TOYOBO, Japan). Each 25-μL reaction mixture contained the following components: 10 μM forward primer (0.75 μL), 10 μM reverse primer (0.75 μL), template DNA (1 μL) or distilled water (1 μL; for the negative control), KOD PCR master mix (12.5 μL), and distilled water (10 μL). The PCR products were electrophoresed on a 1.5% agarose gel mixed with RedSafe™ (iNtRON Biotechnology, South Korea), and the expected bands were visualized using a UV transilluminator. The oligonucleotide primers, product sizes, and cycle conditions required to identify tick species and pathogens within ticks are detailed in Table 1.

Positive PCR products showing DNA bands of the expected size were cut from the agarose gel and purified using the GenepHlow™ Gel/PCR cleanup Kit (Geneaid Biotech, Taiwan) following the manufacturer's protocols. The PCR products were then submitted to a commercial service, U2Bio (Bangkok, Thailand), for DNA sequencing. The quantity and quality were assessed using the NanoDrop™ 1,000

TABLE 1 Primers used for tick and pathogen identification.

Identification	Target gene	Primer name	Oligonucleotide primer (5′... 3′)	Product size (bp)	References
Tick	*16S rRNA*	16S + 1 16S-1	CTGCTCAATGATTTTTTAAATTGCTGTGG CCGGTCTGAACTCAGATCAAGT	401–460	(16)
Babesia	*18S rRNA*	Bab-F Bab-R	GTTTCTGMCCCATCAGCTTGAC CAAGACAAAAGTCTGCTTGAAAC	420–440	(17)
Theileria	*18S rRNA*	989-F 990-R	AGTTTCTGACCTATCAG TTGCCTTAAACTTCCTTG	1,098	(18)
Anaplasmataceae Family	*16S rRNA*	EHR16SD EHR16SR	GGTACCYACAGAAGAAGTCC TAGCACTCATCGTTTACAGC	345	(19)

Spectrophotometer, and only samples with Genomic DNA (gDNA) concentration exceeding 30 ng/μL were chosen for sequencing.

2.3 DNA sequencing and phylogenetic analysis

The nucleotide sequencing results were aligned and trimmed using the ClustalW multiple alignments tool (20) on the Molecular Evolutionary Genetics Analysis software version 10.0 X (MEGA X) (21) and compared with reference DNA sequences in the GenBank database. BLASTn was used to identify genera and/or species of ticks and pathogens (22). The sequences were subjected to genetic diversity analysis using the DnaSP6 software[1] to determine haplotype or nucleotide sequence type diversity. The identified haplotypes were uploaded to the GenBank database. To visualize the sequences of different haplotypes, we used Population Analysis with Reticulate Trees version 1.7 (PopART 1.7) (23), a software for population genetics analysis that generates haplotype networks. These networks implement minimum spanning and median-joining network methods (24). For phylogenetic analysis, the optimal model of nucleotide substitution was determined using the Find Best DNA/Protein Model implemented in MEGA X (21). The best-fitted model selected for constructing the phylogenetics of each dataset was the one with the lowest Bayesian Information Criterion score. The phylogenetic of the protozoal, bacterial, and tick DNA sequences identified were generated using the maximum likelihood method based on the best-fitted models using MEGA X (21). The robustness of the phylogenetic was estimated using 1,000 bootstrap replicates.

2.4 Statistical analysis

Statistical analysis was performed using the Chi-square test and two-way ANOVA implemented in GraphPad Prism version 9.4.1 (GraphPad Software Inc., La Jolla, CA, United States). Statistical significance was set at p-value <0.05.

1 http://www.ub.edu/dnasp/

3 Results

3.1 Tick collection and identification

A total of 10,436 ticks were collected during the study. The vast majority, comprising 97.26% (10,150 out of 10,436), were obtained through vegetation dragging, whereas the remaining 2.74% (286 out of 10,436) were directly collected from hosts. Among these, most ticks were retrieved from the soft areas of wildlife, notably from regions such as the inguinal area, front and hind legs, ears, and neck (Supplementary Figure 1A). Of the ticks collected, larvae were the most abundant (96.4%, 10,060/10,436), followed by nymphs (1.86%, 194/10,436), adult females (1.24%, 129/10,436), adult males (0.51%, 53/10,436; Supplementary Figure 1B). In total, 449 pool samples were established, of which 48.1% consisted of larvae (216/449), 28.73% of adult females (129/449), 11.8% of adult males (53/449), and 11.36% of nymphs (51/449; Supplementary Figure 1C).

3.1.1 Phylogenetic analysis of collected ticks

NCBI BLASTn was used to generate mitochondrial *16S rRNA* gene sequences from 114 samples (1,338 ticks), all of which contained pathogens. All *16S rRNA* gene sequences generated were aligned using MEGA X, and analyses were conducted using BLASTn and DnaSP6. The outcomes of BLASTn and haplotype analyses are presented in Table 2, which identified 16 haplotypes across three genera and four species: *Rhipicephalus microplus* 35.96% (41/114) was grouped in haplotype 1–2 (OQ918450-51), *Dermacentor auratus* 2.63% (3/114) was grouped in haplotype 3 (OQ918452), *Haemaphysalis* (*H.*) *wellingtoni* 0.88% (1/114) was grouped in haplotype 4 (OQ918453), and *H. lagrangei* 60.53% (69/114) was grouped in haplotype 5–16 (OQ918454-64). These results were used to construct a phylogenetic for comparisons with other ixodid tick species registered on the GenBank database.

The phylogenetic tree presented in Figure 2 was generated using the Tamura 3-parameter model (25), derived from *16S rRNA* gene sequences. The phylogenetic tree revealed three groups of tick species: *Haemaphysalis*, *Dermacentor*, and *Rhipicephalus* group. Of the 16 haplotypes, *H. lagrangei* was the most abundant (haplotypes #5–16), followed by *R. microplus* (haplotypes #1–2), *D. auratus* (haplotype #3), and *H. wellingtoni* (haplotype #4). First, *Haemaphysalis* were classified into two clades in which *H. lagrangei*, *H. bispinosa*, *H. longicornis* were closer to one another, whereas *H. wellingtoni* was distinct. Second, *Dermacentor* were classified into two clades and our sequences were closer to *D. auratus*. Finally, *Rhipicephalus* were classified into two clades and our sequences were closer to *R. microplus*. The haplotype

TABLE 2 Identification of tick species collected from Khao Kheow Open Zoo, Chonburi Province, Thailand based on nucleotide sequence analysis using the nucleotide basic local alignment search tool.

Haplotype	No. of sequences (N = 114)	BLASTn			Length (bps)	Submitted sequences accession number
		Closest sequence	Species	% identity		
1	20	MN650726	*Rhipicephalus microplus*	100	409	OQ918450
2	21	MN650726	*R. microplus*	99.75	409	OQ918451
3	3	MT371592	*Dermacentor auratus*	99.75	409	OQ918452
4	1	MG874023	*Haemaphysalis wellingtoni*	100	409	OQ918453
5	40	MG788690	*H. lagrangei*	100	409	OQ918454
6	4	MZ490779	*H. lagrangei*	99.75	409	OQ918455
7	3	MG788690	*H. lagrangei*	99.75	409	OQ918456
8	2	MG788690	*H. lagrangei*	99.75	409	OQ918457
9	7	MZ490779	*H. lagrangei*	99.75	409	OQ918458
10	3	MG788690	*H. lagrangei*	99.51	409	OQ918459
11	2	MZ490779	*H. lagrangei*	99.51	409	OQ918460
12	2	MZ490779	*H. lagrangei*	99.51	409	OQ918461
13	2	MG788690	*H. lagrangei*	99.51	409	OQ918462
14	2	KC170731	*H. lagrangei*	99.27	409	OQ918463
15	1	MZ490779	*H. lagrangei*	99.27	409	OQ918464
16	1	MG788690	*H. lagrangei*	99.26	409	OQ918465

BLASTn, nucleotide Basic Local Alignment Search Tool.

networks presented in Figure 3 were generated using a median-joining network in PopART 1.7, incorporating a total of 16 haplotypes derived from 114 taxa of *16S rRNA* gene sequences. The network classified the haplotypes into three distinct groups consisting of *Haemaphysalis*, *Dermacentor*, and *Rhipicephalus* group. First, *H. lagrangei* were found in all wildlife hosts and habitats employed in this study and *H. wellingtoni* were found in a hybrid cow. Second, *D. auratus* were found in spotted deer and tapir. Finally, *R. microplus* were found in all Eld's deer habitats, tapir, and hybrid cow.

3.1.2 Association between season and number of collected ticks

The association between the season and number of questing ticks collected by vegetation dragging was determined. Following the seasonal classifications in Thailand,[2] three seasons were established: summer (March–June), rainy (July–October), and dry season (November–February). Our findings indicated a relationship between rainfall and the collection of questing ticks in the Khao Kheow Open Zoo. Interestingly, the summer and dry seasons exhibited a more pronounced impact on the active questing ticks collected via vegetation dragging in wildlife areas than in the rainy season. To comprehensively assess the influence of weather variables on tick collection, we analyzed the correlation between the number of ticks collected in each season and specific meteorological data such as rainfall, temperature, and relative humidity. We found while the rainfall significantly negatively affected the quantity of ticks collected (Figure 4A), the temperature and the relative humidity did not (Figures 4B,C). This demonstrated that the trend in the number of questing ticks collected varied relative to the amount of rainfall in each season.

3.2 Pathogen detection in collected ticks

The overall infection rate for the 449 samples (10,436 ticks) was 66.37% (298/449), with the highest infection rate by Anaplasmataceae family at 55.23% (248/449), followed by *Babesia* at 29.62% (133/449) and *Theileria* at 16.26% (73/449). Of the 449 samples, 114 positive sequences were retrieved which all of which contained pathogens. Among these, 73 sequences, originating from individual tick samples, were analyzed for the prevalence of infection. The highest infection rate in these samples was *Anaplasma* at 100% (73/73), followed by *Theileria* at 9.58% (7/73), and *Babesia* at 5.47% (4/73). The type of infection was classified into single-, co-, or triple-infection. In a single infection, *Anaplasma* was the most abundant at 90.41% (66/73). Nevertheless, no single infection by *Theileria* and *Babesia* was identified. Coinfection of *Anaplasma* and *Theileria* was the most abundant at 5.47% (4/73), followed by *Anaplasma* and *Babesia,* and *Theileria* and *Babesia*, both at 1.36% (1/73). Triple infection of *Anaplasma*, *Theileria*, and *Babesia* was found at 2.73% (2/73). The results are presented in Table 3.

3.2.1 *Anaplasma* detection and phylogenetic analysis

A chi-square test was performed to determine the parameters associated with the rate of *Anaplasma* detection (Table 4). In terms of tick collection method, we observed a higher detection rate in ticks collected through dragging than through direct sampling

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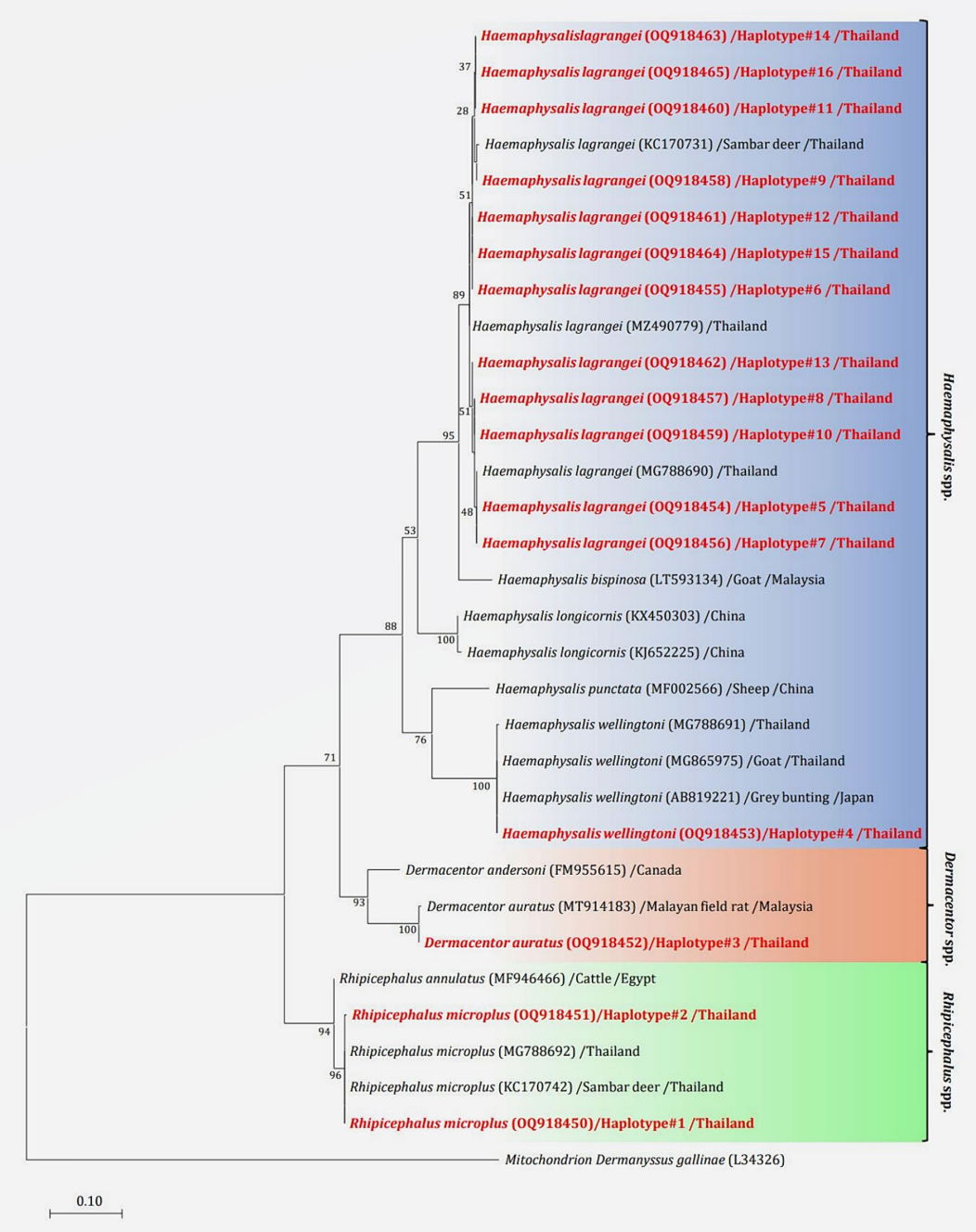

FIGURE 2
Maximum likelihood phylogenetic tree (413 nucleotide sites) from partial mitochondrial *16S rRNA* genes of the tick species found in this study (highlighted in red) and global isolates. Nucleotide sequences using the Tamura 3-parameter model with 1,000 bootstrap replications and using mitochondria from *Dermanyssus gallinae* as the outgroup. The tree is drawn to scale, with branch lengths indicating the number of substitutions per site.

(*p*-value =0.0002). The season of collection was significantly associated with the rate of *Anaplasma* detection, particularly during the dry season (*p*-value =0.0001). The tick development stage was significantly associated with *Anaplasma* detection, particularly larvae (*p*-value <0.0001). Among adult ticks, *Anaplasma* was more frequently detected in males than in females (*p*-value =0.0011). The PCR technique employing EHR16SD and EHR16SR primers indicated the presence of *Anaplasma* in 248 of 449 samples (equivalent to 6,971 ticks out of 10,436 ticks). Of these positive samples, 90 samples (1,327 ticks) were utilized for further DNA sequencing. Four species of *Anaplasma*, namely, *A. capra* 90% (81/90), *A. bovis* 6.67% (6/90), unidentified *Anaplasma* spp. 22.22% (2/90), and *A. phagocytophilum* 1.11% (1/90). The nucleotide sequences from the 90 samples were grouped into seven nucleotide sequence types (ntSTs): ntST#1 (two sequences; OQ352827) showing a 100% match with unidentified *Anaplasma* spp. (KY766243); ntST#2 (79 sequences; OQ352818) showing a 100% match with *A. capra* (OQ552619); ntST#3 (one sequence; OQ352831) showing a 100% match with *A. capra* (LC432126); ntST#4 (one

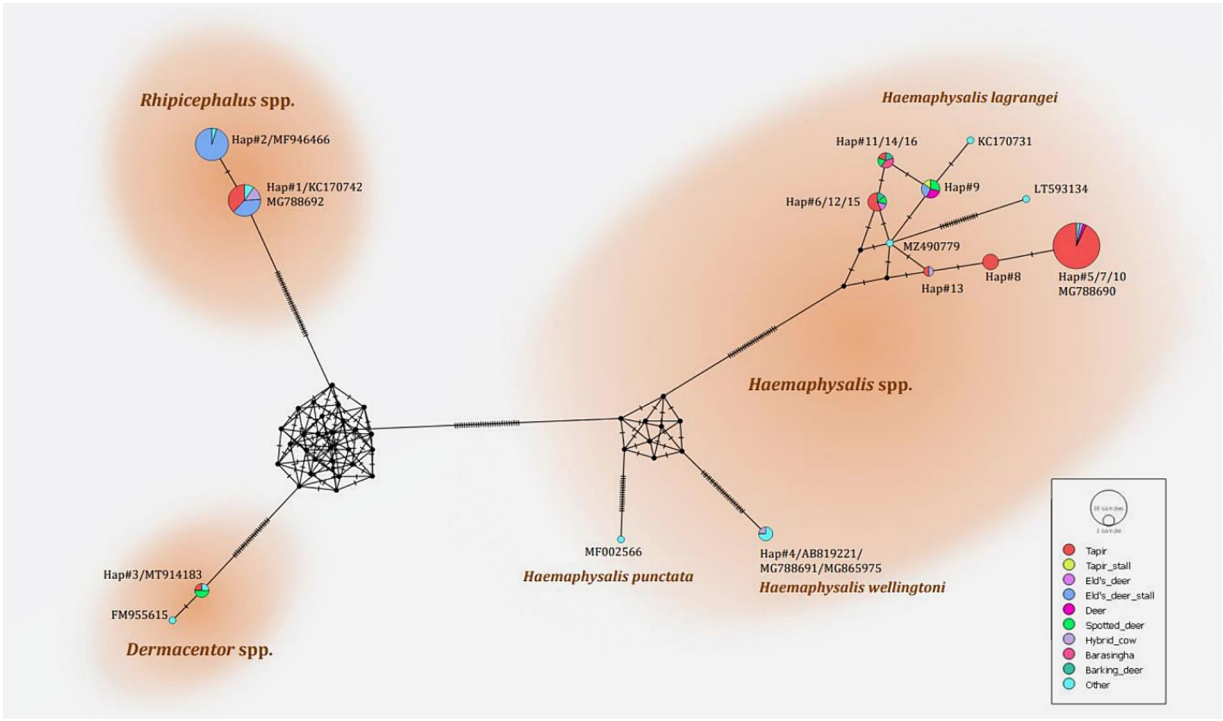

FIGURE 3
Haplotype networks of the partial mitochondrial *16S rRNA* gene of the tick species identified (413 nucleotide sites) were constructed using a median-joining network in PopART 1.7. Each circle represents a single nucleotide sequence. The size of the circle represents the frequency of each nucleotide sequence, whereas the colors represent the sources or hosts of the ticks collected. "Other" refers to the reference genes.

sequence; OQ352828) showing a 100% match with *A. capra* (MH762073); ntST#5 (one sequence; OQ352830) showing a 99.67% match with *A. bovis* (MK028574); ntST#6 (5 sequences; OQ352829) showing a 100% match with *A. bovis* (MK028574); and ntST#7 (one sequence; OQ352832) showing a 100% match with *A. phagocytophilum* (MK394178). The results are presented in Table 5.

The phylogenetics presented in Figure 5 were generated using the Kimura 2-parameter model (K2) model (26). The *Anaplasma 16S rRNA* gene sequences were used to generate a phylogenetic to compare them with the sequences for 21 *Anaplasma* strains registered in the GenBank database. The phylogenetics are characterized into three clusters. Eighty-three DNA sequences (ntST#1–4) were placed in the same cluster at 97% bootstrap value. Within this cluster, ntST#2 was categorized among the branches of two *A. centrale* (KC189842 and MH588233), two *A. ovis* (KJ639880 and KC484563), two *A. marginale* (OP851751 and FJ226454), and one *A. capra* (OQ552619). ntST#1 was divergent and was categorized with two *Anaplasma* spp. (KY66243 and KX505303) and ntST#3 (LC432126) and ntST#4 (MH762073) with *A. capra*. One *Anaplasma* DNA sequence of ntST#7 was classified in the same cluster at 72% bootstrap value with two *A. phagocytophilum* (KR611719 and KT454992). Five *Anaplasma* DNA sequences of ntST#6 were placed in the same cluster at 81% bootstrap value with several *A. bovis* (KJ659040, AB983376, KP062958, and MK028574), whereas one sequence ntST#5 was found to be divergent. The networks of nucleotide sequence types presented in Figure 6 were generated using a median-joining network in PopART 1.7 and identified seven haplotypes derived from 90 taxa of *16S rRNA* gene sequences were identified. The network classified the nucleotide sequence types into three distinct groups: *Anaplasma* spp., *A. bovis*, and *A. phagocytophilum*. *Anaplasma* spp. were found in four species of ticks, namely, *H. lagrangei, H. wellingtoni, R. microplus,* and *D. auratus*. Furthermore, *A. bovis* was found in *H. lagrangei, R. microplus,* and *D. auratus*, wheeas *A. phagocytophilum* was only found in *R. microplus*.

3.2.2 *Theileria* and *Babesia* detection and phylogenetic analysis

The chi-square test was performed to determine the parameters associated with the rate of detection of tick species. *Theileria* was observed more frequently in ticks collected by vegetation dragging than by direct sampling (p-value = 0.0001; Table 6). Furthermore, we observed a higher incidence of *Theileria* in larvae than that in the other stages (p-value <0.0001). However, the rate of *Theileria* detection was not significantly associated with tick sex or season of collection. The rate of *Babesia* detection was significantly associated with the season, particularly the dry season (p-value = 0.0069; Table 7). The detection frequency of *Babesia* was significantly higher with vegetation dragging than with direct sampling (p-value <0.0001). Additionally, we noted a significantly higher incidence of *Babesia* in larvae compared to other developmental stages (p-value <0.0001). Nevertheless, no significant association was observed between tick sex and rate of *Babesia* detection.

The PCR results for *Theileria* spp. (Table 5) using the partial *18S rRNA* gene, 989-F and 990-R primers, revealed that 16.26% (73/449) of the samples were positive for the protozoal species. Two species of *Theileria* were identified including, unidentified *Theileria* spp. 88.89% (16/18) and *T. equi* at 11.11% (2/18). In terms of *Babesia* spp. (Table 5), the PCR results using the partial *18S rRNA* gene, Bab-F and Bab-R

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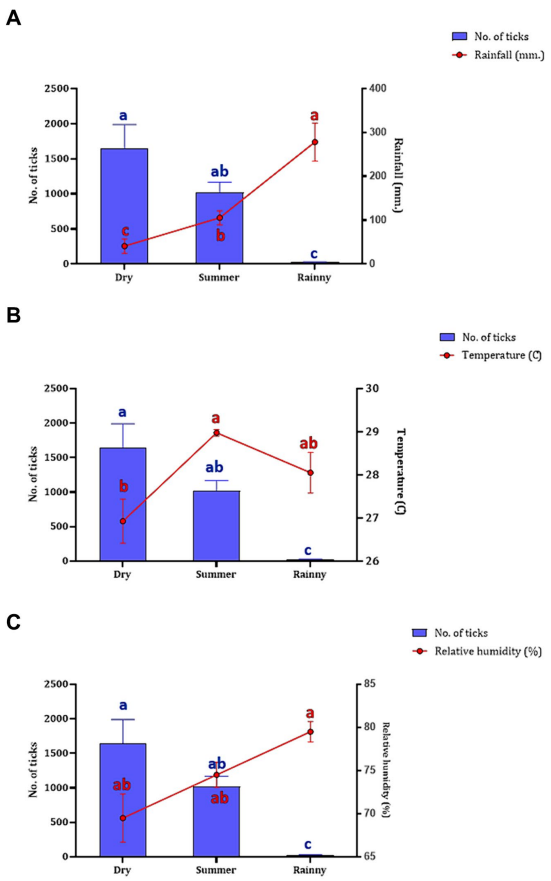

FIGURE 4
The number of questing ticks collected in each season (represented by blue bar) relative to the weather variables (represented by red circle and line), including rainfall **(A)**, temperature **(B)**, and humidity **(C)**. The seasons in Thailand, namely, summer (collected in March–June 2021), rainy (collected in July–October 2021), and dry (collected in November 2021–February 2022). Data are presented as means with SEM. The blue bars with distinct blue letters and the red circles with distinct red letters denote significant differences between each season in a two-way ANOVA.

primers, revealed that 29.62% (133/449) of the samples were positive for the species. The protozoal DNA sequences were grouped into six ntSTs: ntST#8–11 (16 sequences; OR003900-03) showing a 96.08–97.96% match with *Theileria* spp. (KP410272 and KP410273); ntST#12 (two sequences; OR003904) showing a 99.71% match with *T. equi* (MT463610); and ntST#13 (six sequences; OR003905) showing a 99.16% match with *Babesia* spp. (KY766213). The DNA sequences are available from GenBank.

The phylogenetics presented in Figure 7 were generated using the Tamura-Nei model (27). Analysis of the *18S rRNA* gene sequences of *Theileria* and *Babesia* was performed for comparison with the 36 *Theileria* and *Babesia* strains registered in the GenBank database. The DNA sequences were phylogenetically characterized into four clusters: a putative novel species of 13 DNA sequences (ntST#8–10) classified into the *Theileria* group with the same cluster at 80% bootstrap value; three DNA sequences of ntST#11 categorized in the same cluster at 100% bootstrap value with five *Theileria* spp., although more divergence was found in this sequence; two DNA sequences of ntST#12 categorized in the same cluster at 100% bootstrap value with

TABLE 3 Prevalence of tick-borne protozoal and bacterial infections in 73 individual tick samples collected from Khao Kheow Open Zoo, Chonburi Province, Thailand.

Tick species	Prevalence of infections (infected/tested samples)			Type of infection							
				Single infection			Coinfection			Triple infection	
	Anaplasma	*Theileria*	*Babesia*	*Anaplasma*	*Theileria*	*Babesia*	*Anaplasma + Theileria*	*Anaplasma + Babesia*	*Theileria + Babesia*	*Anaplasma + Babesia + Theileria*	
Rhipicephalus microplus	100% (11/11)	9.09% (1/11)	9.09% (1/11)	90.9% (10/11)	- (0/11)	- (0/11)	- (0/11)	- (0/11)	- (0/11)	9.09% (1/11)	
Dermacentor auratus	100% (3/3)	66.67% (2/3)	- (0/3)	33.33% (1/3)	- (0/3)	- (0/3)	66.67% (2/3)	- (0/3)	- (0/3)	- (0/3)	
Haemaphysalis wellingtoni	100% (1/1)	100% (1/1)	- (0/1)	- (0/1)	- (0/1)	- (0/1)	100% (1/1)	- (0/1)	- (0/1)	- (0/1)	
Haemaphysalis lagrangei	100% (58/58)	5.17% (3/58)	5.17% (3/58)	94.82% (55/58)	- (0/58)	- (0/58)	1.72% (1/58)	1.72% (1/58)	1.72% (1/58)	1.72% (1/58)	
Total	100% (73/73)	9.58% (7/73)	5.47% (4/73)	90.41% (66/73)	- (0/73)	- (0/73)	5.47% (4/73)	1.36% (1/73)	1.36% (1/73)	2.73% (2/73)	

TABLE 4 The parameters associated with the detection of the Anaplasmataceae family based on PCR diagnostics in tick (Chi-square test; Confidence interval 95%).

Parameters	No. tested samples	Rate of detection (infected/tested samples)	*p*-value
Tick collection method ($n = 449$ samples)			
Dragging	234	63.68% (149/234)	0.0002
Picking	215	46.05% (99/215)	
Season ($n = 234$ samples)			
Summer	129	41.86% (54/129)	<0.0001
Rainy	7	28.57% (2/7)	
Dry	98	94.89% (93/98)	
Stage of tick ($n = 449$ samples)			
Larva	216	66.67% (144/216)	<0.0001
Nymph	51	9.8% (5/51)	
Adult (Female and Male)	182	47.25% (86/182)	
Sex of tick ($n = 182$ samples)			
Female	129	39.53% (51/129)	0.0011
Male	53	66.04% (35/53)	

ns, non-significant; *p-value < 0.05, **p-value < 0.01, ***p-value < 0.001, ****p-value < 0.0001.

two *T. equi* (MT463610 and MN625897); and six DNA sequences of ntST#13 classified in the same cluster at 91% bootstrap value with two *Babesia* spp. (KY766213 and KJ486569). The networks of nucleotide sequence types presented in Figure 8 were constructed using the median-joining network function in PopART 1.7 from a total of six ntSTs (24 taxa of *18S rRNA* gene sequences). The nucleotide sequence types were classified into four groups: unidentified *Theileria* spp. were found in two species of ticks, namely, *H. lagrangei,* and *R. microplus*; *Theileria* spp. found in *H. lagrangei*; *T. equi* found in *R. microplus*; and *Babesia* spp. found in *H. lagrangei* and *R. microplus*.

4 Discussion

In this study, various protozoa and bacteria species were identified from 10,436 tick specimens collected. The larva was the predominant specimen type, followed by the nymph, female, and male specimens, respectively. Our field data indicated that a higher quantity of ticks can be collected during the dry season (November–February) at the Khao Keow Open Zoo in Thailand. Conversely, in the Amazon, tick density is higher during the rainy season (July–November) (28). We also observed significant detection rates for tick pathogens during the dry season. This concurs with a previous study in 2023 that also observed significant infection rates of *Anaplasma* spp. in beef cattle during the dry season in Thailand, which was linked to ticks or blood-sucking flies (29). Since animals and wildlife in the open zoo have free access to the wild, the high tick infection rates in our study could be partially explained by interactions with wildlife.

Four tick species, namely, *H. lagrangei*, *H. wellingtoni*, *D. auratus*, and *R. microplus*, were identified in this present study, which confirmed the previous reports of tick species occurring in many parts of Thailand (15, 30, 31). Ticks play a crucial role as ectoparasites affecting wildlife, livestock, and companion animals in Thailand (32, 33). The present study found that all four tick species (*H. lagrangei*, *H. wellingtoni*, *D. auratus*, and *R. microplus*) were all infected by bacteria in the genus *Anaplasma*, including unidentified *Anaplasma* spp., *A. phagocytophilum,* and *A. bovis.* Based on the results of *16S rRNA* gene sequencing, the harbored *Anaplasma* species in this study matched with *A. marginale*, *A. ovis*, *A. centrale*, and *A. capra*, which were collected from tapirs, deer, spotted deer, Eld's deer, hybrid cows, and vegetation. A previous study based on the results of *16S rRNA* gene sequencing reported four *Anaplasma* spp. (*A. marginale*, *A. bovis*, *A. phagocytophilum*, and *A. centrale*) detected in *R. microplus* specimens collected from tapirs, cows, and surrounding vegetation (34). Nevertheless, limitations were found when using the *16S rRNA* sequence for species classification of *Anaplasma* samples (35). The PCR primers used in the present study, specifically designed for amplification of the *16S rRNA* gene, showed limited accuracy in distinguishing *Anaplasma* isolates at the species level. Careful consideration needs to be exercised in the design of PCR primers by incorporating the alignments of diverse target genes and alternative genetic markers. This strategic approach is imperative for improving the precision and specificity of identifying and characterizing *Anaplasma* species.

We identified *A. phagocytophilum* in the *R. microplus* ticks collected from Eld's deer. This finding aligns with the observations of Zhang et al. (36), who reported *A. phagocytophilum* infection in *R. microplus* ticks collected from 10 provinces in China. This important human pathogen has a broad host range and can cause severe infections in various mammalian species (37). Furthermore, we identified *Anaplasma bovis* in *H. lagrangei, R. microplus*, and *D. auratus* ticks collected from deer, spotted deer, and tapir. *A. bovis* causes diseases in both ruminants and small mammals, with transmission facilitated by *Haemaphysalis, Rhipicephalus, Amblyomma*, and *Ixodes* (7). Previous studies have identified certain species of *Haemaphysalis,* including *H. lagrangei, H. megaspinosa*, and *H. longicornis*, as potential vectors of *A. bovis* in East and Southeast Asia (31, 38). Given the widespread distribution of these tick species in Southeast Asia, infection of domestic cattle and wildlife, including various deer species, poses a significant concern (2). While most studies have confirmed *A. bovis* infection across a diverse range of ruminant hosts, its presence in ticks collected from tapirs has not been previously reported. Confirming *A. bovis* infection in ticks obtained from tapirs is crucial to determine whether the ticks acquired the pathogen from the tapir or if they were already harboring the pathogen before contact.

The present study found that *H. lagrangei* and *R. microplus* were infected with *Theileria* and *Babesia 18S rRNA* gene sequencing revealed *Theileria* and *Babesia* spp. in ticks collected from tapir and vegetation. Another study that performed *18S rRNA* gene sequencing reported *Theileria* and *Babesia* spp. in *H. lagrangei* (15). Furthermore, we discovered that some sequences of unidentified *Theileria* spp. were found in the sister clade of *T. equi*. However, the bootstrap value was relatively strong; hence, the sequences might indicate putative novel species, and other gene markers should be considered. In Thailand, *T. equi* is a tick-borne parasite that is considered endemic in equines and mules (39). However, the present study confirmed the natural occurrence of *T. equi* in *R. microplus* collected from tapirs in Chonburi, Thailand. To the best of our knowledge, the occurrence of *T. equi* and

TABLE 5 Analysis of protozoal and bacterial nucleotide sequences using the nucleotide Basic Local Alignment Search Tool.

ntST	No. of sequences (*N* = 114)	BLASTn			Length (bp)	Positive tick samples			Vertebrate hosts	Submitted sequences accession no.
		Closest sequence	Species	Identity (%)		No. of sequences	Tick species	Stage		
1	2	KY766243	*Anaplasma* sp.	100	305	1	*Haemaphysalis lagrangei*	F	Tapir	OQ352827
						1	*Dermacentor auratus*	F	Tapir	
2	79	OQ552619	*A. capra*	100	305	13	*H. lagrangei*	F	Tapir	OQ352818
						1	*H. lagrangei*	F	Spotted deer	
						22	*H. lagrangei*	M	Tapir	
						1	*H. lagrangei*	M	Deer	
						1	*H. lagrangei*	M	Spotted deer	
						1	*H. lagrangei*	N	Eld's deer stall	
						2	*H. lagrangei*	N	Spotted deer	
						15	*H. lagrangei*	L	Tapir stall	
						1	*H. lagrangei*	L	Eld's deer stall	
						1	*H. wellingtoni*	M	Hybrid cow	
						1	*Rhipicephalus microplus*	M	Hybrid cow	
						5	*R. microplus*	N	Tapir	
						1	*R. microplus*	N	Hybrid cow	
						1	*R. microplus*	N	Eld's deer	
						1	*R. microplus*	N	Eld's deer stall	
						3	*R. microplus*	L	Eld's deer stall	
						8	*R. microplus*	L	Eld's deer stall	
						1	*D. auratus*	N	Spotted deer	
3	1	LC432126	*A. capra*	100	305	1	*R. microplus*	L	Eld's deer stall	OQ352831
4	1	MH762073	*A. capra*	100	305	1	*H. lagrangei*	F	Tapir	OQ352828
5	1	MK028574	*A. bovis*	99.67	305	1	*H. lagrangei*	F	Tapir	OQ352830

(Continued)

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TABLE 5 (Continued)

ntST	No. of sequences (*N* = 114)	BLASTn			Length (bp)	Positive tick samples			Vertebrate hosts	Submitted sequences accession no.
		Closest sequence	Species	Identity (%)		No. of sequences	Tick species	Stage		
6	5	MK028574	*A. bovis*	100	305	1	*H. lagrangei*	M	Deer	OQ352829
						1	*H. lagrangei*	F	Deer	
						1	*H. lagrangei*	F	Tapir	
						1	*R. microplus*	F	Tapir	
						1	*D. auratus*	N	Spotted deer	
7	1	MK394178	*A. phagocytophilum*	100	305	1	*R. microplus*	N	Eld's deer	OQ352832
8	4	KP410273	*Theileria* sp.	97.96	508	1	*R. microplus*	L	Eld's deer stall	OR003900
						1	*H. lagrangei*	F	Tapir	
						2	*H. lagrangei*	M	Tapir	
9	7	KP410272	*Theileria* sp.	96.47	508	7	*R. microplus*	L	Eld's deer stall	OR003901
10	2	KP410272	*Theileria* sp.	96.08	508	2	*R. microplus*	L	Eld's deer stall	OR003902
11	3	KP410272	*Theileria* sp.	96.47	508	3	*R. microplus*	L	Eld's deer stall	OR003903
12	2	MT463610	*T. equi*	99.71	508	2	*R. microplus*	L	Tapir stall	OR003904
13	6	KY766213	*Babesia* sp.	99.16	479	3	*R. microplus*	L	Eld's deer stall	OR003905
						1	*H. lagrangei*	F	Tapir	
						2	*H. lagrangei*	M	Tapir	

BLASTn, nucleotide Basic Local Alignment Search Tool. ntST, nucleotide sequence type.

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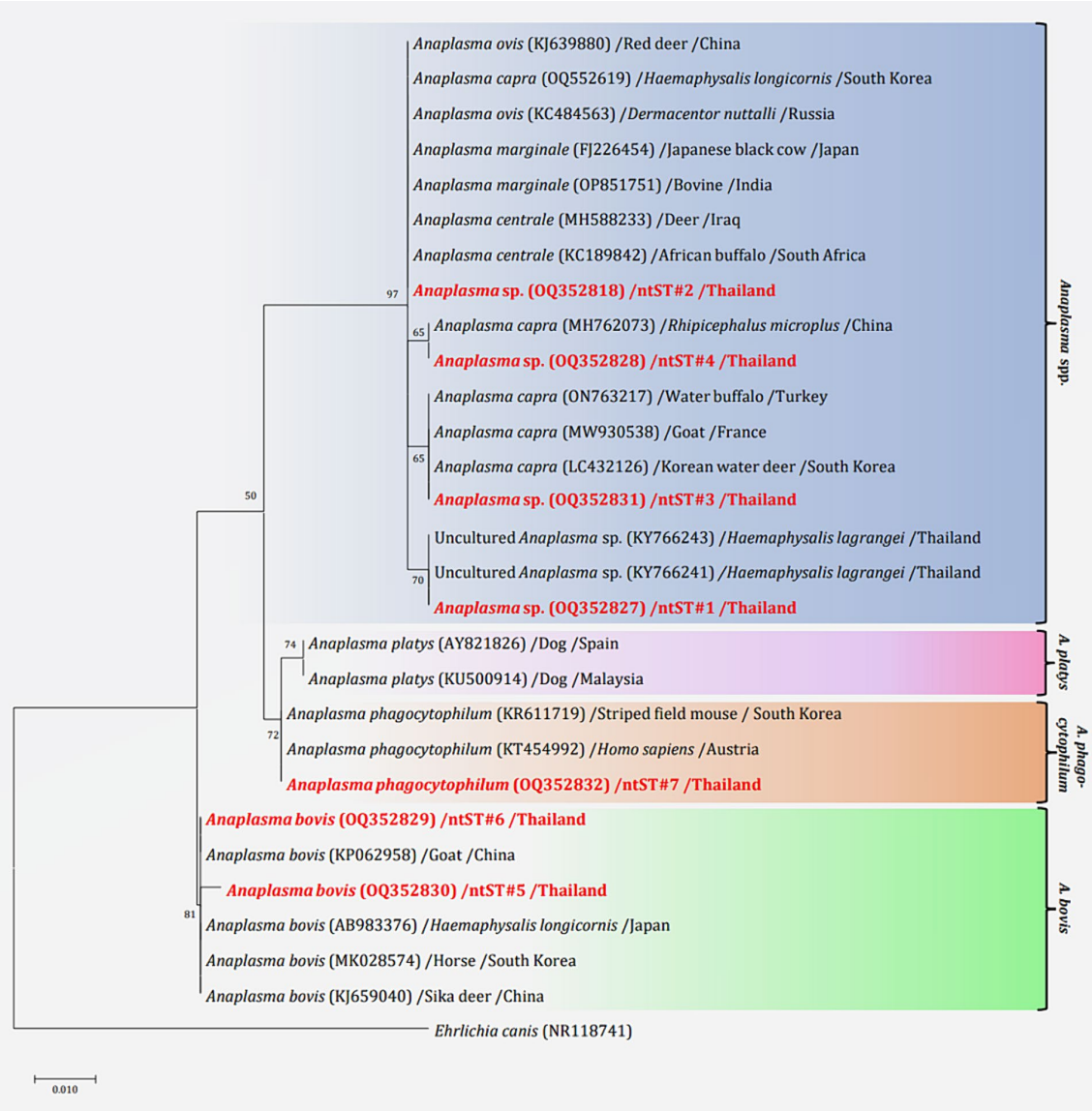

FIGURE 5
Maximum likelihood phylogenetic tree of 306 nucleotide sites from partial mitochondrial *16S rRNA* genes of the *Anaplasma* spp. found in this study (highlighted in red) and the global isolates. Nucleotide sequences were determined using the Kimura 2-parameter model (K2) with 1,000 bootstrap replications and *Ehrlichia canis* (NR118741) as the outgroup. The tree is drawn to scale, with branch lengths indicating the number of substitutions per site.

closely related genotypes in Thailand has not been previously reported in tapirs or ticks removed from tapirs. Nevertheless, this protozoan species naturally occurs in the South American tapir in Brazil (40). The housing of tapirs in Khao Kheow open zoo areas connects to wildlife sanctuary areas in Thailand, which may promote close contact between different animal species, vector sharing, and consequently pathogen transmission. Thus, our findings corroborate the cross-transmission of pathogens between domestic and wild animals and provide evidence of ticks as possible vectors for diseases in Thailand's wildlife habitats. Although we detected *Theileria* in tick larvae, this study could not confirm the transovarial transmission of *Theileria*. Additional evidence is required to address this question. However, our findings align with those of Wattanamethanont et al. (15), who identified *Theileria* in the larvae of ixodid ticks collected by vegetation dragging in a national park in Thailand. These findings imply that ixodid ticks actively searching for hosts in wildlife habitats might be potential vectors for *Theileria* in Thailand.

Interestingly, our findings demonstrated coinfection with *Anaplasma* spp., *Theileria* spp., and *Babesia* spp. Ticks infesting wild animals in their natural habitats can become co-infected, transmitting two or more pathogens (41). Co-infection with more than one tick-borne pathogen is a common occurrence that amplifies pathogenic processes and consequently increases the risk of disease severity (11, 42). Furthermore, pathogen interactions can also increase the risk of infection in wildlife (43). Despite the lack of blood samples collected from wildlife to assess for infection in the present study, data on the various pathogens in ticks observed in this study can be used to assess the well-being of

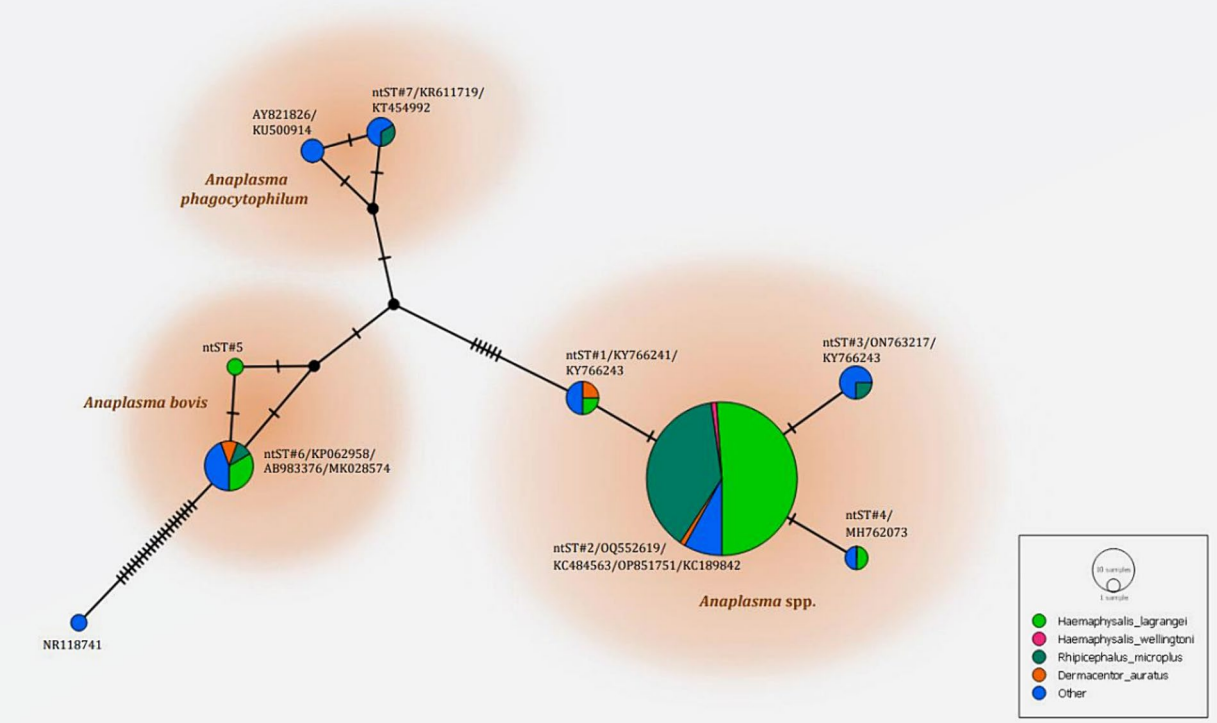

FIGURE 6
Nucleotide sequence type (ntST) networks of the *16S rRNA* gene (306 nucleotide sites) of *Anaplasma* spp. The network was constructed using a median-joining network in PopART 1.7. Each circle represents a different haplotype. The size of the circle represents the frequency of each ntST, while the colors represent the tick species. "Other" refers to the reference genes.

TABLE 6 The parameters associated with the detection of the *Theileria* genus based on PCR diagnostics in tick (Chi-square test; Confidence interval 95%).

Parameters	No. tested (samples)	Rate of detection (infected/tested samples)	*p*-value
Tick collection method ($n = 449$ samples)			
Dragging	234	22.65% (53/234)	0.0001
Picking	215	9.3% (20/215)	
Season ($n = 234$ samples)			
Summer	129	20.16% (26/129)	0.5898 (ns)
Rainy	7	28.57% (2/7)	
Dry	98	25.51% (25/98)	
Stage of ticks ($n = 449$ samples)			
Larvae	216	24.07% (52/216)	<0.0001
Nymphs	51	1.96% (1/51)	
Adults (Female and Male)	182	8.79% (16/182)	
Sex of ticks ($n = 182$ samples)			
Females	129	8.53% (11/129)	0.8444 (ns)
Males	53	9.43% (5/53)	

ns, non-significant; **p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001, *****p*-value < 0.0001.

TABLE 7 The parameters associated with the detection of the *Babesia* genus based on PCR diagnostics in tick (Chi-square test; Confidence interval 95%).

Parameters	No. tested (samples)	Rate of detection (infected/tested samples)	*p*-value
Tick collection method ($n = 449$ samples)			
Dragging	234	48.72% (114/234)	<0.0001
Picking	215	8.84% (19/215)	
Season ($n = 234$ samples)			
Summer	129	48.06% (62/129)	0.0069
Rainy	7	- (0/7)	
Dry	98	53.06% (52/98)	
Stage of ticks ($n = 449$ samples)			
Larvae	216	51.39% (111/216)	<0.0001
Nymphs	51	5.88% (3/51)	
Adults (Female and Male)	182	7.14% (13/182)	
Sex of ticks ($n = 182$ samples)			
Females	129	5.43% (7/129)	0.1607 (ns)
Males	53	11.32% (6/53)	

ns, non-significant; **p*-value < 0.05, ***p*-value < 0.01, ****p*-value < 0.001, *****p*-value < 0.0001.

wildlife and monitor diseases. Additionally, the cross-transmission of ticks between humans and wildlife increased with the rise in outdoor human activities in natural wildlife habitats. Thus, awareness of the risks of zoonotic diseases should be increased. Our findings also demonstrated that certain tick species found in Thailand are possible vectors of tick-borne diseases in wildlife at

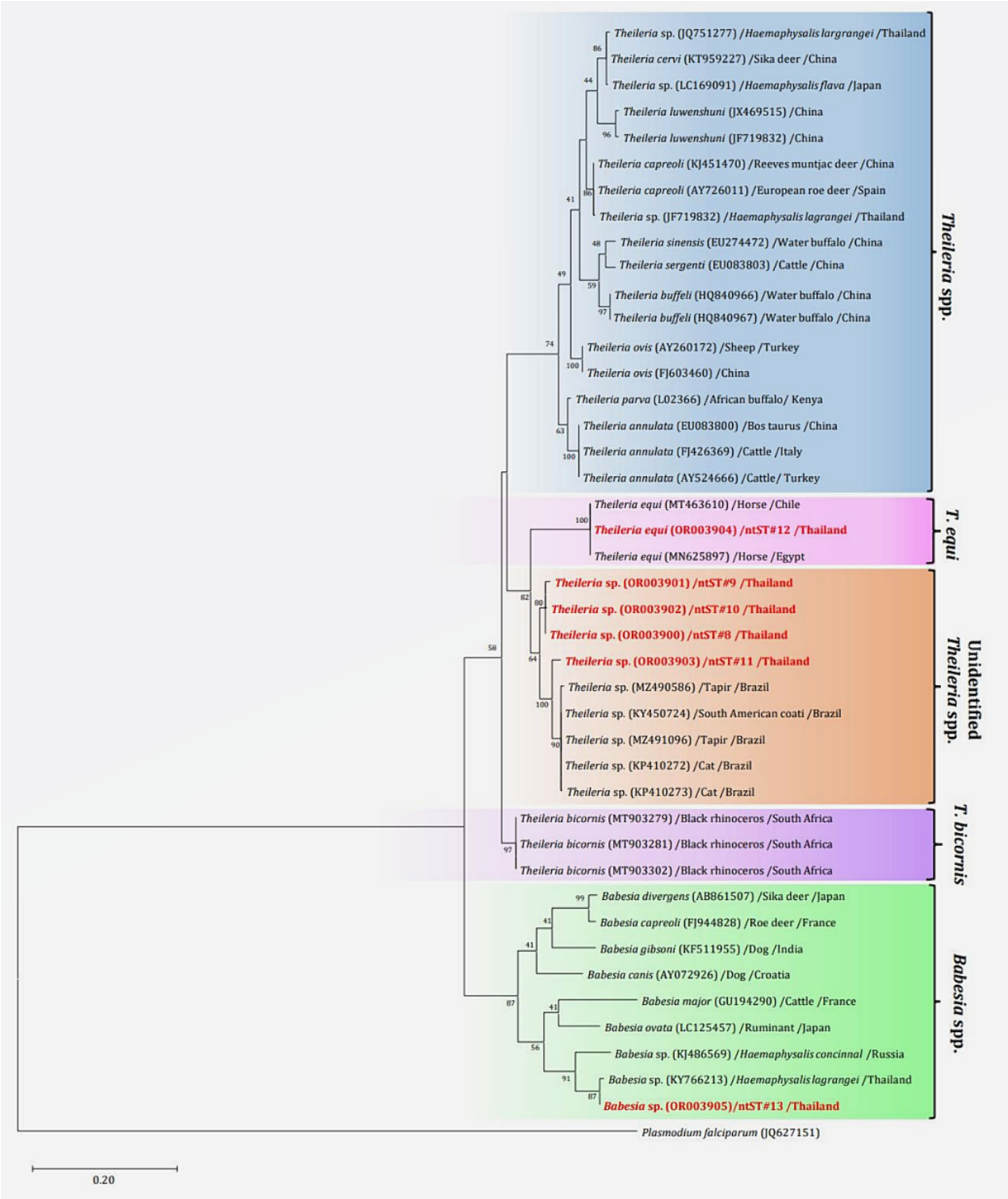

FIGURE 7
Maximum likelihood phylogenetic tree of the partial *18S rRNA gene* (603 nucleotide sites) of *Theileria* and *Babesia* spp. found in this study (highlighted in red) and the global isolates. Nucleotide sequences were generated using the Tamura–Nei model with 1,000 bootstrap replications, with *Plasmodium falciparum* (JQ627151) as the outgroup. The tree is drawn to scale, with branch lengths indicating the number of substitutions per site.

the Khao Kheow Open Zoo, as confirmed by the detection of pathogens in the areas studied. The present study provided valuable insights for the effective treatment, prevention, and planning of annual tick control and surveillance in open zoo areas to prevent tick-borne illnesses. However, additional research is required to determine the ability of each species to transmit such diseases and to enhance the understanding of the relationships among pathogens, ticks, and hosts.

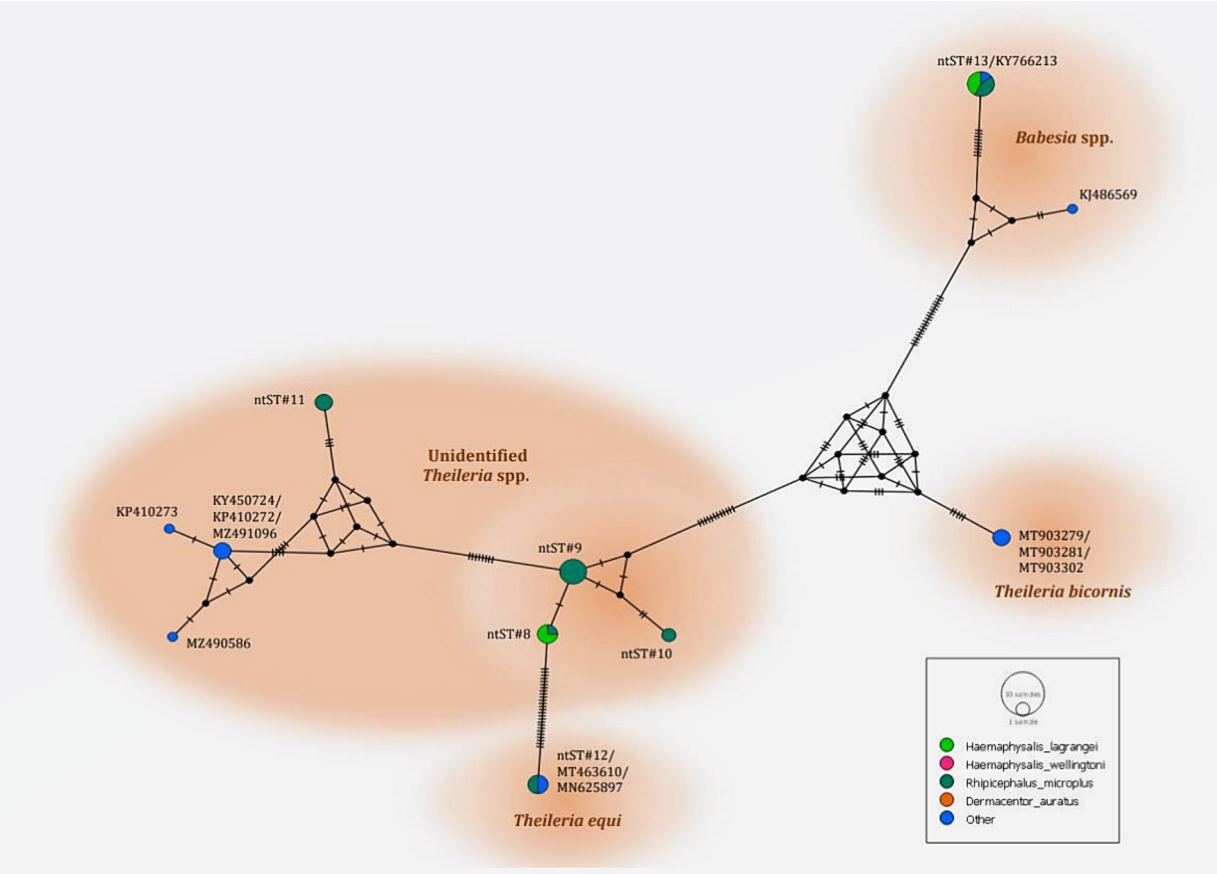

FIGURE 8
Nucleotide sequence type (ntST) networks of the *18S rRNA* gene (603 nucleotide sites) of *Theileria* and *Babesia* spp. were constructed using the median-joining network function in PopART 1.7. Each circle represents a different haplotype. The size of the circle represents the frequency of each ntST, and the colors represent the tick species identified. "Other" indicates the reference genes.

Data availability statement

The data supporting the results of this study can be obtained from the corresponding author upon request. Moreover, all DNA sequences have been deposited in GenBank® under the following accession numbers: the 16S rRNA gene from collected ticks (OQ918450-OQ918465), the 16S rRNA gene obtained from Anaplasma isolates (OQ352818, OQ352827-OQ352832), and the 18S rRNA gene obtained from protozoa isolates (OR003900-OR003905).

Ethics statement

The animal study was approved by Chulalongkorn University Animal Care and Use Committee (Animal Use Protocol no. 2131007 and 2231058) and the Animal Conservation and Research Institute Committee, Zoological Park Organization of Thailand. The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

CS: Investigation, Methodology, Writing – original draft. KT: Investigation, Writing – review & editing. WW: Investigation, Writing – review & editing. NY: Investigation, Writing – review & editing. GR: Investigation, Writing – review & editing. CA: Investigation, Writing – review & editing. NB: Investigation, Writing – review & editing. NS: Investigation, Writing – review & editing. ER: Investigation, Writing – review & editing. TB: Investigation, Writing – review & editing. PaK: Investigation, Writing – review & editing. UM: Investigation, Writing – review & editing. PiK: Investigation, Writing – review & editing. AS: Investigation, Writing – review & editing. LB: Writing – review & editing. ST: Funding acquisition, Methodology, Supervision, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets.2024.1430892/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Parts of whole tick collection methods **(A)**, tick stages **(B)**, and pool samples 622 **(C)** established in this study.

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EDITED BY
Rosa Estela Quiroz Castañeda,
Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Mexico

REVIEWED BY
Maria Primo,
CONICET Santa Fe, Argentina
Hugo Aguilar-Díaz,
Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Mexico

*CORRESPONDENCE
Tian-yin Cheng
✉ hn5368@163.com

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What are the main proteins in the hemolymph *of Haemaphysalis flava* ticks?

Dan Li, Lei Liu, Zi-ling Liu, Yuan Tian, Xin Gao and Tian-yin Cheng*

Research Center for Parasites and Vectors, College of Veterinary Medicine, Hunan Agricultural University, Changsha, Hunan, China

Background: *Haemaphysalis flava* is a notorious parasite for humans and animals worldwide. The organs of *H. flava* are bathed in hemolymph, which is a freely circulating fluid. Nutrients, immune factors, and waste can be transported to any part of the body via hemolymph. The main soluble components in hemolymph are proteins. However, knowledge of the *H. flava* proteome is limited.

Methods: The hemolymph was collected from fully engorged *H. flava* ticks by leg amputation. Hemolymph proteins were examined by both blue native polyacrylamide gel electrophoresis (BN-PAGE) and sodium dodecyl sulfate PAGE (SDS-PAGE). Proteins extracted from the gels were further identified by liquid chromatography–tandem mass spectrometry (LC–MS/MS).

Results: Two bands (380 and 520 kDa) were separated from tick hemolymph by BN-PAGE and were further separated into four bands (105, 120, 130, and 360 kDa) by SDS-PAGE. LC–MS/MS revealed that seven tick proteins and 13 host proteins were present in the four bands. These tick proteins mainly belonged to the vitellogenin (Vg) family and the α-macroglobulin family members. *In silico* structural analysis showed that these Vg family members all had common conserved domains, including the N-terminus lipid binding domain (LPD-N), the C-terminus von Willebrand type D domain (vWD), and the domain of unknown function (DUF). Additionally, two of the Vg family proteins were determined to belong to the carrier protein (CP) by analyzing the unique N-terminal amino acid sequences and the cleaving sites.

Conclusion: These findings suggest that the Vg family proteins and α-macroglobulin are the primary constituents of the hemolymph in the form of protein complexes. Our results provide a valuable resource for further functional investigations of *H. flava* hemolymph effectors and may be useful in tick management.

KEYWORDS

***Haemaphysalis flava*, hemolymph, vitellogenin, carrier protein, α-macroglobulin**

1 Introduction

Diseases caused by *Haemaphysalis flava* can influence human and animal health and have a major economic impact. *H. flava* is commonly found in the Asian continent, including China, South Korea, Vietnam, and Japan (1). *H. flava* can infect a wide range of animals, including humans, domestic animals (dogs, pigs, horses, sheep, and cattle), and wildlife

(hedgehogs, water deer, Raccoon dogs, eastern roe deer, and pandas) (2–4). *H. flava* plays the role of a vector in the transmission process of bacterial pathogens, parasites, and viruses in these animals (5–7).

Hemolymph is an important exchange and transport medium in ticks. It is a circulating fluid that bathes all internal tissues and organs in ticks, similar to the blood and lymph in vertebrates. It is composed of hemocytes and plasma (8). The plasma contains proteins, lipids, carbohydrates, and hormones and is involved in the transport of nutrients, metabolites, hormones, and even specific pathogenic microorganisms (9). Proteins are the main soluble component in the hemolymph and are crucial in many physiological processes, such as the regulation of osmotic pressure and innate immunity (10, 11). However, studies on the main proteins in tick hemolymph remain relatively limited.

Proteomics has continued to be the preferred method for studying tick proteins in recent years. Numerous researchers have attempted to identify and isolate hemolymph proteins in ticks. At the very beginning, Belozerov and Luzev separated 25 protein bands from the hemolymph of the tick *Dermacentor marginatus Sulz* using polyacrylamide gel electrophoresis (12). At present, the proteins studied exclusively are two storage proteins, namely, carrier protein (CP) and vitellogenin (Vg). Because of the similarities between CP and Vg, they have been thought to have a common evolutionary origin (13, 14). In our study, CP and Vg are collectively referred to as the Vg family. The role of Vg in ticks is to supply nutrients to growing embryos as a yolk protein precursor (15). CP is involved in the storage and transportation of carbohydrates and lipids such as free fatty acids, free cholesterol, and monoacylglycerol (16). In addition to Vg and CP, many proteins, such as α-macroglobulins, lectins, and antimicrobial peptides, are associated with tick immunity and are secreted into the hemolymph in response to injury and pathogen invasion (17–19). In spite of the importance of these proteins, our knowledge about them is limited relative to ticks.

Previously, we provided an overview of the components in the hemolymph of *H. flava* (8). There are 312 proteins, including tick-derived proteins and host-derived proteins. Unlike previous studies, we further separated the biomacromolecules of hemolymph mainly by blue native polyacrylamide gel electrophoresis (BN-PAGE) and sodium dodecyl sulfate PAGE (SDS-PAGE). Furthermore, the composition of the biomacromolecules was determined and analyzed by LC–MS/MS. These findings add to our understanding of the vital role of these proteins in the physiology of the tick circulatory system and provide additional potential targets for the development of tick control methods.

2 Materials and methods

All procedures performed in this study involving animals were in accordance with the ethical standards of the Hunan Agricultural University Institutional Animal Care and Use Committee (No. 2021085).

Abbreviations: BN-PAGE, blue native polyacrylamide gel electrophoresis; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LC–MS/MS, liquid chromatography–tandem mass spectrometry; LPD_N, the N-terminus lipid binding domain; vWD, the C-terminus von Willebrand type D domain; DUF, domains of unknown function; Vg, vitellogenin; CP, carrier protein; Vn, vitellin; ACN, acrylonitrile; DDT, dithiothreitol; NH_4HCO_3, ammonium bicarbonate; IAA, iodoacetamide; TFA, trifluoroacetic acid; TAM, tick a-macroglobulin.

2.1 Tick hemolymph collection

Ticks were collected and identified on the basis of morphology and molecular biology, as previously described (20). The fully engorged females (300–350 mg in weight) were picked from hedgehogs in Xinyang City, Henan Province, China (31°44′N, 114°10′E).

After being rinsed three times with distilled water and sterilized with 70% ethanol, 50 ticks were immobilized on a sterile glass slide with tape. The femurs were cut off, and the hemolymph was collected with a pipette pre-filled with a protease inhibitor cocktail (Phygene, Fujian, China). The mixture was centrifuged at 14,000 *g* for 10 min at 4°C, and the supernatant was stored at 80°C for subsequent analysis.

2.2 Electrophoresis analysis of hemolymph proteins

Protein concentrations were measured using a BCA protein assay kit (Applygen, Beijing, China). For BN-PAGE, the sample preparation and procedures were carried out according to the Blue/Clear Native PAGE Electrophoresis Kit (Real Times, Beijing, China). Briefly, 6.5 μL samples (6.04 μg/μL) were mixed with 2.5 μL of 4 × BN/CN-PAGE protein loading buffer (Real Times, Beijing, China) and 1 μL of 5% G-250 dyestuff (Real Times, Beijing, China). After mixing, each sample was subjected to BN-PAGE (4–16%, Real Times, Beijing, China) at 4°C at 5–15 V for 3 h. After electrophoresis, proteins in the gels were stained with Coomassie brilliant blue and scanned with a ScanMaker i800 plus (Microtek, Shanghai, China). For SDS-PAGE, BN-PAGE lanes were cut out of the gels with a razor blade, transferred to sterile microtubes, and incubated in the SDS protein loading buffer (TransGen, Beijing, China). They were ground and centrifuged at 14,000 × *g* at 4°C for 10 min. The mixture was subjected to a boiling-water bath for 5 min. After being cooled and centrifuged at 14,000 × g for 10 min at 4°C, samples were loaded into the wells of precast gels (4–12%, Epizyme, Shanghai, China). Electrophoresis was conducted at 150 V for 1 h. After electrophoresis, gels were stained with Coomassie brilliant blue for proteins. The target bands were cut, and the proteins were digested in the gels and analyzed by LC–MS/MS.

2.3 Digestion of the proteins in the gels

The clear and highly dyed protein bands were cut from the gels and put into tubes. Then, acrylonitrile (ACN) was added to decolorize the gels, which were cleaned with ultrapure water until transparent. After adding 10 μL of 100 mM dithiothreitol (DTT) and 90 μL of 100 mM ammonium bicarbonate (NH_4HCO_3) to each tube, the liquid was incubated at 56°C for 30 min. Then, it was set in a dark room for 30 min with 30 μL of 200 mM iodoacetamide (IAA), 70 μL of 100 mM NH_4HCO_3, and further rinsed with 100 mM of NH_4HCO_3 and ACN. The mixture was incubated overnight at 37°C after adding 12.5 ng/mL of trypsin and 25 mM of NH_4HCO_3 mixture. The peptides were extracted with 60% ACN and 0.1% trifluoroacetic acid (TFA) three times. The extracts were pooled and dried completely by vacuum centrifuge (21).

2.4 Analysis by LC–MS/MS

MS analyses were carried out on a Q Exactive mass spectrometer coupled to Easy nLC (Thermo Fisher Scientific, Waltham, MA, United States) following the same conditions previously described (8). MS data were obtained using data-dependent top 10 methods, which dynamically selected the most abundant precursor ions from the survey scans (300–1800 m/z) for higher-energy collisional dissociation (HCD) fragmentation. The target value was determined based on predictive automatic gain control. The dynamic exclusion duration was set to 25 s. The resolution for survey scans and HCD spectra was set to 70,000 at 200 m/z and 17,500 at 200 m/z, respectively. The normalized collision energy was set to 30 eV. The fill ratio was defined as 0.1%.

2.5 Sequence database searches and data processing

MS data were handled using MaxQuant software (version 1.6.14.0).[1] The MS/MS raw files were searched against the self-constructed *H. flava* protein database deduced from transcriptomic data[2,3,4,5] for identifications of tick proteins. An initial search was set with a precursor quality window of 6 ppm. The search followed the enzymatic cleavage rule of trypsin/P and allowed a maximum of two missed cleavage sites and a mass tolerance of 20 ppm for fragment ions. Carbamidomethylation of cysteines was defined as a fixed modification, while protein N-terminal acetylation and methionine oxidation were defined as variable modifications. The cutoff of the global false discovery rate for peptide and protein identification was set at 0.01. Protein abundance was calculated based on the normalized spectral protein intensity. The sequence analysis was conducted using DNAMAN and IBS.[6]

3 Results

The proteins in *H. flava* hemolymph were measured by a BCA assay. The protein concentration of tick hemolymph was 6.04 μg/μL. To examine the main proteins of the hemolymph in more detail, we used BN-PAGE to separate protein complexes by their size. The result showed that there were two bands with proteins isolated from the hemolymph; we called them A and B. The native protein complexes were found to have molecular weights of 520 and 380 kDa. SDS-PAGE, followed by BN-PAGE, indicated an enrichment of specific proteins. The protein complex A (520 kDa) was separated into band C (130 kDa) and D (105 kDa), while the protein complex B (380 kDa) was separated into band E (360 kDa) and F (120 kDa) (see Figure 1).

Bands C, D, E, and F were sliced, digested with trypsin, and analyzed by LC–MS/MS. Protein annotation using UniProt confirmed the presence of seven tick-derived proteins and 13 host proteins. These tick-derived proteins mainly belong to the Vg family and the α-macroglobulin family (see Table 1). The host protein types were as follows: histone H4, desmoplakin isoform X2, serum albumin, ATP-binding cassette sub-family A member 1, actin, phospholipid scramblase, synaptotagmin-16-like, myelin-associated oligodendrocyte basic protein, transthyretin, thymosin beta-4-like, zinc finger protein 414, insulin-like growth factor I, and Tudor domain-containing protein 7 (see Table 2).

To better understand the function of the Vg family proteins, we analyzed the structure of Vg identified in our study using DNAMAN and the IBS software analysis tools. The Vg family proteins have three conserved regions, namely, the N-terminal lipid binding domain (LPD_N), the C-terminal von Willebrand type D domain (vWD), and the domain of unknown function (DUF) (see Figure 2). In addition, the RXXR highly conserved unique N-terminal amino acid sequences (FEVGKEYVY), and GLCC are partially present in the Vg family of proteins.

4 Discussion

4.1 Overview of the hemolymph protein complexes of *Haemaphysalis flava*

The biomacromolecules in *H. flava* by Native PAGE have not been reported, but they have been reported in *Dermacentor variabilis* and *Ornithodoros parkeri* (22). Thompson et al. found a Vg protein with a molecular weight between 198 and 443 kDa by Native PAGE in pre-ovipositing and ovipositing replete (mated) females (15). Gudderra et al. purified and characterized a novel lipoglycoheme-carrier protein (CP) in *D. variabilis* by Native PAGE from partially fed virgin female hemolymph. The CP has a molecular weight of 200 kDa by Native PAGE and 340 kDa by gel filtration chromatography (16). Meanwhile, a high molecular weight protein (500 K) was found in *O. parkeri* (15, 16). These studies agree that Vg and CP are the main proteins in tick hemolymph. In addition, the three most abundant host-derived proteins in the hemolymph of *H. flava* are hemoglobin subunit-α, subunit-β, and albumin and the tick-derived proteins are Vg, microplusin, and α-2macroglobulin (8). Consistent with these studies, our study also found two high molecular weight proteins (520 kDa/380 kDa), and their main components were identified as Vg, α-macroglobulin, serum albumin, etc.

4.2 Vg family proteins

Protein domains are substructures of proteins that may give clues to their functional analysis. Therefore, we analyzed the structure of the Vg family of proteins characterized in the current study. Previous studies have shown that Vgs are proteins of 400–600 kDa and exist in oligomeric forms in the insect hemolymph (15, 23, 24). However, in *H. flava*, no such similar rule was observed in terms of the molecular weight and the subunit number of Vg. In the current study, three conserved regions (LPD_N, vWD, and DUF) exist in the Vg family of proteins. LPD-N is the most significant phosphorylation site of Vg and also an important protein modification region. It plays an important role in Vg splicing, Vg-VgRs recognition, and its mediated nutrient

1 https://maxquant.net/maxquant/
2 https://www.ncbi.nlm.nih.gov/bioproject/PRJNA756707/
3 https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA600997
4 https://www.ncbi.nlm.nih.gov/bioproject/?term=GSE69721
5 https://www.ncbi.nlm.nih.gov/bioproject/?term=GSE67247
6 http://ibs.biocuckoo.org/

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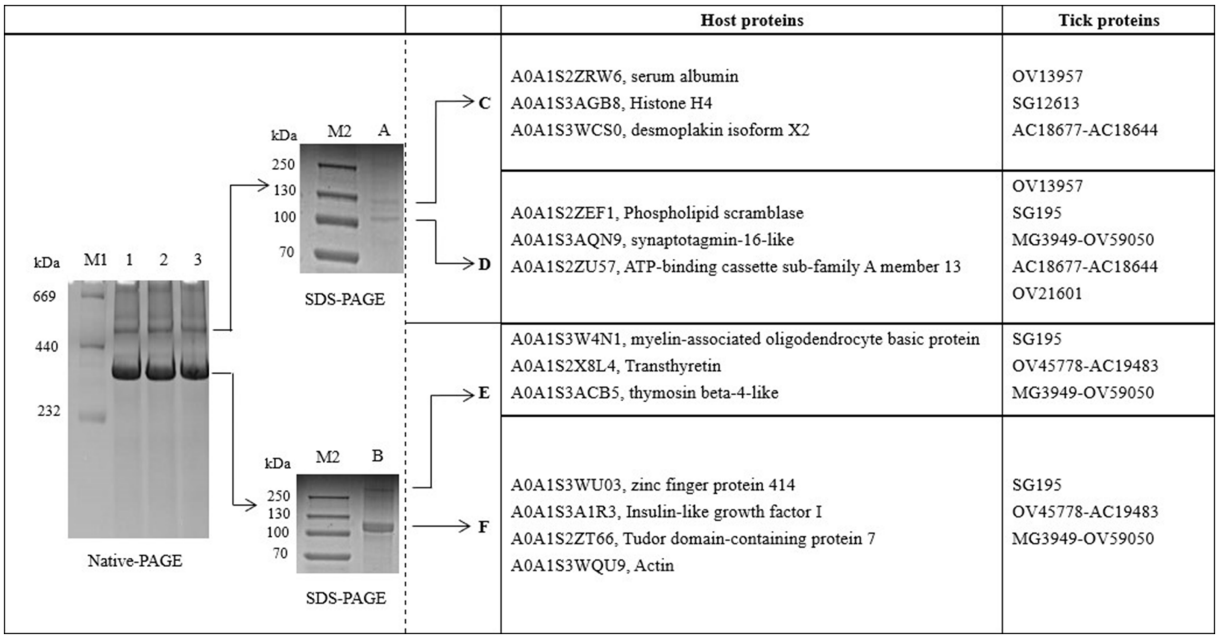

FIGURE 1
BN-PAGE and SDS-PAGE analysis of hemolymph proteins in *Haemaphysalis flava*. OV13957, SG195, OV45778-AC19483, MG3949-OV59050, OV21601, and AC18677-AC18644 are protein IDs in the self-constructed *H. flava* protein database. They can be annotated as Vg-1, Vg-1, Vg-2, Vg-2, Vg-2, α-macroglobulin, and α-1-macroglobulin.

TABLE 1 High confidence tick proteins in the hemolymph of *Haemaphysalis flava* ticks.

Polypeptide ID	UniProt accession	Protein name	Organism	*E*-value	Score	Identity (%)
OV13957	A0A346JM05	Vitellogenin-1	*Haemaphysalis flava*	0	8,535	92.9
SG12613	A0A346JM05	Vitellogenin-1	*H. flava*	0	8,294	100
SG195	A0A346JM06	Vitellogenin-2	*H. flava*	0	5,619	100
OV45778-AC19483	A0A346JM06	Vitellogenin-2	*H. flava*	0	5,408	64.3
MG3949-OV59050	A0A346JM06	Vitellogenin-2	*H. flava*	0	2,524	85.2
OV21601	A0A1E1X3E1	Alpha-macroglobulin	*Amblyomma aureolatum*	0	4,969	81.4
AC18677-AC18644	A0A023FY55	Alpha-1-macroglobulin	*Amblyomma parvum*	0	7,027	89.1

TABLE 2 High confidence host (*Erinaceus europaeus*) proteins in the hemolymph of *Haemaphysalis flava* ticks.

Protein name	UniProt Accession	Organism	No. of unique peptides	Coverage (%)
Histone H4	A0A1S3AGB8	*Erinaceus europaeus*	4	42.7
Desmoplakin isoform X2	A0A1S3WCS0	*Erinaceus europaeus*	3	1.3
Serum albumin	A0A1S2ZRW6	*Erinaceus europaeus*	3	4.3
ATP-binding cassette sub-family A member 1	A0A1S2ZU57	*Erinaceus europaeus*	3	0.3
Actin	A0A1S3WQU9	*Erinaceus europaeus*	3	9.3
Phospholipid scramblase	A0A1S2ZEF1	*Erinaceus europaeus*	1	4.5
Synaptotagmin-16-like	A0A1S3AQN9	*Erinaceus europaeus*	1	2.3
Myelin-associated oligodendrocyte basic protein	A0A1S3W4N1	*Erinaceus europaeus*	1	7.3
Transthyretin	A0A1S2X8L4	*Erinaceus europaeus*	1	9
Thymosin beta-4-like	A0A1S3ACB5	*Erinaceus europaeus*	1	25
Zinc finger protein 414	A0A1S3WU03	*Erinaceus europaeus*	1	2.1
Insulin-like growth factor I	A0A1S3A1R3	*Erinaceus europaeus*	1	7.8
Tudor domain-containing protein 7	A0A1S2ZT66	*Erinaceus europaeus*	1	1.1

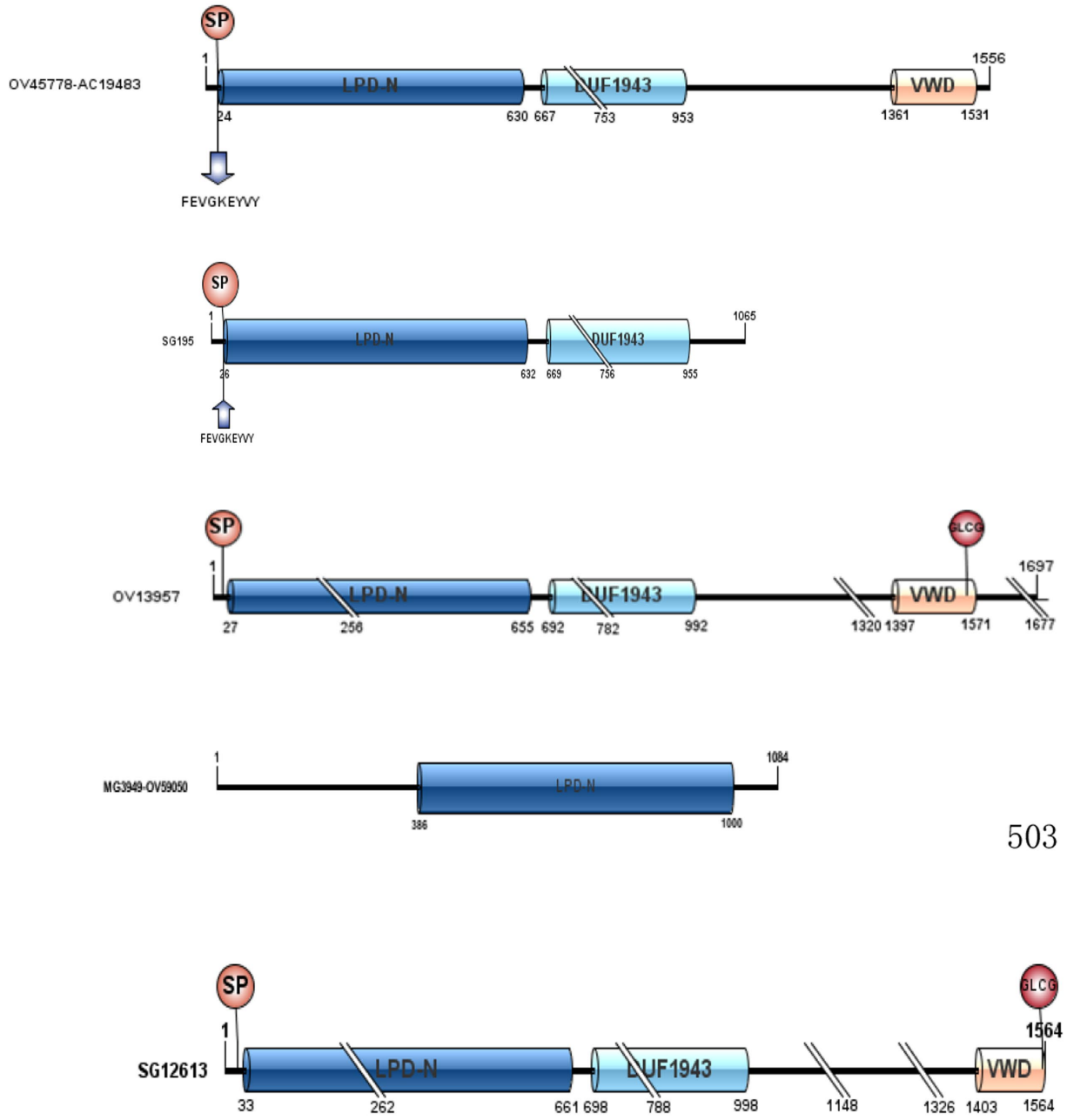

FIGURE 2
LPD_N, DUF1943, and vWD of the five Vg family proteins in *Haemaphysalis flava* hemolymph.

transport. vWD and DUF interact with pathogenic microorganisms, such as viruses or bacteria, which in turn eliminates pathogenic microorganisms (25). In 1988, Baker et al. noticed that the vWD domain might be involved in binding the Vg receptor present on the surface of oocytes by drawing comparisons between the vWD domain of Vgs and the vW factor (26). Interestingly, these structures can be found in comparable locations to DvCP, AaCP, and HlVg-C (14). In addition, we found that RXXR exists in these proteins. The RXXR can be recognized by subtilisin-like proprotein convertases, and the protein can be cleaved into multiple subunits. Thompson et al. showed that DvVg has an RXXR and would be split into two subunits (15). Therefore, this may be related to the fact that Vg appeared in different bands in our study. Meanwhile, SG195 and OV45778-AC19483 were discovered to have highly conserved unique N-terminal amino acid sequences (FEVGKEYVY) and a single cleavage site (RXXR)., which could be called CP (23). Khail et al. studied CP and Vg in detail. They reported that Vgs usually have many subunits, whereas CPs only have two subunits. In other words, the CP usually has one RXXR, and Vg has multiple RXXRs. SG12613 and OV13957 contain one to several cleavage sites and have non-conservative N-terminal amino acid sequences (Figure 2), which could be called Vg. This is consistent with our previous research. In our laboratory, we found that four proteins (Cl-33,847.0, Cl-k.7718, Cl-k.65658, and Cl-k.17389) were consumed in *H. flava* eggs, and we speculated that these proteins supported the

embryonic development as precursors of Vn (27). SG195 and OV45778-AC19483 were not present in these proteins, which meant they were not Vg but CP.

Both Vg and CP belong to the Vg family. Vg is known to be a yolk protein precursor supplying nutrients to growing embryos (28). CP is thought to be a transporter of carbohydrates and lipids (16). Vg is mainly synthesized by the fat body and gut cells, then expelled into the hemolymph and finally taken up by the oocytes (29). Similar to Vg, CP is synthesized in the fat body and found in the midgut, salivary glands, hemolymph, and ovary (13, 22, 30–34). In our study, Vg and CP exist in the hemolymph as a protein complex. Some studies suggest that the abundance of CP was affected by blood feeding in females. Compared to unfed adult females, the abundance of CP declined in partially fed females and replete females. In addition, CP was found in 1- and 9-day-old eggs (35). Therefore, CP may act as a carrier for Vg to transport it to the egg to play a role.

4.3 Tick α-macroglobulin

An essential component, α-macroglobulin, was identified in the electrophoretic strip. TAM is considered to be a universal protease inhibitor, including serine-, cysteine-, aspartic-, and metalloproteases, on which the digestion of blood meal depends (36). In invertebrates, our knowledge of the α-macroglobulin in ticks is minimal. The α2M family of protease inhibitors so far are single chains with a molecular mass of approximately 180 kDa, and they can form tetramers or dimers or remain as monomers (17). The structure is similar to that of αM purified from the soft tick (17); α-macroglobulin in our study was a protein of approximately 520 kDa in the native state and dissociated on reducing SDS-polyacrylamide gels into two subunits with a mass of 130 and 105 kDa. Kopáček also reported that TAM inhibits trypsin and thermolysin cleavage of the high-molecular-weight substrate azocoll in a manner similar to that of bovine α-2-macroglobulin. In other studies, TAM protects multiple proteins from enzymatic hydrolysis (37) and is related to hormone transportation (38). Meanwhile, TAM is also a transmembrane molecule related to a low-density lipoprotein. Therefore, we speculate that TAM may be involved in lipoprotein transport with CP in tick hemolymph.

Recent discoveries have revealed hundreds of different proteins in tick hemolymph. The most prominent proteins are Vg, microplusin, macroglobulin, metalloproteinase, and serpins (8, 39). However, apart from Vg and macroglobulin, we did not detect any other proteins. Because these small molecular proteins are dispersed in bands and do not bind to larger proteins, such as Vg, these proteins cannot be seen and identified in electrophoretic bands.

The biomacromolecular in *H. flava* by Native PAGE has not been reported, but it has been reported in *Dermacentor* var*iabilis* and *Ornithodoros parkeri* (22). Thompson et al. found a Vg protein with a molecular weight between 198 and 443 kDa by Native PAGE in pre-ovipositing and ovipositing replete (mated) females (15). Gudderra et al. purified and characterized a novel lipoglycoheme-carrier protein (CP) in *D.* var*iabilis* by Native PAGE from partially fed virgin female hemolymph. The CP has a molecular weight of 200 kDa by Native PAGE and 340 kDa by gel filtration chromatography (16). Meanwhile, they found a high molecular weight protein (500 K) in *O. parkeri* (15, 16). These studies agree that Vg and CP are the main proteins in tick hemolymph. The top three abundant host-derived proteins in the hemolymph of *H. flava* are hemoglobin subunit-α, subunit-β, and albumin and tick-derived proteins are Vg, microplusin, and α-2macroglobulin (8). Consistent with these studies, our study also found two high molecular weight proteins (520 kDa/380 kDa), and their main component was identified as Vg, α-macroglobulin, serum albumin, etc.

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4.4 Host proteins

Ticks are obligatory blood-feeding ectoparasites that ingest a significant volume of host blood. Therefore, the host proteins are abundant in tick hemolymph. Liu et al. revealed that 40 host-derived proteins, such as hemoglobin subunit-α and subunit-β, albumin, serotransferrin-like, and ubiquitin-like, were present in the hemolymph of *H. flava*. It is worth mentioning that hemoglobin is the most abundant host protein; however, it was not found in our study. At present, we have found that the majority of the hemoglobin present in ticks is related to antimicrobial peptides (40, 41). Therefore, hemoglobin may degrade into small antimicrobial peptides without our detection. In our study, we found an important host protein, serum albumin, present in hemolymph at a higher percentage. Serum albumin is the main protein in the blood plasma and functions as a carrier, chaperone, antioxidant, source of amino acids, osmoregulatory, etc. (42). Serum albumin is also a fatty acid-binding protein. Fatty acids are one of the most important sources for maintaining metabolism and energy homeostasis in cells (43). Serum albumin possesses approximately 7 binding sites for fatty acids with moderate to high affinity (44). Hence, it can transport bulk amounts of FA into the hemolymph. Moreover, serum albumin plays a very important role in the uptake of FA by organs. This study could be useful in understanding the fatty acid metabolism and transport in ticks and presents a potentially major contribution to research on this molecule.

Finally, these bands were found to contain 12 other host proteins, namely, histone H4, desmoplakin isoform X2, ATP-binding cassette sub-family A member 1, actin, phospholipid scramblase, synaptotagmin-16-like, myelin-associated oligodendrocyte basic protein, transthyretin, thymosin beta-4-like, zinc finger protein 414, insulin-like growth factor I, and Tudor domain-containing protein 7 (Table 2). These proteins are not only involved in the functions of maintaining cellular morphology, mechanical support, and

load-bearing but also play roles in defense, protection, nutrition, and repair (45–47). Nevertheless, their biological functions after being absorbed by ticks are unknown.

5 Conclusion

Overall, tick hemolymph has a powerful transport function. Its transport function is largely dependent on its internal proteins. Our data indicated that 7 tick proteins and 13 host proteins were identified from hemolymph protein complexes by electrophoretic technique and the LC–MS/MS method. The five Vg family proteins and host serum albumin make up a large portion of hemolymph proteins. It is possible that these two types of proteins work together to participate in the transport of a variety of substances, such as fatty acids. This finding may offer fresh insights into the biology of ticks and, consequently, novel approaches to tick management.

Data availability statement

The data presented in the study are deposited in the iProX repository, accession number PXD053899.

Ethics statement

The animal study was approved by the Hunan Agricultural University Institutional Animal Care and Use Committee (No. 2021085). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

DL: Writing – original draft. LL: Writing – review & editing. Z-lL: Data curation, Writing – review & editing. YT: Data curation, Writing – review & editing. XG: Supervision, Writing – review & editing. T-yC: Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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EDITED BY
Rosa Estela Quiroz Castañeda,
Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Mexico

REVIEWED BY
Saeid Fathi,
Razi Vaccine and Serum Research Institute, Iran
Hugo Aguilar-Díaz,
Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Mexico

*CORRESPONDENCE
Heba F. Alzan
✉ heba.alzan@wsu.edu
Carlos E. Suarez
✉ carlos.suarez@usda.gov

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Current vaccines, experimental immunization trials, and new perspectives to control selected vector borne blood parasites of veterinary importance

Heba F. Alzan[1,2]*, Mona S. Mahmoud[2] and Carlos E. Suarez[1,3]*

[1]Department of Veterinary Microbiology and Pathology, College of Veterinary Medicine, Washington State University, Pullman, WA, United States, [2]Parasitology and Animal Diseases Department, Veterinary Research Institute, National Research Center, Giza, Egypt, [3]Animal Disease Research Unit, Agricultural Research Service, United States Department of Agriculture, Pullman, WA, United States

Parasite infections transmitted by vectors such as ticks and blood-sucking arthropods pose a significant threat to both human and animal health worldwide and have a substantial economic impact, particularly in the context of worsening environmental conditions. These infections can manifest in a variety of symptoms, including fever, anemia, jaundice, enlarged spleen, neurological disorders, and lymphatic issues, and can have varying mortality rates. In this review, we will focus on the current state of available vaccines, vaccine research approaches, and trials for diseases caused by vector-borne blood parasites, such as *Babesia*, *Theileria*, *Anaplasma*, and *Trypanosoma*, in farm animals. Control measures for these infections primarily rely on vector control, parasiticidal drug treatments, and vaccinations for disease prevention. However, many of these approaches have limitations, such as environmental concerns associated with the use of parasiticides, acaricides, and insecticides. Additionally, while some vaccines for blood parasites are already available, they still have several drawbacks, including practicality issues, unsuitability in non-endemic areas, and concerns about spreading other infectious agents, particularly in the case of live vaccines. This article highlights recent efforts to develop vaccines for controlling blood parasites in animals. The focus is on vaccine development approaches that show promise, including those based on recombinant antigens, vectored vaccines, and live attenuated or genetically modified parasites. Despite intensive research, developing effective subunit vaccines against blood stage parasites remains a challenge. By learning from previous vaccine development efforts and using emerging technologies to define immune mechanisms of protection, appropriate adjuvants, and protective antigens, we can expand our toolkit for controlling these burdensome diseases.

KEYWORDS

vaccine, farm animals, vector borne, ticks, blood sucking flies, parasites

1 Introduction

The impact of blood parasites is scaling up globally. The increasing costs of control using acaricides, insecticides, and treatments of infected animals, associated with the emergence of drug resistance and environmental hazards, accelerated efforts to find effective vaccines against hemoparasites using rational approaches. Moreover, it is known that animals that resolve and recover from acute blood-borne infectious diseases, usually become asymptomatic carriers for

the pathogens and may act as reservoirs of the infective agents that cannot be clinically differentiated from the non-infected ones (1). Alternative methods for the control of hemoparasites usually involve targeting their vectors, which is frequently a very difficult approach due to the existence of wildlife reservoirs and the development of acaricide resistance, but vector control could also be achieved by developing effective anti-vector vaccines.

Despite many efforts on the development of efficient vaccines, there are still many factors that can impact the efficacy of vaccines. These combined factors may include issues associated with the hosts, human-related activities, and the types of vaccines.

Among host related factors, it should be considered: (i) animal breed variations; (ii) age-related factors; and (iii) incubation periods in case of infected animals. Important human related factors are those related to vaccine design, formulation, and administration. This includes the nature of vaccine, antigen definition and concentration, vaccine stability, expiration date, vaccine material reconstitution, different administration routes (*i.e.*: intranasal, subcutaneous, intramuscular) and schedules of vaccination. Users will also need to be aware of appropriate vaccine storage, shelf life, and the logistics of shipment to different locations. Regarding vaccine-related factors, we should consider first the nature of the antigenic components of the vaccine which may include, the use of infectious pathogens or distinct noninfectious components of the pathogens, purified recombinant antigens representing molecules of the pathogens, or other components, including nucleic acids encoding for protective antigens. Using attenuated forms of infectious pathogens may elicit strong and long-lasting immunity, but this approach may result in undesirable spreading of the infectious agents among animals in the field and may convey the risk for reversion to virulence. Also, the degree of attenuation, either *in vitro* or *in vivo* using live animals, is considered an important feature in the preparation of these types of vaccines. On the other hand, using noninfectious forms, materials, or antigens derived from the pathogens, including killed parasites, purified or partially purified antigen extracts, toxoids, defined recombinant or native subunit vaccines, or nucleic acid-based vaccines, is usually considered safer than the infectious forms, although usually, these approaches are generally less effective compared to live vaccines. Another important factor is the definition of proper, non-toxic, and cost-effective adjuvants, since most non-living vaccines usually require them in order to stimulate adequate and strong immune responses. Finally, deciding on the possible need for vaccine booster doses requires, definition of the characteristics of the vaccine booster, and the number of vaccine inoculations and the frequency required for optimal coverage and efficiency. Importantly, determining these parameters also requires a previous assessment of the quantitative and qualitative responses of the immune systems of vaccinated animals. In addition, the costs associated with the applications of vaccine boosters could be an important consideration to evaluate vaccine practicality.

Achieving, licensing, and marketing efficient vaccines against hemoparasites is also not a trivial endeavor. The relatively slow pace for developing efficient vaccines is due, at least in part, to the typical complex life cycles of the responsible agents, our limited knowledge of protective immune mechanisms, and the fact that parasites are well adapted to their hosts and evolved complex and efficient mechanisms of immune evasion, including antigenic variation. The following sections describe features of selected blood-borne parasites of high impact currently occurring in animals globally (*Babesia*, *Theileria*, *Anaplasma* and *Trypanosoma*), their associated diseases, and different approaches employed for developing vaccines against these pathogens. Also, this review will briefly discuss the current vaccine and control measures of tick and blood-sucking vectors.

There is an urgent need for more research seeking effective strategies to produce durable and effective vaccines for better disease control. Understanding the parasite biology, the host immune responses against the infection, and the smart use of recently developed omics tools will undoubtedly facilitate vaccine development for improved disease control and parasite transmission.

2 Brief outlook on selected blood borne pathogens of veterinary relevance

2.1 *Babesia* parasites

Babesia (B) spp. are apicomplexan parasites that cause Babesiosis and can be transovarial transmitted by different kinds of Ixodes ticks. The disease can affect many mammals, including cattle, horses, sheep, goats, swine, cats, and dogs, and can also be fatal to humans (2). Babesiosis is considered as the second most important blood-borne parasitic disease of animals of veterinary importance, only behind Trypanosomiasis (3). *Babesia* sp. are transovarially transmitted parasites with a complex life cycle involving intracellular stages in at least two types of hosts, vertebrates, and tick vectors. Importantly, *Babesia* parasites undergo sexual reproduction in the gut of their definitive tick host, where they are also able to invade distinct types of cells (such as gut epithelial, ovary, and salivary gland cells), but they are only able to invade the erythrocytes of their vertebrate hosts, which can aid the parasites by providing adequate nutrition and a relatively protected environment for parasite expansion though asexual reproduction. *Babesia* comprises more than 100 species that infect erythrocytes in many vertebrates (4). Babesiosis can affect different livestock in tropical and semitropical regions worldwide, including ovine and caprine, but globally effective and fully safe vaccines against these species remain unavailable. Importantly, *Babesia* parasites have a life cycle that includes sexual reproduction in the midgut of their ixodid tick vectors, which eventually leads to transovarial transmission and the expansion of the parasites in the tick vectors. In this review we will focus mainly on *Babesia* species that infect cattle, equids, and dogs, since they have received more attention for vaccine development.

Bovine babesiosis can be caused by different *Babesia* spp. such as *B. bovis*, *B. bigemina*, *B. divergens*, *B. orientalis*, and *B. major*. In addition, *B. divergens*, which is prevalent in Europe, occasionally can also infect humans (5). However, *B. bovis* is considered as the most virulent *Babesia* spp. responsible for bovine babesiosis (6). This acute and persistent disease poses a significant global challenge for cattle production. Clinical signs range from mild to severe, depending on animal age, impacting animal health and productivity (7), and may include anemia, fever, ataxia, cerebral signs, abortions, anorexia and kidney damage, among others. Young calves ranging from 6–8 months old show some sort of increased resistance likely because they can generate more efficient innate immune responses against the parasites that prevent the devastating effects of acute infections. In contrast, infected adult cattle are more prone to display clinical signs of acute

babesiosis, which are presented mainly as fever, anemia, loss of appetite, and weight loss (8). In severe cases of infection by *B. bovis*, animals suffer from neurological manifestations, due to parasite sequestration in the brain blood vessels, which may eventually lead to animal death (7, 8). *Babesia* species can also infect dogs and/or cats, as well as many wild animals, but none of them are known to be of zoonotic importance (9). Canine babesiosis is also a significant tick-borne disease caused by various species of the protozoan genus *Babesia* (10). Large and small forms of *Babesia* species (*B. gibsoni*, *B. canis*, *B. vogeli*, and *B. microti*-like isolates also referred to as "*B. vulpes*," "Spanish dog isolate," "*Babesia cf. microti*" and "*Theileria annae*") can infect dogs with different levels of clinical signs (11, 12). Although canine babesiosis is mostly transmitted by tick vectors, it can also be transmitted to healthy hosts directly by blood transfusion, vertically (13), and by direct contact, as in case of fighting dogs, through wounds, saliva, or blood ingestion (14, 15). Equids also can be infected by *Babesia* spp., *B. caballi*, as one of etiological agents of equine piroplasmosis (EP) which is considered as an economically important disease of equids (16). In addition, EP can be caused by the *Babesia*-related *Theileria equi* parasites, as it will be discussed below, and vaccines remain needed for controlling this disease (16, 17). The small ruminant industry is a very significant component of livestock production especially in developing countries and in poor rural communities worldwide. *B. ovis* is the main causative agent of ovine babesiosis which has major economic impact on small ruminant (sheep and goat) industry in tropical and subtropical areas. Ovine babesiosis is transmitted by *Rhipicephalus* ticks, in particular *R. bursa* (18) and it could result in mortality if animals remain untreated (19, 20).

2.2 *Theileria* parasites

Theileria (*T.*) spp. are tick-borne apicomplexan parasites responsible for theileriosis, an important disease that affects cattle, equids and other animals worldwide with variable clinical signs. *Theileria* parasites are piroplasmid parasites which are closely related to *Babesia* that can also be transmitted by ixodid ticks. However, there are two key differences among these two parasites: one is the ability of *Theileria* parasites to infect more than a single type of cells in their vertebrate hosts, and another one is their transstadial, rather than transovarial, mode of transmission by ticks. *Theileria* parasites also have complex life cycles, and similar to *Babesia* parasites, they also develop sexual stages, kinetes and sporozoites inside its tick vectors. However, after the infection through the tick bite, *Theileria* parasites develop schizonts and piroplasms once established in their vertebrate hosts (21, 22). In the infected animals, schizonts form merozoites, which in turn parasitize the erythrocytes, which then develop into piroplasms. The piroplasms undergo sexual reproduction after being acquired by ticks during feeding on an infected host. Infected ticks can later infect another host with *Theileria* sporozoite stage parasites by transstadial transmission, regardless of their status of naïve or infected (21). *T. annulata*, *T. parva and T. orientalis* are cattle parasites (23) while *T. equi* and *T. haneyi* infect equines (16). *T. lestoquardi* and *T. ovis* can infect small ruminants (24). *Theileria parva*, *T. annulata*, and *T. orientalis* are the major causes of East Coast fever (ECF), tropical theileriosis, and oriental theileriosis, respectively (23). Based on their ability to infect leukocytes, *Theileria* parasites can be classified as host-cell transforming and non-transforming species. Thus, in the case of *T. annulata*, and *T. parva* infective sporozoites infect leukocytes, to develop into macro-schizonts causing uncontrolled leukocyte proliferation (25, 26). Eventually, these parasites produce merozoites that invade erythrocytes. In contrast, *T. equi* and *T. orientalis* do not induce uncontrolled leukocyte proliferation in horses and cattle, respectively (27). However, *T. orientalis* merozoites invade the host red blood cells, leading to anemia which is associated with the clinical signs of acute oriental theileriosis (28).

2.3 Anaplasma

Anaplasma (A) spp. is a vector-transmitted rickettsia, that resides in blood cells of its vertebrate hosts, leading to the disease anaplasmosis in tropical and semitropical areas (29). Anaplasmosis can be transmitted among animals by mechanical and/or biological vector transmission. Mechanical transmission may occur at least via two ways, either through contaminated surgical equipment (fomites) carrying the infected blood cells, or by mouthparts of biting flies who carry an *Anaplasma* species (30). Biological vector transmission mainly occurs by the bite of ticks (many different tick species) infected with the blood parasite. Inside the ticks, *Anaplasma* can survive and multiply, or it can stay dormant for months till transmission to another animal through a tick bite (31).

The clinical manifestations of anaplasmosis caused by *A. marginale* and *A. centrale* can be variable and similar to those caused by *Babesia* parasites. Depending on several factors, the intensity of disease can range from mild, lacking clinical signs, to severe, with elevated morbidity and mortality in affected ruminants. However, the infected animals become lifelong carriers that may become reservoirs for the pathogen (32). In addition, *A. ovis* can infect sheep, goats and some wild ruminants causing anaplasmosis (33, 34). Infections by *A. phagocytophilum* (transmitted by *Ixodes ricinus*) in dogs have been described mainly in northern and central Europe while infection with *A. platys* (transmitted by *Rhipicephalus sanguineus*) was identified in dogs from Mediterranean basin Romania, Turkey, Greece, Italy, France, Spain and Portugal (35). Both *A. phagocytophilum* and *A. platys* can infect other animals in addition to dogs, such as cats, sheep, goats, cows, equines, rodents, roe deer, deer, as well as other wild mammals, and even birds, as in case of *A. phagocytophilum* (36, 37). In general, it's difficult to control anaplasmosis efficiently using vaccination approaches because of the ability of the responsible organisms for undergoing antigenic variation and their genetic variability. Also, the occurrence of multiple hosts and arthropod vectors, as well as the different mechanisms of transmission (biological, mechanical, and transplacental) may also impose important challenges for efficient control (38, 39).

2.4 *Trypanosoma* parasites

Trypanosoma (T) spp. is also an important blood parasite causing trypanosomiasis in animals and humans. These parasites are kinetoplastids, a monophyletic group of unicellular parasitic flagellate protozoa. Trypanosomiasis is a disease geographically constrained due to the nature of its arthropod vectors with strict requirements in terms of climate and environment. It is known as Surra diseases in South and

Central America, Northern Africa, the Middle East, Asia, Indonesia, and Philippines, but it is also known as African animal trypanosomiasis in Central and Southern Africa (40). This disease is considered endemic in at least 37 of 54 countries in Africa (41). It can be transmitted biologically and mechanically by hematophagous insects by biting. The vectors involved in transmission include *Stomoxys*, *Tabanids,* and *Hippoboscids*. However, the parasite can also be sexually transmitted as in equine species infected with *T. equiperdum*. The signs of disease ranged from acute, with high mortalities, to chronic forms, which are frequently concomitant with reduction in body weight, anemia, and infertility. Also, the *T. evansi*'s may cause immunosuppression, usually accompanied with secondary infections, which makes clinical identification more difficult (42). It affects horses, camels, cattle, sheep, goats, pigs and humans. Wild animals also can be infected with *Trypanosoma* parasites, but they rarely suffer from the disease, and can act as infection reservoirs for domestic animals.

In Africa, *T. congolense*, *T. vivax*, and *T. brucei brucei* are the most important trypanosome species affecting domestic livestock in cattle, sheep, and goats, while *T. simiae* can infect pigs, and *T. evansi* infects camels. In South America, *T. vivax* also has an impact on cattle production. While *T. evansi* affects camels in Asia and horses, cattle, and domestic buffalo in South America, India, and Southeast Asia (43). It is interesting to note that *T. evansi* is more common in camels, in contrast to dogs, donkeys, and horses. This may be due to the chronic nature of trypanosomiasis in camels. While infected camels may become weaker and emaciated as the disease progresses, the infection manifests acutely and is usually fatal in dogs and equines (44, 45). Interestingly, although camels as well as donkeys, dogs and horses are similarly exposed to vector challenges, those animals are less prone to be infected by trypanosomes. These may be due to feeding preference of vectors for camels, or the greater ability of those animals to put away the flies through head movements, skin rippling, and other behavioral avoidance mechanisms (46).

In Egypt, *T. brucei evansi* (*T. b. evansi*) is an enzootic organism found in Egyptian camels, which is genetically classified into types A and B (47). *T. evansi* can cause high parasitemia, especially in camels, horses, and dogs (sometimes cattle and buffaloes), and might act both as blood and tissue parasite, because of its ability to invade the nervous system not only in horses and dogs, but also in cattle, buffaloes, and pigs (48, 49). Data on the presence of *T. evans* has been reported in many regions of Upper and Lower Egypt, however, its epidemiological significance in Egypt remains scarce (50).

3 Preventive control measures against tick-borne pathogens of veterinary relevance

Vaccines are among the most efficient methods available to prevent infectious diseases. Ideally, vaccines should fully protect the recipient against infections, but this goal is very difficult to achieve against the selected blood borne pathogens discussed hereby. However, current vaccines help ameliorate the risk and impact of acute diseases caused by these groups of selected parasites in animals. Effective stimulation of protective immune responses by vaccines may lead later to a more complete protection in the field, once the animals are exposed to the pathogens. In some specific cases, it could be at least possible to develop vaccines that can prevent infections, for example, by developing vaccines that can block the invading sporozoites, such as is the case of *Babesia* and *Theileria* parasites, preventing the vectors to infest their hosts, or prevent infection of the vectors by the parasites, such as in transmission blocking vaccines. As an alternative (or complementary) to these approaches, there are also research efforts focused on achieving vaccines that can block or control the development of the arthropod vectors, which ideally, could also be combined with pathogen-specific protective antigens.

So far, there are not globally effective, commercially available, vaccines against any of these blood parasites. In the next sections we will address vaccine trials performed against selected parasites of veterinary importance, and some new perspectives in vaccine development against these important blood parasites, mainly inspired by innovations and advancements in cell biology, vaccinology, immunology, and molecular biology, such as gene editing and recombinant protein production. The newly developed experimental vaccines discussed in the sections below typically consist either of subunit vaccines including native or recombinant versions of parasite proteins, crude antigen extracts derived from the parasites, or live parasites that are either attenuated or genetically edited. The goals pursued in the vaccine trials discussed in the next sections vary. While some of these vaccines are aimed to prevent acute disease symptoms, others are designed to prevent parasite transmission (also known as transmission blocking vaccines ["TBV"]). We will also address other approaches aimed at controlling both the parasite and its associated vector, known as dual vaccines (Figure 1).

3.1 *Babesia* parasite vaccines

Current measures aimed at the control of bovine babesiosis include individual or combined use of at least three approaches: tick vector control, animal therapeutic treatments, and vaccinations. However, each of these measures has its own pitfalls and limitations. Tick control using acaricidal drugs is a major approach to control babesiosis, but its intensive use invariably leads to the emergence of acaricide-resistant tick strains (51). In addition, this method of control may cause environmental hazards, and the introduction of dangerous chemical residues in the food chain (52). Babesicidal drugs currently used for the treatment of *Babesia* infected cattle, such as imidocarb, may also lead to the surge of drug-resistant parasites, especially when used in suboptimal doses, and worrisomely, the generation of drug residues or metabolites in milk and meat, This features make drug treatments expensive and inefficient as a disease-preventive tool in extensive production systems. Due at least in part, to these limitations, imidocarb is not licensed for use in some European countries (51).

Currently, live vaccines based on attenuated *Babesia* parasites are not available or allowed in many endemic countries. However, some countries with large cattle populations at risk, like South Africa, Argentina, Brazil, Uruguay and Australia opted for producing and using such attenuated vaccines. An important limitation of live attenuated vaccines is their safety, since that they can be potentially virulent, mainly for adult animals, and thus, these vaccines are in use for less than one-year-old calves (53). Moreover, some countries, including Australia and Argentina, use a trivalent vaccine which contain not only attenuated strains of *B. bovis* and *B. bigemina,* but also *A. centrale,* an organism of low virulence originally identified in

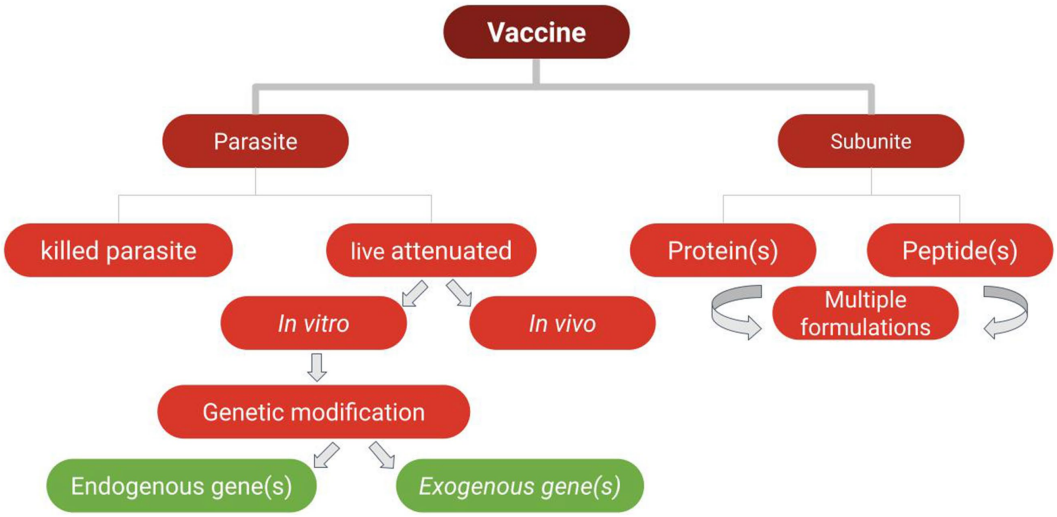

FIGURE 1
Types of vaccines used in vaccine trials against the selected parasites of veterinary importance addressed in this study.

South Africa (54), that can elicit partial protective responses against *A. marginale*. Trials involving live *Babesia* vaccines are discussed in detail below.

There are also a few vaccine options that are commercially available to prevent canine babesiosis in different countries. The Pirodog® (Merial) vaccine, currently available in the European market is based on soluble parasite antigens obtained from culture media supernatants (55, 56). This vaccine induces partial protection and can reduce severe clinical signs but does not prevent infections. The vaccine is recommended for administration into 5 months of age dogs, with annual booster doses (57, 58). It is worth mentioning that chemoprophylactic approaches are also used as alternatives to vaccination in dogs. For instance, the carbanilide derivative Imidocarb dipropionate has been shown to be effective against *B. canis* infection. The application of a single subcutaneous dose (6 mg/kg) of this drug demonstrated protection for two weeks (59). Doxycycline (5 mg/kg/day) also reduces the severity of disease in dogs that experimentally infected with an extremely pathogenic isolate of *B. canis* (60).

The following sections describe recent efforts toward developing vaccines against *Babesia* parasites using different approaches, including subunit, whole parasite, and culture supernatant vaccines (Figure 1).

3.1.1 Vaccine trials against *Babesia* based on subunit and native crude extracts antigens

Researchers have identified several potential recombinant and native subunit and crude antigenic extracts that have been proposed as vaccine candidates for bovine babesiosis, since they can prevent severe clinical signs (Table 1). Crude extract antigen and subunit vaccines have been designed and tested in several reported *in vivo* trials.

Typically, a subunit vaccine contains selected purified molecules derived from a target pathogen that are antigenic and able to elicit a protective immune response. These antigens may be either purified native or recombinant proteins, or in other forms, as described below. Unlike live attenuated or non-viable parasite derived vaccines (such as irradiated, or chemically inactivated), subunit vaccines typically include only specific and selected components such as proteins, polysaccharides, or peptides (61). As such, subunit vaccines are considered safer and more stable than live vaccines because they do not contain infectious components.

Because this property applies to these two types of antigens, vaccine trials involving subunit and native crude extracts derived from the parasites, are included together in this section.

Subunit vaccine candidates can take various forms, including purified recombinant protein(s), and/or synthetic peptide(s) representing relevant B and T cell epitopes, or mixed formulations. Also, custom designed synthetic genes encoding for a combination of several protective B and/or B and T cell epitopes can also be used for expression in prokaryotic or eukaryotic systems (62), or even for direct injection of coding DNA into the animals using specific devices such as gene guns (Table 1). In addition, animal vaccine trials have also been performed for experimental subunit vaccines based on recombinant proteins aimed at blocking pathogen transmission (Table 2).

Several strategies for selecting protective blood stage or transmission-blocking antigens were proposed, with some focused on functionally relevant molecules and others using pragmatic approaches like previously defined surface exposed, neutralization sensitive and conserved molecules. Protection in blood stage vaccines is typically defined by the ability of vaccinated animals to survive standardized challenges from *Babesia* merozoites from virulent strains, and the results and efficiency in these trials are usually compared to protection resulting from live vaccinations. The following Tables 1, 2 illustrate different vaccination trials for blood and sexual stage subunit and crude antigenic extracts so far described in the literature.

Regarding blood stage vaccines, none of the subunit vaccination trials targeting blood stage parasites described in the literature were able to elicit fully efficient and long-lasting protection against challenge with virulent *Babesia* strains. Some of these unsuccessful results may be due, at least in part, to the structural and antigenic differences between native

TABLE 1 Different forms of subunit and native crude extract vaccine trials against blood stage of *Babesia* parasites.

Vaccine form	Efficacy	Reference
Native protein		
B. bovis native extract: Combination of two affinity chromatography purified antigens (11C5 and 12D3)	Immunization of cattle resulted in decreased parasitaemia upon challenge with homologous *but* not with heterologous virulent *B. bovis* strain indicating these antigens induce strain-specific immunity.	(148, 149)
Adult sheep vaccinated twice with crude extracts of either *B. bovis* or *B ovis* parasites (*In vitro* culture extract)	Both vaccinated groups had significantly reduced parasitaemia upon challenge with *B. ovis* organisms.	(150)
Exoantigen-containing supernatant fluids of *in vitro B. bovis* and *B. bigemina* cultures	Vaccination reduced the incidence of clinical disease among vaccinated animals and complete protection against mortality caused by babesiosis.	(151)
Recombinant protein		
B. ovis apical membrane antigen-1 (rBoAMA-1)	Experimentally vaccinated sheep generated a specific response against the recombinant protein, but the antibody response did not associate with protection upon challenge with *B. ovis* infected cell culture.	(20)
Recombinant Rhoptry associated protein 1 (RAP-1) of *B. bovis*.	Vaccination did not elicit protective immunity against challenge with the virulent *B. bovis* strain.	(152–156)
Recombinant Merozoite surface antigen-1 (MSA-1) of *B. bovis*.	Vaccination did not protect calves after challenge with the *B. bovis* T2Bo strain	(157)
Apical membrane antigen 1 domain [rBbAMA-1(I/II)]	Antigens induced strong Th1 cell responses. No challenge with virulent *B. bovis* reported.	(158, 159)
Mixture of MSA-1, MSA-2c and 12D3 recombinant proteins emulsified with the Montanide adjuvant, administered in two doses.	No signs of effective protection after challenge with the *B. bovis* Yucatan strain	(160)
B. bovis RON2 containing conserved B-cell epitopes	Vaccine stimulated antibody production in cattle which inhibited *in vitro* culture parasite invasion. No challenge with live pathogenic *B. bovis* reported.	(161)
Peptide form		
Traditional form		
B. bovis AMA-1, MSA-2c and RAP-1 containing conserved B and T-cell epitopes	Production of neutralizing antibodies in cattle and durable Th1 immune response. No challenge with live pathogenic *B. bovis* reported.	(162)
B. bovis merozoite surface antigens: MSA-2a1, MSA-2b, and MSA-2c	The vaccine elicited immune stimulation in vaccinated cattle. No challenge with live pathogenic *B. bovis* reported.	(163)
Chimeric multigene vaccine (viral vector)		
Chimeric multi-antigen of DNA fragments containing B- and T-cell epitopes of merozoite surface antigen 2c (MSA-2c), rhoptry-associated protein 1 (RAP-1) and heat shock protein 20 (HSP20) genes.	All vaccinated cattle showed clinical signs of disease upon challenge with virulent *B. bovis* strain	(164)
Poly-N-acetylglucosamine (PNAG)		
Synthetic ß-(1 → 6)-linked glucosamine oligosaccharides conjugated to tetanus toxoid (5GlcNH2-TT)	The vaccinated calves were not protected upon challenge with virulent *B. bovis* parasites.	(62)

TABLE 2 Different forms of subunit vaccine trials against sexual stage of *Babesia* parasite (Transmission blocking vaccine).

Antigen(s)	Efficacy	Reference
Mixed recombinant proteins		
B. bovis 6cys A and B	Failed to block transmission *in vivo*	(52)
Single protein		
B. bovis HAP2 (hapless2)	Vaccination blocked *Babesia* transmission via tick in *in vivo* experiments performed on immunized cattle.	(147)
B. bigemina HAP2	Anti-HAP2 specific antibodies from immunized animals were able to block zygote formation *in vitro* for *B. bigemina*. No *in vivo* application followed by a challenge described.	(165)
Peptides		
B. bovis 6cys A and B	Rabbit anti *B. bovis* 6cys A and B were able to block *B. bovis* gametes development *in vitro*.	(52)

and recombinant versions of the antigens, including parasite-specific protein folding and post-translational modifications of the native proteins, which may result in critical differences in their antigenicity, when compared to recombinant proteins produced in heterologous expression systems (63). For instance, immunization experiments using native purified *B. bigemina* antigens, including RAP-1 of *B. bigemina* resulted in partial protection (64).

There are other issues that might significantly influence the outcome of the blood stage vaccine trials involving recombinant proteins and synthetic peptides described in Table 1. One important factor is the nature and severity of the challenge used. While in nature animals get usually infected by ticks, which inoculate variable amounts of antigenically distinct sporozoite stage parasites in the blood of the vertebrate host, the challenges in all these trials were invariably performed using a large, fixed, amounts of blood stage merozoites from a defined highly virulent strain of *Babesia*. The current method of challenge using large numbers of virulent merozoites may be realistic for assessing immunity elicited by live vaccines, but that may not be the case for other cases involving recombinant proteins. We thus suggest that a more realistic and standardized method of parasite challenge, preferably using sporozoite stage parasites or natural *Babesia*-infected tick field challenge, should be also developed and adopted for future trials.

However, in case of TBV, while the full-size *E. coli*-derived recombinant version of the *B. bovis* Hap2 protein was able to block transmission via ticks, that was not the case for full size eukaryotic system-derived r6cys proteins. It was hypothesized that the transmission-functional regions of these proteins were poorly immunogenic when presented in the context of the full- size 6cys A and B proteins. This notion was supported by additional experiments that identified specific and poorly immunogenic transmission-sensitive regions of these proteins that may contain epitopes responsible for transmission reduction function (52). Future vaccination experiments using these specific regions might result in the definition of alternative candidates for TBVs based on 6cys proteins.

Worthy of note that there are other promising functional approaches toward *Babesia* vaccine candidate definition that had been proposed in the last few years (65). One approach is based on the selection of critical protein-derived conserved regions related to parasite adhesion. Interestingly, conserved high activity binding peptides (cHABPs) to the erythrocytes were identified in the genome of *B. bovis* using intensive *in silico* analysis (66). Similarly, using a bioinformatics approach researchers identified a sub-immunodominant B-cell epitope in a highly conserved 15 amino acid region of the Rhoptry Associated Protein Related Antigen (RRA) of *B. bovis*, that is also a candidate for inclusion in peptide-based subunit vaccines (67). These *in silico* subunit vaccine antigen candidate identification approaches, could also be extended not only against *Babesia* but also against other blood parasites, due to the availability of annotated genomes for most parasites of medical importance, with the addition of other omics analysis. Moreover, exploiting exosomes, which consists of small extracellular vesicles that serve as carriers for proteins, nucleic acids, and lipids in host-*Babesia* or parasite–parasite interaction should be also considered (68, 69). This approach was successfully applied in *Plasmodium* spp., and it allowed the identification of novel subunit vaccine candidates involved in non-classical protein secretion, pathogenesis, immune modulation, and parasite–host interactions (70). Its potential in disease pathogenesis was explored in two major human *Babesia* species, *Babesia divergens*, from *in vitro* culture and those from an *in vivo B. microti* mouse infection (71). Thus, further similar studies using this approach in *Babesia* vaccination are warranted.

3.1.2 Vaccine trials based on whole *Babesia* parasites

Using whole *Babesia* live or inactivated parasites in vaccination approaches has important advantages, since they should include most, if not all, antigens expressed at least in certain stages of the organisms, and in some cases, such as in live attenuated vaccines, they may overcome limitations due to antigenic variation and natural polymorphism (72), although this may depend on the method used to reduce the virulence of the parasites. These types of vaccines may contain live attenuated or genetically modified parasites, or whole inactivated (killed) parasites.

3.1.2.1 Inactivated (killed) parasite

Killed vaccines were achieved in different ways. One of those is the freeze-dried suspension of *Babesia argentina* (*B. argentina*) (currently known as *B. bovis*) parasite (73) and *B. microti*-killed parasites enclosed within liposomes which incorporated a mannosylated lipid core peptide. In both cases the vaccination induced protective immunity in cattle and in mice against *B. argentina* or *B. microti, respectively.* Also, chemical attenuation was performed on *B. microti* parasitized red blood cells from infected mice by using Tafuramycin-A (TF-A). A culture-based liposomal vaccine, a liposome containing killed parasite material, was also used as a vaccine in mice. The parasitemia was reduced in vaccinated animals upon challenge with the *B. microti* (74).

3.1.2.2 Live attenuated vaccines

Live vaccines based on attenuated parasites remain as the only preventive measure against bovine babesiosis caused by *B. bovis* and *B. bigemina,* and for canine babesiosis caused by *B. gibsoni*.

These live vaccines have several significant limitations, including the need of cold chain for transportation and short shelf-life, which is only about of 4 days from the production date when stored between 2 and 8°C. Also, live attenuated parasites strains may pose the risk of reversion to virulence upon exposure to the natural conditions in the field (52). Moreover, there is also the risk of transmitting other contaminating blood-borne pathogens during vaccination. This may occur because the process of attenuation requires serial infections passages on several splenectomized or spleen intact calves (for *B. bovis* and *B. bigemina* respectively) (75), and the large-scale production of the vaccine in some countries (*i.e.*: Australia) involves infecting splenectomized cattle as well. Yet, the method of vaccine production varies among countries, and in some cases such as in Argentina, the attenuated strains are expanded in *in vitro* cultures, rather than in splenectomized cattle, for vaccine production purposes. Finally, as mentioned before, there is an age restriction for the vaccination, and issues regarding dose standardizations (54).

Attenuation can be achieved from virulent *B. bovis, B. bigemina,* and *B. gibsoni* parasites by using different approaches (Tables 3, 4). Attenuation can be generated *in vivo* through successive rapid passages in splenectomised calves, in the case of *B. bovis* (76), or by slow passages in non-splenectomized calves for *B. bigemina* (77).

Attenuation can also be achieved in *B. bovis* by long term *in vitro* culturing of virulent parasites (6), by deep freezing for long periods in liquid nitrogen (78), using chemical treatments, or by irradiation (79).

TABLE 3 *In vivo* live attenuated parasite used in vaccine against *Babesia*.

Vaccine component	Efficacy	Reference
South African S24 vaccine attenuated strain (with 24 passages in cattle) of *B. bovis*	Vaccination conferred protection in cattle and the attenuated strain was not transmissible by ticks. Co-transmission of the attenuated strain together with field strains resulted in the emergence of a new transmissible parasite population with a distinct hybrid genotype.	(166, 167)
Australian *B. bovis* vaccine attenuated strains (Ka strain), was produced by rapid 20–30 passages in cattle.	This strain is used as a component of a trivalent live vaccine which also contains an attenuated *B. bigemina* Australian strain, and *Anaplasma centrale* which adds protection against *Anaplasma marginale*.	(54)
Test the infectivity of a vaccinal and a pathogenic strain of *Babesia bovis* for the tick *Boophilus microplus*. Vaccine strainR1A isolated from clinical case and attenuated and produced by 30 passages in splenectomised cattle. Pathogenic strain of *B. bovis* S2P isolated from splenectomised calf infected naturally via tick larvae by *B. bovis*.	Engorged female ticks fed on calves inoculated with the S2P strain were able to transmit the infection to splenectomised calves. This vaccine strain was shown not to be transmissible by ticks under natural conditions.	(168)
Attenuated *B. bigemina* (*in vivo* and *in vitro*) were used to vaccinate two groups of Holstein Friesian heifers. Another group of heifers was vaccinated twice with purified soluble antigens obtained from the supernatant of *in vitro* culture combined with saponin.	Heifers vaccinated with attenuated *B. bigemina* either *in vivo* or *in vitro* resisted challenge without specific treatment, whereas the opposite occurred in heifers group vaccinated with culture soluble antigens. Antibody titers were higher in heifers inoculated with soluble antigens than in heifers inoculated with *in vivo* live *B. bigemina*, suggesting that antibody titers may not be a proper indicator of animal protection.	(169)
An australian *B. bigemina* vaccine strain (G strain) of reduced virulence was tested in animal against a virulent South African strain	The strain caused mild reactions in 10 animals and afforded good protection to challenge with a virulent strain.	(170)
An Australian vaccine strains (including *B. bovis*, *B. bigemian* and *A. marginale*) was test their safety in pregnant Holando heifers and their efficacy against challenge from inoculated local field strains of the three parasites from Paraguay	The *Babesia* strains, but not *A. marginale*, provided good protection against field challenge and were safe to be used in highly susceptible cattle.	(171)

Tables 3, 4 show previously published vaccination trials performed using *in vivo* and *in vitro* live attenuated parasites in vaccine trials.

In general, live vaccines against cattle babesiosis are not recommended for use in adult animals, since they may induce acute disease. However, the three experimental *in vitro* culture immunizations (6–8) performed in cattle described in Table 4 suggest that the attenuated S74-T3Bo is also a potentially efficient and sustainable attenuated candidate vaccine strain not only because was able to prevent acute bovine babesiosis upon challenge with a homologous virulent strain in highly susceptible adult cattle and young animals, but also because it was shown to be non-tick transmissible. However, the effectiveness of this vaccine still needs to be tested against heterologous virulent strains. In summary, this *in vitro* cultured attenuated strain might become an optimal choice as a component of attenuated live vaccine because it is a clonal-like strain (6), and thus might be unlikely to revert to virulence. Nonetheless, more experimental vaccinations tests using the LTCP attenuated S74-T3Bo strain are still needed in a larger number of animals and using different challenge strains. Similarly, *in vitro* culture attenuated strains of *B. bigemina* and *B. gibsoni* (Table 4), may be also optimal candidates for "universal" and more standardized effective *in vitro* culture attenuated vaccines, but again, more testing, including studies on tick transmissibility, are warranted on these strains.

3.1.2.3 Genetically modified *Babesia* parasites

The advancements in the field of the gene editing, including transfection technologies and CRISPR/cas9, in combination with *in vitro* culture systems, and other related technologies, may lead eventually to the generation of genetically modified live vaccines. This type of vaccines can serve as dual vaccines, targeting both the parasites and their vectors, in an integral approach of control. Still, there are important challenges for approving, producing, and commercializing this type of vaccines. In addition, there is the need for large scale cultivation to produce the parasite, and being a live vaccine, its distribution may also generate costly and cumbersome logistic requirements, such as the need for a cold chain (80). Another limitation of this approach includes concerns derived from the application of the genetic modified vaccine into the field animals. Despite these potential obstacles, this approach may prove to be cost-effective under certain circumstances. The following scientific trials shown in Table 5 exemplify the innovations achieved in this area of research.

Both trials described in Table 5 presented evidence for the ability of transfected live attenuated parasites to protect against challenge with a virulent parasite strain. At the same time, the parasites used in these vaccination trials will not be able to be transmitted by competent ticks because these experimental vaccines are based on long term *in vitro* cultured *B. bovis* that likely lost their transmission phenotype (6). Moreover, the addition of a gene expressing protective antigens derived from the tick vectors to the *B. bovis* vaccine strain, increases the advantages of this live-vaccine approach by providing a dual effect against the pathogen and its vector.

3.2 *Theileria* parasite vaccines

The main strategies currently used to control theileriosis are based on the use of anti-*theilerial* drugs. Also, in the case of bovines, improved control includes the use of indigenous and cross breeds of

TABLE 4 *In vitro* live attenuated parasite lines used in vaccines against *Babesia*.

Vaccine component	Efficacy	Reference
Infected erythrocytes of *B. ovis*/Akçaova stabilate (passage 5 in *in vitro* culture) was used for sheep immunization, either alone or mixed with *B. ovis* rBoAMA-1 protein.	Sheep co-immunized with sublethal dose of *B. ovis*/Akçaova stabilate and *B. ovis* rBoAMA-1 protein did not show clinical signs and/or changes in hematological parameters following challenge with *B. ovis* parasites.	(20)
A *B. bovis* isolate was cloned by *in vitro* cultivation and compared to the original cultured isolate for pathogenicity by yearling heifers inoculation. Four of them were inoculated with cloned material and the other 4 with the original culture.	The four animals receiving the cloned parasite showed comparatively minor hematologic changes and no clinical signs. One animal died in the group vaccinated with the original culture. All the animals receiving the cloned parasites were totally immune with no significant change in temperature or decrease in PCV upon challenge 100 days after vaccination.	(172)
Attenuated *B. bovis* strain by *in vitro* culturing using equine and bovine serum	All four splenectomized vaccinated calves recovered from mild clinical disease signs, and developed solid immunity upon challenge with virulent strain.	(173)
Possible cross-protection conferred by strains of *B. bigemina* or *B. bovis* derived from *in vitro* culture was tested in cattle by vaccination of calves using individual (*B. bovis or B. bigemina*) or combined (*B. bovis* and *B. bigemina*) live parasites.	The resulted protection was 25, 50, and 100% for cattle immunized with *B. bigemina, B. bovis*, and *B. bigemina/B. bovis*, respectively upon natural challenge via *Boophilus microplus* tick in the field. The mixed vaccinated animals were challenged under control conditions with virulent strains of both protozoan species. All vaccinated animals survived and showed a slight decrease in PCV with unchanged rectal temperature.	(174)
In vitro culture derived *Babesia bovis* and *B. bigemina* vaccine to susceptible *Bos taurus* bulls in a babesiosis endemic area was evaluated. Animals were over 18 months of age	After vaccination for 21 days, all animals under the study were released to a tick infested paddock where they remained until the end of the study. Results showed that a combined *B. bovis* and *B. bigemina* vaccine can confer a 70% protection to bovines introduced to *Babesia* infected areas.	(175)
Co-immunization of cattle with a vaccine against babesiosis (*B. bovis* and *B. bigemina*) and *Lactobacillus casei*	Results suggested that the vaccine efficiency was in part improved due to the *L. casei* boost of IgG1 over IgG2 antibodies agaisnt *B. bovis* and *B. bigemina*.	(176)
Vaccine with *Babesia bovis* and *B. bigemina* cultured *in vitro* maintained in a bovine. Serum and serum-free medium	The vaccine derived from *in vitro* culture in bovine serum-free medium reached 100% protection *vs* 83.3% protection with a vaccine derived from *in vitro* culture in bovine serum medium.	(177)
Long-term (LTCP) cultured *B. bovis* parasites [Attenuated S74-T3Bo-12 years]	Cattle immunized by this strain showed mild clinical signs, and the parasite wasn't transmissible in a tick transmission experiment (TBV).	(6)
	The immunized 6 months old calves survived superinfection with Vir-S74-T3Bo without displaying signs of acute babesiosis.	(7)
	In a separate experiment, immunized adult (>1.5 year of age) cattle displayed self-limiting signs of acute infection and protected against challenge with the homologous virulent *B. bovis* strain Vir-S74-T3Bo.	(8)
Culture-derived attenuated live vaccine against *B. bigemina*	A single shot of attenuated vaccine was capable of complete protection and parasitic clearance after the challenge with a lethal intravenous challenge virulent calf-derived *B. bigemina*	(178)
B. gibsoni attenuated Oita isolate 400 *in vitro* culture passages	Vaccinated dogs with the attenuated strain were protected against the challenge with *B. gibsoni* virulent Oita isolate	(179)

cattle that are known to be more resistant to the parasites and its vectors, such as *Bos indicus* (21). Historically, vaccines based on attenuated macro-schizont cell lines were successfully used in case of *T. annulata*. However, since these are attenuated parasite lines, there is a concern of spreading infections in the field (81). Table 6 shows different antigens used in subunit, or combined subunit-live vaccine trials against *Theileria* parasites.

Because of the current lack of commercial vaccines to control animal theileriosis, the variable therapeutics effectiveness (82), and the results derived from unsuccessful previous vaccination attempts, the main current control strategies against *T. parva*, *T. annulata*, and *T. orientalis,* in cattle, and *T. equi* in equines, still rely mainly on the control of their tick vectors. Ideally tick control should be done using environmentally friendly acaricidal drugs or tick vaccines, which can be complemented by parasite control using anti-*theileria* drugs. However, none of these options are currently available and more research in these fields is required.

Although the current use of ITM and cell line vaccines to control ECF and tropical theileriosis, respectively, are relatively effective, the procedure still has drawbacks. This includes the need for a cold chain for distribution, high costs, and the potential risk of tick transmission of *Theileria* parasites (83). Since ITM depends mainly on the use of oxytetracycline, there is also the concern of developing and expanding antibiotic resistances through food and milk contamination (84). The most widely used vaccines against *T. annulata are attenuated* schizont cell culture. The methodology

TABLE 5 Gene modification studies in vaccine development against *Babesia* parasite.

Type of vaccine	Study	Target	Reference
Stable transfected strain of *B. bovis* expressing an enhanced GFP (eGFP) and a chimeric version of Bm86 (*B. bovis*/Bm86/eGFP)	Splenectomized calves immunized with *B. bovis*/Bm86/eGFP showed mild signs of acute disease after challenge with *B. bovis* and generated antibodies that recognized native Bm86, a vaccine candidate protein expressed in the vector tick midgut. The number of ticks that fully developed and detached as engorged females was reduced (70%) in vaccinated calves. PCR analysis for *B. bovis* in ovaries and eggs of female ticks fed on immunized calves was negative.	*B. bovis* blood stage. *R. microplus* & *R. annulatus*	(180)
Live *B. bovis* vaccine expressing the protective tick antigen glutathione-S-transferase from *Haemaphysalis longicornis* (HIGST)	Cattle inoculated with transfected parasites developed mild babesiosis upon challenge with a virulent strain of *B. bovis* and produced antibody responses against the tick antigen expressed by the transfected parasites. Challenge of vaccinated cattle with heterologous *R. microplus* ticks, resulted in reduction of egg fertility and weight of fully engorged female ticks. Reduction in tick size and fecundity of *R. microplus* was also observed.	*B. bovis* blood stage and *R. microplus* ticks.	(181)

of production and its safety evaluation had been evaluated (85, 86). This kind of vaccine is used in different countries such as Israel, Iran, Turkey, Spain, India, northern Africa, central Asia and the People's Republic of China (87). A recent study in Egypt reported a vaccination trial in cattle using culture-attenuated schizont-infected cell lines isolated from Egypt. The vaccinated groups were inoculated with 4 mL (1×10^6 cells/ml) of the attenuated cell line. Three weeks after vaccination, calves of vaccinated and control non vaccinated groups were transported to the New Valley Governorate (Egyptian oasis), where they were kept under field conditions and exposed to natural *Theileria annulata* challenge. All animals in the control unvaccinated group showed severe clinical signs and died despite treatment with buparvaquone. In contrast, animals in the vaccinated group became seropositive without developing severe clinical signs other than transient fever. These findings indicate that the Egyptian attenuated cell line was successful in protecting against tropical theileriosis under field conditions (88). Although, cell culture vaccine against *T. annulata* has been recognized for more than three decades and has proven to be effective in the field, it still has limitations (87), and each country developed the vaccine from local isolates (89). Because of the continuous attenuation, some of the attenuated lines lost the ability to differentiate to erythrocytic merozoites (piroplasms) when inoculated to cattle, one example of that when *Hyalomma* nymphs fed on vaccinated cattle did not become infected (90). This pitfall, in addition to the problem in standardization of the antigenic composition of the cultured parasites and the need of a cold chain for distribution of the vaccine to the field are limiting factors in commercialization of this vaccine (89).

T. equi and *B. caballi* are both responsible for equine piroplasmosis, a disease that limits horse movement worldwide and seriously affects the development of horse industries and equestrian sports. Equine theileriosis caused by *T. equi* is mainly prevented in non-endemic areas worldwide by regulating the movement of horses from endemic countries. Also, for some countries, animals must be tested negative for *T. equi* serologically, as an obligatory procedure in animals' importation (91). Thus, in order to generate potent vaccines, research should be focused ideally on targeting different parasite developmental stages, such as blood and sexual stages, and avoiding, if possible, antigens that may interfere with the current mandatory tests required for exportation of horses. Also, advances in reverse vaccinology should be applied. The similarities among different species of *Theileria* parasites genomes should be considered during vaccine approach. Importantly, identification of host immune correlates which are associated with protection against the parasite infection, would be of high value for vaccine design. Using effective adjuvants and innovative and practical vaccine delivery systems should also be considered as crucial aspects of vaccine development.

3.3 *Anaplasma* vaccines

Although *Babesia* parasites are apicomplexan protozoa and *Anaplasma marginale* is a rickettsia, they were historically linked since they form part of a complex of diseases also known as cattle tick fever or Bovine parasitic sadness (BPS) in several countries, sharing important features: they both infect exclusively erythrocytes in the vertebrate hosts causing a clinically related acute disease, and they are all transmitted by *Rhiphicephallus* (*Boophilus*) ticks, often co-occurring in endemic areas.

There are currently no worldwide effective vaccines to prevent canine anaplasmosis, caused by *Anaplasma phagocytophilum* and *A. platys,* nor for bovine anaplasmosis caused by *A. marginale* (92, 93). *A. phagocytophilum* has zoonotic potential and is responsible for human granulocytic anaplasmosis.

Many vaccine candidates have been evaluated for the control of bovine anaplasmosis, but the antigenic diversity of the pathogen has impaired many efforts to control the disease (92). However, a live heterologous vaccine based on *A. centrale* have been used since the early 20th century. *A. centrale,* is an organism isolated in South Africa, which is less virulent for bovines than *A. marginale,* and provides some degree of cross-protection against this pathogen in naive cattle. In some countries, *A. centrale* is added to live *Babesia* spp. vaccines, to make a trivalent vaccine (including *B. bovis, B. bigemina*, and *A. centrale*). This trivalent vaccine is in use as a live vaccine in countries such as South Africa, Australia, Argentina, Brazil, Uruguay (94). Despite the success of this vaccine to some extent, there are few reports indicating *A. centrale* was the cause of vaccine outbreaks, with fatalities (95). Furthermore, in some cases the vaccine failed to induce immunity against *A. marginale* challenge (96). Moreover, University

TABLE 6 Vaccine trials based on *Theileria* antigens associated with different parasite developmental stages.

Form	Efficacy	Reference
T. annulata		
Recombinant SPAG-1 [Surface sporozoite antigen] & TAMSA-1 [Merozoite surface antigen]	Vaccination resulted in significant levels of protection in cattle when the two antigens (SPAG-1& TAMSA-1) were used in the form of a cocktail. The immunization results indicated a potential synergistic effect between both antigens in inducing protection against *T. annulata*. Also, in contrast to using SPAG-1 alone, immunization with TAMS-1 reduced the severity of several disease parameters compared to non-immunized control animals.	(182, 183)
Combination of SPAG-1 and the attenuated parasite cell line	All immunized animals survived challenge with virulent parasites. Protection provided by combining sporozoite and schizont antigens in vaccination against the disease was considered as effective.	(184)
Ta5 and Ta9	These antigens proved that the role of cytotoxic cells is less evident in the protection to *T. annulata* than to *T. parva*. There was no parasite challenge reported.	(185)
6-cys antigen	Candidates for transmission blocking vaccine were identified by bioinformatic analysis, but without animal trial experiments.	(186)
T. parva		
Infection and treatment method (ITM)	ITM is considered a primary choice to prevent the dramatic effects of East Coast Fever. The procedure involved inoculation with a cocktail of *T. parva* strains followed by administration of long acting oxytetracycline. This method induces protective immune responses.	(81, 187)
Polymorphic immunodominant molecule (PIM). An expressed protein by both the sporozoite and schizont stages of *T. parva*. Used with different delivery platforms *i.e.* nanoparticles	This vaccine elicits strong humoral and cellular immune responses in vaccinated animals, but no protection was reported.	(188, 189)
T. parva p67 (A surface sporozoites antigen)	Immunized animals showed variable levels of protection in field trials following challenges with parasite sporozoites.	(190, 191)
Polypeptide derived from p67 C-terminal by using nanotechnologies	Although vaccination showed variation in the response among the immunized animals, it displayed significant protection	(192)
Tp1	This antigen induced schizont-specific CD8 (+) central memory T cells with partial protection against a lethal challenge (36% survival)	(193)
T. orientalis		
Live vaccine using piroplasm parasites	Has low efficacy and represent a risk for parasite transmission	(23)
Full-length or immunogenic segments of the *T. orientalis* major piroplasm surface protein (MPSP). This protein is highly diverse and involved in immune evasion.	The immunized animals show no clinical signs with lower parasitemia level when challenged with sporozoites	(194)
T. equi		
Passively transferred merozoite-specific IgG3 [immune plasma containing *T. equi* merozoite-specific antibodies infused into young horses (SCID foals)]	Although the immunized animals were infected after intravenous challenge with homologous *T. equi* merozoite stabilate, foals show a delayed time to peak of parasitemia and significant delay in the clinical signs.	(195)
T. equi recombinant EMA-2 [geldings- pregnant mares and foals]	The vaccinated animals showed humoral and cellular immunity responses similar to those observed in natural parasite infections. Vaccinated animals survived challenges with *T. equi*.	(196)

Products LLC (Louisiana, USA) currently offers the only inactivated commercial vaccine against bovine anaplasmosis based on *A. marginale*[1] (97). This vaccine has been field-tested for over 20 years (98). Although this vaccine does not prevent infection with virulent *A. marginale*, it induces enough protection in cattle against acute anaplasmosis (92). This vaccine requires only two doses in the first year, followed by one annual booster each year, and it is safe to be used in any stage of bovine pregnancy (98). Still this inactivated vaccine is not approved in many countries, but it is approved and established for use in the United States. The following sections provide a glimpse of some vaccine development efforts in the field of *Anaplasma* (Figure 1).

1 https://www.prnewswire.com/news/university-products-llc/

3.3.1 Whole organism vaccines

In this section, *A. marginale* vaccines based on whole organisms were introduced, which can be inactivated or live vaccines. Inactivated vaccine trials are shown in Table 7. The live vaccine contained the virulent form of the pathogen in a low dose, which may be derived from infected animals, or attenuated through multiple animal passages, or in long term *in vitro* culture. In case of *Anaplasma,* the mammalian *in vitro* culture system efforts, based on erythrocytes, have been explored without any success, either using bovine erythrocytes alone, or co-cultured with endothelial cells [bovine pulmonary artery] (99). Only several tick cell lines were successful to establish the *Anaplasma in vitro* culture for both *A. marginale and A. centrale*, but long-term *in vitro* cultures of these organisms in erythrocytes was not yet achieved (100, 101). However, cattle immunization with *in vitro* cultured *A. marginale* induced an antibody immune response but without the expected protection level (102). The trials of live *Anaplasma* vaccines are shown in Table 8.

3.3.2 Subunit vaccines against *Anaplasma* organisms

Novel omics technology has yielded a list of antigens that could help the researchers to explore new possible vaccine candidates. Protein function, localization, conservation, and either their dominant or subdominant antigenic characteristics, are the main criteria, among others, for antigen selection. Proteins as major surface proteins (MSPs), outer membrane proteins (OMPs) and several type 4 secretion system (TFSS) proteins (103) were intensively explored. Those proteins are involved in different function as adhesins to red blood cells and tick epithelial cells (MSP) (104), Adhesin/invasion (OMPs) (105) proteins can mediate transfer of DNA and proteins into eukaryotic host cells, may interfere and are important for the survival of intracellular bacteria (TFSS) (106, 107). The antigens were tested as vaccine candidates in a form of recombinant protein(s), DNA, plasmids, or synthetic peptides and even in some cases, as mixtures of different forms. Recently "quantum vaccinomics" was applied to identify and characterize *A. phagocytophilum* MSP4 protective epitopes by a microarray epitope mapping. The identified candidate protective epitopes, or immunological quantum, were used to design and produce a chimeric protective antigen that was used in vaccination trials of rabbits and sheep. The resulting antibodies from the two types of immunized animal hosts were equally effective to block cell infections in an *in vitro* inhibition assay of *A. phagocytophilum*. The results from these experiments supported the use of quantum vaccinomics as an effective tool for the design of new chimeric candidate protective antigens, as a better alternative to the use of a full-size single protein (MSP4) for vaccine development (108).

3.3.3 Genetically modified *Anaplasma* pathogens

Genetic modification was achieved in *Anaplasma* pathogens either by knocking out biologically important proteins, or by introducing an exogenous gene into its genome to express a protein able to generate protective immune responses. These modifications were performed in *in vitro* culture to achieve genetically modified organisms. A mutant strain was attained in *A. marginale* by transposon mutagenesis of the *A. marginale* Virginia strain to reduce the expression of OMP (109). Although this mutant strain can be transmitted by ticks, it showed reduced infectivity in both intact and splenectomized cattle (110). However, this mutant was not tested for protection against homologous or heterologous challenge. Another immunization trial was performed by the

TABLE 7 Inactivated vaccines against cattle anaplasmosis.

Form	Efficacy	Reference
Lyophilized highly infected RBCs in oil adjuvant and inoculated into susceptible hosts	Not a practical formulation due to high content of erythrocyte stroma.	(94, 197)
Inactivated preparations of purified initial bodies from bovine RBCs	The vaccine was not effective in immunized animals against a heterologous strain.	(198)
Vaccination with three strains that shared major surface proteins Msp1a and Msp4	Vaccinated animals that received the challenge with the homologous strain in the form of inoculum showed no protection and chemotherapy was required to prevent death. In contrast, animals that received the challenge by infected ticks (gradual infection), were protected against naturally tick-transmitted anaplasmosis.	(199, 200)

TABLE 8 Live vaccines against cattle anaplasmosis.

Form	Efficacy	Reference
live virulent *A. marginale* paired with treatment premunition	Variable. Some vaccinated animals were infected with a delayed incubation period, while other animals showed no clinical signs after vaccination.	(201)
Virulent strains of *A. marginale* attenuated by passaging organisms in unnatural hosts (splenectomized sheep and deer)	Variable. The deer-passaged strain vaccine failed to induce solid protection in vaccinated animals while sheep-attenuated strains induced protection.	(201, 202)
Fresh or frozen infected RBCs with *A. marginale* strains of naturally low virulence	Induced a mild clinical syndrome in the immunized animals	(203, 204)

mutant of *A. centrale* with a transposon-mediated insertion of a construct containing Turbo GFP as a marker, and antibiotic resistance genes for selection. Upon animal immunization, it provided immunity and showed clinical signs like the infection with unmodified *A. centrale* but with lower percentage of infected RBCs (110). Both trials were considered as an advance for disease control, but the possibilities of other pathogen transmission like mycoplasmas and even viruses present in the culture systems should also be taken into consideration (92).

No universal vaccine is yet available to protect against diverse geographic strains of *A. phagocytophilum* and *A. platys*, *Anaplasma* species which are known to infect dogs (39) and other animals. *Anaplasma* spp. can be transmitted mainly by *Rhipicephalus* and *Ixodes* for *A. platys* (33, 111) and *Dermacentor, Hyalomma* and *Rhipicephalus* in case of *A. phagocytophilum* (112). Whole genome sequencing of both *A. phagocytophilum* and *A. platys*, allowed for the identification of several potential candidates for vaccine development. It was found that nine of proteins that have an immunogenic potential like the Asp14, Asp55, Msp5, Msp2, AipA, OmpA, APH 0032, and APH 1384 antigens of the type IV secretion system of *A. phagocytophilum* (113–117) can be rational candidates for vaccine development.

3.4 *Trypanosoma* parasite vaccines

Currently there is no available vaccine for trypanosomiasis prevention. One of the main challenges to develop a vaccine is the mechanism that *Trypanosoma* parasites use to evade the host immune system, by constantly varying the structure of their surface coating. The antigenic variation operated by the parasites makes it difficult to identify appropriate candidates for vaccine development, especially for the case of subunit vaccines.

There are limited options to prevent trypanosomiasis within vertebrate hosts. In addition, the increase of resistance toward trypanocidal drugs makes chemotherapy, the major means to control infection, difficult to use. Additionally current drugs have various other shortcomings, including toxicity and limited efficacy (118). The drugs commonly used for the treatment of infected domestic animals with *T. evansi* are Diminazene aceturate, which causes some toxicity to the host (119) and isometamidium chloride (120). In this scenario, vector control remains a very important step for disease prevention (121). Another important challenge, despite sporadic reports, is the lack of full knowledge on the geographic distribution of the disease and its transmitted vectors, which affects the control initiatives that require reliable information.

To control this disease, different approaches should be addressed, but the main and more practical strategy is just minimizing contact with the vector tsetse flies. The principal control method is targeting the tsetse fly using insecticides, which has environmental drawbacks. These drawbacks led to the development of bait technologies which include traps and insecticide-impregnated targets (122). Another approach is to use naturally trypano-tolerant breeds of livestock, which is considered as an economical addition to the intervention tools (41).

Previous attempts to develop subunit vaccines against African trypanosome infections have highlighted the difficulties in overcoming the immune evasion mechanisms that have been evolved by these parasites for survival (123). Many antigens were chosen as vaccine candidates. Such is the case of the variable surface glycoprotein (VSG) (124), which provided partial protection, but became non-effective after some time (125). Also, vaccine formulations based on invariant surface glycoproteins and subcellular proteins of the cytoskeleton, like actin and tubulin (123) generated unsatisfactory and non-protective immune responses. However, the advancement in the field of bioinformatics, together with the availability of omics data from different organisms is allowing the design of new generation vaccines that may offer better antigenicity and safety profile. The so-called reverse vaccinology approach, which depends mainly on omics data, permits the design of vaccines that can involve many antigens of different expected important functions. Here are some examples of vaccination trials performed against *Trypanosoma* parasites.

3.4.1 Subunit vaccines against *Trypanosoma* parasites

One vaccine trial was based on a single recombinant protein comprising the extracellular region of a conserved cell-surface protein that is localized to the flagellum membrane invariant flagellum antigen from *T. vivax*. When this protein was used in vaccination in mice it resulted in survival of 10 out of the 15 mice which also were protected beyond at least day 170 (126). This formulation is considered as the first ever successful vaccine trial against this devastating disease caused by *Trypanosoma* parasites (127).

3.4.2 Chimeric vaccine against *Trypanosoma* parasites

Advancements in computational modeling coupled with the availability of large amounts of omics data from different organisms have allowed the design of new generation vaccines. A multi-epitope vaccine (MEV) designed from a collection of antigenic peptides from conserved hypothetical plasma membrane proteins of *Trypanosoma brucei gambiense*. It was expected to give adequate immune stimulation but this vaccine was not tested with parasite challenge (128).

3.4.3 Genetically engineered *Trypanosoma* parasites

Since it is predicted that *T. cruzi* cyclophilin-19 (Cyp19) protein is important for parasite growth, a mutant parasite line lacking the Cyp19 gene was generated. The mutant parasites failed to replicate when inoculated into host cells *in vitro* or in mice, confirming that Cyp19 is critical for infectivity as well. Moreover, immunization with a *T. cruzi* Cyp19 deletion mutant protects 100% upon challenge of the mutant-immunized mice with virulent wild-type parasites, indicating the effectiveness of this line at preventing death from acute disease in mice. In addition, this mutant line did not cause clinical disease in immuno-deficient mice confirming their lack of virulence (129).

3.4.4 mRNA-based *Trypanosoma* vaccines

It is now known that there are many benefits of mRNA-based vaccines, which led researchers to use this approach into vaccine development against *Trypanosoma* parasites. The mRNA-based vaccine approach could prevent disease, but so far, no study was conducted regarding testing this concept (125).

4 Vaccines against vectors

4.1 Tick vaccines

Tick-borne diseases (TBDs), particularly those caused by blood parasites, pose a significant threat to global livestock industries. The widespread distribution of various tick species, combined with climate change and human activities, contributes to the outgoing expansion of tick populations. Anti-tick vaccines (ATVs) offer a promising, safe, and environmentally friendly approach to tick control. These vaccines not only reduce vector infestations on vertebrate hosts but also hinder the transmission of blood parasites, thereby mitigating the economic impact of tick-borne diseases at both herd and regional levels. ATVs also serve as an effective alternative to chemical tick control, as they are cost-efficient and can be applied across different host species (130). By minimizing the need for acaricides, ATVs help to prevent the emergence of acaricide-resistant tick strains, reduce contamination in the food chain, and promote environmental safety.

Two commercial vaccines were developed based on the glycoprotein Bm86 tick midgut protein, TickGARD Plus (131) and Gavac Plus (132) for use in cattle. This protein was derived from the *Rhipicephalus microplus* ticks, but it is also conserved in other related *Rhipicephalus* ticks. Those vaccines were developed and tested in Australia and Cuba, respectively (133), and were also used in other countries like Mexico and Venezuela against the cattle ticks *R. microplus*, *R. australis* and *R. annulatus* with different levels of efficacy, ranging between 10 and 89% (134, 135). In Cuba and Colombia, those vaccines were generally effective to reduce tick populations and the number of acaricide treatments, in addition to babesiosis and anaplasmosis infections, respectively (136). The TickGARD Plus vaccine is no longer used since 2021 in Australia because of the need for numerous applications (3–4 booster doses per year) (137). So, currently, the only commercially available anti-tick vaccine is Gavac® (138, 139).

Developing effective vaccines against ticks requires intensive research and costly experimental trials. Current approaches in tick vaccine research are focused on identifying potent candidates playing a critical role in tick biology that are capable of inducing cross-reactive immunity against multiple tick species. This requires the identification of highly conserved and functionally relevant antigens that are also exposed to antibodies in the host. It can be anticipated that recent progress in tick omics in addition to other novel molecular biology approaches, and a better understanding of tick biology, will be helpful for the identification of such antigens.

As it was described in the *Babesia* parasite vaccines section above, there is also the hope of developing dual anti-tick and anti-*babesia* vaccines.

4.2 Blood sucking flies' control

To date, there is not a specific vaccine against blood-sucking flies. However, there are several other alternative methods available that can be applied and used in combination to approaches aimed at controlling the blood parasite transmission and its causing diseases, in order to protect livestock. Such methods include the following:

1-Physical barriers: Blood-sucking insects can cause allergic reactions in animals, and can also cause different diseases by transmitting several pathogens that are able to propagate in them. Physical barriers can be designed to prevent direct access of flies to animal skin (140). This approach includes the use of protective gear, typically fly sheets, leg wraps and masks. These methods are considered an easy, affordable, and reliable system to prevent insect bites and stings effectively and may prevent the use of chemicals such as insect repellents or insecticides decreasing the environmental hazard.

2-Insecticides: Insecticides can be applied to animals and their surroundings either to repel or to kill the blood-sucking flies. Application of insecticides should be used with caution and following the recommended doses to avoid the emergence of insect resistance strains and environmental pollution. Repellents can also be used effectively to prevent flies approaching the skin of target animals. Application of pesticides in animal treatments can be done using automatic sprayers, back rubbers and ear tags (141).

3-Biological control: this method, considered as one of the most environmentally friendly approaches, can potentially reduce the current reliance on insecticide-based control. This approach is based on the use of known natural predators or parasites that target blood-sucking flies. Non-insecticide-based strategies have been implemented in different blood sucking as mosquito (142) and ticks by entomopathogenic organisms such as nematodes (143) and protists or mites as biocontrol agents. However, little attention has been given so far to control methods based on biological control, and further exploration studies on the biocontrol of immature and adult stages of blood sucking flies are needed (144).

4-Environmental management: This approach involves adequate waste disposal and maintaining clean surroundings, which in turn discourages fly breeding. More importantly, both negative and positive impacts of insects on human and animal health and on the environment need to be addressed by public health professionals. It is also crucial to balance important aspects and goals in insect management approaches, which include regulating their production, exploiting their potential, and limiting their potential negative effect on animals and humans, and on the environment (145).

5 Conclusion

The more rational way to avoid blood parasites causing disease is to apply preventive measures against ticks and other transmitting vectors. To have effective and deployable vaccines is not a straightforward effort. There are many obstacles in the way of vaccine development, including the identification of proper vaccine antigen candidates. However, this task is currently facilitated by the availability of data derived from the application of available omics and other approaches, which can provide information on vaccine-targetable proteins that are exposed to the effectors of the immune system and may play key roles in the pathogens' biology. Ideally, those antigens, in conjunction with proper adjuvants, should be able to induce desirable protective immune responses in immunized animals. Moreover, other aspects such as practicality of vaccine production, the choice of efficient and safe adjuvants (if needed), feasibility of shipment, shelf life, safety of the vaccine, licensing, and commercialization are also important points that also need to be considered for successful vaccine design.

Recombinant protein production is a technology usually applied to vaccine development. However, confirming that the structure of the

recombinant protein faithfully resembles that of the native protein should be considered, so the relevant epitopes, including conformational ones, will be antigenic and reachable by the protective antibodies. With the advancements in bioinformatics, we can now predict quite reliably the presence of T or B cell epitopes, but that does not necessarily mean that these antigens will be immunogenic enough to be immunoreactive in the actual immunization trials. In addition, recent developments in vaccinology imply that researchers should also consider other alternative methods of vaccine delivery that do not require the production of recombinant antigens, including DNA and RNA vaccines.

Although live vaccines with or without the addition of genomic modifications had achieved promising results in several vaccine trials, they should be used with caution to avoid massive outbreaks in non-endemic areas populated with animals that were never exposed to such pathogens before. Also, there are still concerns regarding the application of GMOs in the vaccination field, fearing that genomic modifications may have unforeseeable consequences, either to the environment, in other unrelated commensal organisms, or in the animal genomes, with the subsequent impact on humans as well.

To test vaccine candidates and to get the relevant and valid results, researchers should be cautious with the route of pathogen challenge, either as an inoculum form, or by natural infection through the vector (ex. tick challenge). While the former one is considered as a fast way, the latter one acts in a more gradual way, representing a simulation of natural infection in the field.

Next,-Generation Technologies and Systems Biology is considered a modern molecular toolkit, and it is a leader guide for the future direction in vaccine candidate design. The traditional way to identify a novel antigen(s) is usually restricted to individual studies, an approach that can be considered as slow and limited. Sequencing technologies, bioinformatics, and statistics analysis facilitate the omics for lots of parasites. Omics such as genomics and transcriptomics have facilitated the functional annotation of the genome for many of these parasites, which significantly improved the understanding of the parasite biology, interactions with the host, as well as virulence and host immune response (146). To identify ideal vaccine component peptides or proteins, a comprehensive identification of the entire gene repertoire through genome, transcriptome, and proteome data mining, followed by the analysis of their encoding sequences using bioinformatics tools. The analysis should focus on *in silico* characteristics and assess both intra-species (from at least five genomes) and inter-species (among phylogenetically related organisms) conservation levels. Moreover, cutting-edge technologies such as gene editing by CRISPR-Cas9 have also allowed the discovery and functional characterization of potential novel vaccine antigens (146).

Finally, even if the vaccine candidates derived from omics technologies, computational approaches, or validated by using genetic manipulation approaches, they still need to be evaluated by clinical or field trial by animal experiments in order to test efficacy and safety. Therefore, systems vaccinology combined with experimental validation and evaluation in animal models through field trials can significantly improve the design of novel vaccines against blood parasites, opening a new era of vaccinology research that could lead to an expansion in licensed products after decades of slow advances (Figure 2).

In the *Babesia bovis* field, there are examples of the use of NGS technologies for the selection of vaccine candidates. The 6cys proteins and Hap2 protein were chosen as transmission blocking vaccine candidates after investigation of the genome annotation since these antigens were known to be involved in sexual stage development in other *Babesia*-related parasites, and then experimentally validated for *Babesia* parasites. Although the recombinant 6Cys proteins used in vaccination trials did not generate an effective transmission blocking response, a vaccine based on recombinant Hap2 was shown to be effective in blocking transmission of *B. bovis* (147), a finding that should be considered for the eventual production of a commercially available TBV against this parasite.

In conclusion, addressing the various challenges in vaccine development is of paramount importance. Recent advances in vaccine technology offer significant opportunities, particularly using multicomponent formulations that incorporate multiple antigens. Utilizing live or whole-cell immunogens, along with a combination of whole attenuated parasites and recombinant sexual stage antigens, generally enhances the effectiveness of vaccines in controlling blood parasite infections and their transmission by vectors. However, it is essential to proceed with caution. The inclusion of a greater number of antigenic subunits may lead to unfavorable three-dimensional structures, which could result in the loss of critical conformational epitopes. Therefore, while the multicomponent approach shows promise, careful attention must be paid to maintaining the structural integrity and immunogenicity of the antigens to ensure the development of an effective vaccine.

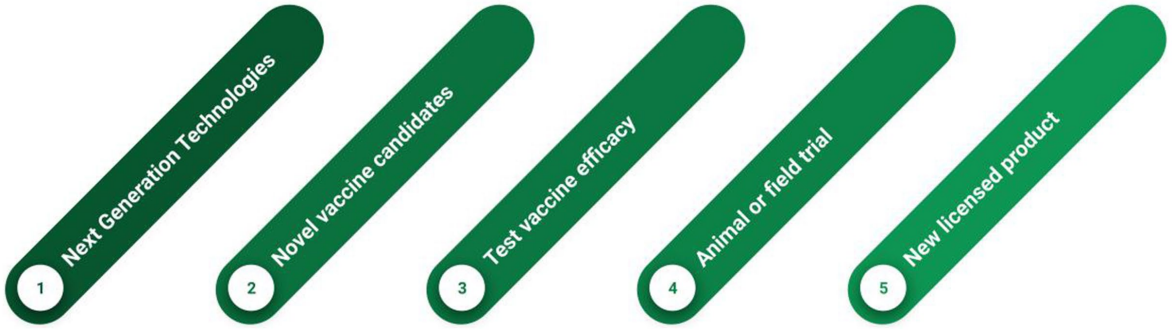

FIGURE 2
Next-generation technologies leading to novel vaccines pathway.

6 Recommendations

- Based on previous experiences, ideally, future research directions toward development of effective vaccines should involve the prevention of the acute disease, as well as prevention of parasite transmission.
- Sequence variations in vaccine target genes may result in vaccine failures. In order to achieve the goal to control blood parasites and its associated vectors using recombinant proteins, it is important to verify sequence conservation of the target antigens among distinct geographic isolates of the parasites, using, for instance, available sequence databases.
- It is likely that using a single antigen as a vaccine is disadvantageous to induce full protective immune responses. Thus, using multiple antigens as a multicomponent formulation of vital functions or using the live or whole-cell immunogens or a consolidation of the whole attenuated parasite in addition to recombinant sexual stage antigens, would be, in general, more effective vaccine to control blood parasites infection and its transmission by vectors.
- Finally, to ensure disease control and to reduce disease impact, it's critical to have environmentally friendly vector control measures applied in addition to the use of safe vaccines, in the vector specific season.

Author contributions

HA: Conceptualization, Data curation, Investigation, Writing – original draft, Writing – review & editing. MM: Validation, Writing – review & editing. CS: Conceptualization, Data curation, Formal analysis, Funding acquisition, Resources, Visualization, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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EDITED BY
Mourad Ben Said,
University of Manouba, Tunisia

REVIEWED BY
Bersissa Kumsa,
Addis Ababa University, Ethiopia
Ayodele O. Majekodunmi,
Food and Agriculture Organisation of the United Nations, Nigeria
Musheerul Hassan,
Ilia State University, Georgia

*CORRESPONDENCE
Solomon Ramagoai Magano
✉ magansr@unisa.ac.za

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Documentation of ethnoveterinary knowledge and alternative practices for cattle tick control in Sekhukhune District, Limpopo Province, South Africa

Confidence Semakane Phaahla[1], Jeremiah Leshweni Shai[2], Vincent Maduna[2], Resoketswe Charlotte Moropeng[2] and Solomon Ramagoai Magano[1]*

[1]Department of Life and Consumer Sciences, Faculty of Agriculture and Life Sciences, University of South Africa, Pretoria, South Africa, [2]Department of Biomedical Sciences, Faculty of Science, Tshwane University of Technology, Pretoria, South Africa

Introduction: The integration of traditional plant-based methods for controlling ectoparasites in the primary healthcare of livestock is progressively emerging as a crucial intervention to enhance livestock productivity in regions with limited resources, particularly in smallholder farming areas facing resource constraints. In Sekhukhune District, where livestock plays a vital role in rural livelihoods, cattle ticks present a significant challenge to cattle farming. This study aimed to document the ethnoveterinary practices employed by local communities to control cattle ticks, highlighting the use of alternative methods rooted in indigenous knowledge (IK).

Methods: Data were collected using a purposive sampling method to select traditional livestock keepers, herders, and community elders to uncover the plant-based treatments and management strategies used in tick control. In addition, a semi-structured questionnaire and a guided field survey were employed to collect data.

Results: A total of 250 participants, with an age range from 18 to over 60 years, were recruited. The result revealed that the elder participants, over 60 years of age, were more knowledgeable compared to the youth and adults. Furthermore, 28 plant species with potential acaricidal properties and other methods aimed at controlling cattle tick infestations were documented. *Cissus quadrangularis* was the most frequently cited species, which was widely distributed throughout the district.

Conclusion: In addition, these results are framed within the larger context of sustainability, promoting eco-friendly cattle farming practices in Sekhukhune District while reducing reliance on conventional acaricides. By documenting this ethnoveterinary knowledge, the study contributes to the preservation of indigenous knowledge while advocating for sustainable approaches to livestock health management in rural areas. The study concludes with valuable insights into the selected local community's traditional methods of managing tick infestations. Furthermore, the study underscores the significance of preserving and understanding indigenous knowledge in livestock health management, particularly in regions where conventional veterinary approaches may face challenges.

KEYWORDS

ethnoveterinary knowledge, indigenous communities, herbal acaricides, ticks, Sekhukhune District

Introduction

Indigenous knowledge (IK) is defined as a systematic body of knowledge developed by local individuals through an accumulation of informal experiments, beliefs, and understanding of their environment. It evolves through an adaptive process and is passed down through generations orally (1–5). In addition, the practice of IK differs across localities and cultural groups, each possessing different systems. Thus, these practices create a distinctive indigenous knowledge system (IKS). As the IKS is multi-cultural and dynamic, it allows for the continuous addition of new wisdom to the existing body of knowledge. As the exchange of the IKS occurs, so do the beliefs and procedures. However, the adaption of new practices is required to maintain a positive impact on the environment (6). Furthermore, the IKS is an important tool for local people as it is integral to the social structures of primary healthcare. Moreover, its contribution to livelihoods in developing countries cannot be undervalued as it is reported that approximately 80% of people in developing countries depend on traditional herbal mixtures to treat different diseases (7).

Indigenous knowledge systems (IKS) are widespread practices that are explored across the world. Furthermore, it has been discovered that selected medicinal plants used to treat human ailments can also be applied in primary healthcare for domestic animals, depending on local knowledge and the availability of natural resources (8). This knowledge could help bridge gaps in treating ailments commonly affecting livestock and distress its production. Recently, scientific reports have shown interest in documenting the IKS and exploring the bioactivities of medicinal plants for ethnoveterinary practices, similar to ethnobotany (9, 10) highlighting the influence of ethnoveterinary practices in rural areas.

Furthermore, ethnoveterinary medicine (EVM) continues to be practiced in developing countries, especially where public veterinary services are considered inefficient and financially unfeasible (11–16). The continued use of EVM is encouraged by the belief that traditional medicine is more effective than conventional medicine, with the presumption that it has no side effects as animals consume selected plants. These treatments are considered environmentally friendly (7, 12, 17, 18).

However, the risk of losing such significant IK may arise due to migration, urbanization, and modern technologies. These factors can interfere with the traditional means of knowledge transfer, leading to knowledge decline or misinformation. This has stimulated researchers to attempt to preserve the fragility of oral knowledge. In Limpopo Province, studies have been conducted on preserving plant species with acaricidal activity (12, 18–22).

Moreover, the studies mentioned above listed numerous plant species from Limpopo Province that were investigated to identify novel plant extracts that could be used as alternatives to conventional cattle acaricides against ticks. Plant species such as *Carica papaya*, *Tagetes minuta*, *Diospyros lycioides*, *Clerodendrum glabrum*, *Terminalia sericea*, *Calpurnia aurea, Lantana camara*, and *Bulbine latifolia* were cited. In addition, certain medicinal plants with acaricidal properties against cattle ticks are expected to become viable alternatives for controlling ticks in the future. Nonetheless, some researchers have expressed concerns about using medicinal plants to control cattle ticks, citing the need for more solid evidence to support their effectiveness. Despite being widely used by farmers globally, ethnoveterinary practices have often been criticized or dismissed. However, other studies (18, 20) have provided scientific evidence that helps identify effective plant extracts under controlled and reproducible conditions. This enables resources to be directed toward developing products based on these promising plant species. For example, medicinal plants such as *Calpurnia aurea* and *Rotheca alabrum* have been tested for biological activities against *Rhipicephalus* species, showing effects similar to those of conventional acaricides (18, 22).

Furthermore, these types of research offer hope and encouragement to rural farmers who continue to rely on indigenous practices for controlling cattle ticks (18–21). In addition, based on the extensive literature review and the ethnoveterinary practices of the rural communities, numerous extracts from the plant species in Limpopo Province were examined to identify novel botanicals that could serve as alternatives to conventional acaricides for cattle tick control (21). Historically, the primary focus of research on plants with acaricidal effects against cattle ticks has been in Vhembe District, Mopani District, and Capricorn District. However, in Sekhukhune District, there is no record of plants being documented for their acaricidal properties. Nonetheless, ethnobotanical practices related to human primary healthcare remain sufficiently documented (2, 23, 24). Therefore, this study was conducted to document the fragility of oral IK regarding the traditional practices of medicinal plants used as alternative acaricides against cattle ticks in Sekhukhune District.

Materials and methods

Study area

The study was conducted in Sekhukhune District Municipality, located in the southeastern part of Limpopo Province, South Africa (24°23′27.52″S and 29°50′06″E), as shown in Figure 1. The district covers approximately 13,528 km^2 of geographical area and is divided into four local municipalities: Makhuduthamaga, Elias Motsoaledi, Ephraim Mogale, and Fetakgomo Tubatse. The regional climate is subtropical, characterized by warm, moist summers and cool, dry winters (23).

The rainfall experienced in the district ranges from 350 mm during the dry season to 650 mm during the wet season, with an average altitude of 494 m above sea level (23, 25). The district is largely rural, with the majority of the indigenous inhabitants in the study area belonging to the Bapedi ethnic group (26).

Ethnoveterinary knowledge survey

A purposive sampling method was used to select localities based on the availability of different plant biodiversity, number of livestock, and topography position. The four local municipalities were selected, with specific villages targeted within these local municipalities named: Ga-Phaahla, Ga-Nkoana Masehlaneng, Mamatsekele, Mampane, Ga-Mampuru, Ga-Malekana, Makgatle, Mohlotsi, Ga-Rakgwadi, and Tafelkop. Furthermore, participants were selected based on socio-demography factors, ensuring an equal chance of inclusion for all groups in the sample. Permission to conduct the research and to access the communities was obtained from traditional leaders. The purpose of the research was explained to both the traditional leaders and participants in the local language, Sepedi.

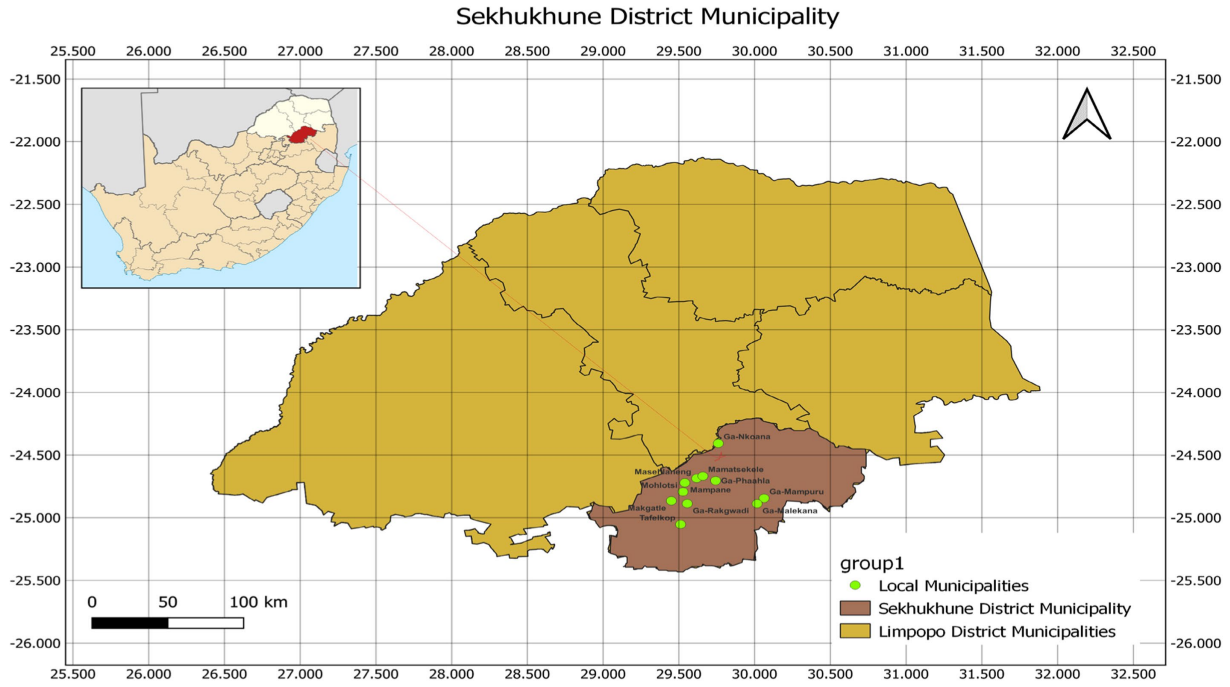

FIGURE 1
A map of Limpopo Province showing the study area (Sekhukhune District) highlighted in brown color.

Indigenous knowledge documentation / data collection

Oral interviews were conducted with approximately 250 participants between July 2021 and June 2022. Data were collected using a semi-structured questionnaire and a guided field survey among the participants. A questionnaire was designed to gather data on the names of plants used for controlling cattle ticks, the source of these plants, the part(s) used, the methods of preparing remedies, and different ways of administering the remedies to cattle. The questionnaire was translated into the local native language to ensure an accurate understanding of the questions asked.

Plant collection and identification

Plants were collected from their natural habitat in Sekhukhune District, under the guidance of the participants. Plant samples were collected when mentioned twice for ethnoveterinary use by at least two participants (27). Botanical data were collected using a collection form prescribed by the South African National Biodiversity Institute (SANBI). Pictures of the plants were taken with a digital camera, and precise coordinates of the locations were recorded using a GPS instrument. Specimens were collected, labeled, and pressed according to methods described in a previous study (28). One specimen was sent to the SANBI for identification. Approximately 2 kg of fresh plant materials was collected, dried in the shade, ground to a powder, and stored in darkened glass jars for subsequent laboratory investigations.

Statistical analysis

All collected data were analyzed using statistical software (Stata 17.0). Descriptive statistics were applied for quantitative analysis. Univariate analyses were presented using frequencies and percentages. Bivariate analysis using cross-tabulation was presented using frequency counts and percentages.

Results

Demographic information of the participants

A total of 250 participants, with an age range from 18 to over 60 years, were recruited into the study. Among these participants, the most dominant group included the male participants, comprising 60.8%, followed by the female participants at 3%. In addition, the male and female participants in the age group of 60 and above were the most dominant, with a frequency of 34 and 22.0%, respectively. The demographic information of the participants during the study period is provided in Table 1.

Level of education in the study area during the study period

The results of the survey revealed the majority of the participants were either secondary school dropouts or still in secondary school, with the male and female participants

comprising 22 and 17.6%, respectively. Moreover, 18.4% of the male participants and 11.2% of the female participants were found to have dropped out of primary school. Among all the participants, 11.2% of the male participants and 3.2% of the female participants were found to either have a tertiary qualification or be studying at the university. Furthermore, 9.2% of the male participants and 7.2% of the female participants were found to have no formal education. Table 2 provides a summary of the level of education during the study period.

An indicative summary of the level of employment in the study area

The survey revealed that a significant proportion (52.8%) of the participants were dependent on social welfare grants, as shown in Table 3. The survey further revealed that 16.8% had professional jobs, while 16% of the participants had non-professional jobs. In addition, 12.8% of the participants were unemployed, with 1.6% having unspecified sources of income.

Ethnoveterinary knowledge and practices involving the locally available medicinal plants

The results of the locally available medicinal plants and practices within the study area are shown in Table 4.

The results of the survey revealed 15 different families of the medicinal plants available within the study area. Among the 15 families, *Fabaceae* was found to have five species of medicinal plants. *Euphorbiaceae* and *Hyacinthaceae* were found to have four species, followed by *Asphodelaceae*, *Asparagaceae*, and *Asteraceae, all of* which were found to have two species of medicinal plants.

Furthermore, nine families—namely, *Araceae*, *Hypoxidaeae*, *Malvaceae*, *Orchidaceae*, *Passifloraceae*, *Rutaceae*, *Solanaceae*, *Verbenaceae*, and *Vitaceae*—were found to have only one species of a medicinal plant. The results further revealed that the majority of the plants were either administered orally or topically in the form of liquid infusions and pastes, respectively. Furthermore, *Cissus quadrangularis* was the most frequently cited species. *Cissus quadrangularis* is widely distributed in the district.

Use of the different plant parts in the preparation of ethnoveterinary medicine

Figure 2 shows the plant parts used in the ethnoveterinary practices within the study area. The results of the survey revealed that the leaves emerged as the predominant part used in preparing EVM, accounting for 31.47%, followed by a combination of medicinal plants (4.74%) and the whole plant (3.02%). The roots were the least used part, accounting for 0.43%.

Different methods for controlling cattle ticks in the study area

Table 5 shows a summary of the methods used to control cattle ticks within the study area. The results revealed that the majority of the participants used a combination of ethnoveterinary medicine and conventional acaricides, with 33.2% of the male participants and 22% the female participants, followed by conventional acaricides, with 19.6% of the male participants and 13.2% of the female participants. The least common method was EVM, with 8% of the male participants and 4% of the female participants.

Comparison of the age and different methods for controlling cattle ticks

Table 6 shows a summary of the methods used for controlling cattle ticks in comparison with the age groups within the study area. The results revealed that the participants shared one commonality: regarding all the methods of controlling ticks, the older participants, aged 60 and above, were more knowledgeable compared to other age

TABLE 1 Demographic information of the participants during the study period.

Characteristic of participants	Category	Sex	Frequency (N = 250)	Percentage
Sex		Male ♂	152	60.8
		Female ♀	98	39.2
Age group (years)	18–29	Male ♂	6	2.4
		Female ♀	1	0.4
	30–39	Male ♂	17	6.8
		Female ♀	4	1.6
	40–49	Male ♂	18	7.2
		Female ♀	14	5.6
	50–59	Male ♂	26	10.4
		Female ♀	24	9.6
	≥ 60	Male ♂	85	34
		Female ♀	55	22

TABLE 2 The level of education in the study area during the study period.

Characteristic of participants	Category	Sex	Frequency (*N* = 250)	Percentage
Level of education	No formal education	Male ♂	23	9.2
		Female ♀	18	7.2
	Primary	Male ♂	46	18.4
		Female ♀	28	11.2
	Secondary	Male ♂	55	22
		Female ♀	44	17.6
	Tertiary	Male ♂	28	11.2
		Female ♀	8	3.2

TABLE 3 An indicative summary of the employment of the participants in the study.

Category		Frequency (*N* = 250)	Percentage
Employed	Professional jobs	42	16.8
	Non-professional jobs	40	16
Unemployed	Social welfare grant	132	52.8
	Unemployed	32	12.8
	Other	4	1.6

groups. This may be due to the perception among the youth that indigenous knowledge is outdated.

Nonetheless, as shown in Table 6, the younger generation had limited knowledge about all the methods, with 0% of them having knowledge about EVM, 1.2% about conventional acaricides, and 1.6% about the combination of ethnoveterinary medicine and conventional acaricides.

Discussion

Demographic information of the participants

Ethnoveterinary practice is a gender-based activity that requires a substantial amount of skills, knowledge, natural resources, and traditional beliefs, with men, women, and children of the household involved in rearing and practicing indigenous primary healthcare of livestock. The ethnic groups inhabiting Sekhukhune District use diverse flora in controlling tick infestations in cattle. Generally, the practice of EVK is mostly limited to the elders of the communities (16, 29, 30). Nonetheless, the participants in the research demonstrated rich EVK regarding the practices of tick control in cattle. As shown in Table 1, the majority of the male participants were the most knowledgeable, with approximately 60.8%, and this observation has also been reported by previous studies (17, 30–32). In addition, it was observed that the local male participants were mostly subjected to inherited livestock ownership from their parents. This, in turn, created a gender-biased system rooted in local rules that disadvantaged female individuals.

Furthermore, male individuals, starting from the age of 18, are taught ethnoveterinary practices as primary healthcare of cattle by the older generation through passive oral communication and practical applications. They (young male individuals) exchange EVK among peers at the dipping station or during herding in the bushes (16). This provided male individuals with significantly greater control over ethnoveterinary knowledge as compared to their female counterparts (16).

Moreover, sociocultural norms in many settings may limit female individuals in cattle care giving (33–35). As shown in Table 1, approximately 39.2% of the female participants were knowledgeable as female individuals are typically subjected to household duties and caregiving for poultry and small-stock (small ruminants). Furthermore, during the interviews, it was observed that the majority of the female participants preferred to let the male participants (old or young) speak as they believed that the male participants had better knowledge of this aspect of tick control in cattle. Moreover, some misconceptions contributed to the limitation of the female participants in cattle caregiving, such as female individuals are not permitted to enter the kraal. Collectively, these findings are supported by those of previous studies (36, 37), which concluded that female individuals have practically minimal knowledge of ethnoveterinary practices.

Currently, female individuals partake in the practices since the majority of households are headed by them. The results of the research correspond with those of a previous study (38). The age group of the knowledgeable participants ranged from 18 to above 60 years, as shown in Table 1. Approximately 2.4% of the participants were young male individuals aged 18–29 years.

They were particularly knowledgeable about the aspect of tick control in cattle and other aspects of primary healthcare in livestock production as they were shepherds. However, only 0.4% of the female participants were knowledgeable. In addition, in the age groups 30–39 years and 40–49 years, 11% of the male participants and 10% of the female participants and 18% of the male participants and 14% of the female participants, respectively, were knowledgeable.

The percentage of the overall knowledgeable participants between the ages of 18 and 49 years was alarming since approximately 90% of the population inhabiting Sekhukhune District is younger than 60 years (39). Furthermore, in the age groups 50–59 years and 60 years and above, 26% of the male participants and 24% of the female participants and 85% of the male participants and 55% of the female participants, respectively, were knowledgeable. Moreover, the oral transfer of IK is under great threat if not conveyed to the right person in time as valuable knowledge is lost with the passing of an elder who carries it (40).

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TABLE 4 Ethnoveterinary knowledge and practices involving the locally available medicinal plants within the study area.

Family name	Scientific name	Vernacular name	Part used	Preparation and administration
1. Asphodelaceae	*Aloe marlothii*	Sekgopha	Leaves	Crushed leaves are mixed with water and applied to infested sites, or infusions are given orally.
	Aloe castanea Schönland	Segafane	Leaves	Crushed leaves are mixed with water and applied to infested sites.
2. Asparagaceae	*Agave americana*	Sekgokgopha	Leaves	Crushed leaves are mixed with water and applied to infested sites.
	Asparagus laricinus Burch.	Leutlwautlwane	Bulbs	Crushed bulbs are mixed with water and applied to infested sites.
3. Asteraceae	*Schkuhria pinnata (Lam.) Kuntze ex Thell.*	Shatume	Whole plant	Crushed whole plant is mixed with water and applied to infested sites.
	Kleinia longiflora DC	Sebale	Stems	Crushed stems are mixed with water and applied to infested sites.
4. Araceae	*Stylochaeton natalensis Schott*	Mokunya	Bulbs	Crushed bulbs are mixed with water and applied to infested sites.
5. Euphorbiaceae	*Croton gratissimus Burch.* Var. *gratissimus*	Mologa	Leaves	Crushed leaves are mixed with water and applied to infested sites.
	Euphorbia cooperi N.E.Br ex A.Berger	Mokgwakgwata	Stems	Crushed stems are mixed with water and applied to infested sites.
	Jatropha zeyheri Sond.	Sefapabadimo	Roots	Crushed roots are mixed with water and applied to infested sites.
	Tragia dioica	Motšhetšherepe / motšhegerepe	Leaves	Crushed leaves are mixed with water and applied to infested sites.
6. Fabaceae	*Elephanttorrhiza elephantine (Burch.) Skeels*	Mošitšane	Leaves and roots	Crushed leaves and roots are mixed with water and applied to infested sites.
	Peltophorum africanum Sond.	Mosehla	Bark	Crushed bark is mixed with water and applied to infested sites.
	Rhynchosia atropurpurea Germish.	Tshokang	Leaves	Crushed leaves are mixed with water and applied to infested sites.
	Senegalia burkei (Benth.) Kyal. & Boatwr.	Mokgwaripa	Thorns	Pull out ticks
	Senegalia mellifera (Vahl) Seigel & Ebinger subsp. detinens (Burch.) Kyal. & Boatwr.	Mongana	Thorns	Pull out ticks
7. Hyacinthaceae	*Drimia altissima (L.f) Ker Gawl*	Sekgaga	Bulbs	Crushed bulbs are mixed with water and applied to infested sites.
	Drimia sanguinea	Sekanama	Bulbs	Crushed bulbs are mixed with water and applied to infested sites.
	Albuca shawii Baker	Serantša	Bulbs	Crushed bulbs are mixed with water and applied to infested sites.
	Ledebouria inquinata (C.A.Sm.) Jessop.	Mantsikinyane	Bulbs	Crushed bulbs are mixed with water and applied to infested sites.
8. Hypoxidaceae	*Hypoxis obtusa Burch. ex Ker Gawl.*	Monnamaledu	Bulbs	Crushed bulbs are mixed with water and applied to infested sites
9. Malvaceae	*Dombeya rotundifolia (Hochst.) Planch. var. rotundifolia*	Mokgoba	Leaves	Crushed leaves are mixed with water and applied to infested sites.
10. Orchidaceae	*Eulophia petersii (Rchb.f.) Rchb.f.*	Mongwang wa taba	Bulbs	Crushed bulbs are mixed with water and applied to infested sites.
11. Passifloraceae	*Adenia fruticose Burtt Davy subsp. fruticosa*	Mopowane	Bulbs	Crushed bulbs are mixed with water and applied to infested sites.
12. Rutaceae	*Verpris reflexa I. Verd*	Pharagobe	Leaves	Crushed leaves are mixed with water and applied to the infested site.
13. Solanaceae	*Solanum litchtensteinii Wild*	Thola	Fruits	Juice of the fruits is applied to infested sites. Apply the fruit extract directly to the tick, repeatedly.
14. Verbenaceae	*Lippia javanica (Burm.f.) Spreng*	Mošunkwane	Leaves	Crushed leaves are mixed with water and applied to infested sites.
15. Vitaceae	*Cissus quadrangularis L.*	Monokelela	Stems	Crushed stems are mixed with water and applied to infested sites. Apply the extract directly to the tick, repeatedly.

The majority of the youth showed no interest in the indigenous practices for animal health caregiving. Nonetheless, adults and elders regularly practice it and are willing to pass on the IK. Similar results were reported by previous studies (40, 41). It was observed that the older participants believed that EVM is more effective compared to conventional acaricides (7). Furthermore, they supported their practice stating that EVM has fewer side effects.

Level of education in the study area during the study period

A small percentage of the participants, approximately 16.4% (male and female), lacked formal education, predominantly within the elderly group, as shown in Table 2. They were observed to rely more on IK compared to those with formal education. These findings are consistent with the results reported by a previous study (42). Approximately 83.6%

(male and female) of the participants had formal education, ranging from primary to tertiary level. Furthermore, it was observed that the selected knowledgeable participants with formal education and access to additional finance tend not to practice the indigenous traditions, and this might have an influence on the abandonment of ethnoveterinary practices (43). In addition, the diminishing of ethnoveterinary knowledge and practices might be a reflection of cultural changes observed in migration or contact with other cultures, changes in the environmental resources and beliefs, and technology interventions. Furthermore, with access to modernized practices, the younger generation tends to use veterinary services and over-the-counter conventional acaricides, neglecting indigenous practices. Thus, the results are supported by those of previous studies (16, 20, 44). Furthermore, employing modernized practices in treating or controlling ticks in cattle production posed challenges for the participants. When utilizing conventional medicine, issues such as skin burns and the development of acaricidal resistance in ticks were observed, particularly with prolonged use, similar results were reported by a previous study (45).

An indicative summary of the level of employment in the study area

The primary household income source was social welfare grants, accounting for approximately 52.8% (male and female), as shown in Table 3. The results of this study agree with those of a previous study (38), which reported that the majority of participants were of the older generation (retirement age). However, these results are in contradiction with those of another study (43), which reported that the majority of participants who used medicinal plants to control parasites in livestock were unemployed; nonetheless, they were elders. This could be attributed to the fact that a significant number of youth and adults might have been occupied with work or school during the day when the interviews were conducted or they might have migrated to urban areas for new opportunities (20). This was followed by the participants in professional jobs (16.8%), non-professionals (16%), unemployed (12.8%), and the smallest group, other (1.6%), which included varsity students. It was observed that the majority of the participants were dependent on government social welfare grants. The participants, therefore, used EMV to provide their livestock with basic healthcare (42).

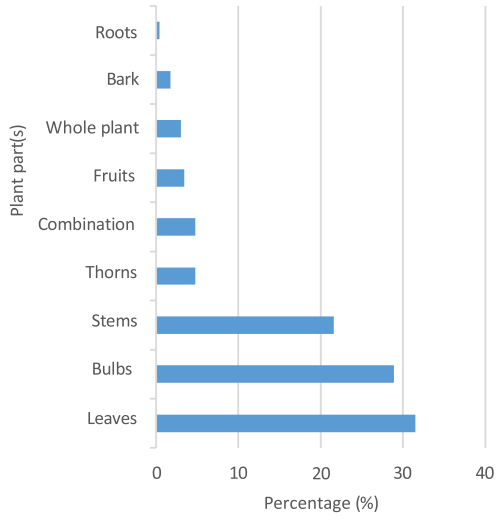

FIGURE 2
Plant parts used in the preparation of ethnoveterinary medicine within the study area.

Ethnoveterinary knowledge and practices involving the locally available medicinal plants within the study area

All plant species referenced in Table 4 were collected from the wild as it is believed that cultivated plants, those near roadsides, or those within homesteads are not as effective as EVM, contrary to the results reported by a study (46), which stated that selected EVM were found on farms. The results of this study revealed a total of 28 plant species from 15 families cited by the participants. *Fabaceae* emerged as the dominant family with five plants, consistent with the results reported by previous studies (20, 32, 47), followed by *Euphorbiaceae* and *Hyacinthaceae* (four plant species each), *Asparagaceae*, *Asphodelaceae,* and *Asteraceae* (two plant species each), and *Araceae*, Hypoxidaceae, *Malvaceae*, *Orchidaceae*, *Pasicfloraceae*, *Rutaceae*, *Solanaceae*, *Verbenaceae,* and *Vitaceae,* each with one plant species cited. However, contrary to the results reported by a previous study (48), which stated that *Lamiaceae* is the dominant family used in EVM, this discrepancy might be due to differences in species abundance and a lack of IK in the location. The majority of the medicinal plants used in controlling cattle ticks were applied as water-based extracts, followed by raw juice and roasted formulations. However, the roasted plant parts were mixed with paraffin to create a paste, as detailed in Table 4. The primary route of administration was topical, followed by oral. Moreover, *Aloe Marlothii* was the only medicinal plant that was used in both forms of administration, similar to the results reported by previous studies (11, 14, 49).

The study identified some selected medicinal plants with precautionary warnings due to skin irritation, while the plants producing milky latex or fruits were deemed highly poisonous. Some selected medicinal plants had thorns that required careful handling. Furthermore, species such as *Euphorbia cooperi* are known for their highly poisonous milky latex to humans and animals. According to previous studies (50–52), *Euphoria cooperi* may have therapeutic potential against inflammatory diseases and possess antioxidant activity. In addition, other studies (53, 54) have reported that *Solanum lichtensteinii* might have therapeutic potential for skin-related diseases. Nevertheless, the majority of the cited medicinal plants in the study are user-friendly and commonly used in human healthcare by the Bapedi ethnic group in Limpopo Province, including *Lippia javanica*, *Schkuhria pinnata*, *Aloe marlothii*, *Kleinia longiflora,* and *Vepris reflexa* (12, 24, 55, 56).

Use of the different plant parts used in the preparation of ethnoveterinary medicine within the study area

The cited plant parts used in ethnoveterinary practices are shown in Figure 2. The leaves were the most commonly used part to prepare medication (31.47%), followed by the bulbs (28.88%), the stems (21.55%), the thorns, a combination of medicinal plants (4.74%), the fruits (3.45%), the whole plant (3.02%), the barks (1.72%), and the

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TABLE 5 Different methods for controlling cattle ticks in the study area.

	Category	Sex	Frequency (*N* = 250)	Percentage
Method of animal treatment	Ethnoveterinary medicine	Male ♂	20	8
		Female ♀	10	4
	Conventional acaricides	Male ♂	49	19.6
		Female ♀	33	13.2
	A combination of ethnoveterinary medicine and conventional acaricides	Male ♂	83	33.2
		Female ♀	55	22

TABLE 6 Comparison of the age and different methods for controlling cattle ticks.

	Category	Age	Frequency (*N* = 250)	Percentage
Comparison of the age and different methods for controlling cattle ticks	Ethnoveterinary medicine	18–29	0	0
		30–39	3	1.2
		40–49	0	0
		50–59	7	2.8
		>60	20	8
	Conventional acaricides	18–29	3	1.2
		30–39	7	2.8
		40–49	10	4
		50–59	18	7.2
		>60	44	17.6
	A combination of ethnoveterinary medicine and conventional acaricides	18–29	4	1.6
		30–39	11	4.4
		40–49	22	8.8
		50–59	25	10
		>60	76	30.4

least used plant part, the roots (0.43%). According to the participants, the leaves are the most commonly used medicinal plant part in EVM applications due to their availability and abundance. Moreover, they are efficient and carry the least risk of damaging the plant. However, the roots were the least used plant part in the study, which might be attributed to the fact that the roots are not easily accessible and their use could endanger the plant species.

According to previous studies (18, 49, 57–61), which reported similar results, the leaves of selected medicinal plants were found to possess acaricidal activity against cattle ticks. Furthermore, other studies recorded that the following plant parts possessed acaricidal activity and mechanisms for controlling ticks: bulbs, stems, bark, roots, thorns, fruits, and the whole plant. The results are supported by those of previous studies (16, 40, 60, 62). However, in this study, seeds were not cited by the participants. Nevertheless, a previous study (61) reported that cumin seeds (*Cuminum cyminum*) possess acaricidal activity against cattle ticks. This information was not cited in the study due to the origin or availability of the plant in the study area. The plant parts were collected from the wild and used fresh, with no reference to storing them for future use. However, a few participants reported that *Stylochaeton natalensis* and *Hypoxis obtuse* were scarcer in their areas, so they tended to store the plant parts. Moreover, the predominance of fresh materials in ethnoveterinary medicine is contrary to human traditional medicine. This is explained by the fact that the participants resort to EVM only when remedies are needed for their animals. The plant parts were prepared as both monotherapy and in combination. In addition, EVM was applied when ticks were noticeable, and oral administration was practiced daily.

Different methods for controlling cattle ticks in the study area

The results revealed that the participants in the study used a combination of EVM and conventional acaricidal methods, with 33.2% of the male participants and 22% of the female participants. This was followed by conventional acaricidal methods, with 19.4% of the male participants and 13.2% of the female participants. The least used method was EVM, with 8% of the male participants and 4% of the female participants, as shown in Table 5. Similar results were reported by a study (63), which stated that in South Africa, indigenous people preferred to use traditional medicine even when conventional medicine was available.

However, the literature indicates that using conventional acaricides for tick control is a widely used method (63, 64). It has several advantages, such as being applicable over a large population with minimal lag time. Moreover, in the district selected, the dipping stations are supplied with conventional acaricides by the government for controlling cattle ticks. Eraditick emerged as the dominant conventional acaricidal method (39%), followed by a combination of conventional acaricides (22%), Deadline (21%), Taktic (10%), Supona aerosol (1.6%), Triatix, Copper supadip, Albotic, Delta dip, and Dazzel NF (1.2%), and the least used conventional acaricidal method was Pro-dip cyperthrin (0.81%).

Comparison of the age and different methods for controlling cattle ticks

As shown in Table 6, the results revealed that a combination of EVM and conventional acaricides was most commonly used by the elderly generation (60 years and above), with a frequency of 30.4%, followed by conventional acaricides (17.6%), and the least used method was EVM (8%). Furthermore, the other age groups were less knowledgeable. However, this result is in contrast with those of a previous study (20), which reported that the majority of the conventional acaricides were used by the youth as they believed that conventional acaricides were more convenient and required less work. The study also reported that ethnoveterinary knowledge resources, such as water and transport, were less utilized unless they used pour-on. In addition, when conventional acaricides are used for prolonged periods, ticks develop resistance to them and the compounds are not easily leashed in the environment. These results are supported by those of previous studies (45, 65).

Conclusion

The four selected local municipalities within Sekhukhune District are predominantly rural, with the majority of the population being Bapedi. Inhabitants of these municipalities possess extensive knowledge of ethnoveterinary practices for controlling cattle ticks and other essential healthcare practices for livestock. Based on the reported results, 28 medicinal plants have been identified as possessing acaricidal activity, underscoring the continued importance of ethnoveterinary practices in livestock farming.

Ethnoveterinary knowledge (EVK) is primarily restricted to herders, farmers, and elderly men in the communities. Although elderly women exhibit moderate knowledge, the majority of the youth remain unaware of these indigenous practices due to modernization trends. Furthermore, given that the majority of the knowledgeable participants were elderly, there is a risk to the preservation of EVK.

Therefore, the study aimed to conserve traditional EVK from extinction and document its knowledge in its purest form. Furthermore, ethnoveterinary practices reflect the unique cultural and ecological context of the region, underlining the need for continued research, documentation, and potential incorporation of these methods into broader tick control strategies to address the challenges posed by chemical resistance and the environmental impacts of conventional acaricides.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The studies involving humans were approved by Department of Life and Consumer Sciences, UNISA-CAES Animal Research Ethics Committee, REC Reference number: 2021/CAES_AREC/106. The studies were conducted in accordance with the local legislation and institutional requirements. The participants provided their written informed consent to participate in this study.

Author contributions

CP: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Validation, Visualization, Writing – original draft, Writing – review & editing. JS: Data curation, Formal analysis, Funding acquisition, Methodology, Resources, Supervision, Writing – original draft, Writing – review & editing. VM: Data curation, Formal analysis, Methodology, Software, Writing – review & editing. RM: Data curation, Formal analysis, Methodology, Writing – review & editing. SM: Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Resources, Software, Supervision, Validation, Writing – original draft, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Appendix. Pictures of the selected medicinal plants used in this study

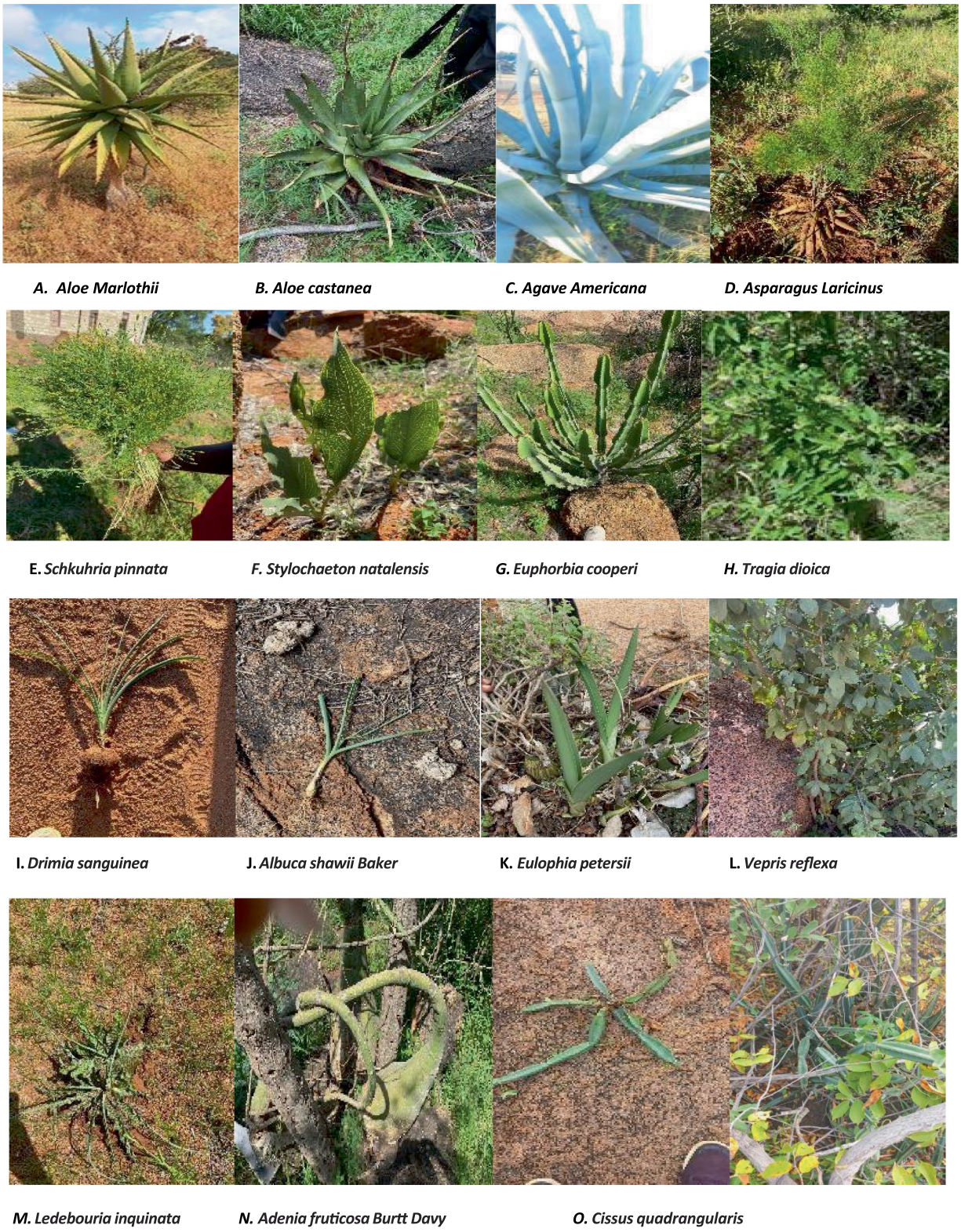

FIGURE A1
Selected medicinal plants available within the study area.

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EDITED BY
Mourad Ben Said,
University of Manouba, Tunisia

REVIEWED BY
Elizabeth Salinas Estrella,
Instituto Nacional de Investigaciones Forestales, Agrícolas y Pecuarias (INIFAP), Mexico
Gloria Castañeda,
Autonomous University of the State of Morelos, Mexico

*CORRESPONDENCE
Sara Salah Abdel-Hakeem
sara_assiut86@aun.edu.eg

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Piroplasm infestations in cattle: exploring tick control using *Chrysanthemum* extract and neem oil emulsion

Salwa Mahmoud Abd-Elrahman[1], Fatma Atea Kamel[1], Sara Salah Abdel-Hakeem[2]*, Abeer A. Khedr[3,4], Shaymaa M. Mohamed[5,6], Ahmed A. Abdelgaber[7], Madeha Darwish[8], Ahmed M. Al-Hakami[9], Abdulah J. Alqahtani[10] and Ahmed Kamal Dyab[11,12]

[1]Department of Parasitology, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, [2]Parasitology Laboratory, Department of Zoology and Entomology, Faculty of Science, Assiut University, Assiut, Egypt, [3]Department of Biosciences, Durham University, Durham, United Kingdom, [4]Department of Parasitology, Faculty of Veterinary Medicine, New Valley University, El-Khargah, Egypt, [5]Department of Pharmacognosy, Faculty of Pharmacy, Assiut University, Assiut, Egypt, [6]Department of Pharmacognosy and Pharmaceutical Chemistry, College of Pharmacy, Taibah University, Medina, Saudi Arabia, [7]Department of Animal, Poultry, and Aquatic Life Behavior and Management, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, [8]Department of Animal and Poultry Behavior and Management, Faculty of Veterinary Medicine, Assiut University, Assiut, Egypt, [9]Department of Clinical Microbiology and Parasitology, College of Medicine, King Khalid University, Abha, Saudi Arabia, [10]Department of Microbiology and Clinical Parasitology, College of Medicine, King Khalid University, Abha, Saudi Arabia, [11]Department of Medical Parasitology, Faculty of Medicine, Assiut University, Assiut, Egypt, [12]Department of Parasitology, School of Veterinary Medicine, Badr University in Assiut, Assiut, Egypt

Introduction: Tick-borne diseases represent a major threat to both animal and human health globally. This study explores the prevalence of tick infestation and associated piroplasm infections specifically *Theileria* and *Babesia* species in cattle, in addition to evaluating the acaricidal effectiveness of *Chrysanthemum* extract (*Dendranthema grandiflora*) and neem oil emulsion (*Azadirachta indica*).

Methods: Among 130 cattle examined, 61 were infested with ticks and subsequently screened for piroplasm infections. Molecular analysis identified infections caused by *Theileria annulata* and *Babesia bigemina*.

Results: A strong association was found between tick infestation and *Babesia* species, while *T. annulata* infection showed a slight correlation. Hemolymph examination confirmed the critical role of ticks in the life cycle of piroplasm infection. *Chrysanthemum* extract and neem oil were tested for their acaricidal properties against adult ticks (*Rhipicephalus annulatus*). *Chrysanthemum* extract (0.5 mg/mL) caused tick mortality within 24 h. However, neem oil induced rapid and significant tick mortality at (20 mg/L) and (15 mg/L), achieving 100% mortality within the same time frame. Both treatments demonstrated high effectiveness, with results indicating strong dose-and time-dependent effects compared to controls. Scanning electron microscopy (SEM) revealed extensive morphological damage to treated ticks. This damage included destruction of the hypostome, loss of surface striations, wrinkling with pore formation, and cracking following exposure to neem oil and *Chrysanthemum* extract.

Discussion: These findings highlight the potential of *D. grandiflora* extract and neem oil emulsion as effective natural acaricides for controlling tick infestations and reducing tick-borne diseases.

KEYWORDS

Theileria annulata, *Babesia bigemina*, *Rhipicephalus annulatus*, hemolymph, acaricides, scanning electron microscope

1 Introduction

Ticks are blood-feeding ectoparasites that pose a global health concern. The cattle tick, *Rhipicephalus annulatus*, is one of the most economically significant vectors affecting human and animal health (1). Distributed across many tropical and subtropical areas, where they naturally contribute to the maintenance of tick-borne diseases in both animals and humans (2). Consequently, ticks may be an effective indicator for tracking tick-borne parasites (TBPs) due to their capacity to transmit blood and pathogens from many hosts. Data on the geographical distribution of pathogens in ticks help assess the risk of exposure to tick bites and, consequently, the risk of disease transmission (3).

Tick-borne diseases such as theileriosis and babesiosis significantly threaten the cattle industry (4). Blood parasites are transmitted when infected ticks inject parasites into the host's bloodstream while feeding (5). In Egypt, *T. annulata* causes tropical theileriosis, characterized by symptoms such as fever, anorexia, jaundice, tachycardia, difficulty breathing, lymphadenopathy, and general weakness, severely impacting cattle productivity (6). Different species of *Babesia*, (*B. divergens*, *B. naoakii*, *B. bigemina*, and *B. bovis*) cause babesiosis, which is characterized by fever, hemolytic anemia complicated with neurological and respiratory disorders, which can be fatal (7). The molecular characterization and phylogenetic analysis of *Babesia* and *Theileria* are essential for understanding the diversity, evolution, and epidemiology of these protozoan parasites (28). This knowledge is crucial for developing targeted control measures, vaccines, and diagnostic tools, which ultimately aid in managing tick-borne diseases. Additionally, genetic characterization can provide insights into host-parasite interactions and help predict how these pathogens may adapt to environmental changes or develop resistance to treatments. This underscores the importance of this research in both veterinary and human health (59).

Therefore, addressing tick control is essential for sustaining cattle health and productivity (8). Currently, the National Drug Authority has registered more than 25 acaricide brands readily available to farmers (9). Regrettably, ticks in Egypt have developed resistance to all available acaricides (10). This resistance and environmental and health concerns have driven a shift toward exploring alternative tick control methods (11). Consequently, there is upwards interest in evaluating the acaricidal efficacy of natural, environmentally sustainable, and safer biological agents, such as essential oils and plant extracts (12).

Natural products often contain bioactive compounds that exhibit insecticidal, repellent, and growth-regulating properties (13). Among these alternatives is Pyrethrum, which is recognized as a substantial reservoir of pyrethrins (14). They are derived from the flowers of *Chrysanthemum cinerariaefolium* and have been used as insecticides for millennia. These natural neurotoxins target the nervous system of insects, causing paralysis and death (15). They are effective against a broad spectrum of pests as they act rapidly upon contact (16). The development of synthetic pyrethroids, such as permethrin, has enhanced the insecticidal properties and environmental stability of these compounds. Consequently, they are extensively utilized in agricultural, veterinary, and household pest control products (17). Other species of *Chrysanthemum* may have insecticidal properties for tick control, but there is limited research on their effectiveness against *R. annulatus* ticks.

Neem oil, derived from the seeds of the neem tree (*Azadirachta indica*), is a natural source of insecticidal compounds. It contains a complex mixture of bioactive compounds. The primary compound, azadirachtin, disrupts various physiological processes in ticks, such as feeding, reproduction, and molting (18, 19). It acts as an insect growth regulator (IGR) and interferes with the synthesis and release of molting hormones, leading to disruption of the molting process and future reproduction (20). Additionally, azadirachtin reduces ticks by deterring them from attaching to and feeding on their hosts due to its anti-feedant and repellent properties. Using botanical insecticides, such as *Chrysanthemum* extract and neem oil, is consistent with the principles of integrated pest management (IPM). IPM underscores the use of multiple, complementary control strategies to attain effective acaricidal management while reducing its environmental impact (21). Despite the potential of *Chrysanthemum* extract and neem oil as tick control agents, significant knowledge gaps need addressing. Research is needed to determine the best formulations, application methods, and dosages for these insecticides to maximize their effectiveness in controlling *R. annulatus* while ensuring the safety of animals. This research aims to provide valuable insights for the development of alternative and eco-friendly strategies for tick control.

2 Materials and methods

2.1 Sample collection and study area

A total of 130 cattle, aged between one and five years old, were examined at the veterinary clinic of the Faculty of Veterinary Medicine at Assiut University in Assiut, Egypt, from January 2023 to December 2023. The examined cattle consisted of 52 males and 78 females. Only infested animals with ticks (61) were included in the study to further assess tick-piroplasm association.

2.2 Blood collection and thin blood film examination

Five milliliters of blood were collected from the jugular vein of all animals using sterile vacutainer tubes containing anticoagulants. The blood samples were then transported on ice to the Parasitology Department for further examination. Fresh thin blood smears were prepared, dried, and fixed in methanol to identify piroplasm infection using a light microscope (Olympus BX43F, Tokyo 163-0914, Japan). Positive samples were used for molecular analysis and species identification (22).

2.3 Genetic characterization of *Theileria* and *Babesia* species

2.3.1 DNA extraction and PCR amplification

All positive samples with same morphological characteristics, obtained from the same host and region, underwent DNA genome extraction using the QIAamp DNA Mini Kit (Catalogue 51304, Qiagen). Two target genes were selected for amplification: *tams1* for *Theileria annulata* and 18S rRNA for *Babesia*. The primer sequences for each gene were as follows: *tams1* primers were 5′-GTAACCTTTAAAAACGT-3′ and 5′-GTTACGAACAT GGGTTT-3′, yielding a 721 bp product (23). The 18S rRNA primers were 5′-GTCTTGTAATTGGAATGATGGTGAC-3′ and 5′-ATGCCCCCAACCGTTCCTATTA-3′, producing a 340 bp product (24). PCR amplification was carried out using the EmeraldAmp GT PCR Master Mix (Takara, Code No. RR310A). Each reaction mixture involved (12.5 μL) of 2× premix, (5.5 μL) of PCR-grade water, (1 μL) of each primer (20 pmol), and (5 μL) of a DNA template, and the total reaction volume was (25 μL). The conditions of the thermal cycling started with initial denaturation for 5 min at 94°C, followed by 35 cycles for 30 s at 94°C, annealing for 40 s at 55°C, and extension for 45 s at 72°C (for *tams1*), and 40 s (for 18S rRNA), final extension for 10 min at 72°C end the PCR. Ten microliters aliquots of each PCR reaction were loaded onto a 1.5% agarose gel in a horizontal gel electrophoresis system (Compact M, Biometric, Germany), stained with ethidium bromide, and visualized under UV light to confirm successful amplification (25).

2.3.2 Purification of PCR products

The QIAquick PCR Purification Kit (Qiagen Inc., Valencia, CA) was used to purify PCR products. The PCR product was mixed with Buffer PB1 (five volumes), the mixture was added to a QIAquick spin column, washed with Buffer PE, and then the purified DNA was eluted with nuclease-free water. This procedure needs approximately 10 min to produce purified DNA suitable for subsequent sequencing applications.

2.3.3 DNA sequencing and analysis

PCR products were sequenced in forward and reverse directions using an automated DNA sequencer (Applied Biosystems, United States). The sequencing was performed with the BigDye Terminator v3.1 Cycle Sequencing Kit (Perkin-Elmer/Applied Biosystems). After sequencing, the NCBI BLAST tool was used to compare the sequences to those in GenBank that already existed. This comparison allowed us to identify similarities and differences between the freshly generated sequences and those already in the database.

2.4 Phylogenetic analysis

Following sequencing, the obtained sequences were aligned using the CLUSTAL W algorithm, integrated into the MegAlign software (version 12.1) (26). MEGA11 software was used to perform Phylogenetic analyses, engaging multiple methods such as maximum likelihood, neighbor-joining, and maximum parsimony (27).

2.5 Ticks' isolation and identification

The ticks were carefully removed using soft forceps and prepared for morphological characterization. They were steeped in potassium hydroxide solution (5%) and then transferred to 2.5% an acid alcohol solution for pH regulation, dehydrated in an escalating sequence of ethanol alcohol, and finally immersed in xylene to become transparent for 24 h (28). Microscopic and molecular identification of tick's species, *Rhipicephalus annulatus*, was previously reported using the *coxI* gene and deposited in the Gene bank under accession number (OR965090) (28).

2.6 Direct examination of hemolymph

The isolated ticks were used to prepare the hemolymph smears, to detect the presence of ookinetes of piroplasm following the method defined by Allam et al. (29). The procedure involved amputating the distal portion of one or more legs of the tick to obtain a small drop of hemolymph on a clean glass slide. The smears were air dried, fixed in absolute methanol for 10–15 min, stained with Giemsa's stain, and examined under an oil immersion light microscope.

2.7 Preparation of *Chrysanthemum* extract

In May 2024, the aerial parts of the *Chrysanthemum* (*Dendranthema grandiflora*) were collected from the Floriculture Farm at the Faculty of Agriculture, Assiut University, Egypt. *Chrysanthemum* is an herbaceous flowering plant known for its ornamental significance. It encompasses a range of varieties displaying notable genetic diversity in traits such as flower yield, quality, and growth parameters. The specific variety under investigation is characterized by its white flowers. The plant material was air-dried, ground into a 200 g powder, and macerated in 70% methanol for 24 h. After filtration and concentration using a rotary evaporator, this process was repeated to obtain a 60 g dry residue. The *Chrysanthemum* extract was then diluted in distilled water and glycerol to create a series of varying concentrations: 0.125, 0.25, and 0.5 g/mL (30).

2.8 Determination of total phenolic acid of *Chrysanthemum* extract

In the investigation of total phenolic acid levels, a stock solution of gallic acid was prepared in methanol at a concentration of 2 mg/mL. A series of dilutions were prepared from the stock solution to achieve concentrations of 1,000, 750, 500, 375, 250, 187.5, and 125 μg/mL. Additionally, a *Chrysanthemum* extract was prepared in methanol at a concentration of 10 mg/mL. Folin–Ciocalteu method was used to quantify the total phenolic content, succeeding the procedure described by Kamtekar et al. (31). Briefly, 10 μL of either the sample or standard solution was mixed with 100 μL of Folin–Ciocalteu reagent (1:10) in a 96-well microplate. After mixing, 80 μL of 1 M Na_2CO_3 was added to the mixture and incubated at 25°C for 20 min in the dark. The resulting blue color complex was quantified at 630 nm using a spectrophotometer. The collected data were analyzed and

presented as means ± SD. We used a FluoStar Omega microplate reader to record the results.

2.9 Determining the total flavonoid content of *Chrysanthemum* extract

For this purpose, the stock solution of standard rutin was prepared in methanol (CH_3OH) at a concentration of 2,000 µg/mL. A series of dilutions were then made from this stock solution to achieve concentrations of 1,000, 500, 250, 125, 62.5, 31.25, and 15.625 µg/mL. Furthermore, the *Chrysanthemum* extract was prepared in CH_3OH at a concentration of 10 mg/mL. The total flavonoid content was evaluated using the $AlCl_3$ colorimetric assay as mentioned by Nurcholis et al. (32). For the assay, 15 µL aliquots of both the sample and standard rutin solutions were dispensed into a 96-well microplate. Then, 175 µL of CH_3OH, 30 µL of 1.25% $AlCl_3$, and 30 µL of 0.125 M CH_3COONa were sequentially added. The mix was then incubated at room temperature for 5 min, the yellow color mix was measured at 420 nm using a UV/VIS spectrophotometer. The data are presented as means ± standard deviation (SD). The results were recorded using a FluoStar Omega microplate reader.

2.10 Preparation of neem oil emulsion

Neem oil was obtained from Centa Kind Pharmaceuticals (RC# 7797, Nefertari, Limited Co., El-Fayoum, Egypt). This oil was derived from the seeds of the neem tree (*Azadirachta indica*, family: Meliaceae) and is known for its high purity level of over 98%. Commercial formulations often contain standardized concentrations of active ingredients, such as azadirachtin, which enhances their effectiveness. This standardization ensures that the product delivers reliable results, making it preferable for therapeutic applications. To prepare the emulsion, the neem oil was combined with the non-ionic surfactants (soap) in a 1:3 ratio. The process began by mixing deionized water and surfactants using a stirrer, followed by the addition of neem oil. Neem oil concentrations of 10, 20, and 25% were prepared according to Gareh et al. (33).

2.11 Acaricidal potential of *Chrysanthemum* extract and neem oil on *Rhipicephalus annulatus* adult stage

The *in vitro* adult immersion test (AIT) technique was used to evaluate acaricides sensitivity assay and water with four replications as described by FAO (34). Briefly, in sterile petri dishes, three concentrations were prepared from *Chrysanthemum* extract (0.5, 0.25, 0.125 mg/mL) and neem oil (20, 15, 10 mg/L). Phoxim (1 mL/L) was used as a positive control, deionized water, and Tween 80% (2:1) were used as the control negative group. Ten moderately engorged females (nearly the same size) were placed in 3 mL of each concentration from each treatment for 5 min. In sterile 6-well plates with filter paper, three replicates for each concentration were used to incubate the treated ticks at 28°C with a relative humidity of (80 ± 5%). Later, ticks were observed at various time intervals (3, 6, 12, 24, 48, and 72 h). Viability checks were regularly performed, if a tick did not respond to needle stimulation, it was considered dead. The mortality rate was calculated using the equation below:

$$\text{Mortality rate}(\%) = \left(\begin{array}{l}\text{Mortality rate in treated group}\\ -\text{Mortality rate in control group}\end{array}\right) / \left(100 - \text{Mortality rate in control group}\right) \times 100.$$

2.12 Evaluation of the acaricidal effect using scanning electron microscopy

R. annulatus adult ticks from the treated groups with neem oil (20 mg/L), *Chrysanthemum* (5 mg/L), phoxim (1 mL/L), and control negative were selected and preserved in 2.5% glutaraldehyde solution within phosphate-buffered saline at pH 7.4 for a minimum of 2 hours. The samples were prepared as previously described (35, 36). The samples were subsequently mounted on double-sided carbon adhesive tape, gold-coated, and analyzed using a scanning electron microscope (Joel, JSM-5400LV, Tokyo 1993, Japan) at Assiut University's Electron Microscopy Unit to assess the surface morphological alterations (37).

2.13 Statistical analysis

The chi-square test was utilized to analyze the prevalence and associated risk factors concerning the sex and age of the studied animals. The *in vitro* investigation results were processed utilizing SPSS software (version 20). Data are presented as mean ± standard deviation (SD), and differences between experimental groups were evaluated using a one-way ANOVA, with a significance threshold of $p < 0.05$ (38). For multiple group comparisons, *post hoc* analysis was performed, specifically utilizing the LSD and Duncan tests.

3 Results

3.1 Tick infestation and piroplasm infections

Out of 130 cattle examined, 61 (46.9%) were found to be infested with ticks. Analysis of thin blood films from these infested animals revealed an infection rate of 81.9% for blood piroplasm. The identified piroplasm infections included two species: *Theileria* and *Babesia*. As shown in Table 1, the chi-square test for *Theileria* infection showed a value of 3.6885 with a $p = 0.05479$, which is marginally significant, suggesting a potential association between *Theileria* infection and tick infestation. In contrast, the chi-square test for *Babesia* infection revealed a highly significant chi-square value of 22.443 with a $p = 0.00002$, indicating a strong association between *Babesia* infection and tick infestation.

3.2 Molecular identification of positive blood samples

3.2.1 *Theileria annulata*

The PCR method was utilized to amplify the *Theileria annulata* tams1 gene, resulting in the identification of 721 bp bands observed under UV light on agarose gel (1.5%) (see Figure 1A). Sequencing of the resulted PCR product confirmed the affiliation of our sample with *Theileria annulata*. The sequence has been deposited in GenBank under the accession number OR987834. The phylogenetic relationship between the Tams1 nucleotide sequences of our isolate and 14 reference isolates of *Theileria annulata* is illustrated in Figure 2. This analysis provides insights into the genetic diversity and geographical distribution of the detected pathogen. The well-supported clade structure indicates that geographical factors significantly influence genetic variation among the isolates. Notably, there is a distinct clustering of isolates from Scotland, India, the Netherlands, Pakistan, and Egypt, highlighting the impact of geographical origins on the genetic makeup of these parasites. There is a robust subgroup, including the Egyptian isolate (OR987834.1) and the isolate from the Netherlands (AF214848.1).

3.2.2 *Babesia bigemina*

The PCR method was used to amplify *Babesia* 18S rRNA, resulting in the detection of 340 bp bands visible under UV light on agarose gel 1.5% (Figure 1B). Sequencing of the resulted PCR product confirmed that our sample is affiliated with *Babesia bigemina*. The corresponding sequence has been cataloged in GenBank under the accession number OR965914. The phylogenetic relationship between our isolate (18S rRNA nucleotide sequences) and 17 reference strains of *Babesia* species is shown in Figure 3. The well-supported clade includes the newly identified *Babesia bigemina* isolates from Egypt (OR965914.1) and the isolates from Turkey (HQ197740.1 and EF446164.1). In addition, the distinct subgroup formed by the *B. bigemina* Colombian isolates (MH194393.1 and MH194392.1) shows regional genetic diversity within this species. The phylogenetic tree further shows distinct differences between various *Babesia* species. *Babesia ovata* and *Babesia major* form a separate clade, while *Babesia crassa* and *Babesia bovis* group together. There is strong support for the clade containing *Babesia orientalis* and *Babesia occultans* isolates from China.

3.3 Hemolymph examination

By light microscope, the tick hemolymph appeared to contain three main groups of cells (hemocytes), which can be classified into (prohemocytes, plasmatocytes, and spherulocytes) based on their structural characteristics. Prohemocytes are small and rounded with a high nuclear-cytoplasmic ratio and deeply basophilic

TABLE 1 Associations between piroplasm infections and ticks.

Piropalsm	Infection status	Animals infested with ticks (61)	Percentage (%)	χ^2	*p*-value
Theileria sp.	Non_infected	23	37.7	3.6885	0.05479
	Infected	38	62.3		
Babesia sp.	Non_infected	49	80.3	22.443	0.00002
	Infected	12	19.7		

Chi-square analysis was used to detect the association between tick infestation and the presence of *Theileria* and *Babesia* infection. A statistically significant difference ($p < 0.05$).

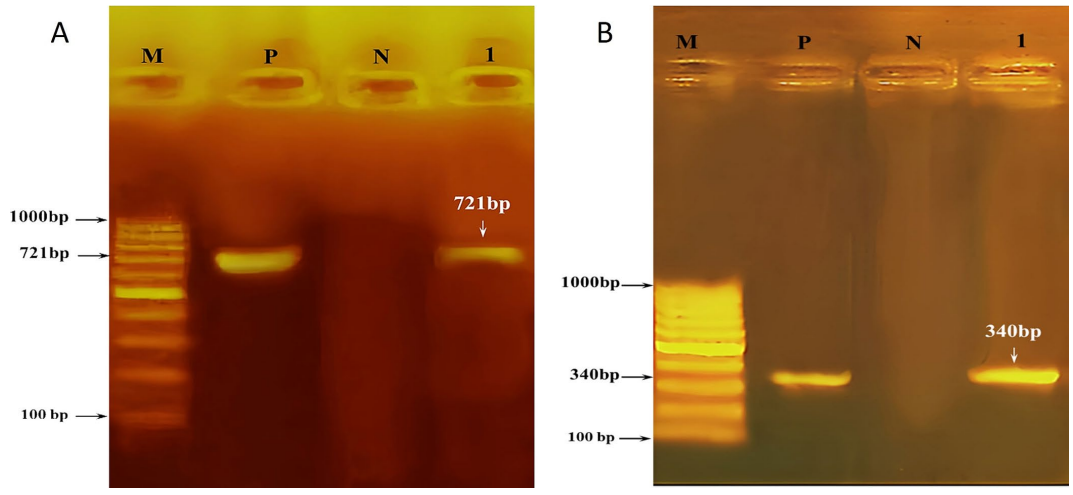

FIGURE 1
Agarose (1.5%) gel showing **(A)** PCR products (721 bp) of amplified *Theileria annulata* samples. **(B)** PCR products (340 bp) of amplified *Babesia bigemina*. Lane (M) is the DNA size marker, lane (P) is the positive control, lane (N) is the negative control, and lane (1) is the positive sample.

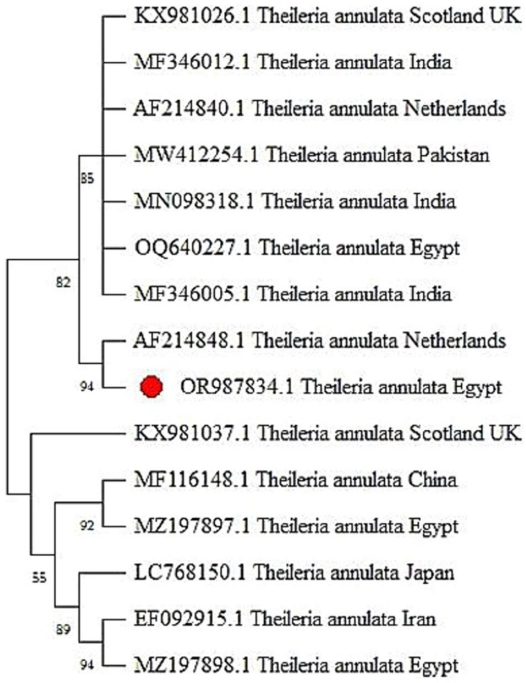

FIGURE 2
The phylogenetic tree generated from tams1 gene sequences, revealing evident clades that define distinct genetic lineages within *Theileria annulata*.

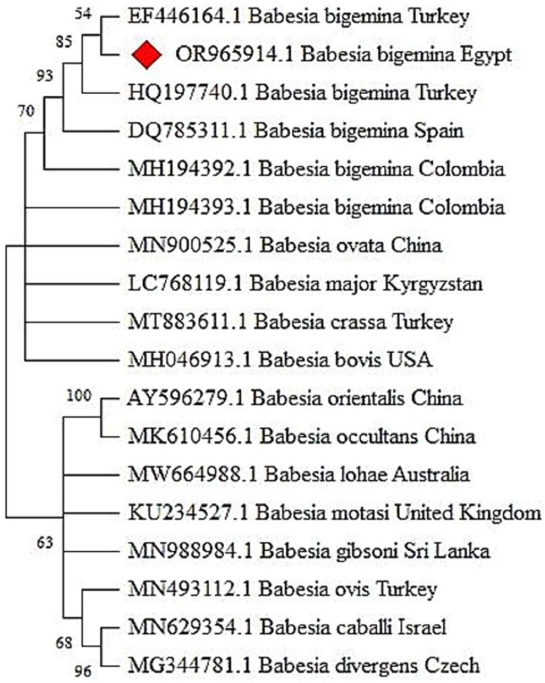

FIGURE 3
The phylogenetic tree constructed from the 18S rRNA gene sequences, unveils clear clades that delineate distinct genetic lineages of *Babesia bigemina*.

cytoplasm. They have a round, single nucleus with condensed homogeneous chromatin. Plasmatocytes are round or ovoid, with a single nucleus. The chromatin is punctate or granular, and the nucleus is usually eccentric in position. Finally, spherulocytes are frequently large, ovoid, or round cells with cytoplasm containing characteristic purple staining clusters of spherules. These spherules are so dense that the cytoplasm and the nucleus are obscured. The infected hemolymph contains ookinetes of piroplasms, which are banana-shaped with curved or semi-curved tails. These ookinetes have an anteriorly positioned nucleus, typically located in the middle of the cell (Figure 4).

3.4 *Chrysanthemum* extract: total phenolic acid content

The analysis of the total phenolic content within the hydro-methanolic extract was accomplished by referencing the gallic acid calibration curve (Figure 5A). The analysis revealed a substantial presence of phenolic compounds, with a measured content of 20.204 ± 1.75 µg gallic acid equivalent (GAE) per 1 mg of dry extract.

3.5 *Chrysanthemum* extract: total flavonoid content

The assessment of total flavonoid content in *Dendranthema grandiflora* aerial parts extract revealed noteworthy findings. Using the curve rutin calibration curve (Figure 5B), the flavonoid level was quantified, showcasing a significant flavonoid content of 34.25 ± 2.55 µg rutin equivalent (RE) per 1 mg of dry extract.

3.6 Acaricidal potential of *Chrysanthemum* extract and neem oil on *Rhipicephalus annulatus* adult stage

Chrysanthemum extract was tested at 0.5 mg/mL, 0.25 mg/mL, and 0.125 mg/mL. The acaricidal effect was increased over time, with concentrations of 0.5 mg/mL and 0.25 mg/mL showing the most pronounced effects. These concentrations induced tick mortality within the first 3 h of application, with mortality percentages of 73.3 and 60%, respectively, reaching complete mortality after 24 h. Phoxim caused mortality in all ticks within 12 h. Significant differences were observed between all concentrations of *Chrysanthemum* extract and the control group ($p < 0.001$). No significant differences were observed between different concentrations at various time points as shown in Table 2.

Neem oil was evaluated at concentrations of 20 mg/L, 15 mg/L, and 10 mg/L. Significant differences were observed between the high and low concentrations of neem oil. The mortality rate increased over time, with concentrations of 20 mg/L and 15 mg/L exhibiting the most significant effects. These concentrations induced noticeable changes and caused tick mortality within the first 3 h of application, with mortality rates of 60%. Complete mortality was achieved after 24 h. Conversely, the minimal effect was observed in the lower concentration10 mg/L, resulted in only 13.3% mortality rate within the

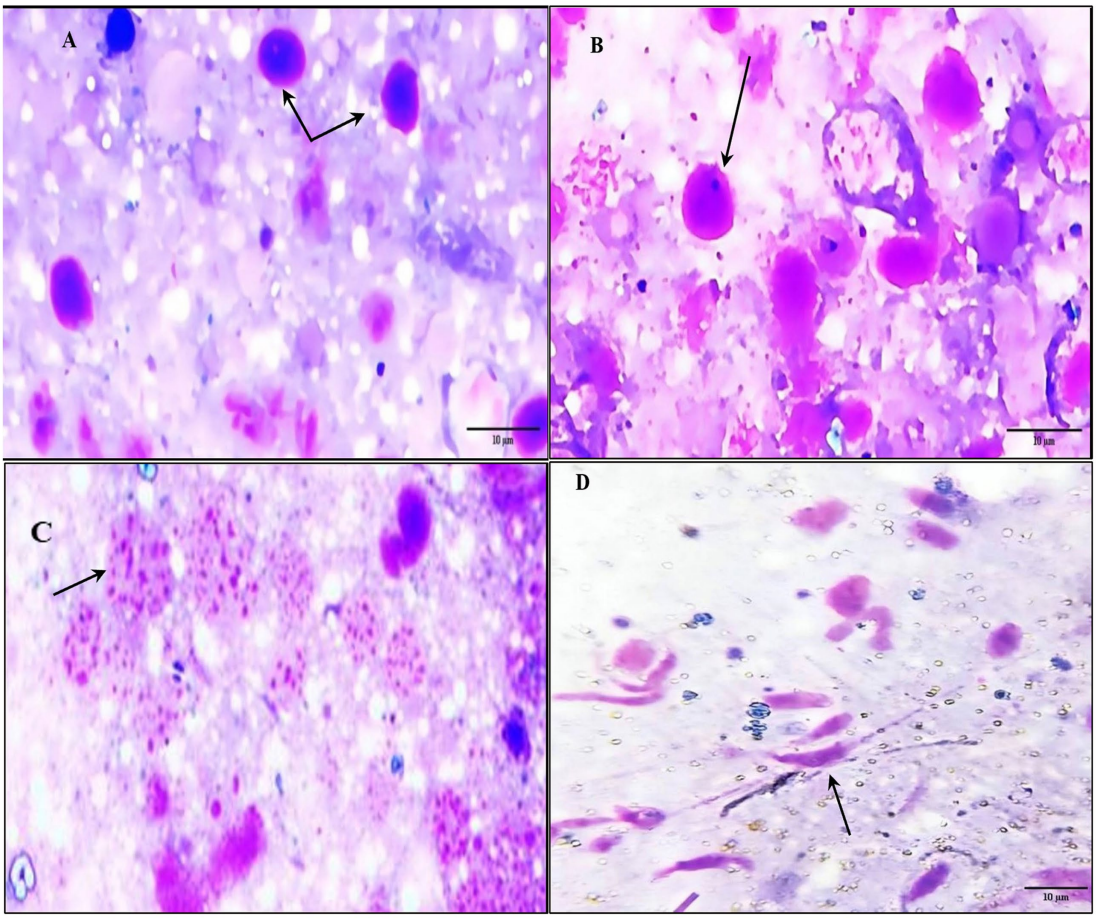

FIGURE 4
Photomicrograph showing different morphological characterization of hemolymph cells and piroplasm ookinetes in the infected samples. **(A)** Prohaemocyte (arrow). **(B)** Plasmatocytes (arrow). **(C)** Spherule (arrow). **(D)** Infected hemolymph containing piroplasm ookinetes (banana-shaped) with curved or semi-curved tails (arrow).

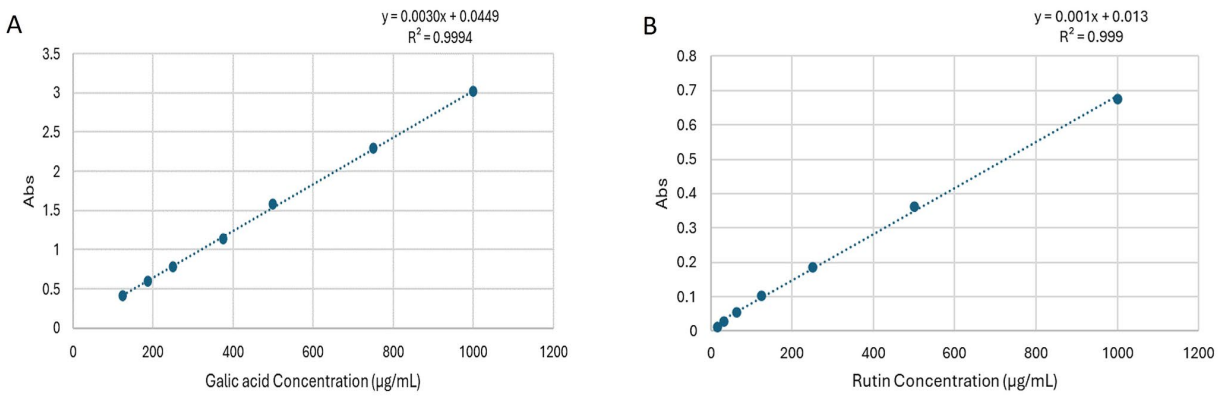

FIGURE 5
(A) Standard calibration curve showing absorbance versus gallic acid concentration for determining the total phenolic content of *Chrysanthemum* extract. **(B)** Standard calibration curve showing absorbance versus rutin concentration for determining the total phenolic content of *Chrysanthemum* extract.

first 3 h, and achieved complete mortality after 72 h of treatment. Significant differences were observed between all concentrations of neem oil and the control group ($p < 0.001$). At various time points, significant differences were also noted between the high concentrations (20 mg/L and 15 mg/L) and the low concentrations (10 mg/L) as shown in Table 3.

TABLE 2 Acaricidal effect of *Chrysanthemum* extract on *R. annulatus* adult mortality.

	Time (hours)					
	3 h	6 h	12 h	24 h	48 h	72 h
Chrysanthemum (0.5 mg/mL)	73.3 ± 11.5^{aA}	80 ± 20^{aA}	86.7 ± 11.5^{abA}	100 ± 0^{cA}	100 ± 0^{cA}	100 ± 0^{cA}
Chrysanthemum (0.25 mg/mL)	60 ± 20^{aA}	73.3 ± 11.5^{abA}	86.7 ± 11.5^{bA}	100 ± 0^{cA}	100 ± 0^{cA}	100 ± 0^{cA}
Chrysanthemum (0.125 mg/mL)	53.3 ± 11.5^{aA}	60 ± 0^{aA}	86.7 ± 11.5^{bA}	86.7 ± 11.5^{bA}	100 ± 0^{cA}	100 ± 0^{cA}
Phoxim	0^{Ab}	60 ± 0^{bA}	100 ± 0^{cA}	100 ± 0^{cA}	100 ± 0^{cA}	100 ± 0^{cA}
Control negative	0^{B}	0^{B}	0^{B}	0^{B}	0^{B}	0^{B}

Small superscript letters denote a significant difference ($p < 0.05$) in the mortality rate during the incubation periods in the same treated group (horizontal comparison). Capital superscript letters denote a significant difference ($p < 0.05$) in the mortality rate of the different treated groups in the same incubation period (vertical comparison).

TABLE 3 Acaricidal effect of neem oil on *R. annulatus* adult mortality.

Treatment	Time (hours)					
	3 h	6 h	12 h	24 h	48 h	72 h
Neem (20 mg/L)	60 ± 0^{aA}	80 ± 0^{bA}	86.7 ± 11.5^{bA}	100 ± 0^{cA}	100 ± 0^{cA}	100 ± 0^{cA}
Neem (15 mg/L)	60 ± 0^{aA}	66.7 ± 11.5^{aB}	80 ± 0^{bA}	100 ± 0^{cA}	100 ± 0^{cA}	100 ± 0^{cA}
Neem (10 mg/L)	13.3 ± 11.5^{aB}	33.3 ± 11.5^{bC}	33.3 ± 11.5^{bB}	86.6 ± 11.5^{cB}	86.6 ± 11.5^{cB}	100 ± 0^{cA}
Phoxim	0^{aC}	60 ± 0^{bB}	100 ± 0^{cC}	100 ± 0^{cA}	100 ± 0^{cA}	100 ± 0^{cA}
Control negative	0^{C}	0^{D}	0^{D}	0^{C}	0^{C}	0^{B}

Small superscript letters denote a significant difference ($p < 0.05$) in the mortality rate during the incubation periods in the same treated group (horizontal comparison). Capital superscript letters denote a significant difference ($p < 0.05$) in the mortality rate of the different treated groups in the same incubation period (vertical comparison).

3.7 Statistical correlation between high concentrations of *Chrysanthemum* extract (0.5 mg/mL) and neem oil (20 mg/L) with phoxim at various time intervals

The scatter plot shows the relationship between *Chrysanthemum* extract (0.5 mg/mL) and phoxim mortality rate (Figure 6A), the correlation coefficient is 0.67, which indicates a moderate positive linear relationship between *Chrysanthemum* extract and phoxim mortality rate, suggesting that higher mortality rates due to *Chrysanthemum* are associated with higher mortality rates due to phoxim. The correlation coefficient is 0.91 between neem oil (20 mg/L) and phoxim mortality rate, indicating a very strong positive linear relationship. As the mortality rate due to neem increases, the mortality rate due to phoxim also tends to increase. The plot contains data points representing pairs of mortality rates for neem and phoxim, most of which lie close to the fitted linear regression line, showing the data trend. Some data points lie further from the line and maybe outliers (Figure 6B). The scatter plot also demonstrates a strong positive correlation ($r = 0.8$) between neem (20 mg/L) and *Chrysanthemum* (0.5 mg/mL) treatments, indicating that as neem's efficacy increases, *Chrysanthemum*'s mortality rate also rises as shown in Figure 6C.

3.8 Evaluation of the acaricidal effect using SEM

SEM was conducted to observe surface changes in various parts of adult female *R. annulatus* specifically the capitulum, dorsal surface, and ventral surface following treatments with neem oil (20 mg/L), *Chrysanthemum* extract (0.5 mg/mL), and phoxim (1 mL/L). In the control negative group, the capitulum maintained its intact structure (Figure 7A). In contrast, the phoxim-treated group showed complete degeneration of the capitulum (Figure 7B). Neem oil treatment caused significant damage, destroying both the hypostome and the capitulum (Figures 7C,D). Similarly, *Chrysanthemum* extract caused severe damage, with complete degeneration of the mouthparts and the capitulum becoming obscured (Figures 7E,F). Upon examining the dorsal surface, the control group (Figure 8A) exhibited an intact anterior region. In contrast, the phoxim-treated group (Figure 8B) revealed numerous cracks in the cuticle. The neem oil treatment resulted in cracking of the cuticle (Figures 8C,D). However, *Chrysanthemum* extract caused more severe damage, featuring visible pores and significant cracks in the cuticle (Figures 8E,F). On the ventral surface around the anal region, the control group exhibited a normal appearance (Figure 9A), while the phoxim-treated group showed a retracted anal region (Figure 9B). However, treatment with neem oil resulted in notable changes, including retraction of the anal region, destruction accompanied by cracks and typical distribution of striations in some area with pores (Figures 9C,D), In contrast, *Chrysanthemum* extract caused wrinkling of the cuticle, leading to the formation of pores or holes (Figure 9E) and a disrupted striation pattern, along with slight degeneration of the anal region (Figure 9F).

4 Discussion

4.1 Prevalence of tick-borne piroplasm infection

This study investigated the prevalence of tick-borne piroplasms infection (*Babesia* and *Theileria*) in cattle and explored the efficacy of *Chrysanthemum* extract and neem oil as potential natural acaricides. Our results showed significant associations between tick infestation and

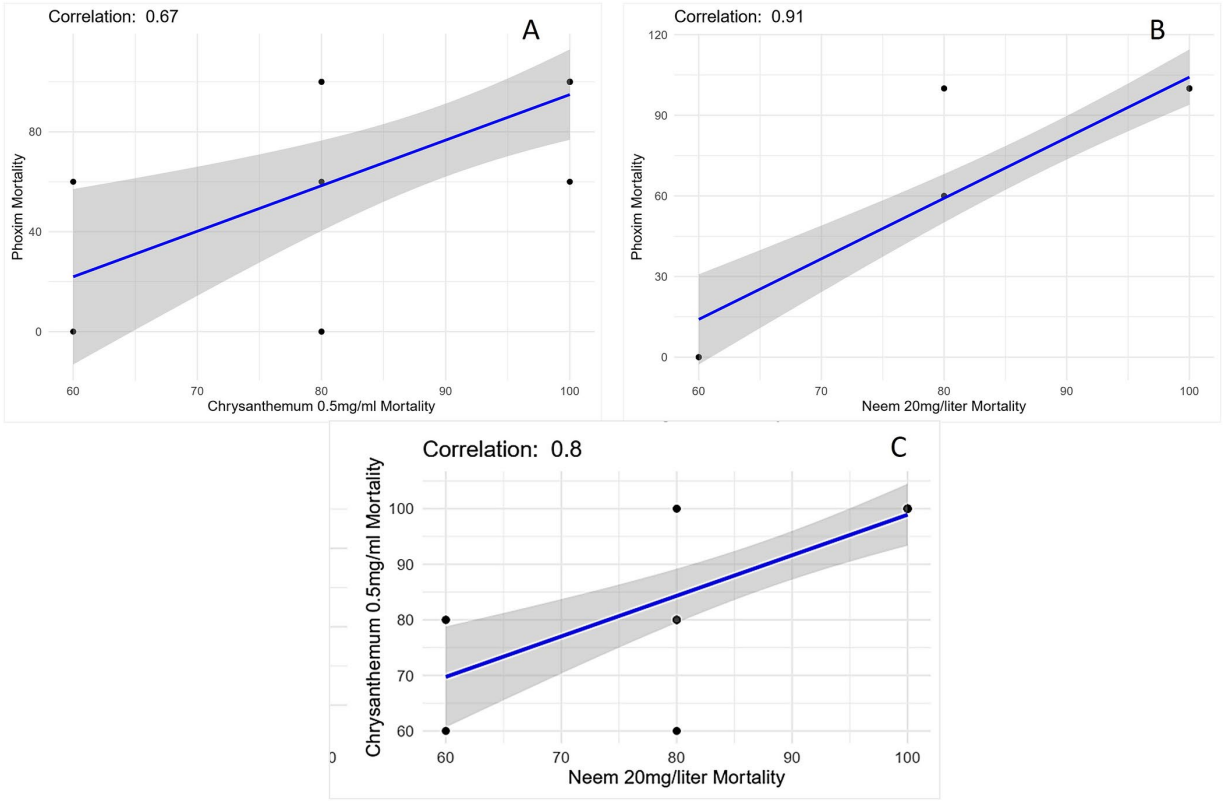

FIGURE 6
(A) Scatter plot illustrating the relationship between *Chrysanthemum* extract 0.5 mg/mL mortality and phoxim mortality with a moderate positive correlation (r = 0.67). **(B)** Scatter plot showing the relationship between neem oil 20 mg/L mortality and phoxim mortality with a strong positive correlation (r = 0.91). **(C)** Scatter plot showing the relationship between neem oil 20 mg/L mortality and *Chrysanthemum* extract 0.5 mg/mL mortality with a positive correlation (r = 0.8). The blue line represents the best-fit linear regression line, and the shaded gray area indicates the confidence interval.

piroplasm infections, with *Theileria* infections being more prevalent (62.3%) than *Babesia* infections (19.7%). These findings align with previous studies in Egypt, where *Babesia* and *Theileria* infections were reported in 11.16 and 10.25% of cattle, respectively, with *B. bigemina* and *T. annulata* being the most common species (39). However, high prevalence of *Theileria* than *Babesia* infection were reported by Prado et al. (40) and Hossain et al. (41), whereas El-Dakhly et al. (42) reported that prevalence of *Theileria* infection was (9.31%) in cattle in El-Wadi El-Gadid province. Additionally, the prevalence of *Theileria* infection was 8.3% in Sudan (43) and Bangladesh (41). In our study, *Theileria* sp. has a higher prevalence than *Babesia* sp. due to a variety of factors, including the availability of suitable tick vectors, reservoirs, and amplification hosts. These factors all contribute to the spread and persistence of *Theileria* in each ecosystem (44), which lower than those reported by El-Metenawy (45) in Saudi Arabia (76.5%) and Al-Emarah et al. (46) in Iraq (69.43%). This study contributes valuable insights into the dynamics of tick-borne diseases and enhances our understanding of their epidemiology (47).

4.2 Molecular characterization of piroplasm infection

To identify piroplasm species, the Tams1 gene for *Theileria* and the 18S rRNA gene for *Babesia* were amplified and sequenced. Phylogenetic analysis revealed genetic diversity within *T. annulata* and *B. bigemina* isolates, suggesting a need for region-specific control measures. Phylogenetic analysis of *T. annulata* revealed significant genetic diversity influenced by geography, with isolates from different regions such as Scotland, India, the Netherlands, Pakistan, and Egypt forming distinct clusters. There are multiple Egyptian isolates in different clades highlighting high genetic variability within the local population aligning with another study in upper Egypt that estimated the presence of *T. annulata* infection in cattle (48). On the other hand, the phylogenetic tree of *B. bigemina* showed a close genetic relationship between the Egyptian isolate and those from Turkey. This indicates regional genetic diversity, with distinct clades for various *Babesia* species and clear evolutionary relationships observed.

In this study, morphological characteristics of piroplasm ookinetes were examined in tick hemolymph. The presence of ookinetes, crucial for active infections, was confirmed using their distinct banana shape, curved tails, and central nucleus. These results align with findings from Martínez-García et al. (49), and are key to recognizing piroplasm ookinetes, which play a crucial role in active infections (49). Our results contribute to the broader understanding of piroplasm morphology and the role of ticks as vectors of hemoprotozoan parasites, offering valuable insights into infection dynamics.

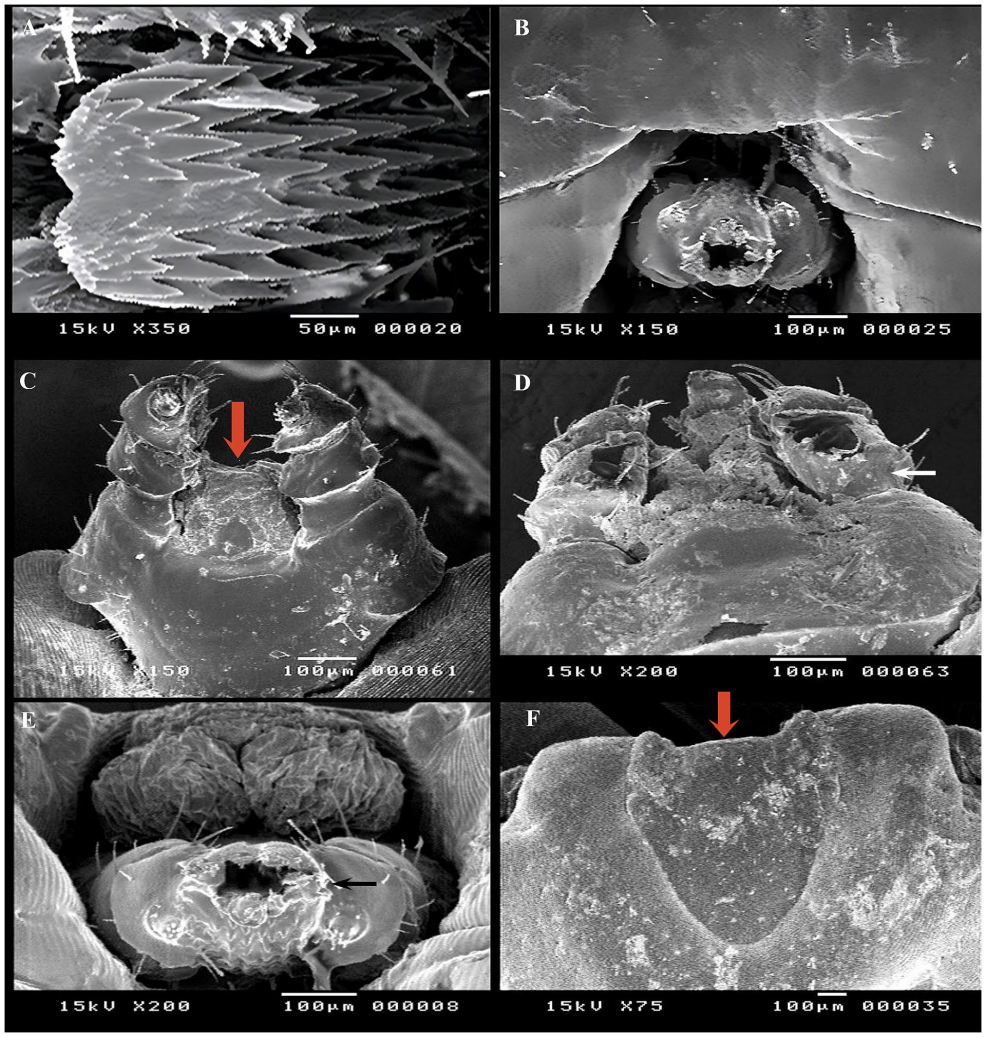

FIGURE 7
Acaricidal effects of the different groups on capitulum of adult female *R. annulatus* ticks. **(A)** Control group showing intact capitulum structure. **(B)** Phoxim-treated group with complete capitulum degeneration. **(C)** Neem oil-treated group displaying complete hypostome destruction. **(D)** Neem oil-treated group with damaged capitulum. **(E)** *Chrysanthemum* extract-treated group showing severe mouthpart damage. **(F)** *Chrysanthemum* extract-treated group with hidden capitulum.

4.3 Acaricidal potential of *Chrysanthemum* extract and neem oil on *Rhipicephalus annulatus* adult stage

Integrating botanical insecticides into tick management programs can contribute to a holistic approach to animal health, addressing ectoparasite control and overall well-being. Given the growing resistance of ticks to commercial acaricides, this study evaluated *Chrysanthemum* extract and neem oil as potential natural eco-friendly alternatives. Our results demonstrate that both extracts had significant acaricidal activity against ticks. *Chrysanthemum* extract, particularly at high concentrations (0.5 mg/mL), showed high efficacy in inducing tick mortality, resulting in 73.3% mortality within the first 3 h, reaching complete mortality at 24 h. Our findings aligned with a study that demonstrated that the effects of *Chrysanthemum roseum* extracts achieved 100% mortality against *Rhipicephalus microplus* at a 5% concentration (50). In this study, the statistical analysis showed a moderate correlation between *Chrysanthemum* extract (0.5 mg/mL) and phoxim mortality rates with significant differences between all concentrations of *Chrysanthemum* extract and the control group. Organic repellent utilizing *Chrysanthemum* oil on 5 mL, resulted in the killing of insects such as ants, cockroaches, and flies after 2.81 min (51). Notably, pyrethrins, derived from Pyrethrum, laid the foundation for the synthesis of more potent synthetic pyrethroids. The insecticidal activity of Pyrethrum, with its rapid biodegradation and its relatively low mammalian toxicity, makes it one of the most widely used non-synthetic insecticides in certified organic agriculture (17). In the present study, the *Chrysanthemum* extract has abundant phenolic and flavonoid contents, which contribute to its potential bioactivity and therapeutic value. In addition, the high flavonoid content in the *Chrysanthemum* extract suggests potential therapeutic value and health benefits. Phenolic compounds are well-known for their antioxidant properties, anti-inflammatory, antimicrobial, and anti-carcinogenic activities (52–54). Flavonoids are

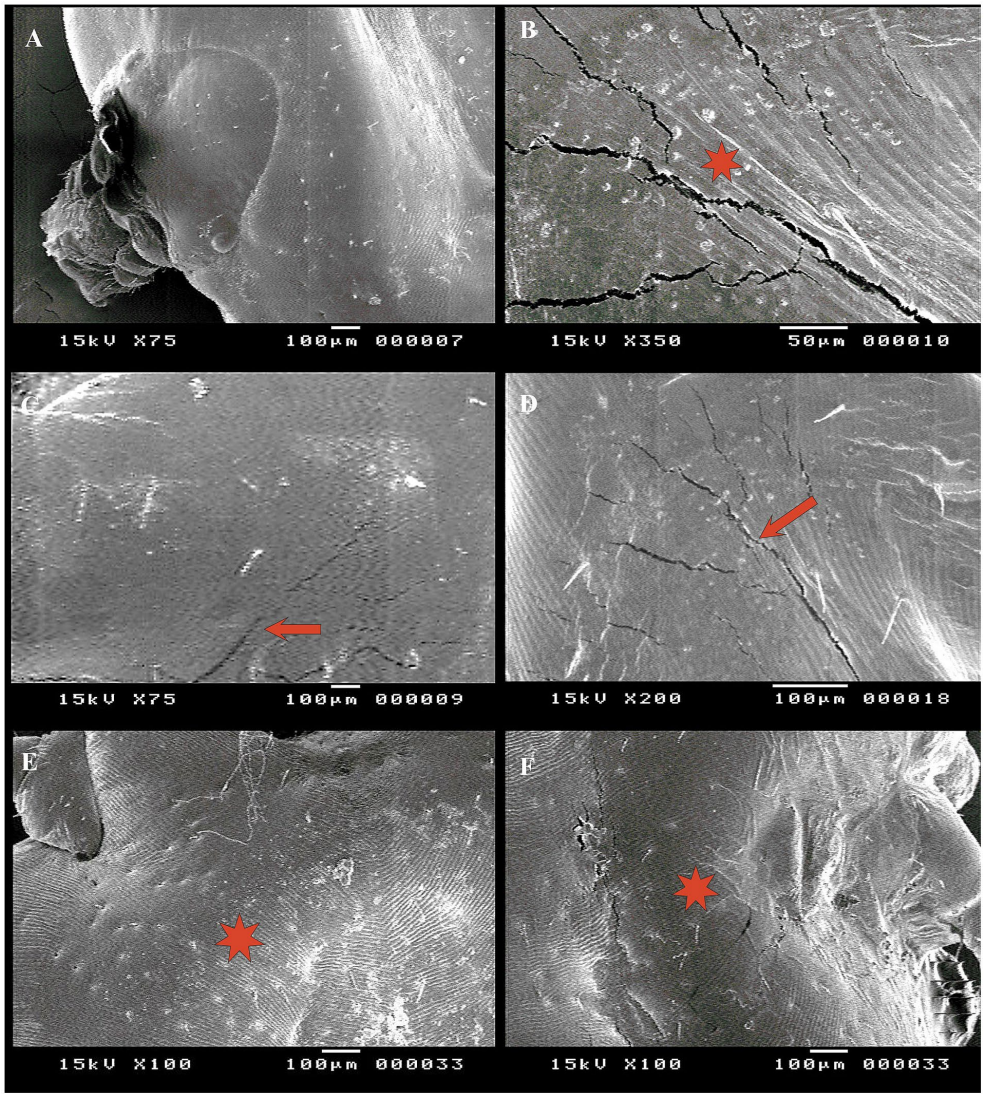

FIGURE 8
Acaricidal effects of the different groups on dorsal surface of adult female *R. annulatus* ticks. **(A)** Control group with intact anterior part. **(B)** Phoxim-treated group displaying cuticle cracks. **(C,D)** Neem oil-treated group showing cuticle cracking. **(E)** *Chrysanthemum* extract-treated group showing pores in the cuticle. **(F)** *Chrysanthemum* extract-treated group with cuticle cracks.

renowned for their diverse pharmacological effects, including antioxidant and anti-inflammatory properties, and anti-cancer effects, among others (55).

Neem oil, at concentrations of 20, 15, and 10 mg/L, exhibited significant acaricidal effects. The highest concentration (20 mg/L) resulted in 60% mortality within the first 3 h and reaching complete mortality after 24 h. Significant differences were observed between all concentrations of neem oil and the control group, as well as between higher and lower concentrations of neem oil at different time points. These results are consistent with the study by Gareh et al. (33), which revealed that 100% mortality of adult ticks on the 1st day post-treatment. The correlation analysis between the high concentration of neem oil (20 mg/L) and phoxim mortality rates showed a strong positive linear relationship, while the mortality rate due to neem oil increased, the phoxim mortality rate also increased. This indicates that *Chrysanthemum* and neem oil could be a powerful alternative to phoxim, as it has also shown insecticidal effects on numerous insect species, such as mosquitoes (56).

4.4 Detection the morphological alteration using SEM

SEM results confirmed that all treatments (*Chrysanthemum* extract, neem oil, and phoxim) caused notable damage to the tick cuticle, including wrinkling, pores, loss of striation, and cracks in some areas. The destruction of the hypostome was particularly evident when neem oil was used. This indicates that neem oil has the potential to interfere with the survival and reproduction of ticks. This effect may be due to the active components of neem, salannin,

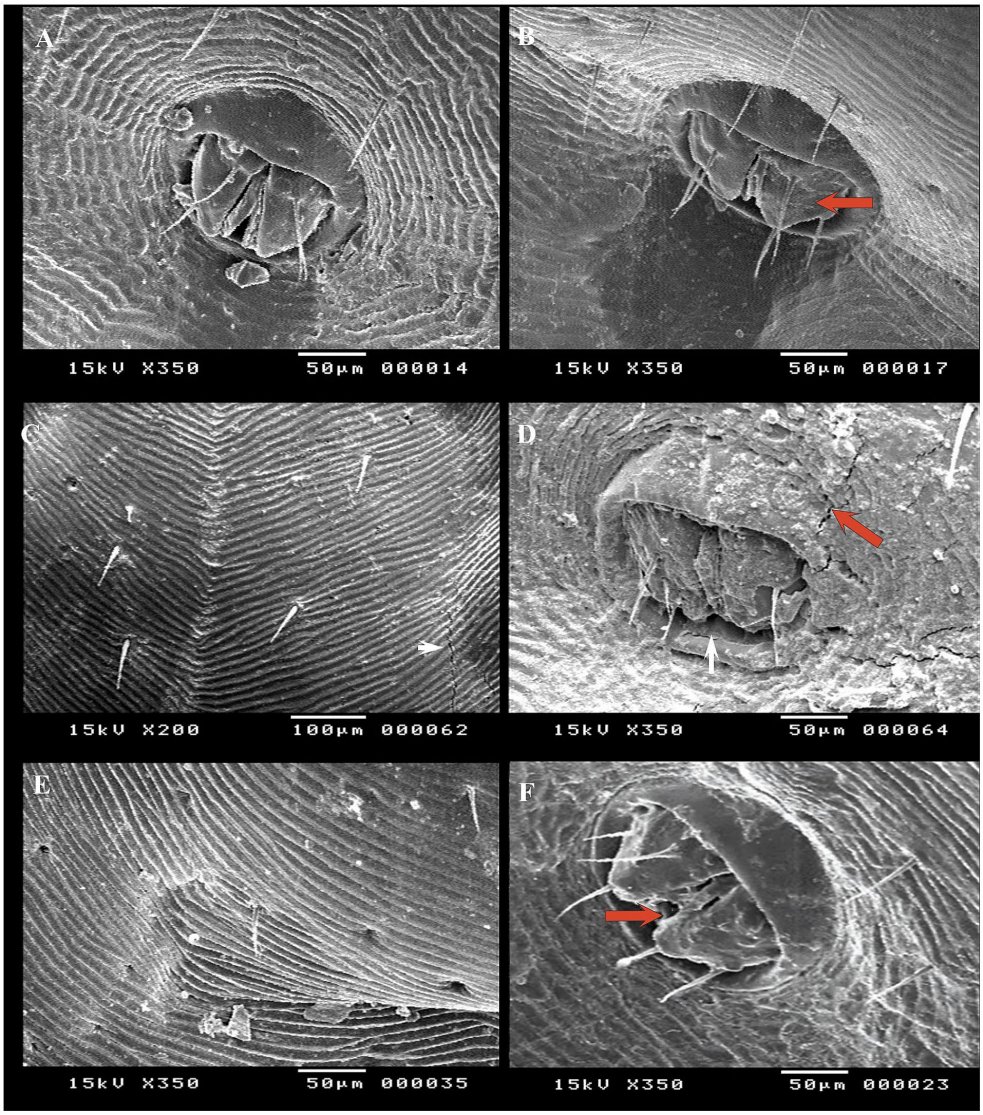

FIGURE 9
Acaricidal effects of the different groups on the ventral surface of adult female *R. annulatus* ticks. **(A)** Control group maintaining normal appearance. **(B)** Phoxim-treated group with retracted anal region. **(C)** Neem oil-treated group showing the typical distribution of striations with some pores. **(D)** Neem oil-treated group showing destruction and cracks around the anal region. **(E)** *Chrysanthemum* extract-treated group with pore formation. **(F)** *Chrysanthemum* extract-treated group with wrinkling and slight degeneration of the anal region.

which have insect growth-regulating and antifeedant activity (33). The destruction of mouthparts with neem oil is similar to the effects noted with *Melia azedarach* and *Artemisia herba-alba* extracts on *Hyalomma dromedarii* (57). On the other hand, *Chrysanthemum* extract induced pronounced damage to the tick cuticle, marked by cracks and pores. Phoxim was effective in causing complete degeneration of the capitulum and extensive cuticle cracks. Also, cracks resulting from *Chrysanthemum* and phoxim were similar to those caused by *Cymbopogon citratus* oil on *Haemaphysalis longicornis* ticks (58). In the neem oil-treated group, the ventral surface and anal region of ticks showed retraction and destruction. In contrast, *Chrysanthemum* extract caused loss of striations and wrinkling with pore formation. These findings suggest that *Chrysanthemum* extract and neem oil demonstrate effective acaricidal effects similar to phoxim, confirming their potential as eco-friendly alternatives in integrated pest management strategies.

5 Conclusion

Our research highlights the strong connection between tick infestations and piroplasm infection in cattle, specifically *Babesia* species. Molecular identification confirmed the presence of *T. annulata* and *B. bigemina*. Phylogenetic analyses revealed significant genetic diversity influenced by geographical factors. *Chrysanthemum* extract, as well as neem oil, demonstrated high acaricidal efficacy, resulting in substantial mortality rates in *R. annulatus* ticks. Both treatments showed time-and dose-dependent effectiveness, causing severe morphological damage to the ticks, as demonstrated by SEM analysis. Overall, the findings support the use of *Chrysanthemum* extract and neem oil as effective natural alternatives for tick control, providing a sustainable solution for livestock health management.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/supplementary material.

Ethics statement

The animal study was approved by Research Ethical Committee, Faculty of Veterinary Medicine at Assiut University (Approval number: 06/2024/0257). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

SA-E: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Validation, Writing – original draft, Writing – review & editing. FK: Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. SA-H: Data curation, Formal analysis, Validation, Writing – review & editing. AK: Data curation, Investigation, Validation, Writing – original draft. SM: Methodology, Writing – review & editing. AAb: Investigation, Methodology, Writing – review & editing. MD: Data curation, Validation, Writing – review & editing. AA-H: Funding acquisition, Validation, Writing – original draft. AAl: Funding acquisition, Investigation, Writing – review & editing. AD: Conceptualization, Data curation, Writing – review & editing.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Generative AI statement

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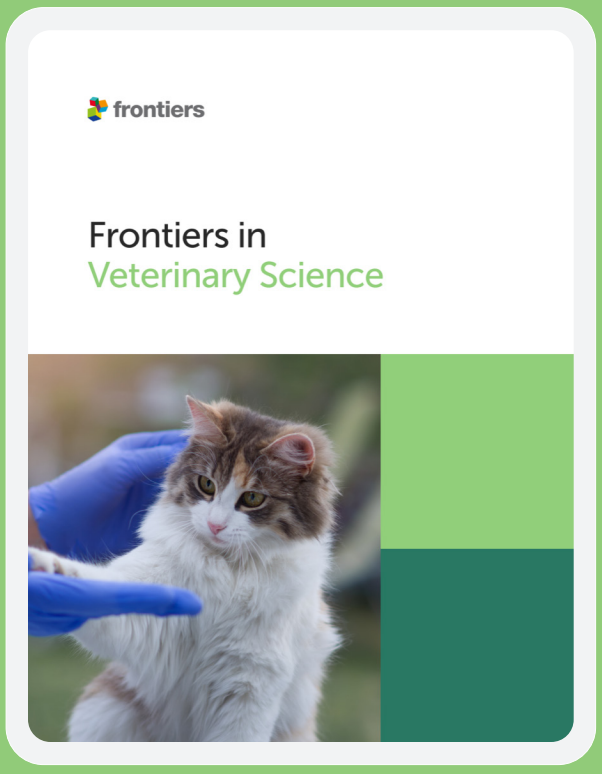

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