CURRENT KNOWLEDGE ON PATHOGENIC AND ENDOSYMBIOTIC TICK-BORNE BACTERIA

EDITED BY: Mourad Ben Said, Armanda Bastos, Sandra Diaz Sanchez and Cornelia Silaghi

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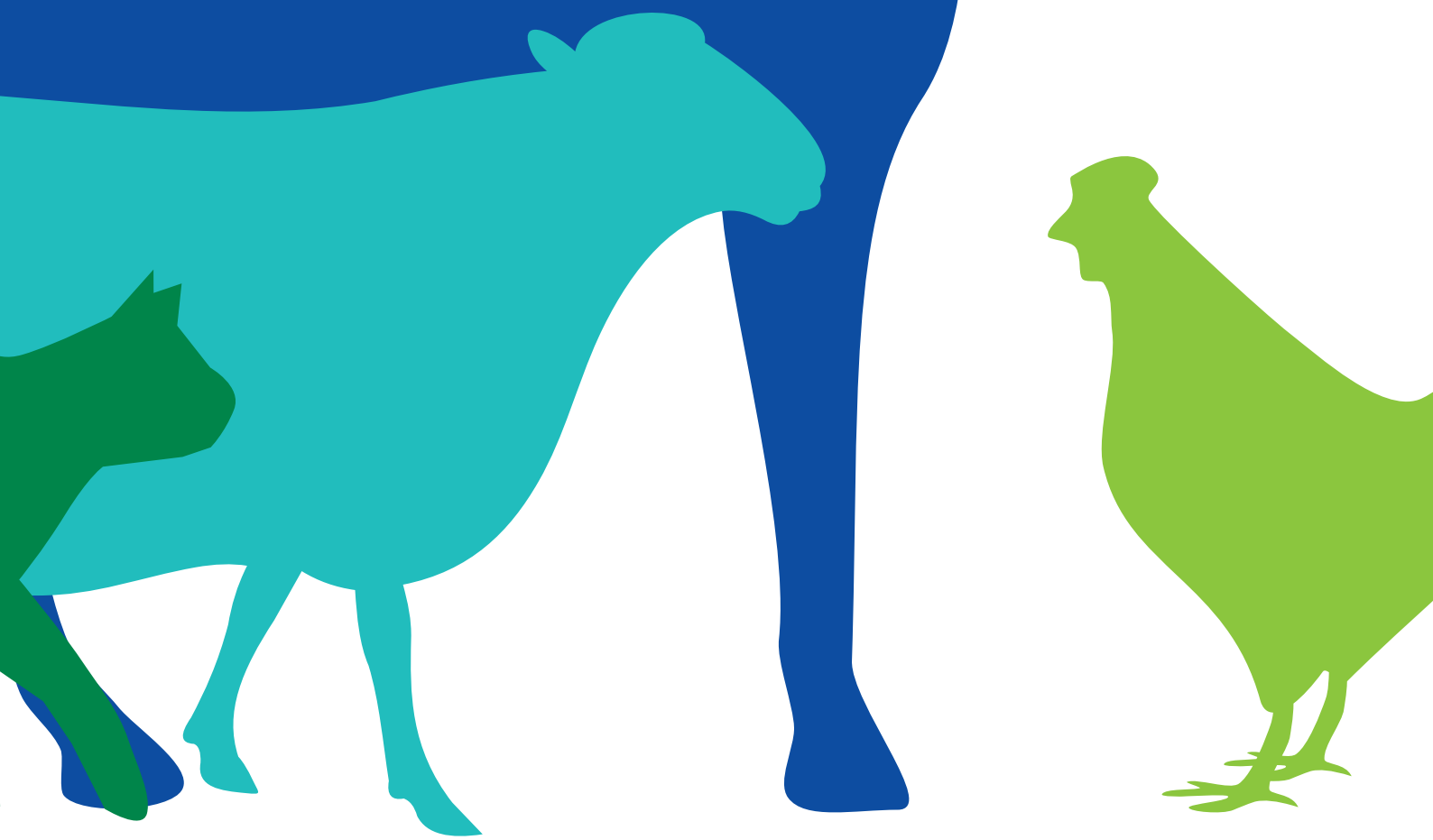

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CURRENT KNOWLEDGE ON PATHOGENIC AND ENDOSYMBIOTIC TICK-BORNE BACTERIA

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EDITORIAL
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Editorial: Current Knowledge on Pathogenic and Endosymbiotic Tick-Borne Bacteria

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Keywords: ticks, hosts, tick-borne pathogens, tick-borne endosymbionts, vectors, tick-borne diseases

Editorial on the Research Topic

Current Knowledge on Pathogenic and Endosymbiotic Tick-Borne Bacteria

This Research Topic converges different original and review contributions highlighting tick associations with microbial communities. These contributions shed light on the fields of (i) diagnosis, epidemiology, and phylogeny of pathogenic and endosymbiotic tick-borne bacteria, and (ii) tick microbiota exploration.

The first contribution to this topic (Körner et al.) reviewed prevalence studies of *Coxiella burnetii* in 25 tick species collected from 23 European countries. The authors show that in half of these studies, no *Coxiella* DNA was detected and that in most studies, no distinction was made between *C. burnetii* and *Coxiella*-like endosymbionts (CLEs), underscoring the need to develop more discriminative methods to better clarify the role of ticks in the transmission of Q-fever.

For the first time, Beliavskaia et al. reported *in vitro* isolation of the bacterial symbiont *Spiroplasma* from third-generation adult male and female *Ixodes persulcatus* maintained in a laboratory colony for over 4 years. This study confirmed that co-cultivation of internal organs with tick cell lines is a simple and effective technique for *in vitro* isolation of intracellular tick symbionts such as *Spiroplasma* species.

To better understand the epidemiology of *Rickettsia* species in Tunisia, Belkahia, Selmi et al. demonstrated the occurrence of rickettsial bacteria in ticks of the *Rhipicephalus* genus infesting small ruminants in Tunisia. The study confirmed the occurrence of human-pathogenic *Rickettsia* species in *Rh. sanguineus* s.l. and *Rh. turanicus* ticks collected from small ruminants in Tunisia. These findings expand knowledge on ticks collected from domestic animals, and highlight the range of infectious agents that may be transmitted with ticks to humans and animals. The presence of *Rickettsia* in ticks is also investigated by Chitanga et al., where the potential risk of human infection by zoonotic *Rickettsia* species in southern Zambia was addressed.

Continuing with epidemiological studies, El Hamiani Khatat et al. presented a systematic review that summarized the wide epidemiological data published on *Anaplasma phagocytophilum* in canine species and described the clinicopathological aspects of canine granulocytic anaplasmosis that are available in the few case series and reports. In this manuscript, the authors gathered all data on *A. phagocytophilum* in dogs that can be valuable for researchers and identified important information gaps to guide future research.

Contributing to shed light on the epidemic situation of tick-borne pathogens, Wang et al. assessed the distribution and risk factors of *Anaplasma* spp. and *Ehrlichia chaffeensis* in yaks

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(*Bos grunniens*) and Tibetan sheep (*Ovis aries*) from Qinghai, China. This first report of *A. capra* and *E. chaffeensis* infection in yaks in China emphasized the need to assess the threat posed by these pathogens to veterinary and public health. Within this context, Belkahia, Ben Abdallah et al. revealed a greater diversity of Tunisian *A. marginale* isolates compared to worldwide isolates and strains by using a single gene typing method. In addition, the analysis of the vaccine candidate OmpA protein demonstrated that this antigen appears to be highly conserved, suggesting that the minimal intraspecific modifications will not affect the potential cross-protective capacity of humoral and cell-mediated immune responses against multiple *A. marginale* strains. To highlight the need for surveillance and control programs for transboundary diseases (TBDs), Galay et al. showed the high prevalence of *A. marginale* in Luzon, Philippines, provided the first molecular evidence of *E. minasensis* in the country, and confirmed the presence of *E. minasensis* in naturally-infected cattle and *Rhipicephalus microplus* ticks.

Regarding the role of the tick microbial community in the success of tick-borne pathogens, Aguilar-Díaz et al. contributed with a review addressing various mechanisms occurring at the microbiota-pathogen interface and its contribution to tick fitness, adaptation, and immunity and identifying potential targets for anti-tick vaccine development. The combination of conventional and high-throughput sequencing methods was implemented by Takhampunya et al. to provide important information on bacterial community composition and co-infection rates in questing ticks in Thailand with implications for animal and human health.

A different approach is presented in Cull et al. Indeed, this research provides evidence that the endosymbiont of *Ixodes scapularis*, *Rickettsia buchneri*, exerts an inhibitory effect on the growth of pathogenic tick-borne bacteria in cell culture and possesses two gene clusters encoding putative antibiotic biosynthesis machinery. This suggests that in addition to being a potential nutritional endosymbiont, *R. buchneri* may also prevent pathogenic *Rickettsia* species from occupying the ovaries that may be detrimental to the tick's biology. Supportive evidence from *in vivo* studies could have important implications for our understanding of rickettsial interference and the vector competence of *I. scapularis* for SFG rickettsiae.

In another context, control of tick-borne pathogens generally depends on three main strategies, namely vector control, vaccine development, and the administration of antimicrobial drugs. For *Babesia* protozoa, one of the promising strategies against species of this genus is to control the receptor-ligand interactions of parasite molecules and their target cells, such as RON-AMA-1. Therefore, in this Research Topic, Li et al. screened 502 compounds from the natural product compounds (NPCs) against the *in vitro B. bovis* growth and against the *in vivo B. microti* growth. Findings obtained by these authors indicate the richness of natural product compounds by new potent anti-babesian candidates, and the identified potent compounds, in particular Narasin, could be used for the treatment of animal babesiosis.

As Editors of this Research Topic, we would like to acknowledge all authors who have contributed high-quality original articles and very interesting reviews. We hope that the reader will find in this Research Topic a useful reference for the state of the art in the knowledge on pathogenic and endosymbiotic tick-borne bacteria on two broad aspects: (i) the epidemiology of pathogenic and endosymbiotic tick-borne bacteria, and (ii) the tick microbiome-pathogen-endosymbiont interface.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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BRIEF RESEARCH REPORT
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Spiroplasma Isolated From Third-Generation Laboratory Colony *Ixodes persulcatus* Ticks

*Alexandra Beliavskaia[1], Vaclav Hönig[2,3], Jan Erhart[2], Tereza Vyhlidalova[2], Martin Palus[2,3], Jiri Cerny[4], Irina Kozlova[5], Daniel Ruzek[2,3], Ana M. Palomar[6] and Lesley Bell-Sakyi[1]**

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Spiroplasma are vertically-transmitted endosymbionts of ticks and other arthropods. Field-collected *Ixodes persulcatus* have been reported to harbour *Spiroplasma*, but nothing is known about their persistence during laboratory colonisation of this tick species. We successfully isolated *Spiroplasma* from internal organs of 6/10 unfed adult ticks, belonging to the third generation of an *I. persulcatus* laboratory colony, into tick cell culture. We screened a further 51 adult male and female ticks from the same colony for presence of *Spiroplasma* by genus-specific PCR amplification of fragments of the 16S rRNA and *rpoB* genes; 100% of these ticks were infected and the 16S rRNA sequence showed 99.8% similarity to that of a previously-published *Spiroplasma* isolated from field-collected *I. persulcatus*. Our study shows that *Spiroplasma* endosymbionts persist at high prevalence in colonised *I. persulcatus* through at least three generations, and confirms the usefulness of tick cell lines for isolation and cultivation of this bacterium.

Keywords: tick cell line, endosymbiont, *Spiroplasma*, tick colony, *Ixodes persulcatus*

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INTRODUCTION

Ixodid ticks naturally harbour a variety of bacterial symbionts that may be obligately or facultatively intracellular and are transovarially transmitted. These include species of the genera *Rickettsia*, *Coxiella*, *Midichloria* and *Spiroplasma* that occur with high frequency (1–4) and less common or well-characterised species of the genus *Francisella* (1, 3) and *Occidentia* (5). The insect symbionts *Cardinium*, *Wolbachia*, *Arsenophonus* and *Rickettsiella* have also been detected in or isolated from ticks (3, 6–10) but it is unclear whether or not their presence results from parasitism by insects such as the wasp *Ixodiphagus hookeri* (7, 9) or cohabiting mites (author's unpublished observations), and they are not known to be transovarially transmitted in ticks. Most studies of occurrence of bacterial symbionts in ticks are based on molecular detection in DNA extracted from individual or pooled ticks sampled directly from the field. Some recognised or putative tick symbionts have been isolated into culture, in either mammalian or tick cells; these include several species of *Rickettsia* (11–16), *Francisella* (17), several strains of *Spiroplasma* (10, 18–21) and one isolate each of *Arsenophonus*, *Occidentia* and *Rickettsiella* (5, 8, 10). In all cases, the unfed or partially-fed ticks had been collected from the field, and bacteria were isolated directly from homogenised/macerated whole ticks or aseptically-dissected internal organs, or from eggs laid by engorged female ticks.

Ixodes persulcatus, a tick species distributed widely from the eastern Baltic coast to Japan (22–25), has been reported to harbour symbionts including the Montezuma agent, now called *Candidatus* Lariskella arthropodarum (26–28), *Coxiella* and *Spiroplasma* spp. (29), as well as human and livestock pathogens including tick-borne encephalitis virus (TBEV), Kemerovo virus, Alongshan virus, *Anaplasma phagocytophilum*, *Candidatus* Neoehrlichia mikurensis, *Ehrlichia muris*, *Rickettsia helvetica*, *Rickettsia heilongjiangensis*, *Candidatus* Rickettsia tarasevichiae, *Borrelia miyamotoi*, *Borrelia burgdorferi* sensu lato, *Theileria equi* and several species of *Babesia* (25, 30–38). Co-infections with multiple pathogens and symbionts are common (29, 35, 39). There is a single report of isolation into culture of bacterial symbionts from Japanese *I. persulcatus*: *R. helvetica* and a *Spiroplasma* were isolated from field-caught adult male ticks into an *Ixodes scapularis* cell line (10).

Research on transmission of tick-borne pathogens of medical and veterinary interest depends largely on ticks maintained in laboratory colonies. However, few studies have assessed such ticks for presence of symbionts, despite the potential influence of the latter on the ability of ticks to harbour (40) and/or transmit pathogens. Prevalence of *Candidatus* Midichloria mitochondrii determined by molecular methods was found to be lower in *Ixodes ricinus* ticks from laboratory colonies than in field ticks, and to decrease (albeit in a small sample size) with increasing numbers of tick generations (2). A subsequent study, using a more sensitive assay, revealed the presence of extremely low levels of *Ca.* M. mitochondrii DNA in 60% of >10th generation laboratory colony *I. ricinus* (41). *Ixodes arboricola* were screened for bacterial symbionts by PCR and higher incidences were found in field-collected ticks than in laboratory colony ticks of three genera: *Rickettsiella* (28.0 vs. 0%), *Midichloria* (1.3 vs. 0%) and *Spiroplasma* (16.0 vs. 5.6%) (3). Both groups harboured similarly high levels of *Rickettsia* (96.0 vs. 100%), suggesting that transovarial transmission was highly efficient for *Rickettsia*, less efficient for *Spiroplasma* and might not occur for *Rickettsiella*. Both lower absolute numbers of bacteria including the symbionts *Spiroplasma* and *Midichloria*, and more limited diversity of bacterial species, were reported in midguts of *I. ricinus* ticks from a laboratory colony compared to wild-caught ticks, and extremely low numbers of bacteria (<100 organisms per midgut) were found in *Rhipicephalus microplus* ticks from a closed colony in Brazil (42). A bacterial symbiont, later identified as a *Cardinium* sp. (43), was isolated from first-generation adult *I. scapularis* reared in the laboratory from field-caught adults (6). *Rickettsia raoultii* was isolated from eggs laid by the first generation of adult *Dermacentor reticulatus* reared in the laboratory from field-caught ticks (21). However, we could not find any report of *in vitro* isolation of a bacterial symbiont from laboratory colony ticks maintained for additional generations.

Here we report isolation and preliminary genetic characterisation of a *Spiroplasma* from third-generation adult male and female *I. persulcatus*, originally collected in Siberia (Irkutsk Oblast, Russian Federation) and maintained in a laboratory colony for over 4 years.

MATERIALS AND METHODS

Ticks

Unfed adult *I. persulcatus* ticks were collected from vegetation by flagging near Irkutsk, (Irkutsk Oblast, Russian Federation) at Talsy (52.024381 N, 104.657681 E) and Ust-Ordynsky (52.700295 N, 104.905164 E) in May 2015. The ticks were subsequently maintained as a laboratory colony through three generations in the tick rearing facility of the Institute of Parasitology, Biology Centre, Czech Academy of Sciences (BCCAS). All animal experiments were in accordance with the Animal Protection Law of the Czech Republic (§17, Act No. 246/1992 Sb) and with the approval of the Czech Academy of Sciences (approval no. 161/2010). All instars were fed to engorgement on guinea pigs or gerbils, incubated for moulting or oviposition at 24°C, 96% relative humidity (RH) and stored following moult or larval hatching under the same conditions. To obtain separate groups of unfed adult male and female ticks, nymphs were visually inspected following engorgement, and males were sorted from females according to their size as male nymphs are approximately one third smaller. Unfed adult male and female ticks were transferred by courier to the Tick Cell Biobank, University of Liverpool, where they were stored at 15°C, 100% RH for 19 days until used for *Spiroplasma* isolation or seven months until used for DNA extraction.

In vitro Isolation of *Spiroplasma*

Five male and five female unfed adult *I. persulcatus* ticks were surface-sterilised by immersion in 0.1% benzalkonium chloride for 5 min, 70% ethanol for 1 min and 2 x 1 min rinses in sterile deionised water. After drying on sterile filter paper, the ticks were embedded in wax and their internal organs (as much as possible of midgut, salivary glands, synganglion, Malpighian tubules, rectal sac, fat body, testes/ovary) were dissected out as described previously (21). Each tick was dissected in a separate drop of Hank's balanced salt solution and the dissecting instruments were sterilised in 70% ethanol between ticks. The internal organs from each tick were inoculated into a separate culture of tick cells in a sealed, flat-sided tube (Nunc, Thermo-Fisher) and incubated at 28°C. Four embryo-derived tick cell lines were used for *Spiroplasma* isolation: *Rhipicephalus microplus* BME/CTVM23 (13) and BME26 (44), *I. ricinus* IRE11 (45) and *Ixodes scapularis* IDE2 (46). BME/CTVM23 and BME26 cells were grown in complete L-15 and L-15B media respectively, (47) and IRE11 and IDE2 cells were grown in complete L-15B300 medium (48); all media contained 100 units/ml penicillin and 100 µg/mL streptomycin. Medium was changed weekly by removal and replacement of ¾ of the medium volume and cultures were monitored by inverted microscope examination. Giemsa-stained cytocentrifuge smears were prepared as described previously (13) from all cultures on day 53 post inoculation (p.i.) and examined for presence of bacteria. All cultures were cryopreserved in vapour phase liquid nitrogen as described previously (21) on day 90 p.i.

Molecular Characterisation of Cultured *Spiroplasma*

On day 65 p.i., the cells in each culture were resuspended and 200 μL aliquots were centrifuged at 15,000×*g* for 5 min. DNA was extracted from the cell pellets using a DNeasy blood and tissue Mini Kit (Qiagen) following the manufacturer's instructions. DNA extracts were screened for presence of *Spiroplasma* using PCR assays amplifying fragments of the 16S rRNA (16S rRNA; ∼500 bp) and RNA polymerase beta subunit (*rpoB*; ∼1443 bp) genes (49, 50). Amplicons were visualised by agarose gel electrophoresis, and positive PCR products were purified using a PureLink Quick Gel Extraction and PCR Purification Combo kit (ThermoFisher) following the manufacturer's instructions and submitted for Sanger sequencing in both directions (Eurofins Genomics, Germany). Phylogenetic analyses were conducted with MEGA X using the maximum likelihood method based on the Kimura 2-parameter model and including all sites (51, 52). The nucleotide substitution model was selected according to the Bayesian information criterion (BIC) implemented in Mega X (53). Confidence values for individual branches of the resulting trees were determined by bootstrap analysis with 500 replicates. Two separate phylogenetic trees based on available 16S rRNA and *rpoB* sequences of *Spiroplasma* spp. isolated or detected in ixodid ticks were inferred. It was not possible to include all these *Spiroplasma* variants in both phylogenies because published sequences of both gene fragments amplified in this study were not available for some of them. Moreover, the *rpoB* analysis was performed with a shorter fragment (<600 bp) corresponding to the fragment available from many of these published sequences. The published sequences used in the analyses are shown in the phylogenetic trees.

Detection of *Spiroplasma* in *I. persulcatus* Colony Ticks

DNA was extracted from 10 ticks (four male and six female) remaining from the batch shipped to Liverpool, 7 months after receipt, using a DNeasy blood and tissue Mini Kit (Qiagen) according to the manufacturer's instructions with overnight lysis. DNA was extracted from a further 17 male and 24 female ticks from the same generation maintained in the BCCAS colony, using a DNeasy blood and tissue Mini Kit (Qiagen) with the following modifications. Briefly, the ticks were homogenised individually in 200 μL of ATL buffer (Qiagen) for 2 min at 30 shakes/s in a Tissue Lyser II (Qiagen). After brief centrifugation and addition of 20 μL of proteinase K, the samples were incubated at 56°C for 30 min. The remaining steps of DNA extraction were done according to the manufacturer's instructions. To confirm species identity of the ticks screened in Liverpool, a fragment of the tick 16S rRNA gene was amplified using primer pairs 16S+1/16S-1 as described previously (54). To detect *Spiroplasma*, DNA from all ticks was PCR-screened using the specific assays for fragments of the *Spiroplasma* 16S rRNA and *rpoB* genes as described above. Randomly-selected positive amplicons were purified and sequenced as above (Liverpool ticks) or enzymatically purified using Exonuclease I FastAP and Thermosensitive Alkaline Phosphatase (ThermoFisher Scientific) and submitted for Sanger sequencing (SeqMe, Czech Republic) (BCCAS ticks), and analysed as described above.

RESULTS

When the tick cell cultures were examined by Giemsa-stained cytocentrifuge smear on day 53 p.i., bacteria resembling *Spiroplasma* were seen in cells that had received organs from 1/5 male and 5/5 female *I. persulcatus* ticks (**Table 1**). In all cases, the *Spiroplasma* were intracellular and concentrated in cytoplasmic vacuoles, but the appearance differed between the various tick cell lines (**Figure 1**). In the *R. microplus* cell lines (**Figures 1A,B**) and IDE2 (not shown), most vacuoles containing *Spiroplasma* also contained homogenous, light blue- or pink-staining background material, whereas in IRE11 cells (**Figures 1C,D**) such material was absent in most vacuoles containing *Spiroplasma*. It was not possible to determine whether this was due to differences between the cell lines or the *Spiroplasma* isolates, although in previous studies background material was visible in *Spiroplasma*-containing vacuoles in cells of the tick cell lines BME/CTVM23 and DALBE3 (21) but not of the tick cell lines IRE11, IRE/CTVM19 or IDE2 (20).

PCR amplification of fragments of the *Spiroplasma*-specific 16S rRNA and *rpoB* genes from DNA extracted on day 65 p.i. confirmed the presence of *Spiroplasma* in the six microscopically-positive cultures, and in both cases failed to amplify any products from DNA extracted from the four microscopically-negative cultures (**Table 1**). To determine the *Spiroplasma* infection rate in adult ticks of the parent colony, the 10 ticks remaining in Liverpool (four males, six females) and a further 41 ticks (12 males, 12 females fed as nymphs on guinea pigs and five males, 12 females fed as nymphs on gerbils) from the same generation of the BCCAS colony were screened using the *Spiroplasma* 16S rRNA and *rpoB* PCR assays. All of the ticks were positive for *Spiroplasma* by one or both assays, and amplification and sequencing of a 430 bp fragment of the tick 16S rRNA gene confirmed the species identity of the ticks tested in Liverpool as *I. persulcatus* (99.8% similarity to *I. persulcatus* from Omsk, Siberia, Russia, Genbank accession no. MH790201.1).

Sequence analysis revealed that, for the *Spiroplasma* 16S rRNA gene, all six culture isolates (designated Irkutsk1-6) and five representative tick samples screened in Liverpool were identical to each other, and identical to eight representative tick samples screened at BCCAS apart from one ambiguous nucleotide at position 105 (**Table 2A**). All sequences showed 99.8% similarity (99.5% query cover) to the only sequence from an *I. persulcatus*-derived *Spiroplasma* available in Genbank at the time of writing (LC388762.1) (10); interestingly, the only mismatch between our sequences and that of the Japanese isolate (10) was also at position 105 (**Table 2A**). The 16S rRNA sequence of the Irkutsk strains isolated from *I. persulcatus* was identical to several other *Spiroplasma* strains isolated from hard ticks: *Spiroplasma* sp. Bratislava 1 (KP967685, from Slovakian *I. ricinus*), *Spiroplasma* sp. 1033 (LC388770, from

TABLE 1 | Detection of *Spiroplasma* by microscopy and PCR analysis of tick cell lines inoculated with internal organs from male and female *Ixodes persulcatus* ticks.

Sample no.	Tick gender	Cell line	Microscopy result	*Spiroplasma* PCR result		Strain designation
				16S rRNA	*rpoB*	
303	Male	BME/CTVM23	None seen	–	–	
304	Male	BME/CTVM23	*Spiroplasma*	+	+	Irkutsk1
305	Female	BME/CTVM23	*Spiroplasma*	+	+	Irkutsk2
306	Female	BME/CTVM23	*Spiroplasma*	+	+	Irkutsk3
307	Male	BME26	None seen	–	–	
308	Female	BME26	*Spiroplasma*	+	+	Irkutsk4
309	Male	IRE11	None seen	–	–	
310	Female	IRE11	*Spiroplasma*	+	+	Irkutsk5
311	Male	IDE2	None seen	–	–	
312	Female	IDE2	*Spiroplasma*	+	+	Irkutsk6

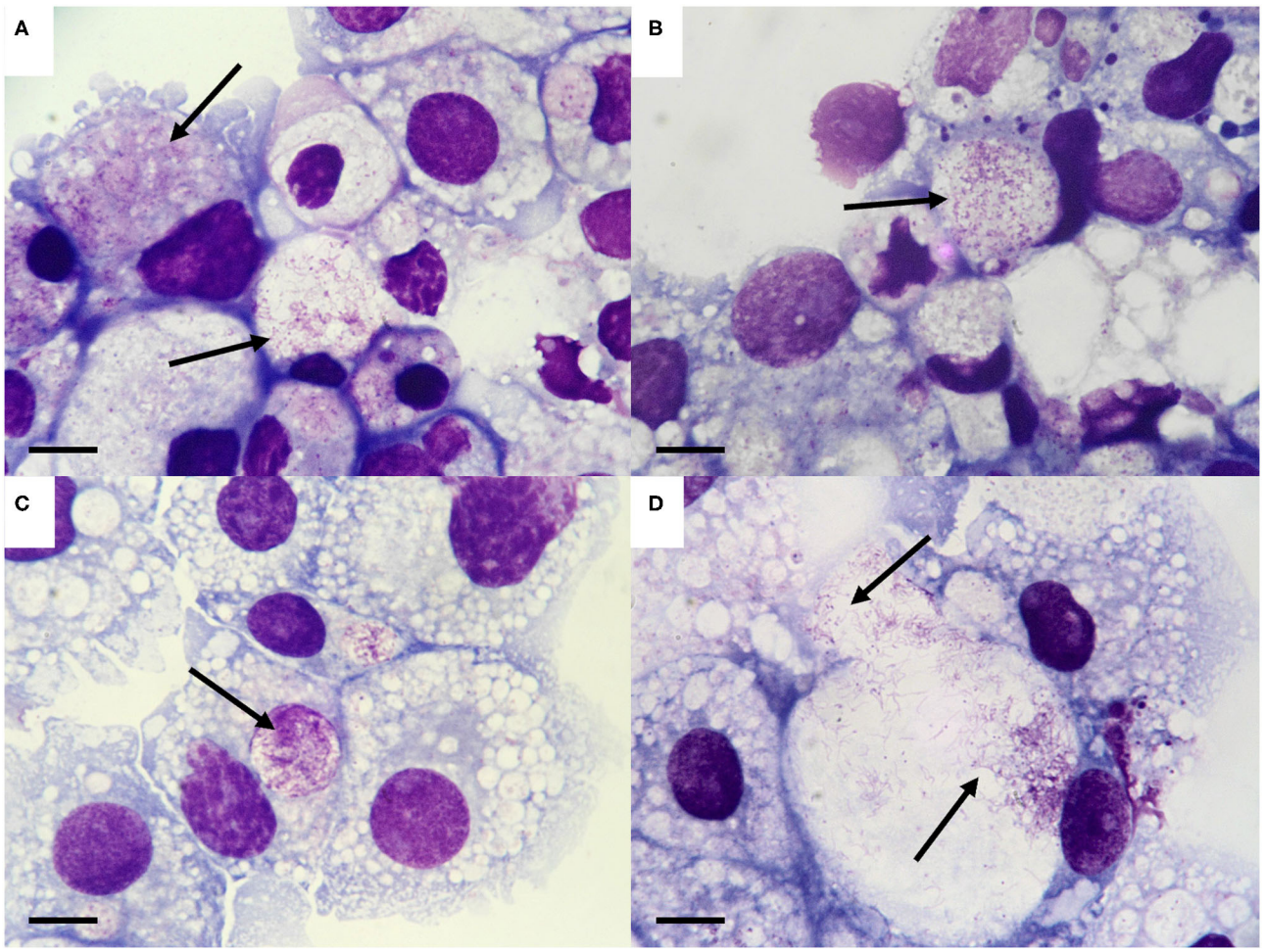

FIGURE 1 | Morphology of the *Spiroplasma* sp. isolated from *Ixodes persulcatus* ticks. **(A–D).** *Spiroplasma* (arrows) in tick cell lines inoculated with internal organs from male and female *I. persulcatus* ticks, day 53 post inoculation. **(A)** BME/CTVM23 cells inoculated with male tick #304 (*Spiroplasma* strain Irkutsk1). **(B)** BME26 cells inoculated with female tick #308 (*Spiroplasma* strain Irkutsk4). **(C,D).** IRE11 cells inoculated with female tick #310 (*Spiroplasma* strain Irkutsk5). Giemsa-stained cytocentrifuge smears; scale bars = 10 μm.

TABLE 2A | Polymorphisms in sequences detected in the *Ixodes persulcatus*-derived *Spiroplasma* sp. in this study compared to other tick-borne *Spiroplasma* spp.

Spiroplasma strain	Tick species	GenBank accession no	Positions[a]														
			55-56[b]	103	105	157	187	209	248	275	298	299	386	400	420	437	449-450[b]
Spiroplasma sp. Liverpool tick	*I. persulcatus*	MW498417	-	T	T	G	G	C	G	C	G	G	C	G	G	G	-
Spiroplasma sp. BCCAS tick	*I. persulcatus*	MW492370	-	T	K	G	G	C	G	C	G	G	C	G	G	G	-
Spiroplasma sp. strain Irkutsk1	*I. persulcatus*	MW498416	-	T	T	G	G	C	G	C	G	G	C	G	G	G	-
Spiroplasma sp. 147_ISE6	*I. persulcatus*	LC388762	-	T	**G**	G	G	C	G	C	G	G	C	G	G	G	-
Spiroplasma sp. 1033_C6/36	*H. kitaokai*	LC388770	-	T	**G**	G	G	C	G	C	G	G	C	G	G	G	-
Spiroplasma sp. 135_C6/36 & ISE6	*I. monospinosus*	LC388760, LC388759	-	T	**G**	G	G	C	G	C	G	G	C	G	G	G	-
Spiroplasma sp. Bratislava 1	*I. ricinus*	KP967685	-	T	**G**	G	G	C	G	C	G	G	C	G	G	G	-
Spiroplasma ixodetis Y32	*I. pacificus*	NR_104852	-	T	**G**	G	G	C	G	C	**A**	G	C	**A**	G	G	**G**
Spiroplasma sp. strain DMAR11	*D. marginatus*	MG859280	-	T	**G**	G	G	C	**R**	C	G	**R**	C	G	G	G	-
Spiroplasma sp. strain DRET8	*D. reticulatus*	MG859282	-	T	**G**	G	**T**	C	**A**	C	G	G	C	G	G	G	-
Spiroplasma sp. Hokkaido IO-1	*I. ovatus*	DQ059993	**G**	**C**	**A**	**A**	G	**T**	**A**	**T**	G	G	**A**	G	**A**	**A**	-

Polymorphisms in the 16S rRNA gene fragment of the Spiroplasma sp. detected in whole third-generation I. persulcatus colony ticks sampled at University of Liverpool (Liverpool tick) and at the Institute of Parasitology, Biology Centre, Czech Academy of Sciences (BCCAS tick) and isolated from I. persulcatus (strain Irkutsk1) compared to other Spiroplasma strains isolated from hard tick species I. persulcatus, Ixodes monospinosus, Ixodes ricinus, Ixodes pacificus, Ixodes ovatus, Haemaphysalis kitaokai, Dermacentor marginatus and Dermacentor reticulatus. [a]*The number corresponds to the positions of nucleotide substitutions with respect to the sequences MW498416 and MW498417 amplified in this study. Corresponding base substitutions are shown. The substitutions compared to the sequences amplified in this study are shown in bold.* [b]*There is an insertion between these two nucleotide bases in one sequence, a gap (-) is marked when this insertion does not occur. K = G or T; R = A or G.*

Japanese *Haemaphysalis kitaokai*) and *Spiroplasma* sp. 135 (LC388760 and LC388759, from Japanese *Ixodes monospinosus*) (10, 20) (**Table 2A**). Moreover, the Irkutsk 16S rRNA sequence showed 99.1–99.3% similarity to those of spiroplasmas isolated from North American *Ixodes pacificus* (*Spiroplasma ixodetis*, NR_104852), Spanish *Dermacentor marginatus* (*Spiroplasma* sp. strain DMAR11, MG859280) and Dutch *D. reticulatus* (*Spiroplasma* sp. strain DRET8, MG859282) (21, 55) (**Table 2A**). For the *rpoB* gene, all sequences obtained from the six culture isolates and 11 representative whole ticks were identical. At the time of writing, we could not find any published *rpoB* sequences from *I. persulcatus*-derived *Spiroplasma* for comparison, and most of those derived from other hard tick species were shorter than 600 bp. Considering the query cover higher than 99%, the *rpoB* sequence of the Irkutsk strains showed 99.9% similarity to the spiroplasmas isolated from *I. ricinus* (*Spiroplasma* sp. Bratislava1, KP967687) and *Dermacentor* spp. (*Spiroplasma* strain DMAR11, MG859278 and *Spiroplasma* strain DRET8, MG859277) (**Table 2B**), and 99.3% similarity to *S. ixodetis* (DQ313832). With a query cover of 43%, the sequences amplified in this study were identical to shorter sequences from spiroplasmas detected by PCR in other hard tick species (GenBank accession numbers MK267073-MK267077, MK267081-MK267085 and MK267097) (4), and also to *Spiroplasma* strain DMAR11 (MG859278) and *Spiroplasma* sp. Bratislava1 (KP967687) that showed polymorphisms in the longer gene fragment (**Table 2B**).

Phylogenetic analysis based on 16S rRNA sequences derived from *Spiroplasma* sp. strain Irkutsk1 and two representative whole ticks revealed that the *I. persulcatus* spiroplasmas clustered together with, but were not identical to, *S. ixodetis* (55) and most of the spiroplasmas from other hard ticks (**Figure 2A**). Similarly, the phylogeny obtained with the *rpoB* sequences showed tight clustering of the *I. persulcatus Spiroplasma* with most other tick-borne *Spiroplasma* sequences (**Figure 2B**).

The *Spiroplasma* 16S rRNA and *rpoB* gene sequences obtained in the present study were deposited in GenBank under accession numbers MW492370, MW498416, MW498417, MW528409-MW528411.

DISCUSSION

Colonisation in the laboratory has been previously reported to result in decrease or loss of the microbial symbiont *Ca.* M. mitochondrii in *I. ricinus* (2, 41), whereas *Coxiella*-like endosymbionts were detected at high prevalence in *Ornithodoros rostratus*, *Amblyomma americanum*, *Dermacentor silvarum* and *R. microplus* ticks maintained in laboratory colonies for unspecified numbers of generations (55). In the case of the *I. persulcatus Spiroplasma* in the present study, after three generations in the laboratory, 100% of whole adult ticks (21 males, 30 females) were PCR-positive for this endosymbiont. Moreover, 5/5 female ticks and 1/5 male ticks harboured sufficient levels of viable bacteria to allow *in vitro* isolation in tick cell lines. Admittedly, the sensitivity of this technique for detection of infection with *Spiroplasma* is unknown, so it is possible that the remaining four male ticks could also have harboured *Spiroplasma* but either at a level insufficient to allow isolation, or in an organ or tissue that was inadvertently not included in the inoculum, or in a state of viability not conducive to *in vitro* isolation. Tissue tropism of the symbiont *Ca.* M. mitochondrii in *I. ricinus* ticks was found to be highly specific to certain organs (56); further study is needed to determine the tissue tropism of *Spiroplasma* spp. in *Ixodes* spp. ticks.

TABLE 2B | Polymorphisms in sequences detected in the *Ixodes persulcatus*-derived *Spiroplasma* sp. in this study compared to other tick-borne *Spiroplasma* spp.

***Spiroplasma* strain**	**Tick species**	**GenBank accession no**	**Positions[a]**														
			188	**226**	**266**	**399**	**402**	**406**	**460**	**533**	**548**	**620**	**650**	**667**	**680**	**719**	**794**
Spiroplasma sp. Liverpool tick	*I. persulcatus*	MW528411	C	A	C	A	A	C	G	C	A	T	G	A	A	C	A
Spiroplasma sp. BCCAS tick	*I. persulcatus*	MW528410	C	A	C	A	A	C	G	C	A	T	G	A	A	C	A
Spiroplasma sp. strain Irkutsk1	*I. persulcatus*	MW528409	C	A	C	A	A	C	G	C	A	T	G	A	A	C	A
Spiroplasma sp. Bratislava 1	*I. ricinus*	KP967687	**T**	A	C	A	A	**T**	G	C	A	T	G	A	A	C	A
Spiroplasma ixodetis Y29	*I. pacificus*	DQ313832	C	**T**	**A**	**G**	**G**	C	G	**T**	A	T	G	A	A	C	A
Spiroplasma sp. strain DMAR11	*D. marginatus*	MG859278	C	A	C	A	A	C	**T**	C	A	T	G	A	A	C	A
Spiroplasma sp. strain DRET8	*D. reticulatus*	MG859277	C	A	C	A	A	C	G	C	A	T	G	**G**	A	C	A
Spiroplasma ixodetis Y32[b]	*I. pacificus*	MK267069	na	na	na	na	na	na	na	**T**	A	T	G	A	A	C	A
S. ixodetis isolate Ixofrob[b]	*I. frontalis*	MK267074	na	na	na	na	na	na	na	C	A	T	G	A	A	C	A
S. ixodetis isolate IxoricR1532[b]	*I. ricinus*	MK267076	na	na	na	na	na	na	na	C	A	T	G	A	A	C	A
S. ixodetis isolate Ixosp1Tickpanthr11[b]	*Ixodes* sp.	MK267080	na	na	na	na	na	na	na	**T**	A	**C**	**C**	A	**T**	C	**G**
S. ixodetis isolate IxospT2641[b]	*Ixodes* sp.	MK267081	na	na	na	na	na	na	na	C	A	T	G	A	A	C	A
S. ixodetis isolate IxouriaeT2631[b]	*I. uriae*	MK267077	na	na	na	na	na	na	na	C	A	T	G	A	A	C	A
S. ixodetis isolate Ixoarbo2[b]	*I. arboricola*	MK267072	na	na	na	na	na	na	na	**T**	**G**	T	G	A	A	**T**	A
S. ixodetis isolate Ixopac2[b]	*I. pacificus*	MK267070	na	na	na	na	na	na	na	**T**	A	T	G	A	A	C	A
S. ixodetis isolate*Rhigeigy3*[b]	*R. geigyi*	MK267085	na	na	na	na	na	na	na	**T**	A	T	G	A	A	C	A
S. ixodetis isolate Rhideco[b]	*R. decoloratus*	MK267084	na	na	na	na	na	na	na	**T**	A	T	G	A	A	C	A
S. ixodetis isolate RhiannBSP21[b]	*R. annulatus*	MK267082	na	na	na	na	na	na	na	**T**	A	T	G	A	A	C	A

***Spiroplasma* strain**	**Tick species**	**GenBank accession no**	**Positions[a]**											
			812	**821**	**845**	**848**	**866**	**869**	**969**	**1022**	**1039**	**1066**	**1118**	**1313**
Spiroplasma sp. (Liverpool tick)	*I. persulcatus*	MW528411	T	A	T	T	A	T	G	C	G	C	C	T
Spiroplasma sp. (BCCAS tick)	*I. persulcatus*	MW528410	T	A	T	T	A	T	G	C	G	C	C	T
Spiroplasma sp. strain Irkutsk1	*I. persulcatus*	MW528409	T	A	T	T	A	T	G	C	G	C	C	T
Spiroplasma sp. Bratislava 1	*I. ricinus*	KP967687	T	A	T	T	A	T	G	C	G	C	C	T
Spiroplasma ixodetis Y29	*I. pacificus*	DQ313832	T	A	T	T	A	T	**A**	C	G	C	C	**C**
Spiroplasma sp. strain DMAR11	*D. marginatus*	MG859278	T	A	T	T	A	T	G	C	G	C	C	T
Spiroplasma sp. strain DRET8	*D. reticulatus*	MG859277	T	A	T	T	A	T	G	C	G	C	C	T
Spiroplasma ixodetis Y32[b]	*I. pacificus*	MK267069	T	A	T	T	A	T	**A**	C	G	C	C	na
S. ixodetis isolate Ixofrob[b]	*I. frontalis*	MK267074	T	A	T	T	A	T	G	C	G	C	C	na
S. ixodetis isolate IxoricR1532[b]	*I. ricinus*	MK267076	T	A	T	T	A	T	G	C	G	C	C	na
S. ixodetis isolate Ixosp1Tickpanthr11[b]	*Ixodes* sp.	MK267080	**C**	**G**	**A**	**A**	**T**	T	**A**	**A**	**A**	**T**	**T**	na
S. ixodetis isolate IxospT2641[b]	*Ixodes* sp.	MK267081	T	A	T	T	A	T	G	C	G	C	C	na
S. ixodetis isolate IxouriaeT2631[b]	*I. uriae*	MK267077	T	A	T	T	A	T	G	C	G	C	C	na
S. ixodetis isolate Ixoarbo2[b]	*I. arboricola*	MK267072	T	A	T	**C**	**T**	**C**	G	**A**	G	C	C	na
S. ixodetis isolate Ixopac2[b]	*I. pacificus*	MK267070	T	A	T	T	A	T	G	**A**	G	C	C	na
S. ixodetis isolate*Rhigeigy3*[b]	*R. geigyi*	MK267085	T	A	T	T	A	T	G	C	G	C	C	na
S. ixodetis isolate Rhideco[b]	*R. decoloratus*	MK267084	T	A	T	T	A	T	G	C	G	C	C	na
S. ixodetis isolate RhiannBSP21[b]	*R. annulatus*	MK267082	T	A	T	T	A	T	G	C	G	C	C	na

Polymorphisms in the rpoB gene fragment of the Spiroplasma sp. detected in the Liverpool tick and the BCCAS tick and strain Irkutsk1 compared to other Spiroplasma strains isolated from hard tick species I. ricinus, I. pacificus, D. marginatus and D. reticulatus, and sequences detected by PCR in I. pacificus, I. ricinus, Ixodes frontalis, Ixodes uriae, Ixodes arboricola, Ixodes sp., Rhipicephalus geigyi, Rhipicephalus decoloratus and Rhipicephalus annulatus. [a]The number corresponds to the positions of nucleotide substitutions with respect to the sequences MW528409 and MW528411 amplified in this study. Corresponding base substitutions are shown. [b]Short sequences. na: Not available. The substitutions compared to the sequences amplified in this study are shown in bold.

The prevalence of *Spiroplasma* in the original Siberian field ticks from which the laboratory colony was initiated in 2015 is unknown, and therefore it is impossible to determine whether colonisation resulted in maintenance of, or increase in, the infection rate. However, it can be concluded that laboratory colonisation does not have a negative effect on occurrence of *Spiroplasma* in *I. persulcatus*, at least over three generations. There have only been two reports of detection of *Spiroplasma* in *I. persulcatus* ticks. Using 16S amplicon pyrosequencing, *Spiroplasma* were detected in salivary glands of at least 5/6 male and 5/6 female unfed *I. persulcatus* collected in the field in Japan (29), and *Spiroplasma* was successfully isolated into arthropod cell culture from 1/30 questing adult *I. persulcatus* collected in Japan (10). In contrast, no *Spiroplasma* or other mollicutes were

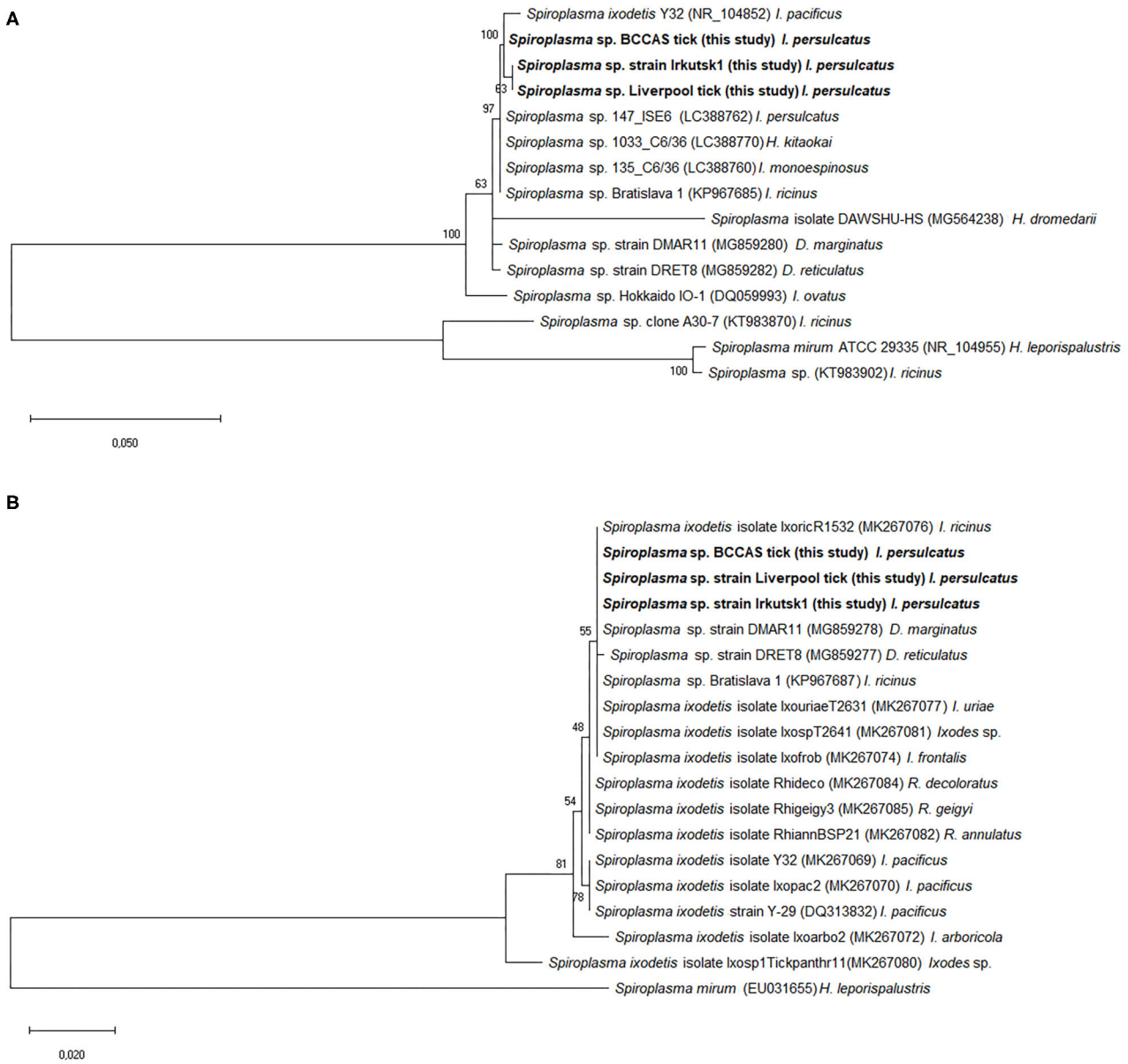

FIGURE 2 | Phylogenetic analysis of *Spiroplasma* gene sequences obtained from third generation *Ixodes persulcatus* colony ticks. Sequences amplified from DNA extracted from representative adult *Ixodes persulcatus* ticks at the University of Liverpool (Liverpool tick) and the Institute of Parasitology, Biology Centre, Czech Academy of Sciences (BCCAS tick) and from a representative *Spiroplasma*-infected tick cell culture previously inoculated with internal organs from an adult *I. persulcatus* (*Spiroplasma* sp. strain Irkutsk1) were compared with published sequences from other *Spiroplasma* spp. or strains derived from ixodid tick species. The evolutionary analysis was inferred using the maximum likelihood method and Kimura 2-parameter model within the Mega X software. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site. GenBank accession numbers of the sequences used in this analysis are shown in brackets following each *Spiroplasma* species or strain and before the tick species host. Sequences obtained in this study are marked in bold. **(A)** Tree constructed from 15 16S rRNA nucleotide sequences and a total of 461 positions in the final dataset. **(B)** Tree constructed from 19 *rpoB* nucleotide sequences and a total of 588 positions in the final dataset.

recorded in questing *I. persulcatus* collected in the Novosibirsk area of Russia and examined by 16S metagenomic profiling (four pools of 87–120 ticks) (32), and *Spiroplasma* were not detected by species-specific 16S rRNA PCR in three questing *I. persulcatus* ticks collected in Finland (3).

Spiroplasma infection rates determined by molecular analysis vary widely in other *Ixodes* spp. ticks collected from the field in different geographical areas. Prevalence of *Spiroplasma* in *I. ricinus* nymphs and adults ranged from 0–0.3% in UK [(21); author's unpublished data] through 5–6% in Hungary and

Czech Republic (57, 58) to 23–30% in The Netherlands, France, Switzerland and Spain (3, 59). The bacterium was detected in 10% of *Ixodes uriae* from Russia (3), 14–16% of *I. arboricola* from Belgium (3, 60) and 100% of *Ixodes ovatus* from Japan (29). The type species *S. ixodetis* was isolated from 7/30 pools representing 600 *I. pacificus* from USA, suggesting a prevalence between 1.2 and 23% (61). Considering this level of variation between species and geographical location, the infection rates of 100% in whole ticks and 60% following *in vitro* isolation in the present study suggest that *Spiroplasma* survives well-under laboratory colony conditions, in both male and female *I. persulcatus* ticks.

Presence of *Spiroplasma* in laboratory colony ticks could affect their ability to be infected experimentally with, and/or transmit, tick-borne pathogens, and therefore their use in this context. A recent study examined correlations between presence of *Spiroplasma* in field-collected *I. ricinus* in Switzerland, and presence in these ticks of bacterial pathogens and symbionts (40). Negative correlations were found between *Spiroplasma* and the pathogens *Rickettsia* spp. and *Borrelia valaisiana* in individual *I. ricinus*, but positive correlations were found between *Spiroplasma* and the symbionts *Lariskella* and *Rickettsiella* at the population level. Further studies are needed to examine whether presence of *Spiroplasma* in *Ixodes* spp. ticks has any effect on acquisition, replication or transmission of tick-borne arboviruses such as TBEV or protozoa such as *Babesia* spp., or indeed any effect on the viability of the ticks themselves.

The molecular analysis revealed almost no differences between the *Spiroplasma* isolated from colonised *I. persulcatus* of Russian origin and cultured for 2.5 months in cell lines derived from heterologous tick species (*I. ricinus*, *I. scapularis* and *R. microplus*), *Spiroplasm*a DNA detected in whole ticks from the same colony and the *Spiroplasma* isolated into *I. scapularis* cells from Japanese *I. persulcatus* (10). Ambiguity was seen in a single nucleotide in the ∼476 bp fragment of the 16S rRNA gene amplified in the present study, and the same nucleotide showed a difference when compared with the sequence from the Japanese isolate. The *rpoB* gene fragment analysed in our study was longer than the 16S rRNA sequences, providing more phylogenetic information, although the shorter length of most of the published sequences from other tick species (4) reduced the coverage available for comparison. The overall topology of the tree and the relationship between strains in the tick-borne *Spiroplasma* branch were very close to the results based on the 16S rRNA gene, although neither of these gene fragments are sufficient to confidently separate *Spiroplasma* strains or species. Nevertheless, as reported previously (21) it is clear that the spiroplasmas harboured by different *Ixodes* spp. ticks are not identical, and also differ from those harboured by *Dermacentor* spp. ticks from broadly contiguous geographic regions.

In conclusion, our study has shown that efficient vertical transmission of *Spiroplasma* can be maintained in *I. persulcatus* ticks under laboratory colony conditions for at least three generations, and has confirmed that co-cultivation of internal organs with tick cell lines is a simple and effective technique for *in vitro* isolation of intracellular tick symbionts such as *Spiroplasma* spp. Further molecular analysis of the cultured *Spiroplasma* strains derived from *I. persulcatus*, either by Sanger sequencing of additional genes or by whole genome sequencing, is required to clarify the phylogenetic relationships between them and *Spiroplasma* harboured by *I. persulcatus* of different geographical origins and by other tick species, and to facilitate an accurate taxonomic classification of these genotypes.

DATA AVAILABILITY STATEMENT

The original sequences generated for this study are publicly available in the NCBI Genbank repository under accession numbers MW492370, MW498416, MW498417, MW528409-MW528411.

ETHICS STATEMENT

The animal study was reviewed and approved by Czech Academy of Sciences (approval no. 161/2010).

AUTHOR CONTRIBUTIONS

AB, VH, JE, TV, MP, and LB-S carried out the experimental work. AB, VH, AP, and LB-S analysed the data. JC, IK, and DR carried out the field work. LB-S conceived the study and drafted the manuscript. AB, VH, JC, DR, and AP revised the manuscript. All authors reviewed and agreed to the final version.

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SYSTEMATIC REVIEW
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The Prevalence of *Coxiella burnetii* in Hard Ticks in Europe and Their Role in Q Fever Transmission Revisited—A Systematic Review

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The zoonosis Q fever is caused by the obligate intracellular bacterium *Coxiella burnetii*. Besides the main transmission route *via* inhalation of contaminated aerosols, ticks are discussed as vectors since the first isolation of the pathogen from a *Dermacentor andersonii* tick. The rare detection of *C. burnetii* in ticks and the difficult differentiation of *C. burnetii* from *Coxiella*-like endosymbionts (CLEs) are questioning the relevance of ticks in the epidemiology of Q fever. In this review, literature databases were systematically searched for recent prevalence studies concerning *C. burnetii* in ticks in Europe and experimental studies evaluating the vector competence of tick species. A total of 72 prevalence studies were included and evaluated regarding DNA detection methods and collection methods, country, and tested tick species. Specimens of more than 25 different tick species were collected in 23 European countries. Overall, an average prevalence of 4.8% was determined. However, in half of the studies, no *Coxiella*-DNA was detected. In Southern European countries, a significantly higher prevalence was observed, possibly related to the abundance of different tick species here, namely *Hyalomma* spp. and *Rhipicephalus* spp. In comparison, a similar proportion of studies used ticks sampled by flagging and dragging or tick collection from animals, under 30% of the total tick samples derived from the latter. There was no significant difference in the various target genes used for the molecular test. In most of the studies, no distinction was made between *C. burnetii* and CLEs. The application of specific detection methods and the confirmation of positive results are crucial to determine the role of ticks in Q fever transmission. Only two studies were available, which assessed the vector competence of ticks for *C. burnetii* in the last 20 years, demonstrating the need for further research.

Keywords: ticks, Coxiella, prevalence, molecular detection, Coxiella-like, endosymbionts, vector

INTRODUCTION

Coxiella burnetii as the causative agent of the zoonosis Q fever is distributed worldwide, except New Zealand. This infectious disease may have a significant impact on animal welfare, human health, and economies (1, 2). *Coxiella burnetii* is a gram-negative bacterium that replicates as an obligate intracellular pathogen under acidic and microaerophilic conditions in phagolysosome-like compartments of cells, predominantly macrophages (3). Infection of hosts mainly occurs due to inhalation of contaminated dust and aerosols (4). During infection, *C. burnetii* shows a tropism for the reproductive tissue and the mammary gland. Therefore, *C. burnetii* is primarily shed during parturition and *via* the milk (5). The considered main reservoirs for human infections are domestic ruminants, whereas other species, such as dogs or horses, can play a minor role as carriers (6). In their birth products, large amounts of infectious agents can be detected (7, 8). As the infective dose by inhalation is <10 bacteria, farmers, and veterinarians are especially at high risk of acquiring this disease through contact with infected animals and their products (9, 10). *Coxiella burnetii* develops spore-like forms, which are resistant to environmental stressors such as desiccation or sunlight. Therefore, the bacteria survive under adverse conditions over long periods in the soil or other dry substances (11).

An infection in ruminants, also termed coxiellosis, is often asymptomatic and not noticed until human Q fever cases occur (12). Decrease in fertility and increase of abortion and stillbirth are common indications of an ongoing Q fever disease in herds or flocks of ruminants (13, 14).

Outbreaks of Q fever, as seen in the Netherlands in 2007–2010, clearly show the huge impact of this infectious disease not only on agricultural economics but also on human health (15, 16). Acute human Q fever infection presents mainly flu-like symptoms with possible complications concerning the lung and liver. Although 60% of acute cases remain asymptomatic, in 1–5% of cases, chronic Q fever may develop (17, 18). This often affects the heart valves as endocarditis. Antibiotic treatment is mandatory over months and, in some cases, up to several years (17–19). Besides the long and difficult treatment, the disease can also be fatal in acute and chronic cases. Although the mortality of acute Q fever was assessed to be 1% in a study on hospitalized patients, the mortality in chronic cases has been reported as 13% and even up to 38% during the Dutch outbreak mentioned earlier (1, 18, 20).

The bacterium is known as an infectious agent since the 1930s when it caused an outbreak of query fever among abattoir workers. In the years after its first isolation from a *Dermacentor andersonii* tick (21), early experimental studies showed horizontal transmission of the agent from ticks to mammalian hosts and suggested that several tick species, e.g., *Haemaphysalis humerosa*, *Haemaphysalis bispinosa,* and *Rhipicephalus sanguineus*, may be capable of transmitting *C. burnetii* (22–25). Although transstadial transmission was shown for most of the examined tick species, the transovarial transmission was reported rarely (25, 26). Later, it was demonstrated that *Hyalomma dromedarii* infected by inoculation excreted *C. burnetii* with their saliva (27). Duron et al. summarized the vector competence data for seven hard tick species (28). Hence, since its first detection, *C. burnetii* has been discussed as a ruminant-associated tick-borne bacterium.

During outbreaks of Q fever, ticks are regularly tested for *C. burnetii*. Until today, no *C. burnetii*-positive ticks associated with outbreaks have been documented. Human infection after tick exposure has been reported, but due to the predominant infection route by inhalation, the source of infection remains unclear (29, 30).

Besides pathogen transmission, ticks are host to many endosymbionts and harbor a diverse microbiome (31). Although *C. burnetii* was the only known member of the genus *Coxiella* for a long time, closely related bacteria were found in recent studies, referred to as *Coxiella*-like endosymbionts (CLEs) (32, 33). This heterogenic group of bacteria with different genome sizes and gene content shows up to 97% genome identity with *C. burnetii* (33–37). Genetically, CLEs are classified into four clades (A–D), and *C. burnetii* belongs to clade A, which otherwise contains CLE associated with soft ticks (38).

Coxiella-like endosymbionts were detected in many different tick species, and for some species, an obligatory mutualism was proven (39–41). As obligate hematophagous arthropods, ticks are dependent on external vitamin B synthesis, likely supplied by the harbored bacteria (40, 42). Furthermore, a positive effect on fecundity was shown for *R. sanguineus*. Treatment with ofloxacin resulted in a significantly lower egg mass, hatching rate, and viability of larvae in this tick species (39). Similar results were shown for *Haemaphysalis longicornis, Rhipicephalus microplus,* and *Rhipicephalus haemaphysaloides*, which were treated with tetracycline and kanamycin, respectively, indicating the influence of CLE on reproduction (43–45).

CLEs seem to be apathogenic, but single studies showed pathogenic potential in particular cases. Fatal infection with CLE was occasionally observed in different bird species (46–48). Furthermore, a skin-associated inflammation caused by the tick-borne bacterium *Candidatus Coxiella massiliensis* was described (49).

The polymerase chain reaction was established in the 1980s and was subsequently used as the predominant detection method. Until then, ticks were primarily tested for the occurrence of *C. burnetii* and related pathogens with staining methods and animal infection experiments (50–52). Because *C. burnetii* is difficult to isolate and cultivate as an obligate intracellular organism, molecular diagnostic using PCR is the method of choice for its detection. Various protocols target several plasmids and chromosomal genes, for example, IS1111, *icd*, *com1*, *sodB*, and GroEL/*htpB* (53–55). The target gene IS1111 is a transposase-like insertion sequence, and the amount of copies from 7 to 110 per genome varies between isolates. Thus, the use for quantification is limited, but due to the higher number of targets per bacterium, this signal amplification leads to a higher sensitivity compared with single-copy targets (53). In contrast, *icd,* encoding for the isocitrate dehydrogenase gene, and *com1*, encoding for the *C. burnetii* outer membrane protein 1, are single-copy genes allowing quantification (53, 54). The commonly used target genes for *C. burnetii* cross-react with

CLE and may lead to misidentification (28, 56). In a study by Duron, roughly one-third of CLE-positive ticks were positive for the IS1111 element (57). Furthermore, IS1111 was shown to be the most unspecific marker for detection of *C. burnetii* in CLE-positive ticks, followed by GroEL/*htpAB* (for chaperone heat shock protein), whereas *icd* was not amplified in the samples (56). However, the *icd* sequence of a novel CLE derived from *Carios capensis* soft ticks was >90% similar to *C. burnetii* (58). As a consequence, usage of a target gene of low specificity could lead to an overestimation of the role of ticks as host and vector of *C. burnetii*. To distinguish between *C. burnetii* and CLE, there is no specific method available, owing to the fact that the group of CLE is very heterogeneous. Therefore, the most reliable method is the sequencing of PCR-positive samples using highly conserved genes such as *rrs* or *groEL* (56, 59).

Ticks (Ixodida) are obligate blood-feeding ectoparasites with a global distribution. A total of 67 tick species are reported in Europe and Northern Africa (60). Out of these, only a small number, i.e., 15–17 hard tick species, depending on their confirmed taxonomic status as a distinct species or subspecies, have been found to harbor either *C. burnetii* or CLE (28, 61). When considering a particular tick species to serve as a vector for a given pathogen, certain aspects of the tick's life cycle should be considered in addition to the sole finding of molecular traces or even morphological structures within an examined tick. This information will help to understand further whether this tick species has vector competence, defined as the capability to transmit a given pathogen horizontally or vertically, which is an essential aspect of the vector capacity. The latter is more relevant for determining the infection risk, as it includes parameters such as longevity, feeding behavior (duration, frequency, and preferred blood source), population density, and frequency of host contact encounters. In this regard, ticks, which are feeding on different hosts in every life stage (three-host-ticks) with a large geographical range, high population densities, and promiscuous feeding behavior are relevant, when it comes to pathogen transmission. The most common hard tick of Europe, the castor bean tick *Ixodes ricinus*, is fulfilling these criteria, making it the most important vector tick species for bacterial and viral tick-borne diseases, e.g., *Borrelia burgdorferi* s.l. or tick-borne encephalitis virus, in Europe (62). Such vector-borne agents, some with a wide range of host species, may have their reservoir in wildlife, leading to a sylvatic transmission cycle between arthropods and wild animals. In addition, transmission can also occur in an urban or domestic cycle, including pets, livestock, or humans. Although *I. ricinus* is ubiquitous in Europe, other relevant tick species are not, and only for some geo-referenced distribution maps are available, e.g., for both *Dermacentor* species (63). However, the geographical distribution of a particular tick species is not static but rather subject to permanent habitat changes due to increased movement of humans, animals, and goods and, very importantly, the increasing ambient temperatures as major drivers. The high frequency of recent introductions of *Hyalomma* spp. ticks into temperate Europe is impressive sign of a very menacing trend of potent vector tick species moving into new areas (64, 65).

Recent prevalence studies using molecular methods aim at determining the percentage of ticks carrying *C. burnetii* among other diverse tick-borne pathogens. This allows surveillance of *C. burnetii* in ticks and benefits the identification of a potential risk of acquiring a Q fever infection by a tick-bite for humans or domestic animals.

Different methods exist for the sampling of ticks. As most European hard ticks seek hosts by questing on the vegetation and naturally intend to be wiped up by a passing host, the mainly used collection method is flagging or dragging. Using this method, a cotton blanket is dragged or flagged over the vegetation, considering that both methods favor different tick species (66). Ticks are stripped off and are hindered in their motion by the weave. The success of this method depends on the texture of the blanket and the structure of the vegetation (67). Activity peaks of most questing ticks are in spring and autumn. However, other studies observed a trend to a unimodal peak with the highest activity in the spring months (68, 69). Ticks can also be removed directly from their host. This method is used to examine the burden of pathogens associated with these animals and for the sampling of hunting ticks, such as *Hyalomma* spp., which are less likely to be collected by flagging (70).

This review gives a comprehensive overview of the present literature on *C. burnetii* in ticks and discusses the vector competence of European tick species for the Q fever agent. To this end, data of prevalence studies conducted in Europe concerning the occurrence of *C. burnetii* in ticks were systematically compiled and analyzed regarding the spatial occurrence, molecular detection method used, and the frequency of detection per tick species, as well as the detection of CLE. Furthermore, experimental approaches to investigate the vector competence of tick species for *C. burnetii* were collected. The determination of the vector competence of ticks is important to assess the role of ticks as a reservoir in Q fever epidemiology.

MATERIALS AND METHODS

Studies about *C. burnetii* in ticks were classified into two different categories. One group investigates the prevalence of ticks for *C. burnetii* in Europe. The other group discusses the vector competence of ticks based on experimental approaches on ticks infected under laboratory conditions. For identification of studies, the databases Pubmed and Web of Science were systematically searched for articles in English, published between 2000 and 2020. Search terms were "*coxiella* tick," "*coxiella*-like," "*coxiella* vector competence." The search was completed on October 27, 2020. Additional studies were included using Google Scholar. References were sorted by the use of Endnote X7 (Clarivate Analytics, Philadelphia, USA). Only studies using DNA detection methods for *Coxiella* spp. on hard ticks obtained in Europe were included as prevalence studies. The definition of Europe used in this study refers to a geographical demarcation as von Strahlenberg's, including all countries of which the major part is situated between the Urals and the Bosphorus. Regions were defined according to the United Nations geoscheme for

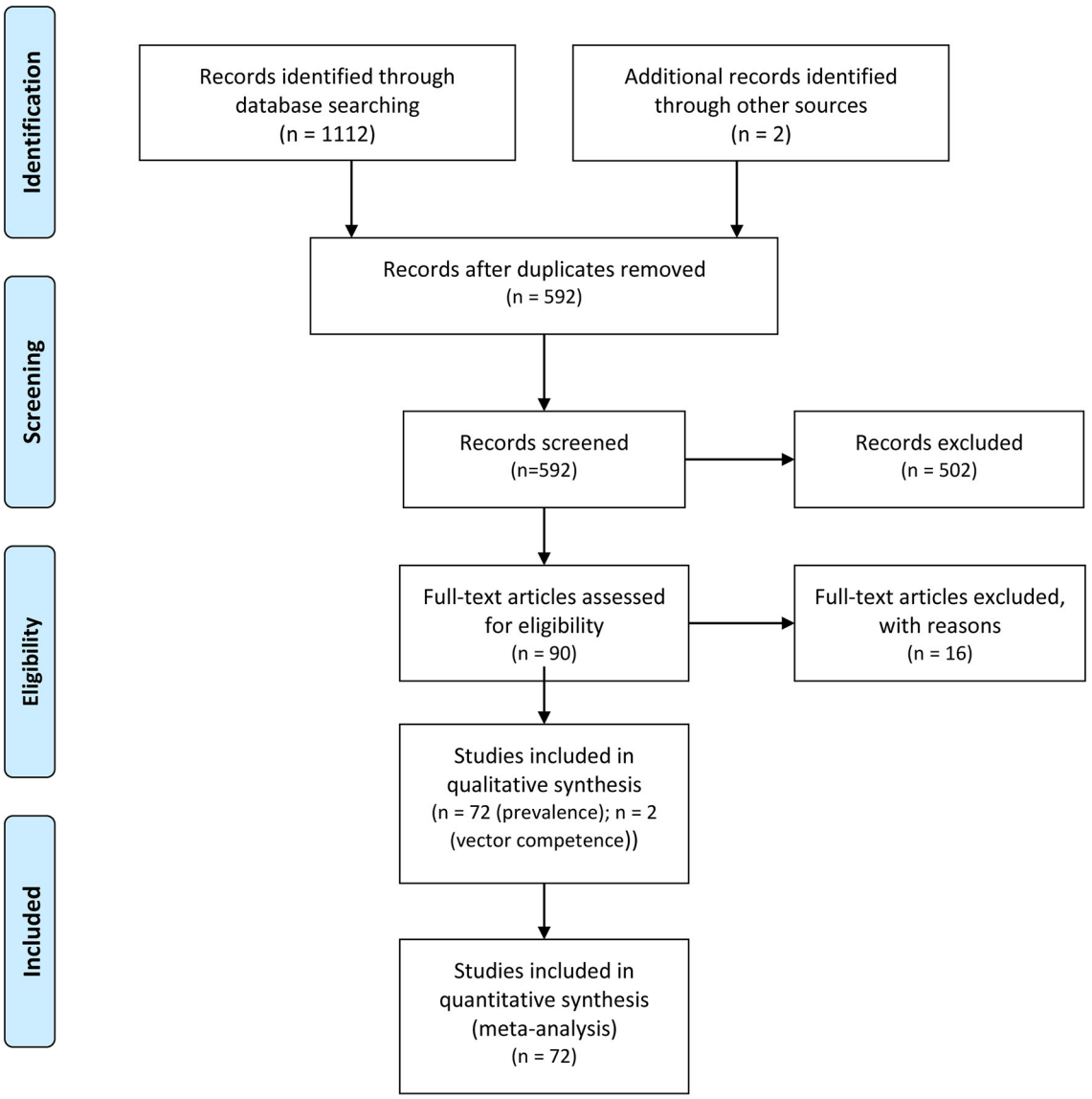

FIGURE 1 | PRISMA flow chart for selection of studies, including the reasons for exclusion from the analysis.

Europe in Southern Europe, Eastern Europe, Northern Europe, and Western Europe (71).

Further criteria for eligibility as prevalence studies were stated information of the number of tested ticks, the number of *Coxiella*-positive ticks, or amount and description of tick pools and at a minimum description of the tick genus or species name.

Data were extracted regarding information about the country in which the ticks were obtained. Furthermore, the number of tested and *Coxiella*-positive ticks and tick pools and the collection and detection method were included in the analysis. All publications were read, and data were extracted by two persons independently.

The proportion of positive ticks was calculated as the ratio of positive ticks to the total number of tested ticks. Prevalence was defined as the proportion of positive ticks multiplied by 100. When ticks were tested in pools, the proportion was estimated as minimum infection rate (MIR) using the formula

$$\text{MIR} = \frac{\text{number of positive pools}}{\text{number of tested specimen}}$$

Studies were evaluated, and confidence interval was calculated using Excel 2016 (Microsoft Corporation, Redmond, USA). For statistical analysis, the Kruskal–Wallis test and Mann–Whitney U test for data lacking normal distribution were performed using SPSS V.22 (IBM, Armonk, USA), and results were considered significant in the case of $p < 0.05$.

RESULTS

A total of 590 studies were found using the mentioned search terms. Additionally, two studies using the platform Google Scholar were included. Based on the title and abstract, 502 studies were excluded. Main reasons for exclusion were non-European studies ($n = 272$), no hard ticks tested ($n = 120$), or reviews ($n = 44$); 66 studies were excluded for other reasons (**Figure 1**: PRISMA analysis). After full-text analysis, 16 further studies were removed. Finally, 72 publications in which the prevalence of *C. burnetii* in ticks collected in European countries were examined, compiled, and evaluated. Additionally, two vector competence studies were included.

Prevalence Studies in Europe—A Systematic Analysis

Prevalence studies were analyzed by country, the number of tested ticks, tick species, the method used for tick collection, and the method used for detection of *C. burnetii* (**Supplementary Table 1**). The ticks tested in these studies were sampled between 1994 and 2018. In more than half of the studies ($n = 44$), ticks were collected between the years 2011 and 2013. Studies were performed in 23 of 45 different European countries.

Most collection areas were rural, single studies collected ticks in recreational areas in urban settings (72, 73). Furthermore, ticks were sampled from vegetation and animals on European islands (74–77).

In total, 115,265 ticks were tested in all 72 analyzed studies, of which 62,889 were sampled in a single and thus largest study performed in Switzerland (78). Excluding this study, on average, 689 ticks were sampled per study, ranging from 18 to 7,050 samples. The mean prevalence of *C. burnetii*-DNA over all evaluated studies was 4.8%. In half of the studies ($n = 38$), no *C. burnetii*-positive tick was identified. The highest prevalence was determined to be 54.2% in a Spanish study (79). Of the 34 studies with positive results, 10 (29.4%) confirmed the results with the sequencing of at least one PCR amplicon. Sequencing could not be performed in some cases because of low DNA yield (80). Ticks were sampled by flagging or dragging from the vegetation or removed from animals.

Most studies simultaneously gathered further information, e.g., about tick infestation of local animal species or other tick-borne pathogens. In addition, the seroprevalence for *C. burnetii* or other tick-borne agents of captive domestic and wild animals was examined (79–82).

Country

There were major differences in prevalence between different countries and regions in Europe (**Figure 2**, **Supplementary Figure 1**). The majority of studies were performed in Southern Europe ($n = 30$), whereas the number of prevalence studies per country was highest for Slovakia ($n = 12$). A high prevalence was found in studies in Southern Europe, especially in Spain (10.1%) and Portugal (10.6%), but also in Poland, a high proportion of ticks was positive (8.5%). In ticks tested in Northern Europe, no *C. burnetii* was detected (83–86).

The highest prevalence within single studies was determined in Spain and Italy. González et al. detected *Coxiella*-DNA in over 50% of the 236 tested ticks (79). In studies from Italy and France, more than 30% of tested ticks were found to be positive for *C. burnetii*-DNA (87–89). The mean prevalence in Southern Europe (8.3%) was significantly higher than in the rest of Europe (Mann–Whitney U test; $p = 0.031$), especially in Western Europe (2.1%) (Mann–Whitney U test; $p = 0.013$).

Tick Species

More than 25 tick species were examined in all studies (**Table 1**). In *Amblyomma* spp., *Haemaphysalis inermis*, *Hyalomma scupense*, *Hyalomma truncatum*, *Ixodes festai,* and *Ixodes hexagonus*, no molecular detection of *C. burnetii*-specific DNA was reported. Furthermore, in *Haemaphysalis hispanica*, *Hyalomma aegyptium*, *Hyalomma lusitanicum*, *Ixodes ventalloi, Rhipicephalus pusillus,* and *Rhipicephalus thuranicus, Coxiella*-DNA was detected, but the results were not sequenced for confirmation or discriminated from CLE.

Differences in detecting *C. burnetii*-positive ticks were noticed for the tick genera tested (**Figure 3**). The highest prevalence was observed in *Hyalomma* spp. ticks (11.3%). Of the six species tested, *Hyalomma rufipes*, *Hyalomma marginatum, H. lusitanicum,* and *H. aegyptium* were positive for *C. burnetii*. The most abundant and most often infected species was *H. lusitanicum*, in which 17.7% of ticks were PCR positive for *C. burnetii*. Furthermore, 6.0% of all tested *Rhipicephalus* spp. were positive for *C. burnetii*-DNA, whereas *Dermacentor* spp. harbored *Coxiella*-DNA in 1.4% of samples. By far, the most tested ticks belonged to the genus of *Ixodes* spp. (81.4%). Of these ticks, only 0.4% carried *C. burnetii*-DNA. The second least *Coxiella*-infested tick species was *Haemaphysalis* spp. with 1.2% positive samples. Of *Amblyomma* spp., only 11 negative ticks were flagged or removed from birds, which is not surprising, as this genus is not endemic in Europe (77, 90).

Collection Method

The most common methods for prevalence studies were sampling from animals ($n = 29$, 40.3%; 11,283 ticks) or from the vegetation by dragging or flagging a piece of cotton ($n =$

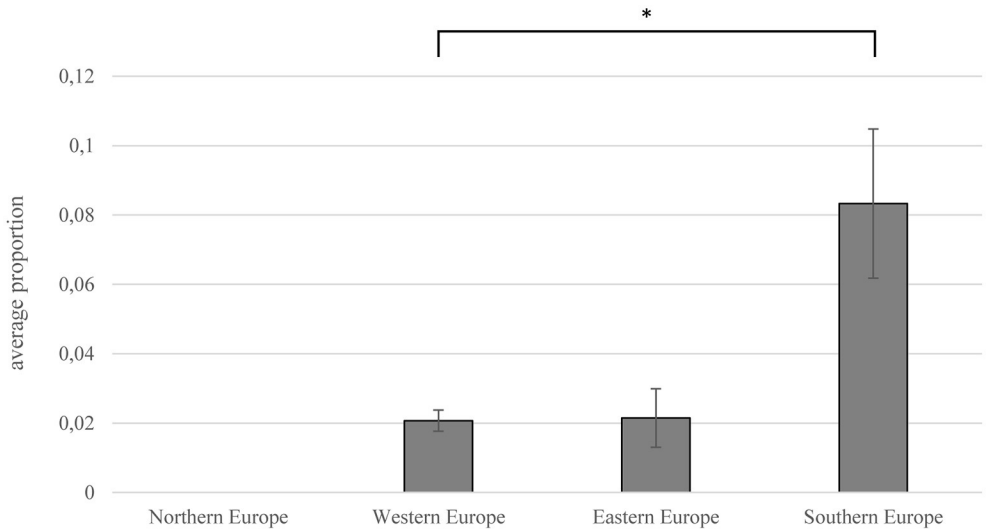

FIGURE 2 | Prevalence (ratio of *C. burnetii*-positive ticks to total number of tested ticks) depending on European regions. Error bars show average confidence interval; significance was proven using Mann–Whitney U-test (*$p = 0.013$).

27, 37.5%, 83,476 ticks). Overall, in the evaluated studies, 72.4% of all tested ticks were derived from vegetation. In 19.4% ($n = 14$; 19,939) of the examined studies, a combination of sampling from animals or humans and flagging was performed. Data in this review were not divided into the different collection methods used. Two studies examined the occurrence of pathogens in ticks exclusively collected from humans (123, 124). The highest mean prevalence for *C. burnetii* was found in studies in which ticks were removed from infected animals with 6.6%, whereas the mean prevalence in ticks collected from vegetation was 2.8% (**Figure 4A**). Of all tested ticks removed by flagging, 0.5% were positive for *C. burnetii*-DNA (**Figure 4B**). Tested animals ranged from pets and livestock to trapped rodents or game. In some studies, ticks were removed from birds to examine their role as hosts and vehicle for tick species (81, 88, 125).

C. burnetii was detected in two *H. marginatum*, feeding on humans in Sardinia. Contrary, in two studies that tested human-derived ticks exclusively, all were negative for *C. burnetii*-specific DNA (76, 123, 124).

The only study that was directly associated with a Q fever outbreak was performed in the Netherlands. Almost 3,000 ticks were sampled from nature, domestic animals, and wildlife. No *C. burnetii*-positive questing tick was found, whereas five female ticks collected from animals were positive for IS1111 and *com1* target sequences, suggesting uptake of blood from a bacteremic host (111). Further investigations revealed that these positive ticks were collected from recently vaccinated sheep. This might indicate a low risk of field infection during the outbreak.

Detection Method

Conventional PCR and real-time PCR were applied as a detection method in most of the studies. The predominant target genes for PCR detection were fragments of the insertion sequence IS1111 ($n = 37$, 51.4%, 33,626 ticks) or the genes *com1* ($n = 14$, 19.4%, 10,725 ticks), *icd* ($n = 12$, 16.7%, 17,631 ticks), *sodB* ($n = 5$, 6.9%, 3,502 ticks), and *htpAB* ($n = 4$, 5.6%, 3,192 ticks). Single studies used more than one target gene for identification (75, 85, 100, 126, 127). Of studies, which used *sodB* as the target gene for *C. burnetii*, an average prevalence of 7.3% was reported (**Figure 5A**). Of the studies using PCR targeting the *com1* fragment, the mean prevalence was 0.8%. The most frequently used target IS1111 resulted in an average prevalence of 5.7%. Of all ticks tested with IS1111, 3.0% were positive, which is the highest percentage (**Figure 5B**). The largest sampling size of 62,889 ticks, exclusively *I. ricinus*, was collected in a study in Switzerland. No *C. burnetii*-positive tick was detected by using *ompA* as the target gene in this study (78). Also, 16S ribosomal RNA (rRNA) sequencing was used for the detection of *Coxiella* spp. in eight studies; one of them detected *C. burnetii*-positive ticks (102).

Before PCR detection, some studies used the hemocyte test for visual detection of *Coxiella* spp. and morphologically similar agents in the hemolymph (91, 104). In these studies, *C. burnetii* was detected by PCR in four hemocyte-positive ticks, but also in two hemocyte-negative ticks.

Furthermore, studies used non-molecular methods in addition to PCR, such as cultivation in cells or embryonated eggs, resulting in the isolation of *Spiroplasma* spp. but not *C. burnetii* (126).

Coxiella-Like Endosymbionts

Some authors considered CLE and searched specifically for these endosymbionts or detected CLE by sequencing positive PCR products. Furthermore, sequencing of 16S rRNA was used to evaluate the tick microbiome (99, 103). In seven studies, CLEs were detected additionally or exclusively using sequencing in a variety of tick species, or *Coxiella* was only identified on genus level (99, 102, 103, 110, 119, 122). Nine tick species were found to harbor CLE in these studies.

TABLE 1 | Selected tick species collected in Europe and detection of *C. burnetii* and CLE (Reference).

Tick species	Molecular detection of *C. burnetii*	samples sequenced	Vector competence studies	Detection of CLE
Dermacentor marginatus	Yes (72, 90–97)	Yes (93)	Fecal excretion (98)	Yes (99)
Dermacentor reticulatus	Yes (82, 92, 100, 101)	Yes (82, 101, 102)	–	Yes (102, 103)
Haemaphysalis concinna	Yes (91, 101, 104, 105)	Yes (101)	–	–
Haemaphysalis inermis	No		–	Yes (102)
Haemaphysalis punctata	Yes (72, 80, 90, 93, 106)	Yes (93)	–	–
Haemaphysalis sulcata	Yes (74, 93, 95)	Yes (93)	–	–
Hyalomma aegyptium	Yes (107)	No	Transstadial transmission (108)	–
Hyalomma lusitanicum	Yes (79, 90, 94)	No		–
Hyalomma marginatum	Yes (76, 87, 88, 97)	Yes (76)	–	–
Hyalomma rufipes	Yes (88)	–	–	–
Hyalomma scupense	No	–	–	–
Hyalomma truncatum	No	–	–	–
Ixodes acuminatus	Yes (109)	Yes (109)	–	–
Ixodes festai	No	–	–	–
Ixodes hexagonus	No	–	–	Yes (99, 110)
Ixodes ricinus	Yes (72, 80, 81, 91, 92, 94, 96, 101, 102, 104, 105, 109, 111–117)	Yes (101, 102, 109, 112)	fecal excretion and transstadial transmission (98)	Yes (99, 102, 110, 118)
Ixodes ventalloi	Yes (96)	No	–	–
Rhipicephalus annulatus	Yes (76, 87)	Yes (76)		
Rhipicephalus bursa	Yes (76, 87, 93, 97)	Yes (76, 93, 97)	–	Yes (99, 119)
Rhipicephalus pusillus	Yes (90, 94)	No	–	–
Rhipicephalus sanguineus	Yes (74, 76, 90, 93, 96, 97, 120, 121)	Yes (76, 93, 95, 97)	historic: transstadial transmission (22)	Yes (99, 110, 122)
Rhipicephalus turanicus	Yes (72, 74)	No	–	Yes (122)

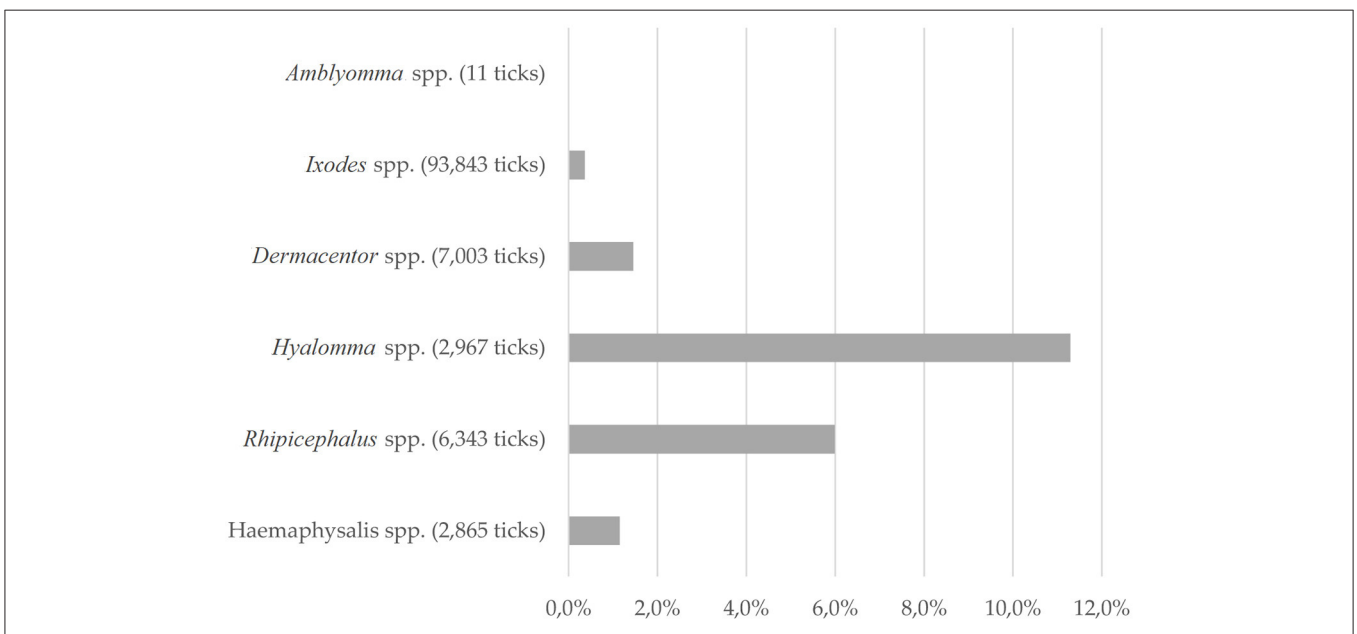

FIGURE 3 | Tick species: Proportion of *C. burnetii*-positive ticks of the ticks tested, depending on tick genera. For 2,233 ticks, only total numbers were described.

Experimental Vector Competence Studies

In the decades after the first isolation of *C. burnetii* from a tick, experimental transmission studies were conducted on various tick species. However, only two recent investigations on the vector competence of ticks under laboratory conditions were found in the literature.

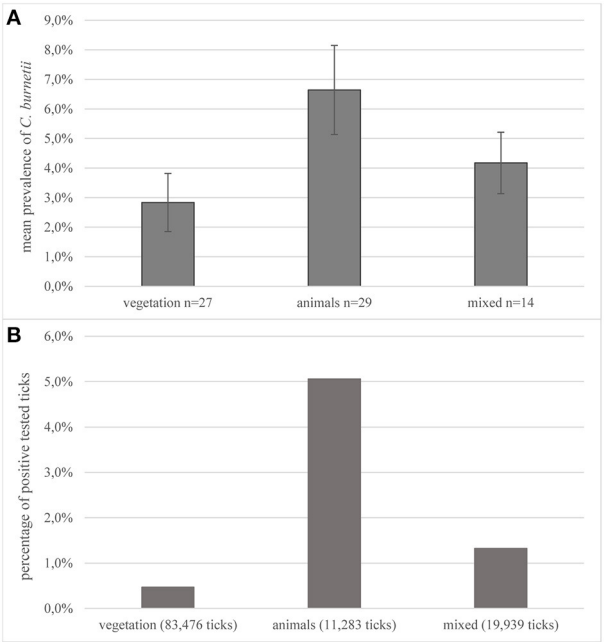

FIGURE 4 | Collection methods. **(A)** Average prevalence of *C. burnetii* in studies on ticks depending on the collection method. Error bars show average confidence intervals. *n* = number of studies; **(B)** percentage of positively tested ticks of the total number of tested ticks in all studies, depending on the collection methods. Number in parentheses = total number of sampled ticks with this method.

In one study, the vector competence of the tick *H. aegyptium* was proven, including the transstadial transmission over all life stages (108). Larvae were fed on infected guinea pigs, and 5.6% tested positive after molting into nymphs. After feeding on an infected host, 28.9% of nymphs molted into adults remained positive. In addition, reinfection of uninfected guinea pigs was shown in this study.

For *I. ricinus*, a transstadial transmission from nymphs to adults was shown using an *in vitro* feeding system, enabling better differentiation between the transmission *via* saliva or feces (98). The transstadial transmission rate from nymphs to adults was determined to be 25%, but horizontal transfer into the blood was not shown, whereas the ticks excreted infectious feces during feeding and after molting.

DISCUSSION

Higher Prevalence in the Mediterranean Region Might Be Associated With Regional Distribution of Different Tick Species

Duron et al. found an average prevalence of 5% of *C. burnetii* in ticks after evaluation of 60 studies conducted worldwide (28). The data of the present study show a similar mean prevalence of studies on ticks in Europe. Here, an update is provided, enhancing the insight into the prevalence of *C. burnetii* in various tick species, demonstrating that there is a huge variation between the results of different studies depending on the country, tested species, method of collection, and *C. burnetii* detection method applied.

Evaluating the studies, a gradient from North to South was noticed, resulting in a higher prevalence in the Mediterranean region, followed by Eastern Europe. Most positive samples were obtained in Southern Europe, especially Portugal, Spain, and Greece. Here, species of the genera *Hyalomma*, *Rhipicephalus*, and *Haemaphysalis* are the most abundant vector ticks. Due to climate change associated with global warming, the spread of arthropods is expected (128), which could possibly lead to an increased role of pathogen transmission by these tick species in Western and Northern Europe. The tick species *H. marginatum* was detected in the last years more often in Central Europe and hibernation of this species was recently observed in Germany (64). Since in most of the studies, no confirmation of positive results by specific methods for *C. burnetii* was performed, an inadvertent detection of CLE cannot be excluded. However, this differentiation is extremely important because CLEs seem to have less if at all pathogenic potential than *C. burnetii*.

Coxiella burnetii is rarely found in ticks in most of the regions in Europe. It is presumed that ticks play a minor role in Q fever transmission, also considering that no validated human cases of *C. burnetii* infection via ticks have been reported. Foci in which ticks may play a role as the natural reservoir for Q fever seem to exist, but these hot spots are hard to detect. Similar patterns of regional foci were described for tick-borne encephalitis virus (129, 130). The absence of *C. burnetii*-infected ticks in endemic areas is often explained by narrow hot spots (131). That causes less frequent surveillance of this pathogen in major prevalence studies, mainly focusing on well-described tick-borne infectious agents such as *Anaplasma phagocytophilum* or *B. burgdorferi* s.l. Under these circumstances, there is a lack of data in certain countries and regions. The evaluation of European studies shows that occurrence of *C. burnetii* is highly dependent on the tick species. The most abundant tick species in Europe, *I. ricinus*, seems to be infected with *C. burnetii* very rarely. Occasional reports of successful isolation of *C. burnetii* from field-collected *I. ricinus* support the theory of less relevance in Q fever transmission (132). In the past, a correlation was presumed between the occurrence of Q fever and the abundance of *Dermacentor marginatus* (133). Considering the low prevalence in this species, this theory was not proven by the data collected here from the analyzed studies. However, there seems to be an association of *C. burnetii* with ticks of the genus *Hyalomma* spp. and *Rhipicephalus* spp., naturally present in regions with ambient temperatures, e.g., the Mediterranean region. This is also supported by the *in vivo* experimental study performed with *H. aegyptium* (108). As *Rhipicephalus* spp. is highly infected with CLE, positive results should be confirmed by sequencing (41, 119).

Prevalence studies targeting *C. burnetii* in ticks are conducted worldwide, and positive ticks were found on all continents except Antarctica (134–138). Comparably with European studies, there are divergent findings in the different investigations. Recently, a study conducted in China using 16S rRNA sequencing reported

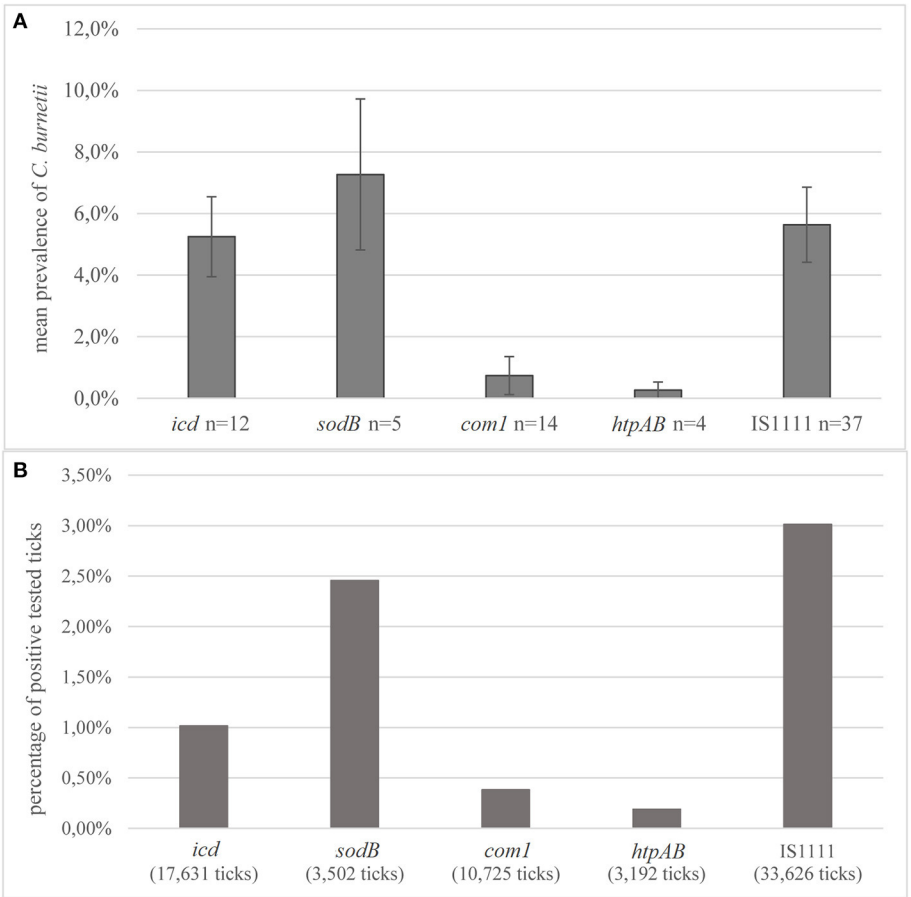

FIGURE 5 | Detection methods **(A)** average prevalence of *C. burnetii* in PCR detection depending on the target genes. Error bars show average confidence intervals. *n* = number of studies; **(B)** percentage of positively tested ticks of the total number of tested ticks in all studies, depending on the detection method. In two studies, 567 ticks were collected from humans and tested negative. Number in parentheses = total number of ticks tested with this method.

a prevalence of 40–96% depending on tested species (139). High prevalence was also found in several countries, e.g., Argentina, Egypt, or Nigeria (140–142). Apart from that, in studies in Japan or Reunion Island, no *Coxiella*-positive ticks were determined (143, 144). Despite the origin of the first isolate, *C. burnetii* is reported rarely from ticks in North America (145, 146).

A Combination of Different Collection Methods Supports a Realistic Depiction of Reality

Different collection methods were used in the studies, but the most common is removing from animals or flagging. Simple detection of the DNA of a pathogen does not prove the vector competence of a certain tick species (92, 147). Ticks can also carry a pathogen without the ability to transmit it (93). These arthropods feed on bacteraemic/viraemic/protozooaemic animals and are likely to take up any agent circulating in the blood of the host. The slightly higher prevalence of *C. burnetii* in ticks collected from animals compared with ticks from vegetation argues in favor of such an uptake. The procedure of flagging of questing ticks, therefore, seems to be more appropriate for information regarding the prevalence and to discriminate between the role of ticks as a reservoir or as an accidental host (148). Additionally, data about the local seroprevalence of *C. burnetii* in animal host species could be included. A combination of both origins, vegetation, and animals, might also be of interest, especially in the context of outbreak surveillance. Moreover, only exophilic and questing ticks can be obtained using the dragging method. That excludes, for example, premature life stages of *Dermacentor* spp. or preferably hunting ticks, e.g., *Hyalomma* spp. As it is known that *C. burnetii* genomes found in ticks genetically cluster with samples isolated from wildlife, the inclusion of hunted animals can increase the knowledge of potentially sylvatic cycles (149). The sampling of ticks from migratory birds can give insight into the movement of ticks and the pathogens or microbial communities they carry. Examination of avian ticks and tick-borne diseases can also help monitor the spread of these ticks and their pathogenic cargo (150). The introduced tick species might not be adapted to the climate and availability of host species in this country; therefore, its survival is unclear. Sampling on farm animals and game favors

a higher rate of adult ticks, as this life stage is more frequently feeding on larger animals (112). Examination of ticks removed from human patients also might be of interest regarding the zoonotic potential of the disease. In a study by Dubourg et al., patients showing scalp eschar were examined, and removed ticks were tested for a wide range of tick-borne pathogens. Of the 11 ticks, mainly *D. marginatus*, two carried *C. burnetii*, whereas *Rickettsia slovaca* was the most prevalent pathogen (151). Simultaneous infection of human patients with *C. burnetii* and other tick-borne pathogens were described, but it cannot be excluded that the temporal connection between the infections is random, as Q fever is endemic worldwide, and infection might have been caused by inhalation (30). To determine the significance of ticks in Q fever transmission, it might be of major interest to perform prevalence studies in areas of active Q fever outbreaks or in known *Coxiella*-positive herds. This is important to prove any association between infected animals or humans and the local arthropods. Furthermore, identification of tick life stage is important to analyze the possibility of transovarial transmission. *Coxiella burnetii* is considered one of the most relevant pathogens and should be prioritized in the examination of wildlife (152).

Prevalence Studies Should Include Specific *C. burnetii* Detection Methods

There was no significant difference noticed between the various target genes used in the evaluated studies. The determined prevalence depends, among other things, on the sensitivity of the used target gene. Some assays, especially when using IS1111, detect lower amounts of DNA and thus are more sensitive than others. This relation can be described more precisely by the use of standardized controls such as plasmids. Because of the close genetic relationship to CLE, the high specificity of molecular methods is required. Because frequently used PCR target gene sequences, e.g., IS1111, were also detected in endosymbionts, there is a need for specific methods to distinguish between these species. This would rule out an overestimation of the dissemination of *C. burnetii* within the tick population. The majority of studies detecting commonly used target genes for the detection of *C. burnetii* did not confirm the results with sequencing. Particularly, the IS1111 fragment is a common target for *C. burnetii* detection in ticks, which is known to be less specific but highly sensitive for the pathogen (57). The specificity for detection of *C. burnetii* in tick samples is limited in all commonly used target genes; thus, the use of single targets is not recommended (56, 58). Positive results in PCR should be interpreted carefully, and sequence confirmation should be mandatory, preferably using long targets for increased specificity (56, 153). Due to low DNA yield, sequencing might not be possible. In these cases, the combination of different target genes for detection could be a valid method to minimize the risk of unspecific results. Thus, there is a need to develop specific assays for differentiation between *C. burnetii* and CLE, which might also be applicable for higher sample sizes and poor DNA yield.

Unclear Pathogenic Potential of *Coxiella*-Like Endosymbionts Should Not Be Neglected

In seven studies, CLEs were detected in tick samples, mainly using sequencing of 16s rRNA. These endosymbionts are distributed in several hard and soft tick species and represent a large proportion of the microbiome of some species (33, 154). This leads to the presumption that the specificity of some molecular methods may not be sufficient to distinguish between *C. burnetii* and endosymbionts. Several *Amblyomma* spp. and *Rhipicephalus* spp. were shown to be CLE carriers in up to 100% of analyzed tick samples (41). In another study with more than 50 different tick species of hard and soft ticks, more than two-thirds of the species were found to harbor CLE (38). *Coxiella*-like endosymbionts seem to be associated with some genera, for example, *Rhipicephalus* spp. or *Ornithodoros* spp., in which CLEs were detected with a high prevalence, whereas only a few positive samples originated from *Ixodes* spp. Binetruy et al. found CLE to be present in 11 of 24 species of the genus *Amblyomma* (155). Recent phylogenetic analysis revealed a close and apparently ancient alliance between *Rhipicephalus* spp. and their CLE (156). The main survival strategy of CLE is the vertical transmission *via* the egg, but also horizontal transfer, possibly *via* co-feeding, was proven (156, 157). Recent results based on genome sequencing have shown that certain CLEs seem to have evolved from an ancestor capable of infection of immune cells (158). Hence, a loss of pathogenic potential was suggested. In contrast, *C. burnetii* was reported to have its phylogenetic origin in CLE (38).

Novel tick-borne pathogens are emerging, and as the evolution of *C. burnetii* is closely linked to endosymbionts, the possibility of other *Coxiella* spp. being pathogenic should not be neglected, considering that bird infections or human skin infections were reported (38, 49, 103, 158). Increasing the knowledge on evolutionary processes and the pathogenic potential of CLE could likewise also contribute to a better understanding of the epidemiology of *C. burnetii*.

Little is known about the impact CLE might have on pathogen transmission. A reduced infection rate of *R. haemaphysaloides* with CLE correlated with a lower rate of transstadial transmission of *Babesia microti*. *Candidatus Midichloria mitochondrii*, another tick endosymbiont, is known to influence the occurrence and ability to detect tick-borne pathogens in *I. ricinus* (159, 160).

Experimental Studies Are Needed to Assess the Vector Competence of Further Tick Species

In the past, seven tick species were shown to be competent vectors for *C. burnetii* (28). There is little recent research exploring the vector competence of ticks under laboratory conditions. Experimental studies prove transstadial transmission and successful reinfection of guinea pigs by *H. aegyptium* ticks (108) and transstadial transmission from nymphs to adults in *I. ricinus* (98). Recently, a transstadial transmission from nymphs to adults and a subsequent excretion with saliva were concluded in naturally infected *H. lusitanicum* (161). There is a lack of vector competence studies on different tick species, focusing

on the transstadial and transovarial transmission of pathogens. However, those studies are limited by the low rate of transmission and consequently incomplete knowledge of epidemiological cycles (162). Furthermore, studies are missing, which describe the level and duration of bacteremia in *C. burnetii*-infected hosts, which are necessary to estimate the actual vector capacity under laboratory conditions. As a mainly airborne pathogen, the potential infection routes may be *via* inhalation of feces besides the injection of saliva during the tick bite (98, 108). Infected feces might also contaminate the wound and thus causing an infection, as it is known from the transmission of *Trypanosoma cruzi* by bed bugs or *Rickettsia prowazekii* and lice (163, 164).

Besides the need for information regarding the prevalence of *C. burnetii* in ticks, the risk of acquiring Q fever by a tick bite cannot finally be determined. In different studies, the correlation between coxiellosis and the abundance of ticks as a risk factor was examined. Predominantly, no significant correlation was found (165–167), whereas other investigations concluded an association between tick infestation and seroprevalence (168, 169).

Most tick species in Europe are spreading, and the increased risk of tick-borne diseases accompanies this. For this reason, extensive and focused monitoring of ticks and their microbial burden is crucial, as well as further research on possibly tick-borne diseases and the pathogenic potential of already known bacteria.

CONCLUSIONS

The evaluation of European studies shows a significantly higher prevalence of *Coxiella* spp. in ticks in Mediterranean countries. This is likely to be driven by the abundance of different tick species in these countries, i.e., *Hyalomma* spp. and *Rhipicephalus* spp. In the context of global warming, the geographical distribution of these tick species changes and thus the epidemiology of Q fever in currently temperate Europe. Based on the large number of studies, which failed to detect *C. burnetii* DNA in tick samples, ticks carrying *C. burnetii* seem to be restricted to certain regions. Positive results have to be interpreted carefully because no distinction between *C. burnetii* and CLE was made in most of these studies. Planning of a prevalence study on ticks should, therefore, in particular, focus on the choice of detection methods for specific results. Methods should aim to differentiate *C. burnetii* and CLE using sequencing or at least to use different target genes in positive samples.

To address the questions on the role ticks play as a reservoir in Q fever transmission, vector competence studies using different and relevant tick species should be performed.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

SK and KM-S designed the study, SK conducted the literature search, SK, GM, SU, MP, and KM-S performed the data extraction, and SK analyzed the data and drafted the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.655715/full#supplementary-material

Supplementary Figure 1 | Forest plots of the included prevalence studies.

Supplementary Table 1 | Overview on included prevalence studies and the collected data.

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Zoonotic *Rickettsia* Species in Small Ruminant Ticks From Tunisia

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Tick-borne rickettsioses present a significant public health threat among emerging tick-borne diseases. In Tunisia, little is known about tick-borne *Rickettsia* pathogens. Therefore, the aim of this study was to investigate the presence of *Rickettsia* species in small ruminant ticks from Tunisia. Adult ticks (n = 694) were collected from goats and sheep in northern Tunisia. Obtained ticks were identified as *Rhipicephalus turanicus* (n = 434) and *Rhipicephalus sanguineus* sensu lato (n = 260). Selected ticks (n = 666) were screened for the presence of *Rickettsia* spp. by PCR targeting a partial sequence of the *ompB* gene followed by sequence analysis. Rickettsial DNA was detected in 122 (18.3%) tested tick samples. The infection rates in *Rh. turanicus* and *Rh. sanguineus* s.l. ticks were 23.4 and 9.5%, respectively. The overall prevalence of rickettsial DNA was markedly higher in ticks collected from goats (23.2%) compared to those infesting sheep (7.9%). The detection of rickettsial DNA was significantly higher in ticks from the governorate of Beja (39.0%) than those from the governorate of Bizerte (13.9%). Two additional genes, the outer membrane protein A gene (*ompA*) and the citrate synthase gene (*gltA*), were also targeted for further characterization of the detected *Rickettsia* species. Genotyping and phylogenetic analysis based on partial sequences (n = 106) of the three different genes revealed that positive ticks are infected with different isolates of two Spotted Fever Group (SFG) *Rickettsia*, namely, *Rickettsia massiliae* and *Rickettsia monacensis*, closely related to those infecting camels and associated ticks from Tunisia, and humans and small ruminant ticks from neighboring countries like Italy, France, and Spain.

Keywords: *Rickettsia* species, *Rhipicephalus* ticks, molecular survey, genotyping, phylogenetic analysis, Tunisia

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INTRODUCTION

Rickettsia species (family Rickettsiaceae; order Rickettsiales) are included into four groups: the spotted fever group (SFG) rickettsiae, the typhus group, the *Rickettsia bellii* group, and the *Rickettsia canadensis* group (1). These pathogens infected several domesticated and wild vertebrate hosts through hematophagous arthropod vectors bites (mainly ticks, fleas, and mites). Besides, tick-borne rickettsioses are considered as one of the most virulent zoonotic diseases affecting humans especially in African countries (2).

Spotted fever group rickettsioses (SFG) are actually considered as emerging and reemerging diseases affecting animals worldwide. They are caused by the pathogenic and zoonotic spotted fever *Rickettsia* bacteria mainly transmitted by ticks. Humans may be accidently infected especially in tropical areas (1, 2).

In Tunisia, several SFG *Rickettsia* species have been previously reported, as *Rickettsia conorii*, that was described for the first time in humans since 1910 (3), and, recently, by Znazen et al. (4) and Khrouf et al. (5). In addition, *R. conorii* subsp. *israelensis* was identified in one human and tick specimens of *Rhipicephalus sanguineus* s.l. complex collected from dogs (4, 6). Furthermore, *R. aeschlimannii*, *R. helvetica*, and *R. africae* were reported from camels' blood samples and infesting *Hyalomma* tick tissues in southern and central Tunisia (7, 8). DNA of *R. helvetica* was also identified in questing *Ixodes ricinus* ticks (9).

Rickettsia massiliae and *Rickettsia monacensis*, belonging to the SFG rickettsiae, are widely identified among animals, humans, and arthropod vectors (1). *Rickettsia massiliae* was firstly isolated in France from *Rhipicephalus turanicus* tick (10). Since then, this pathogen has been transmitted by and/or isolated from *Rhipicephalus* ticks like *Rh. turanicus*, *Rh. sanguineus* sensu lato (s.l.), *Rh. bursa*, and *Rh. pusillus* collected from domestic and wild animals such as cattle, goats, horses, dogs, cats, hedgehogs, red foxes, and hares in different worldwide countries (11–16). In Tunisia, *R. massiliae* was previously detected in *Rh. sanguineus* s.l. ticks collected from dogs (6), in peripheral blood of camels (8), and in skin biopsy of one patient (5). Interestingly, this bacterium is recognized as pathogenic in human and may be clinically expressed as a febrile illness with maculopapular rash, fever, night sweats, headache, and necrotic eschar at the tick bite site (17, 18).

Rickettsia monacensis was earlier detected in *I. ricinus* ticks from several European countries like Italy, Spain, Romania, Bulgaria, Hungary, and Serbia (1, 12). In our country, the first identification of *R. monacensis* was also reported in *I. ricinus* ticks by Sfar et al. (9). Additionally, this human-pathogenic species was recently detected not only in Tunisian camels but also in associated *H. impeltatum* ticks removed from uninfected animals (8). This bacterium causes from moderate to severe infections in humans including fever, rash on palms and soles, and inoculation eschar (19, 20). To better understand the epidemiology of *Rickettsia* species in Tunisia, we investigated, in the present molecular survey the occurrence of rickettsial bacteria in small ruminant ticks according to potential risk factors. Molecular characterization and phylogenetic analysis of revealed *Rickettsia* spp. isolates were also performed by using three different gene fragments.

MATERIALS AND METHODS

Study Area Description

A cross-sectional study was carried out in five localities of Northern Tunisia (**Figure 1**). El Alia 37°16′ N; 10°03′ E and Khetmine 37°16′ N; 9°99′ E fall in the sub-humid bioclimatic zone with an average annual rainfall of 400 mm and a mean temperature of 18.4°C while Joumine 36°92′ N; 9°38′ E, Sejnane 37°15′ N; 9°23′ E, and Amdoun 36°76′ N; 9°08′ E are characterized by humid climate with an average annual rainfall of 650 mm and a mean temperature of 14.4°C.

Tick Collection and Identification

Ticks were collected from 303 apparently healthy goats (233 doe and 70 buck) and 160 healthy sheep (110 ewes and 50 rams). Goats were originated from 16 herds located in Sejnane ($N = 3$), El Alia ($N = 4$), and Joumine ($N = 5$) belonging to the Bizerte governorate and in Amdoun ($N = 4$, Beja governorate). Sheep derived from nine herds from El Alia ($N = 4$) and Khetmine ($N = 5$) in the governorate of Bizerte.

All partially engorged ticks were collected by using a clamp from different preferred sites of small ruminant body (ears, neck, udder, and external genitalia) and separately categorized according to the examined animal host. Obtained specimens were morphologically identified using the taxonomic key of Walker et al. (21) and then classified according to tick species, life stage, and gender. Each tick specimen was individually conserved in a tube containing 70% ethanol and stored at −20°C.

Total DNA Extraction and Tick DNA Amplification

Each identified tick was washed with sterile water, dried, and crushed individually using an automated TissueLyser LT system (Qiagen, Hilden, Germany). Genomic DNA extraction was performed from each tick sample using the DNeasy tissue kit (Qiagen, Hilden, Germany). Obtained DNA extracts were stored at −20°C. DNA extraction efficiency was validated by PCR amplification step targeting the ribosomal RNA subunit (16S rRNA) gene using the tick-specific primers TQ16S+1F and TQ16S-2R as described by Black and Piesman (22) (**Table 1**).

Molecular Detection of *Rickettsia* spp.

In order to identify all species of the *Rickettsia* genus, tick DNA samples were subjected to nested PCR targeting a fragment (425 bp) of the rickettsial outer membrane protein B (*ompB*) gene (23) (**Table 1**). For further characterization, the outer membrane protein A (*ompA*) and the citrate synthase protein (*gltA*) gene fragments (532 and 381 bp, respectively) were amplified by using nested and endpoint PCR, respectively (**Table 1**). PCR reactions were performed in an automated DNA thermal cycler. Thermal cycling profiles were as described by Oteo et al. (24), and Regnery et al. (25), respectively.

The PCR reactions were carried out in a final volume of 50 µl composed of 0.125 U/µL of Taq DNA polymerase (Biobasic Inc., Markham, Canada), 1× PCR buffer, 1.5 mM MgCl2, 0.2 mM of dNTP, 3 µL of genomic DNA (50–150 ng) in the first PCR and 1 µL in the second PCR (for nested PCR), 0.5 µM of the primers, and autoclaved water. PCR products were visualized using electrophoresis in 1.5% agarose gels stained with ethidium bromide and observed under UV transillumination.

Statistical Analysis

Exact confidence intervals (CI) at the 95% level were estimated for prevalence rates according to different considered factors.

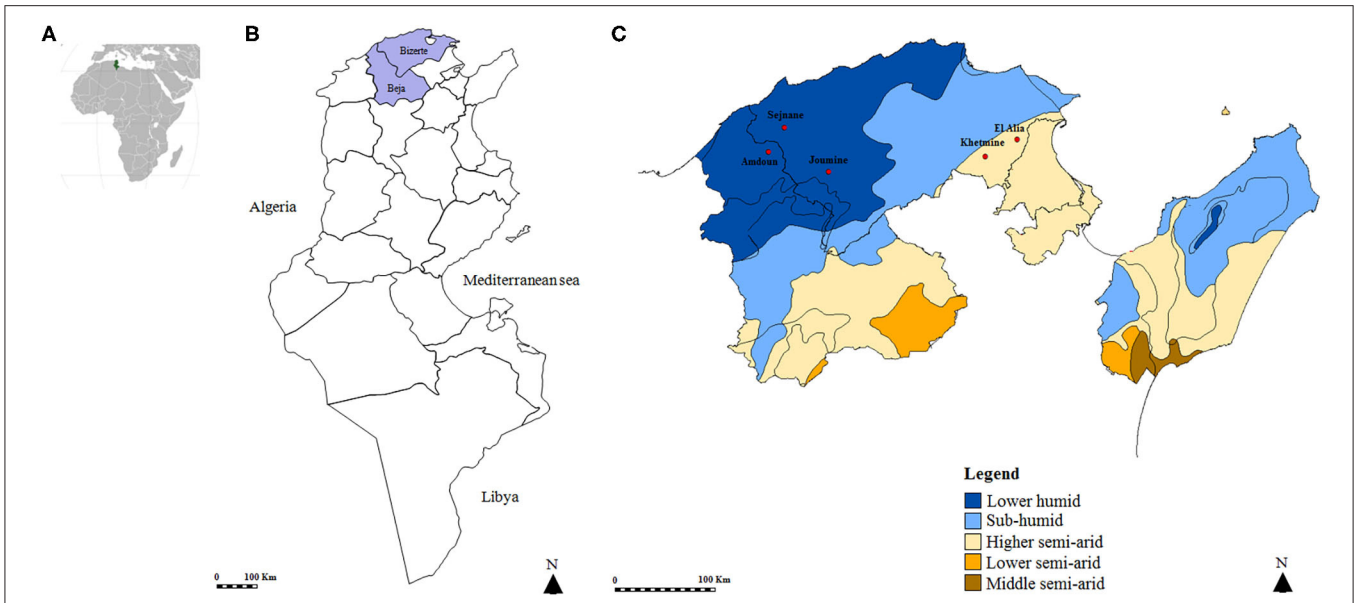

FIGURE 1 | Map of the Tunisian studied regions. **(A)** Geographical position of Tunisia in the African continent. **(B)** Map of Tunisia showing investigated governorates. **(C)** Position of studied localities according to bioclimatic areas.

TABLE 1 | Primers used for the identification and/or genetic characterization of *Rickettsia* species infecting ticks collected in this study from small ruminants.

Assays	Target genes	Primers	Sequences (5′-3′)	Amplicon size (bp)	References
Single PCR[a]					
	16S rRNA	TQ16S+1F	CTGCTCAATGATTTTTTAAATTGCTGTGG	324	(22)
		TQ16S-2R	ACGCTGTTATCCCTAGAG		
Nested PCR[b]					
First PCR	*ompB*	rompB_OF	GTAACCGGAAGTAATCGTTTCGTAA	511	(23)
		rompB OR	GCTTTATAACCAGCTAAACCACC		
Second PCR		rompB_SFG_IF	GTTTAATACGTGCTGCTAACCAA	425	
		rompB SFG-IR	GGTTTGGCCCATATACCATAAG		
Semi-nested PCR[c]					
First PCR	*ompA*	Rr190.70p	ATGGCGAATATTTCTCCAAAA	631	(24)
		Rr190.701n	GTTCCGTTAATGGCAGCATCT		
Second PCR		Rr190.70p	ATGGCGAATATTTCTCCAAAA	532	
		Rr190.602n	AGTGCAGCATTCGCTCCCCCT		
Single PCR[c]					
	gltA	RpCS.877p	GGGGGCCTGCTCACGGCGG	381	(25)
		RpCS.1258n	ATTGCAAAAAGTACAGTGAACA		

[a] *Single PCR based on the 16S rRNA gene allowing the selection of tick samples with DNA extraction efficiency.*
[b] *Nested PCR based on the ompB gene allowing the detection and/or characterization after sequencing of Rickettsia species.*
[c] *Single and semi-nested PCR based on gltA and ompA genes, respectively, allowing the characterization after sequencing of Rickettsia species.*

A comparison of the prevalence of *Rickettsia* species in ticks according to abiotic factors (geographic location and bioclimatic conditions) and factors related to ticks (gender, age, and host origin) was carried out using the Epi Info 6 software 01 (CDC, Atlanta, USA) and the χ^2-test. A difference is considered statistically significant when the degree of significance p is ≤ 0.05. In order to assess possible confusion between the risk factors, a Mantel–Haenszel χ^2-test was performed.

DNA Sequencing and Obtaining Final Sequences

A total of 106 positive PCR products obtained after *ompB*, *ompA*, and *gltA* PCRs were randomly selected and purified using the GF-1 Ambi Clean kit (Vivantis, USA), according to the manufacturer's instructions. Purified DNA amplicons were sequenced in both directions, using the same primers as for the single *gltA* PCR and the second PCR of each

nested PCR amplification targeting *ompA* and *ompB* genes. 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). To obtain maximal data accuracy, sequences were determined on both forward and reverse strands. Indeed, the complementary strands of each sequenced product were manually assembled by using the DNAMAN software (Version 5.2.2; Lynnon Biosoft, Que., Canada). The primer region sequences were automatically removed and the overlapping parts were selected.

Sequence Alignment and Phylogenetic Study

Multiple-sequence alignments and sequence similarities were calculated using the CLUSTAL W method (26). BLAST analysis was performed to assess the level of similarity with previously reported sequences (http://blast.ncbi.nlm.nih.gov/). By using the DNAMAN software, genetic distances among the operational taxonomic units were computed by the maximum composite likelihood method (27) and were used to construct neighbor-joining trees (28). Statistical support for internal branches of trees was evaluated by bootstrapping with 1,000 iterations (29).

RESULTS

Tick Species Recognition

A total of 694 ticks were collected from goats (460/694, 66.3%) and sheep (234/694, 33.7%) from a higher semiarid area (374/694, 53.9%) and a low humid area (320/694, 46.1%). Almost all specimens were removed from animals located in the governorate of Bizerte (82%) while ticks collected from small ruminants in El Alia were the most numerous (43.5%) compared to those in other localities (**Figure 1** and **Table 2**). The sex ratio of ticks collected from these animals (M/F) was 1.14. The intensity of tick infestation is estimated at 1.52 and 1.46 ticks/animal for goats and sheep, respectively. Two tick species belonging to *Rhipicephalus* genus were identified, namely, *Rh. turanicus* (434/694, 62.5%) and *Rh. sanguineus* s.l. (260/694, 37.5%) (**Table 2**).

Efficiency of DNA Isolation

DNA extracts were tested and validated in 666 samples (96%). No amplification products were obtained for 28 samples, reflecting a probable failure of the DNA extraction, and were thus excluded from the analysis. Thereby, a total of 666 ticks were selected from goats (452/666, 67.9%) and sheep (214/666, 32.1%) from the higher semiarid area (357/666, 53.6%) and the low humid area (309/666, 46.4%). Almost all analyzed ticks were collected from small ruminants located in the governorate of Bizerte (82.3%) while ticks collected from animals in El Alia are the most numerous (43.4%) compared to those in other localities (**Figure 1** and **Table 2**). The sex ratio of tested ticks (M/F) was 1.15. After the validation of DNA extracts, a total of 423 *Rh. turanicus* (63.5%) and 243 *Rh. sanguineus* s.l. (36.5%) were subjected to *Rickettsia* spp. screening (**Table 2**).

Rickettsia spp. Screening and Risk Factor Analysis

Based on *ompB* gene analysis, DNA of *Rickettsia* spp. was identified in 122 tick samples (18.3%) (**Table 2**). Infection among *Rh. turanicus* ticks is statistically more prevalent (23.4%) compared to *Rh. sanguineus* s.l. (9.5%) ($p < 0.001$). Ticks collected from goats were statistically more infected with *Rickettsia* spp. (23.2%) than those from sheep (7.9%) ($p < 0.001$; **Table 2**). Ticks removed from small ruminants located in the governorate of Beja were statistically more infected with *Rickettsia* spp. (39.0%) ($p < 0.001$) than those in the governorate of Bizerte (13.9%) ($p < 0.001$). Specimens from Amdoun (39.0%) and El Alia (24.6%) localities were more infected with *Rickettsia* spp. than those from Sejnane (3.0%), Khetmine (1.5%), and Joumine (0%) ($p < 0.001$; **Table 2**). In contrast, no statistically significant differences in *Rickettsia* spp. infection rates were observed according to tick gender and bioclimatic areas ($p < 0.05$, **Table 2**).

Identification of *Rickettsia* Species Infecting Ticks

Two rickettsial species were identified in small ruminants' ticks, namely, *R. massiliae* and *R. monacensis* (**Table 3**). Based on *ompB* gene analysis, 40 PCR products (32 from *Rh. turanicus* and eight from *Rh. sanguineus* s.l.) were sequenced successfully. *Rickettsia massiliae* was identified in *Rh. turanicus* ($n = 32$, 100%) and *Rh. sanguineus* s.l. ($n = 6$, 75%). However, *R. monacensis* DNA was found in *Rh. sanguineus* s.l. ($n = 2$, 25%). Based on *ompA* and *gltA* gene analysis, PCR products were sequenced successfully from 41 and 25 positives samples, respectively. *Rickettsia massiliae* was detected in *Rh. turanicus* ($n = 31$, 100%) and *Rh. sanguineus* s.l. ($n = 10$, 100%) based on *ompA* partial sequence analysis. However, using the *gltA* gene, DNA of this bacterium was found in *Rh. turanicus* ($n = 25$, 100%) (**Table 3**).

Molecular Characterization and Phylogenetic Analysis

Out of 122 *Rickettsia*-positive samples, 94 gave a clear band in the correct nucleotide size of the partial genes (*ompA*, *ompB*, and *gltA*) in at least one of the three genotyping PCRs. Partial sequences ($n = 106$) of the three analyzed genes were deposited under GenBank accession numbers presented in **Table 4**. Based on all revealed sequences of the three analyzed genes, we precisely selected *Rickettsia* spp. genotypes according to infecting tick species, and they differ from each other by at least one mutation in the nucleotidic sequence.

Rickettsia spp. *ompB* Genotypes

Rickettsia infection was confirmed by sequencing of 382-bp *ompB* fragments from randomly selected 32 *Rh. turanicus*- and eight *Rh. sanguineus* s.l. *Rickettsia*-positive samples (**Tables 3**, **4**). Alignment of these sequences revealed two *R. massiliae* genotypes from *Rh. sanguineus* s.l. (ompBRmasRs1 and ompBRmasRs2; GenBank accession numbers MN311185 and MN311189,

TABLE 2 | Molecular prevalences of *Rickettsia* spp. according to tick species, tick gender, infested host, bioclimatic zone, governorate, and locality.

Factors	Number of collected ticks (%)[a]	Number of analyzed ticks (%)[b]	Positive[c] (% ± C.I.[d])	*P*-value (Khi2)
Tick species				0.000* (20.02)
Rh. turanicus	434 (62.5)	423 (63.5)	99 (23.4 ± 0.04)	
Rh. sanguineus s.l.	260 (37.5)	243 (36.5)	23 (9.5 ± 0.04)	
Tick gender				0.519 (0.42)
Male	370 (53.3)	356 (53.4)	62 (17.4 ± 0.04)	
Female	324 (46.7)	310 (46.6)	60 (19.4 ± 0.04)	
Infested host				0.000* (22.65)
Goats	460 (66.3)	452 (67.9)	105 (23.2 ± 0.04)	
Sheep	234 (33.7)	214 (32.1)	17 (7.9 ± 0.03)	
Bioclimatic zone				0.185 (1.76)
Higher semi-arid	374 (53.9)	357 (53.6)	72 (20.2 ± 0.04)	
Lower humid	320 (46.1)	309 (46.4)	50 (16.2 ± 0.04)	
Governorate				0.000* (40.87)
Bizerte	569 (82.0)	548 (82.3)	76 (13.9 ± 0.03)	
Beja	125 (18.0)	118 (17.7)	46 (39.0 ± 0.09)	
Locality				0.000* (87.96)
El Alia	302 (43.5)	289 (43.4)	71 (24.6 ± 0.05)	
Khetmine	72 (10.4)	68 (10.2)	1 (1.5 ± 0.03)	
Sejnane	137 (19.7)	133 (20.0)	4 (3.0 ± 0.03)	
Amdoun	125 (18.0)	118 (17.7)	46 (39.0 ± 0.09)	
Joumine	58 (8.4)	58 (8.7)	0 (0)	
Total	694 (100)	666 (100)	122 (18.3 ± 0.03)	

Rh. Turanicus, Rhipicephalus turanicus, Rh. sanguineus s.l., Rhipicephalus sanguineus sensu lato.
[a]*Number of collected ticks submitted to PCR performed for the confirmation of the DNA extraction efficiency.*
[b]*Number of included ticks for Rickettsia spp. survey selected after the confirmation of the DNA extraction efficiency.*
[c]*Ticks positive to Rickettsia spp. according to the total number of analyzed ticks.*
[d]*C.I.: 95% confidence interval.*
**Statistically significant test.*

TABLE 3 | *Rickettsia* species identified by the sequencing of *ompB*, *ompA*, and *gltA* partial sequences in *Rhipicephalus* ticks.

Tick species	Number	*ompB* PCR positive (%)	*ompB* PCR positives/sequencing	*ompA* PCR positives/sequencing	*gltA* PCR positives/sequencing	*Rickettsia* spp.
Rh. turanicus	423	99 (23.4 ± 0.04)	32	31	25	*R. massiliae*
			0	0	0	*R. monacensis*
Rh. sanguineus s.l.	243	23 (9.5 ± 0.04)	6	10	0	*R. massiliae*
			2	0	0	*R. monacensis*
Total	666	122 (18.3 ± 0.03)	40	41	25	*Rickettsia* spp.

Rh. turanicus, Rhipicephalus turanicus; Rh. sanguineus s.l., Rhipicephalus sanguineus sensu lato.

respectively) and two *R. massiliae* genotypes from *Rh. turanicus* ticks (ompBRmasRt1 and ompBRmasRt2; GenBank accession numbers MN311191 and MN311211, respectively) (**Table 4**). In addition, two *R. monacensis* genotypes from *Rh. sanguineus* s.l. (ompBRmonRs1 and ompBRmonRs2; GenBank Accession Numbers MN311223 and MN311224, respectively) were also recorded (**Table 4**).

A phylogenetic analysis based on the alignment of Tunisian genotypes with 31 *Rickettsia* spp. *ompB* sequences obtained from GenBank shows the assignment of revealed genotypes to *R. massiliae* and *R. monacensis* clusters. The *R. massiliae* cluster is formed by three subclusters supported by robustness node rates ≥ to 81% (**Figure 2**). Tunisian strains were assigned to the first and third subclusters. Genotypes ompBRmasRs2 and ompBRmasRt2 were assigned to the first subcluster and clustered with strains isolated from *H. impeltatum* infesting camels in Tunisia and from *Rh. sanguineus* s.l. ticks located in Mediterranean countries such as Italy and Spain (**Figure 2**). Genotypes ompBRmasRs1 and ompBRmasRt1 were assigned to the third subcluster and clustered with strains isolated from *Rh. sanguineus* s.l. and *Rh. turanicus* ticks originated from North-Mediterranean countries (**Figure 2**). The *R. monacensis* cluster is also formed by three subclusters supported by robustness rates of nodes ≥ to 81% (**Figure 2**). Genotypes ompBRmonRs1

TABLE 4 | Designation and information about sequencing of *Rickettsia* spp. genotypes identified in this study.

Gene	*Rickettsia* sp.	Genotype	Number[a]	Potential vector	Location[b]	GenBank[c]	BLAST analysis
ompB	*R. massiliae*	ompBRmasRs1	4	*Rh. sanguineus* s.l.	Bizerte	MN311185	100% *R. massiliae* (CP000683)
		ompBRmasRs2	2	*Rh. sanguineus* s.l.	Bizerte	MN311189	100% *R. massiliae* (KJ663751)
		ompBRmasRt1	20	*Rh. turanicus*	Bizerte and Beja	MN311191	100% *R. massiliae* (CP000683)
		ompBRmasRt2	12	*Rh. turanicus*	Bizerte and Beja	MN311211	100% *R. massiliae* (KJ663751)
	R. monacensis	ompBRmonRs1	1	*Rh. sanguineus* s.l.	Bizerte	MN311223	100% *R. monacensis* (EU883092)
		ompBRmonRs2	1	*Rh. sanguineus* s.l.	Bizerte	MN311224	99.4% *R. monacensis* (EU883092)
ompA	*R. massiliae*	ompARmasRs1	6	*Rh. sanguineus* s.l.	Bizerte	MN311225	100% *R. massiliae* (MH532237)
		ompARmasRs2	2	*Rh. sanguineus* s.l.	Bizerte	MN311229	100% *R. massiliae* (KJ663747)
		ompARmasRs3	1	*Rh. sanguineus* s.l.	Beja	MW026194	99.8% *R. massiliae* (MH532237)
		ompARmasRs4	1	*Rh. sanguineus* s.l.	Beja	MW026195	99.8% *R. massiliae* (MH532237)
		ompARmasRt1	16	*Rh. turanicus*	Beja	MN311231	100% *R. massiliae* (MH532237)
		ompARmasRt2	4	*Rh. turanicus*	Beja	MW026200	100% *R. massiliae* (KJ663747)
		ompARmasRt3	5	*Rh. turanicus*	Beja	MW026204	99.8% *R. massiliae* (MH532237)
		ompARmasRt4	2	*Rh. turanicus*	Beja	MW026209	99.8% *R. massiliae* (MH532237)
		ompARmasRt5	2	*Rh. turanicus*	Beja	MW026211	99.6% *R. massiliae* (MH532237)
		ompARmasRt6	1	*Rh. turanicus*	Beja	MW026213	99.8% *R. massiliae* (MH532237)
		ompARmasRt7	1	*Rh. turanicus*	Bizerte	MW026214	99.6% *R. massiliae* (KJ663747)
gltA	*R. massiliae*	gltARmasRt1	25	*Rh. turanicus*	Bizerte and Beja	MW026215	100% *R. massiliae* (KJ663740)

R. sanguineus s.l., Rhipicephalus sanguineus sensu lato; R. turanicus, Rhipicephalus turanicus.

[a] *Number of sequenced Rickettsia positive samples.*

[b] *Geographical location.*

[c] *GenBank accession number.*

All information about the GenBank accession numbers represented in the Blast analysis is shown on the phylogenetic trees presented in ***Figures 2–4****. Genotypes ompBRmasRs1, ompBRmasRs2, ompBRmasRt1, and ompBRmasRt2 were also represented by GenBank accession numbers MN311186–MN311188, MN311190, MN311192–MN311210, and MN311212–MN311222, respectively. Genotypes ompARmasRs1, ompARmasRs2, ompARmasRt1, ompARmasRt2, ompARmasRt3, ompARmasRt4, and ompARmasRt5 were also represented by GenBank accession numbers MN311226–MN311228, MW026192, MW026193, MN311230, MN311232–MN311242, MW026196–MW026199, MW026201–MW026203, MW026206–MW026208, MW026210, and MW026212, respectively. Genotype gltARmasRt1 was also represented by GenBank accession numbers MW026216–MW026239.*

and ompBRmonRs2 were assigned, respectively, to the first and second subclusters. Genotype ompBRmonRs1 was closely related to isolates found in Tunisian camels and their infesting *H. impeltatum* ticks, and strains infecting human and ticks from different countries (**Figure 2**).

Rickettsia spp. *ompA* Genotypes

By using the *ompA* partial sequence, the infection with *R. massiliae* was revealed by sequencing of 490 bp of the *ompA* gene from selected 31 *Rh. turanicus*- and 10 *Rh. sanguineus* s.l. *Rickettsia*-positive samples (**Tables 3**, **4**). Alignment of these sequences confirmed the occurrence of four distinct genotypes from *Rh. sanguineus* s.l. ticks (ompARmasRs1 to ompARmasRs4; GenBank Accession Numbers MN311225, MN311229 MW026194, and MW026195, respectively) and seven genotypes from *Rh. turanicus* ticks (ompARmasRt1 to ompARmasRt7; GenBank Accession Numbers MN311231, MW026200, MW026204, MW026209, MW026211, MW026213, and MW026214, respectively) (**Table 4**).

For this gene, a phylogenetic tree based on the alignment of *ompA* partial sequences of *Rickettsia* spp. found in GenBank showed the presence of our sequences in the three subclusters that formed the *R. massiliae* cluster and supported by robustness node rates $\geq$ to 84% (**Figure 3**). Genotype ompARmasRt7 formed separately subcluster 1, and genotypes ompARmasRt2 and ompARmasRs2 were assigned to the last subcluster and clustered with strains isolated from *Rh. sanguineus* s.l. located in different worldwide countries such as Italy, Austria, Argentina, and the USA. The remaining genotypes were clustered together in the second subcluster with several isolates infecting ticks from China and European countries (**Figure 3**).

Rickettsia spp. *gltA* Genotypes

Sequencing of 341 bp of the *gltA* partial sequence obtained from 25 specimens of *Rh. turanicus*-positive to *Rickettsia* spp. confirmed the infection with only one genotype (gltARmasRt1, GenBank accession number KJ663740) of *R. massiliae* (**Tables 3**, **4**). This revealed that the genotype was 100% identical to strain 60B infecting *Rh. sanguineus* s.l. tick collected from Italian human (GenBank Accession Number KJ663740) (**Table 4**).

Phylogenetic tree based on the *gltA* gene revealed that the gltARmasRt1 genotype clustered in the *R. massiliae* cluster especially in the first subcluster 1 with strains infecting *Rh. sanguineus* s.l. ticks from Italy and Argentina, *Hyalomma asiaticum* ticks from China, and *R. turanicus* tick specimens collected from birds in Portugal (**Figure 4**).

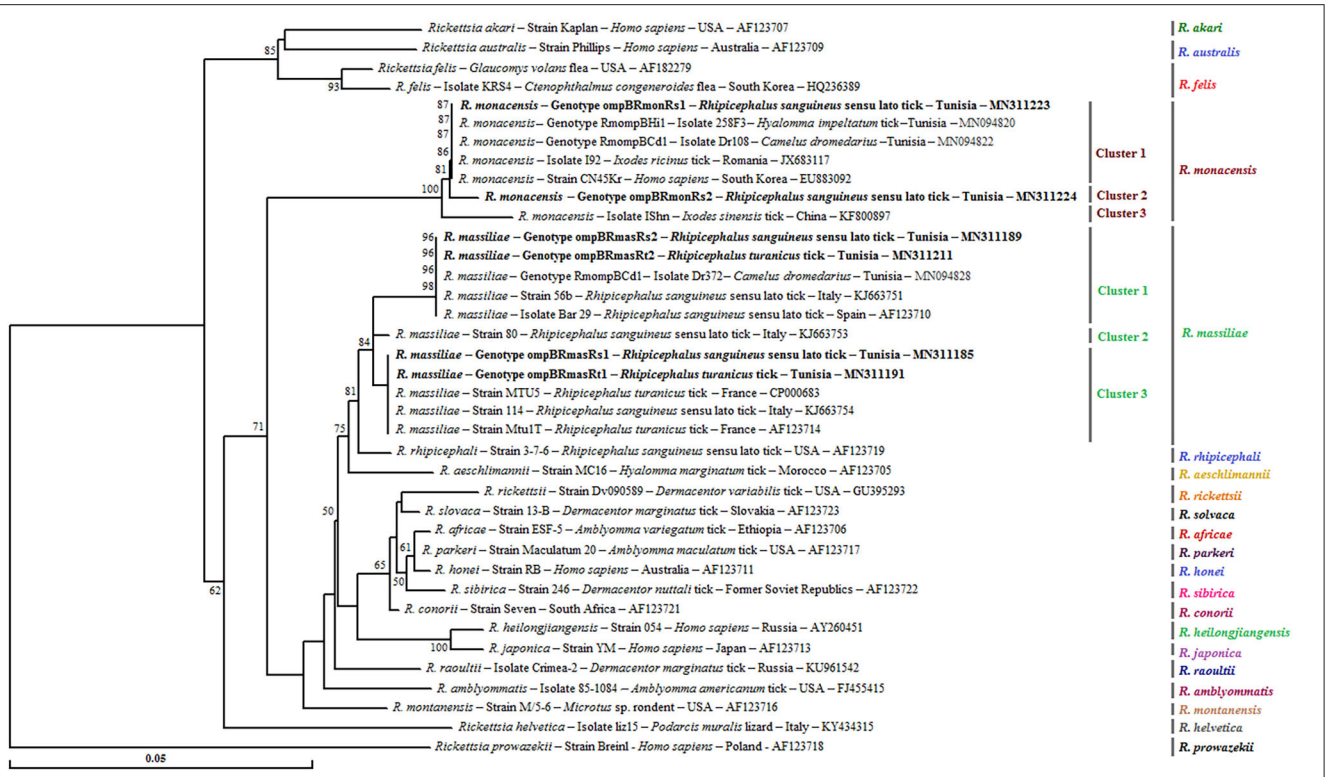

FIGURE 2 | Neighbor-joining tree based on the alignment of partial *ompB* sequences (382 bp) of *Rickettsia* spp. obtained in this study with selected sequences representative of the *Rickettsia* genus. Numbers over the branches indicate the percentage of replicated trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates, only percentages >50% were represented). The six partial *ompB* sequences representative of different *Rickettsia* spp. genotypes obtained in this study are indicated in bold. The host or vector, the genotype, strain or isolate name, the country of origin, and the GenBank accession number are indicated. One *R. prowazekii ompB* partial sequence was added as an outgroup.

DISCUSSION

Data about the occurrence and the genetic diversity of *Rickettsia* species in ticks is limited in North African countries (30, 31), especially in Tunisia (6, 8, 9). In this report, adult ticks infesting small ruminants in northern Tunisia were examined and two species of *Rhipicephalus* genus (*R. turanicus* and *R. sanguineus* s.l.) were identified. This result is in agreement with other surveys which considered these two tick species as major ectoparasites of small ruminants in Tunisia (32, 33).

To our knowledge, we report here for the first time the detection of SFG *Rickettsia* DNA in ticks collected from small ruminants raised in the north of Tunisia. Although this study does not conclude on the competence of these potential vectors, given that these results do not suggest that the tick species mentioned in this report can serve as a competent vector for detected bacteria, this study made a contribution to the knowledge of the presence of SFG rickettsiae in Tunisia. In addition, present data showed the need to search these bacteria in animal hosts and to increase the investigated areas, the potentially incriminated risk factors, and the number of analyzed tick samples, including questing ticks and different life stages. All these information may facilitate future prevention against SFG *Rickettsial* diseases in the country.

Specifically, the detection of *Rickettsia* spp. DNA in *Rh. turanicus* (23.4%) and *Rh. sanguineus* s.l. (9.5%) provides evidence that these tick species may be among the main vectors of *Rickettsia* species in northern Tunisia. These results are consistent with those reported by Khrouf et al. (6) who suggested a possible incrimination of *Rhipicephalus* ticks infesting dogs and sheep in the transmission of *Rickettsia* species in central Tunisia. Furthermore, according to Psaroulaki et al. (34), *Rickettsia* spp. were detected in *Rhipicephalus* ticks collected from domestic animals in Greece. Additionally, Germanakis et al. (35) reported that *Rh. turanicus* has been implicated as a potential vector transmitting to humans several pathogens including *Rickettsia* species. In the Northwest of China, Wei et al. (36) suggested that *R. massiliae*, *R. aeschlimannii*, and *R. sibirica* variants co-circulate in *R. turanicus* ticks. This data was confirmed by another study conducted by Song et al. (37) in the same country that indicates the occurrence of several SFG rickettsiae in *Rh. turanicus* collected from several ruminants. *Rickettsia massiliae* DNA was previously found in the salivary glands, and saliva of *Rh. turanicus* and its specific antibodies were also detected in patient sera. This may suggest, firmly, that *Rh. turanicus* act as a potential vector and reservoir for this bacterium (38).

Furthermore, analysis of potential risk factors demonstrated three interesting facts related to geographic regions, potential tick

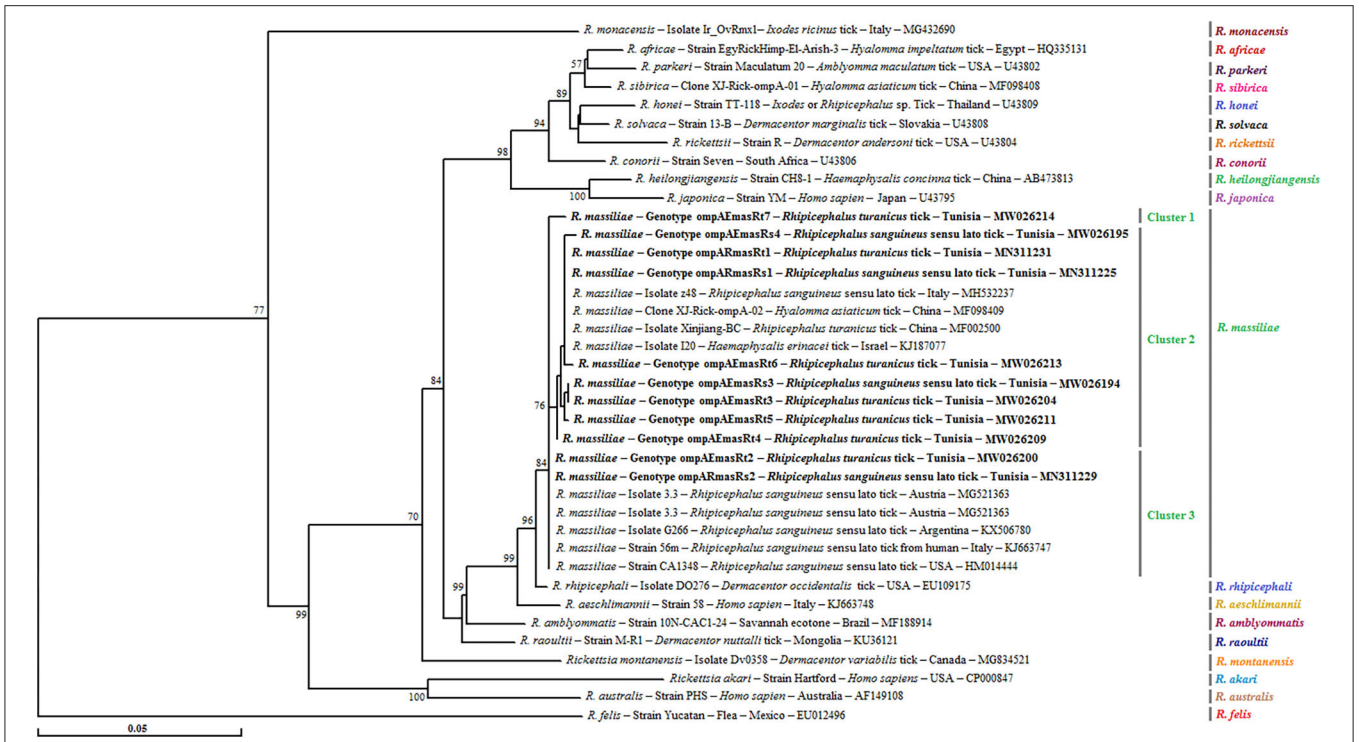

FIGURE 3 | Phylogenetic tree of *Rickettsia* species inferred with partial sequences (490 bp) of the *ompA* gene using the neighbor-joining method showing the novel obtained sequences ($n = 11$) from Tunisian small ruminant ticks. Bootstrap values (1,000 replicates) are indicated in each node (only percentages >50% are shown). The 11 genotypes of *Rickettsia* spp. obtained in the present study are indicated in bold. The host or vector, the genotype, strain or isolate name, the country of origin, and the GenBank accession number are represented. One *R. felis ompA* partial sequence was added as an outgroup.

vector species, and infested hosts. Firstly, the positive rates of SFG *Rickettsia* in ticks were significantly higher in Beja (39%) than in Bizerte (13.9%) governorate. This discrepancy in prevalence rates according to geographic regions could be mainly explained by the diversity and heterogeneity of livestock population especially in El Alia locality and differences in husbandry practices, farm organization, wildlife reservoir hosts, and/or abiotic factors like the air temperature and the relative humidity that significantly affect the distribution of potential tick vectors. In addition, the higher rate of *Rickettsia* spp. observed in the governorate of Beja exclusively represented by the locality of Amdoun may be partly explained by the abundant presence in this region of *I. ricinus* considered to be one of the most important vectors of rickettsiae around the world (9). The infection of *Rhipicephalus* ticks with *Rickettsia* species may therefore come from infected small ruminants earlier infested with *Rickettsia*-positive *I. ricinus* ticks during wet seasons (9). Secondary, the positive rate in *Rh. turanicus* ticks (23.4%) was significantly higher compared to *Rh. sanguineus* s.l. (9.5%). This result is in line with those presented by Ghafar et al. (39) indicating a higher prevalence of *R. massiliae* and *R. slovaca* infections in *Rh. turanicus* ticks from Pakistan compared to other tick species. Furthermore, risk factor analysis showed that ticks collected from goats (23.2%) were more infected with *Rickettsia* spp. than those infesting sheep (7.9%) which is consistent with the same result of Ghafar et al. (39) in Pakistan.

In this study, *R. massiliae* was detected in *Rh. turanicus* and *Rh. sanguineus* s.l., thus confirming its occurrence especially in the north of Tunisia. In our country, previous studies have reported the presence of *R. massiliae* in *Rh. sanguineus* s.l. ticks collected from sheep situated in the center (6) and more recently in camels located in the center and the south (8). Similarly, *R. massiliae* has been also identified in *Rh. turanicus* and *Rh. sanguineus* s.l. from Algeria, Italy, Cyprus, and Greece (15, 34, 40), in *Rh. sanguineus* s.l. ticks from Morocco (41, 42), Spain, and Italy (12, 43), and in *Rh. turanicus* ticks from China (36) and Pakistan (39).

In the present study, *R. monacensis* DNA was detected in *Rh. sanguineus* s.l. tick specimens removed from goats. These results consolidate previous data describing the presence of this bacterium in questing *I. ricinus* ticks (9), and in camels and their infesting *H. impeltatum* ticks (8). Besides, wide geographical distribution of this pathogen was noted particularly in the Mediterranean region (Italy and Spain) and from other countries like Costa Rica and Nicaragua (44–46). Interestingly, this species was identified as a zoonotic pathogen able to cause from moderate to severe illness in humans (19). The detection in Tunisia of *R. monacensis* DNA in *Rh.*

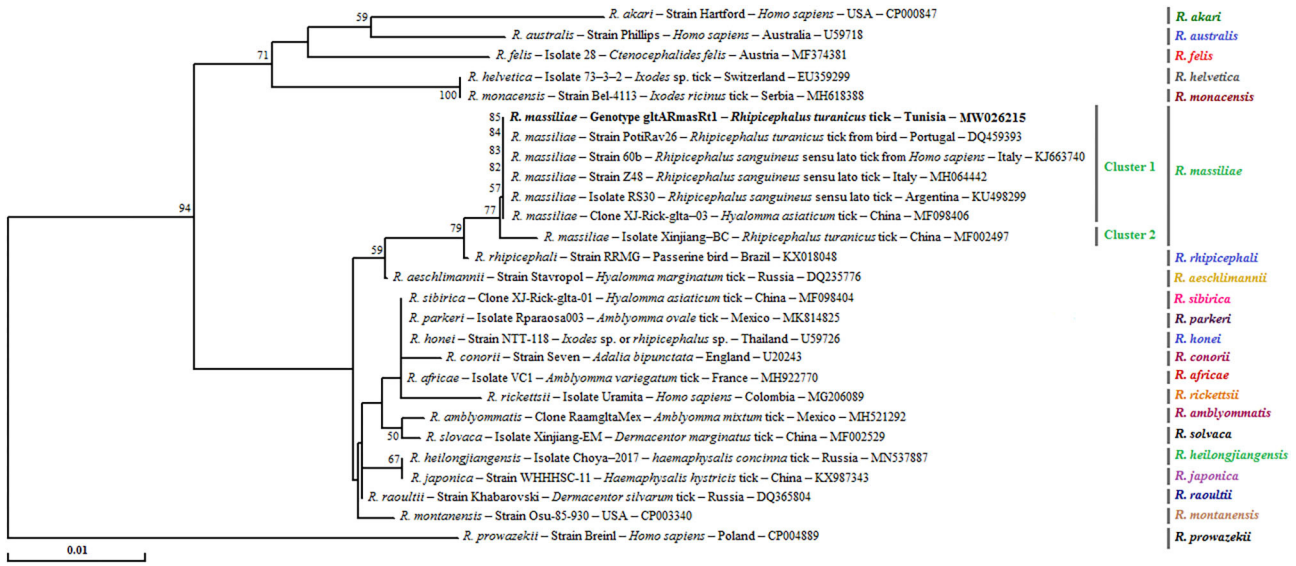

FIGURE 4 | Phylogenetical relationships based on nucleotide multiple alignments of partial *Rickettsia* spp. *gltA* sequences (341 bp). Numbers over the branches indicate the percentage of replicated trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates, only percentages >50% were represented). The only *R. massiliae gltA* genotype revealed in this study from 25 positive samples is represented in bold. The host or vector, the genotype, sequence type, strain or isolate name, the country of origin, and the GenBank accession number are indicated. One *R. prowazekii gltA* partial sequence was added as an outgroup.

sanguineus s.l. ticks collected from goats suggests that, even if the circulation in the environment is essentially maintained by *I. ricinus* ticks, there may be other species incriminated in the transmission of this bacterial species as suggested in other reports from several countries (19, 47). Our findings highlight the need of extensive studies in the *Rh. sanguineus* s.l. tick complex collected from small ruminants and other domestic animals principally dogs to assess and predict the potential risks for humans.

However, given the growing occurrence of novel *Rickettsia* species with unidentified pathogenicity, it will be essential to carry out supplementary genetic characterization of the revealed *Rickettsia* spp. by using a combination of genetic markers such as *ompA*, and *gltA*, in addition to the *ompB* gene. In the present study, phylogenetic trees based on the three gene fragments showed higher genetic diversity among the revealed *R. massiliae* isolates by using *ompA* and *ompB* genes compared to the *gltA* gene. This result is in line with those presented by Ereqat et al. (11) and Chisu et al. (48) investigating Palestinian and Sardinian ticks, respectively.

By analyzing *ompB* partial sequences, two genotypes (ompBRmasRs1 and ompBRmasRt1) infecting *Rh. turanicus* and *Rh. sanguineus* s.l. tick specimens were found similar to that isolated from *R. massiliae* strain MTU5 (CP000683) recovered from *Rh. turanicus* ticks collected on horses in Camargues, France (49), suggesting its potential spread in several Mediterranean countries. The remaining genotypes (ompBRmasRs2 and ompBRmasRt2) also infecting both tick species were found identical to *R. massiliae* Bar29 (AF123710) earlier identified in *Rh. sanguineus* s.l. ticks from Spain based on the same gene (50) and from Tunisia based on the 23S-5S intergenic spacer (6). Additionally, on the basis of the *ompA* phylogenetic tree, we found that *R. massiliae* isolated from *Rhipicephalus* ticks showed genetic divergence with novel genotypes, which indicates that these isolates infecting different tick species may come from various origins, hosts, and reservoirs. Thus, this finding needs to be further investigated.

Based on *ompB* phylogeny, low genetic diversity was observed among *R. monacensis* genotypes identified in this study. Indeed, one genotype (ompBRmonRs1) was found to be 100% similar to the corresponding sequence of *R. monacensis* strain CN45Kr (EU883092) infecting a patient from South Korea (51), revealing its widespread distributions and potential risk for human. Thus, for a more accurate classification of our revealed *R. monacensis* isolates, further testing and phylogenetic analysis with additional genes are needed since no sequences of the two other genes isolated from this *Rickettsia* species were obtained in this study.

Therefore, the observation of these two zoonotic *Rickettsia* species, *R. massiliae* and *R. monacensis*, in investigated regions indicates a possible threat to resident humans. Indeed, infected tick species can also infest various domesticated animals and therefore constitute a possible risk for transmission of SFG rickettsiae to humans (3). However, the pathogenicity of this bacterium to humans is not well-understood (48). Consequently, supplementary trials are needed to investigate the pathogenicity of the revealed *Rickettsia* species and whether found tick species can transmit these pathogens in humans.

CONCLUSIONS

The present study confirms the occurrence of human-pathogenic *Rickettsia* species in *Rh. sanguineus* s.l. and *Rh. turanicus* ticks collected from small ruminants in Tunisia. Our findings expand knowledge on ticks collected from domestic animals and highlight the range of infectious agents that may be transmitted by ticks to humans and animals.

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 reviewed and approved by The Ethics Committee of the National School of Veterinary Medicine of Sidi Thabet, University of Manouba. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

HB, LM, and MB conceived the idea. HB and MD-J carried out the fieldwork. HB, RS, and SZ performed the experiments. HB and MB performed risk factor analysis, genotyping, and phylogenetic study. HB and MB wrote the manuscript and HB, RS, LM, and MB finalized it. All authors read and approved the final version.

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Molecular Detection and Characterization of *Rickettsia* Species in *Ixodid* Ticks Collected From Cattle in Southern Zambia

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Tick-borne zoonotic pathogens are increasingly becoming important across the world. In sub-Saharan Africa, tick-borne pathogens identified include viruses, bacteria and protozoa, with *Rickettsia* being the most frequently reported. This study was conducted to screen and identify *Rickettsia* species in ticks (Family *Ixodidae*) infesting livestock in selected districts of southern Zambia. A total of 236 ticks from three different genera (*Amblyomma*, *Hyalomma*, and *Rhipicephalus*) were collected over 14 months (May 2018–July 2019) and were subsequently screened for the presence of *Rickettsia* pathogens based on PCR amplification targeting the outer membrane protein B (*ompB*). An overall *Rickettsia* prevalence of 18.6% (44/236) was recorded. Multi-locus sequencing and phylogenetic characterization based on the *ompB*, *ompA*, 16S *rRNA* and citrate synthase (*gltA*) genes revealed the presence of *Rickettsia africae* (*R. africae*), *R. aeschlimannii*-like species and unidentified *Rickettsia* species. While *R. aeschlimannii*-like species are being reported for the first time in Zambia, *R. africae* has been reported previously, with our results showing a wider distribution of the bacteria in the country. Our study reveals the potential risk of human infection by zoonotic *Rickettsia* species and highlights the need for increased awareness of these infections in Zambia's public health systems.

Keywords: *Rickettsia*, ticks, tick-borne zoonoses, Zambia, febrile illness

INTRODUCTION

Vector–borne zoonotic pathogens are of increasing importance worldwide, with many reports of emerging and/or re-emerging pathogens being detected in invertebrate hosts (1). This increase in reports has been attributed to factors such as climate change, land-use changes as well as various anthropogenic activities (2). Amongst the emerging and re-emerging vector-borne pathogens, are those which are transmitted by ticks (3).

Ticks are considered to be amongst the main vectors of zoonotic pathogens (4), and rank only second to mosquitoes in terms of importance as vectors of human pathogens (5, 6). Ticks serve as reservoirs, vectors or amplifying hosts for a variety of pathogens, with a number of these ticks reportedly infesting humans (7–12). Tick-borne zoonoses are emerging across the world (13–15), with their public health impact being on the rise in tropical and subtropical regions (3, 16–18).

Within southern Africa, several human infections by tick-borne zoonotic pathogens have been reported. These include viral (Crimean-Congo hemorrhagic fever), bacterial (*Borrelia duttonii*, *Anaplasma phagocytophilum*, *Ehrlichia ruminantium*, *Ehrlichia canis*, *Rickettsia africae*, *Rickettsia aeschlimannii*, and *Rickettsia conorii*) and protozoal (*Babesia microti*) infections (19). The most commonly reported tick-borne pathogen in southern Africa is the obligate intracelluar bacteria of the *Rickettsia* genus (spotted fever group—SFG), which cause febrile illnesses in humans (20, 21). Across Africa, more than 10 SFG Rickettsiae have been reported in ticks, humans and animals (21). Despite these reports of the pathogens in Africa, the diseases they cause are still neglected (22).

Many human rickettsial infections have been reported within southern Africa, mostly in tourists. Countries within the region which have reported active human infections include South Africa (23–34), Botswana (35), Mozambique (36), and Zimbabwe (37, 38). Although there have been no confirmed clinical cases reported in Zambia, serological evidence of human infection exists (39). Furthermore, infection by rickettsial pathogens has been reported in non-human primates in the country (40). However, information on rickettsial pathogens in ticks is very limited. Presently, to our knowledge, there are only two published reports of *Rickettsia* in Zambian ticks: one from a tick survey (41), and the other from a tick collected from an exported reptile (42). Considering that ticks play a significant role in the epidemiology of these pathogens, it is essential that they are surveyed to assess the presence of pathogens, which can inform on the potential risk for human infections. Therefore, this study sought to screen and to phylogenetically characterize rickettsial pathogens from ticks collected from cattle in southern Zambia.

MATERIALS AND METHODS

Tick Collection, Identification, and DNA Extraction

During May 2018 to July 2019, Ixodid ticks were collected from cattle in three districts in the southern part of Zambia (Chirundu, Namwala, and Livingstone; **Figure 1**). The sampling areas were purposively chosen because the southern region has the highest livestock density in the country, with the majority of the people practising subsistence farming. There is therefore close interaction between the people and the animals. Cattle sampling was chosen as it widely reared and ensured easy collection of the tick samples. The ticks were placed in aerated tubes provided with moisture and transported live to the laboratory for morphological identification using identification keys (43). After morphological identification, the ticks were stored at −80°C awaiting further analysis. For molecular analysis, individual ticks were sterilized by washing briefly in 70% ethanol after which they were washed in phosphate buffered saline (PBS) and transferred into homogenizing tubes containing 200 μL of Dulbecco's modified eagle medium (DMEM) (Sigma–Aldrich®–USA). The ticks were then homogenized in a MicroSmash™ MS-100R homogenizer (TOMY Digital Biology Co., Ltd., Japan), after which the homogenate was centrifuged, with the supernatant separated into a clean micro-centrifuge tube. DNA was extracted from the tick homogenate using the TRI Reagent® protocol (Sigma–Aldrich, USA) according to the manufacturer's recommendation. The extracted DNA was used in subsequent polymerase chain reactions (PCR) to screen for the presence of rickettsial DNA as well as sequencing.

Molecular Screening and Phylogenetic Analysis of *Rickettsia*

For initial PCR screening, OneTaq® Quick-Load® 2X Master Mix with Standard Buffer (New England BioLabs® Inc., USA) was used to amplify a 429-bp region of the outer membrane protein B (*ompB*), with cyclic conditions used previously (44). A negative control (using nuclease–free water in place of template DNA) was included in the PCR assays. The PCR primer pairs, expected amplicon size in base pairs (bp) and annealing temperatures of the assays are as shown in **Supplementary Table 1**. PCR products were electrophoresed on ethidium bromide stained 1.5% agarose gel and then visualized under ultraviolet (UV) light.

For sequencing, all samples positive on the *ompB* gene were purified using Wizard® SV Gel and Clean-Up System (Promega, Madison, WI, USA). Bidirectional Sanger sequencing was conducted with the purified DNA as a template using Brilliant Dye™ Terminator Cycle Sequencing Kit v3.1 (NimaGen®) according to the manufacturer's protocol. Nucleotide sequences were assembled and edited using GENETYX ATGC software version 7.5.1 (GENETYX Corporation, Tokyo, Japan). For phylogenetic analysis, reference sequences were retrieved from GenBank and aligned along with those determined in this study using ClustalX2. Phylogenetic trees were constructed in MEGA version 6.0 (45) using the Maximum Likelihood method based on the Tamura 3-parameter model and topological support was assessed using the bootstrap method with 1,000 replicates as a confidence interval.

Based on the results from phylogenetic analysis of the *ompB*, representative samples from each identified species were selected for amplification of a 532-bp fragment of the *ompA*, a 985-bp fragment of the 16S ribosomal ribonucleic acid (16S *rRNA*) and a 589-bp fragment of the citrate synthase (*glt*A) genes for further confimation of the species identity. The amplified segments were subsequently sequenced and phylogenetically analyzed as described for the *ompB* gene. All the primers used in this study are shown in **Supplementary Table 1.** The nucleotide sequences obtained in this study have been deposited in the GenBank with accession numbers LC565644–LC565678, LC565679–LC565695, LC565696–LC565701, and LC565702–LC565706 for *ompB*, *ompA*, *gltA*, and 16S rRNA genes, respectively.

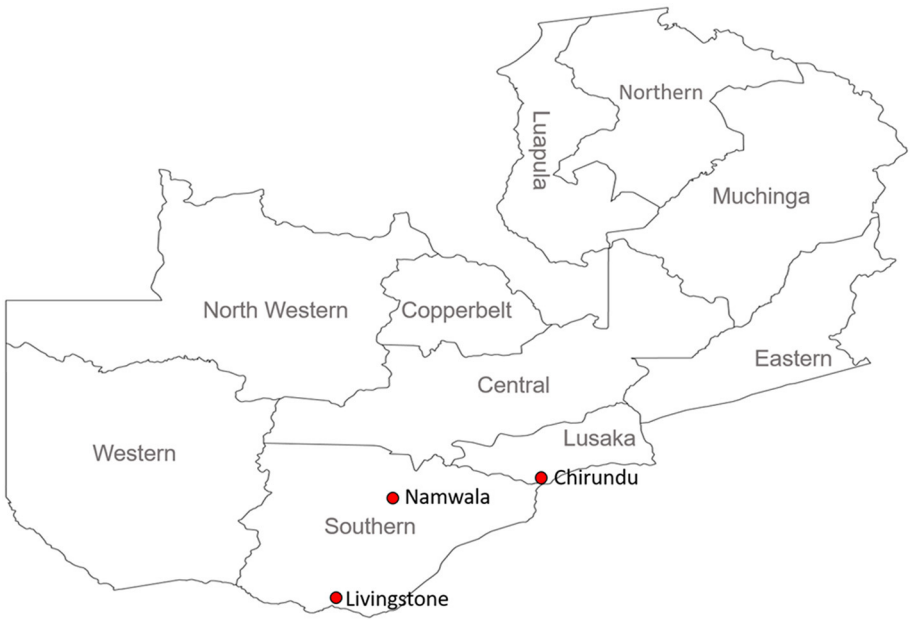

FIGURE 1 | Map of Zambia showing the study sites (Red dots) in southern Zambia. Sampling sites covered Lusaka (Chirundu) and Southern Provinces (Namwala and Livingstone).

TABLE 1 | Number of ticks collected and the prevalence of infection in the different sampling areas by genus.

Tick genus	Number of ticks analyzed (Prevalence)			Total number of ticks (Prevalence)
	Chirundu	Namwala	Livingstone	
Amblyomma sp.	1 (0%)	14 (28.6%)	4 (75%)	19 (36.8%)
Hyalomma sp.	31 (9.7%)	0 (0%)	68 (42.6%)	99 (31.3%)
Rhipicephalus sp.	4 (0%)	86 (5.8%)	28	118 (5.8%)
Total	36 (8.3%)	100 (9%)	100 (32%)	236 (18.6%)

RESULTS

Tick Collection and Prevalence of Infection

A total of 236 engorged and semi-engorged adult ticks were collected (Chirundu-36, Namwala-100, and Livingstone-100). On morphological identification, these included *Amblyomma* spp. (19), *Hyalomma* spp. (99), and *Rhipicephalus* spp. (118). The distribution of tick genera in the study areas is shown in **Table 1**.

Molecular Detection of *Rickettsia*

Based on the initial screening targeting the *ompB* gene, the observed overall prevalence of *Rickettsia* in the ticks was 18.6% (44/236). All the sampled tick genera were found to harbor *Rickettsia* pathogens, with all sampling areas reporting a different prevalence of infection (**Table 1**).

Rickettsia Species Identification

Rickettsia specific *ompB* nucleotide sequences obtained from all the 44 rickettsia-positive tick samples were compared to sequences in GenBank using the basic local alignment search tool (BLAST; https://blast.ncbi.nlm.nih.gov/Blast.cgi) (46). The sequences showed high similarity to *R. africae* (40.9%; 18/44), *R. aeschlimannii* (52.3%; 23/44), and *R. parkeri* (6.8%; 3/44), with sequence identity ranging from 98.3 to 99.77% (**Supplementary Table 2**).

On BLAST analysis of selected *ompA* sequences, there was an agreement between the *ompA* and *ompB* analysis on all samples which on the latter gene had shown close sequence similarity to *R. africae* (N383, N384, N385, N386, and N387), with 100% sequence similarity to *R. africae* KZN26 detected in South Africa (accession no. MH751466). The samples which had shown high sequence similarity to *R. parkeri* on *ompB* analysis (CT36, CT40, and CT43), showed 100% sequence similarity to *R. africae* which was found in India [accession no. MK905242]. Meanwhile, samples which had shown close sequence similarity to *R. aeschlimannii* on *ompB* analysis (N320, N323, N330, N345, N349, N356, N358, and N377), showed high sequence similarity (99.81–100%) to a *Rickettsia* endosymbiont from Turkey (accession no. KT279888), with the exception of sample N381 which showed 100% sequence similarity to *R. aeschlimannii* from Turkey [accession no. MG920562] (**Supplementary Table 3**).

BLAST analysis on *gltA* sequences showed agreement with the *ompB* sequence identity for *R. aeschlimannii* (N381), displaying 100% sequence similarity *R. aeschlimannii* from China [MH267736]. Meanwhile, CT36 which had shown high sequence similarity to *R. parkeri* on the *ompB* analysis, showed high sequence similarity to *R. africae* detected in Egypt

TABLE 2 | *Rickettsia* identity based on the gltA gene.

Area	Tick species	DNA ID	Reference strains of *Rickettsia* species (GenBank Accession Number)	Nucleotide percent identity (%)
Chirundu	*Hyalomma* spp.	CT36	*R. africae* Egypt (HQ335126)	99.87
Namwala	*Amblyomma* spp.	N28	*Rickettsia* sp. Slovakia (HM538186)	99.74
Namwala	*Rhipicephalus* spp.	N61	*Rickettsia* sp. Slovakia (HM538186)	99.61
Livingstone	*Hyalomma* spp.	N381	*R. aeschlimannii* China (MH267736)	100
Livingstone	*Amblyomma* spp.	N383	*Rickettsia* sp. Slovakia (HM538186)	99.87
Livingstone	*Hyalomma* spp.	N385	*Rickettsia* sp. Slovakia (HM538186)	99.61

TABLE 3 | *Rickettsia* identity based on the 16S rRNA gene.

Area	Tick species	DNA ID	Reference strains of *Rickettsia* species (GenBank accession number)	Nucleotide percent identity (%)
Chirundu	*Hyalomma* spp.	CT36	*Rickettsia* sp. Strain Bel–4109 (MH618379)	100
Namwala	*Rhipicephalus* spp.	N61	*R. africae* Ethiopia (L36098)	100
Livingstone	Hyalomma spp.	N323	*R. aeschlimannii* China (MH923218)	100
Livingstone	Hyalomma spp.	N381	*R. aeschlimannii* China (MH923218)	100
Livingstone	Amblyomma spp.	N383	*R. africae* Ethiopia (L36098)	99.78

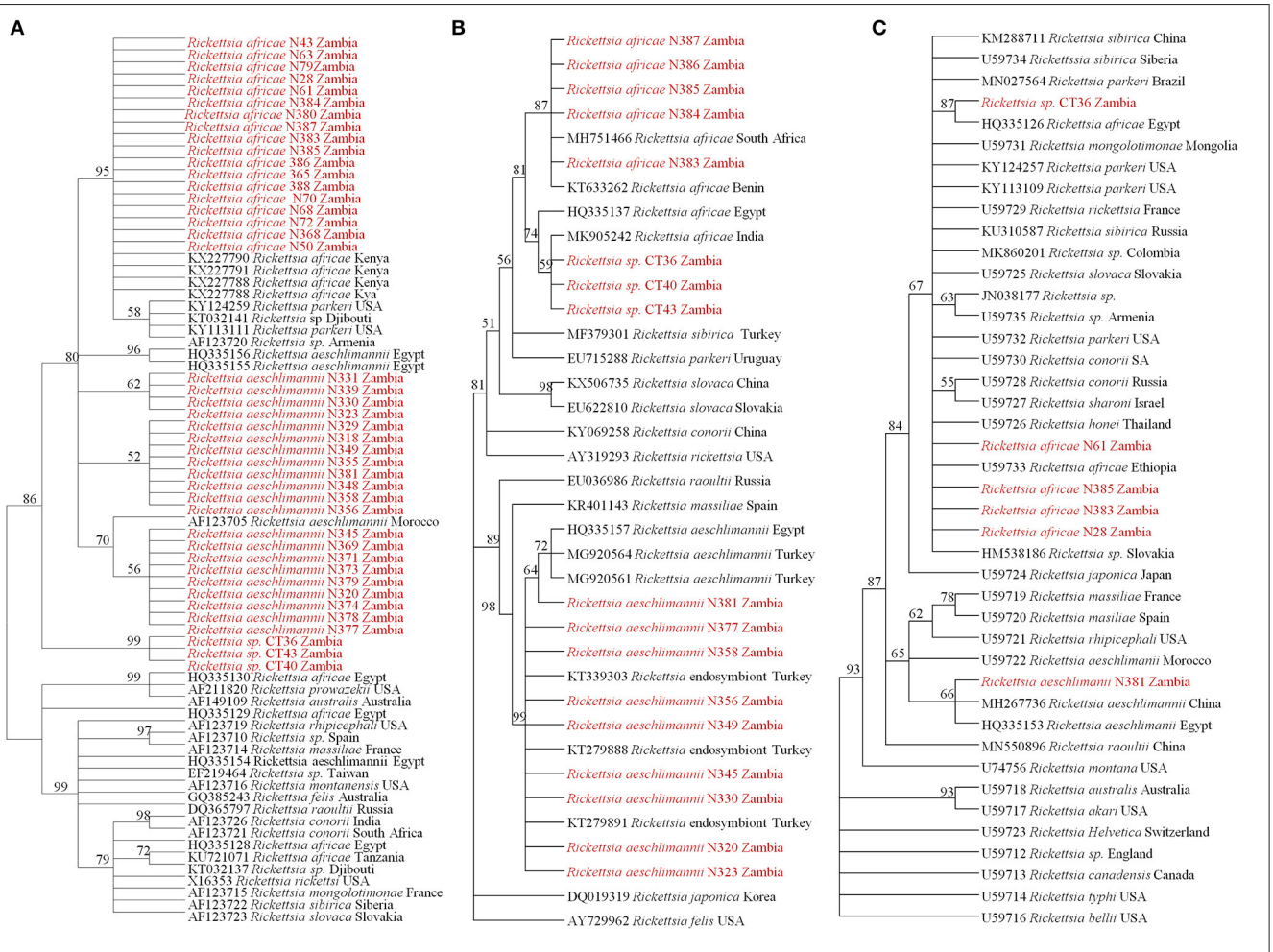

FIGURE 2 | Phylogenetic relationships of the *ompB* **(A)**, *ompA* **(B)**, and *gltA* **(C)** genes of *Rickettsiae* detected from ticks in Zambia. Phylogenetic analysis was based on 397, 460, and 596 bp of the *ompB*, *ompA*, and *gltA* genes, respectively. Numbers at branch nodes indicate bootstrap values ≥50%. The reference sequences included in the analyses are shown by their GenBank accession number, species, and country of origin. The *Rickettsiae* characterized in the present study are in red text.

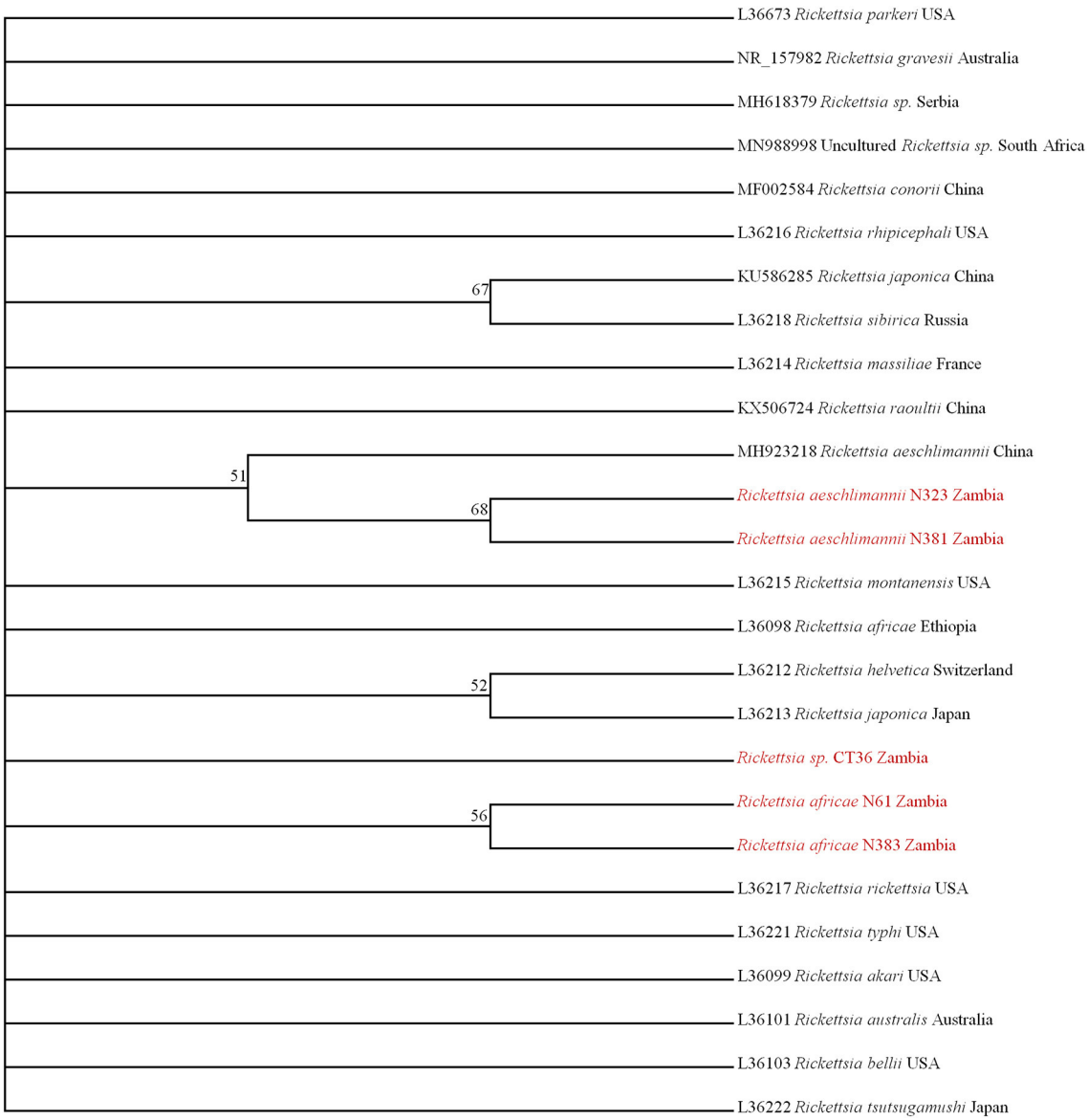

FIGURE 3 | Phylogenetic relationships of the *16S rRNA* genes of rickettsiae detected in ticks in Zambia. Phylogenetic analysis was based on 795 bp of the 16S *rRNA* gene. Numbers at branch nodes indicate bootstrap values ≥50%. The reference sequences included in the analyses are shown by their GenBank accession number, species, and country of origin. The *Rickettsiae* characterized in the present study are in red text.

[accession no. HQ335126] (99.87% identity) and those that had shown close sequence similarity to *R. africae* on *ompB* analysis (N28, N383, and N385), displayed 99.61% sequence similarity to *Rickettsia* sp. Identified in Slovakia [accession no. HM538186] (**Table 2**).

The *16S rRNA* gene BLAST sequence analysis results revealed agreement with the findings of *ompB* analysis for samples with close sequence similarity to *R. africae* (N61, N383) and *R. aeschlimannii* (N323, N381) with sequence identity ranging from 99.78 to 100% to those detected in Ethiopia [L36098] and China [MH923218], respectively. Meanwhile, the sample that had shown close sequence similarity to *R. parkeri* on *ompB* analysis (CT36), showed highest sequence similarity to an uncultured *Rickettsia* sp. Strain Bel-4109 (MH618379) from Serbia (**Table 3**).

Phylogenetic Analysis

On phylogenetic analysis based on the *ompB* sequence, the Zambian sequences under study formed three distinct clusters, namely *R. africae*, *R. aeschlimannii*, and *Rickettsia* sp. (**Figure 2A**). It was noteworthy that the Zambian *Rickettsia* sp. did not cluster with any of the reference sequences from GenBank. Phylogenetic analysis based on the *ompA* sequence showed Zambian sequences forming two clusters; one grouping with *R. africae* and another clustering with

R. aeschlimannii and *Rickettsia* endosymbiont (**Figure 2B**). In addition, phylogenetic analysis of selected samples, based on the *gltA* sequence showed that Zambian samples clustered with *R. africae* (CT36, N61, N385, N383, and N28) and *R. aeschlimannii* (N381) (**Figure 2C**). Based on 16S *rRNA* gene sequences, phylogenetic analysis of the Zambian samples clustered with *R. africae* (N61, N383), *R. aeschlimannii* (N323, N381) with CT36 not clustering with any reference sequences from GenBank (**Figure 3**).

Taken together, based on nucleotide sequence similarities as determined by BLAST analysis and phylogenetic analysis of four different genes, there were three distinct *Rickettsia* species identified, namely *R. africae*, *R. aeschlimannii*-like species and *Rickettsia* sp.

Distribution of *Rickettsia* Species by Sampling Area and Tick Genera

By area of sampling, ticks from Chirundu were infected with *Rickettsia* sp. (3/3), those from Namwala were infected by *R. africae* only (9/9), whilst those from Livingstone were infected by *R. africae* (9/32) and *R. aeschlimannii*-like species (23/32)]. By tick genera, *Amblyomma* were infected with *R. africae* (6/7) and *R. aeschlimannii*-like species (1/7), *Hyalomma* were infected with *R. aeschlimannii*-like species (22/32), *R. africae* (7/32), and unidentified *Rickettsia* sp. (3/32), with *Rhipicephalus* ticks being found only with *R. africae* (5/5).

DISCUSSION

This study found an overall *Rickettsia* prevalence of 18.6% in the ticks sampled from the southern part of Zambia. The previous report (41) on *Rickettsia* prevalence in ticks in Zambia showed a much lower prevalence of 4.6% and the difference in prevalence between the two studies could be attributed to the differences in the proportions of ticks sampled. The study conducted in eastern Zambia by Chitimia-Dobler et al. (41) was predominated by ticks of the *Rhipicephalus* genus (96% *Rhipicephalus*, 4% *Amblyomma*, and *Hyalomma*), which are considered to always have lower rickettsial infections (41). In contrast, our study had relatively more *Amblyomma* and *Hyalomma* species (50% *Amblyomma* and *Hyalomma*, 50% *Rhipicephalus*), tick species considered amongst the principal *Rickettsia* vectors in the region (21). Indeed, our findings on infection rates in the three tick genera confirm the observations by Parola et al. (21) that *Amblyomma* and *Hyalomma* have the highest infection rates of *Rickettsia*, with *Rhipicephalus* being amongst those with low infection rates (21). However, since we collected ticks from cattle, our finding of *Rickettsia* pathogens in the tick species cannot be conclusively used as an indicator of their potential vector role. This is because the identified pathogens could have been picked from cattle during feeding.

In this study, we report for the first time the presence of *R. africae* and *R. aeschlimannii*-like species in Ixodid ticks from the southern part of Zambia. Whilst *R. africae* has been reported before in ticks from the eastern part of Zambia (41, 42), this study reports for the first time the presence of *R. aeschlimannii*-like species in the country, adding to the number of SFG Rickettsiae which are reported in Zambia. The previously reported SFG Rickettsiae include *R. africae*, *R. massiliae*, *R. conorii* and *R. felis* (40–42, 47). To our knowledge, Zambia becomes the third country in southern Africa to report the presence of *R. aeschlimannii*-like species, with previous reports being in South Africa (48) and Zimbabwe (49). We also report unidentified *Rickettsia* sp. which showed distinct phylogenetic clustering pattern based on the four genes analyzed in this study, and thus could not be assigned a specific species. This observation further emphasizes the recommendations by Fournier et al. (50) to use multiple genes to ensure proper speciation of *Rickettsia* species. Alternatively, speciation could be achieved by sequencing the entire full lengths of several diagnostic genes. For example, Moron et al. (51) sequenced the full length *ompB* gene (∼6,000 bp) to phylogenetically compare *R. felis* with its homologs in spotted fever group (SFG) and typhus group (TG) *Rickettsiae*.

Our findings add to available data on the prevalence of rickettsial pathogens in ticks within the southern African region. Other countries in the region which have reports of tick surveys for *Rickettsia* infection include Zimbabwe (49), Botswana (52), Mozambique (53), and South Africa (54–58) with prevalence ranging from as low as 3% to as high as 77%. This study also adds to the growing data on tick-borne (41, 42, 59–61) and tick-associated (62, 63) zoonotic pathogens within Zambia. It further highlights the potential threat posed by tick-borne pathogens to human health and the need to strengthen surveillance of tick-borne diseases in the country.

In conclusion, we have shown the presence of zoonotic SFG *Rickettsiae* in ticks from the southern part of Zambia, indicating the wider geographical spread of these pathogens within the country and the potential threat they pose to human health. It is therefore important to screen for these pathogens in patients reporting with febrile illnesses of unknown origin. We also report on the presence of *R. aeschlimannii*-like species in Zambia, an indication of the expanded geographical spread of this pathogen within the southern African region.

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 reviewed and approved by ERES Converge (IRB - 00005948, FWA - 00011697), under reference number 2017-Jul-021. Written informed consent for participation was not obtained from the owners because verbal consent was sought for collection of ticks from the animals.

AUTHOR CONTRIBUTIONS

SC and ES conceived the study. SC, KChi, KCha, and ES developed the laboratory methodology. KChi and KS conducted most of the laboratory experiments. SC, KN, KCha, and ES supervised the work. SC, KCha, and ES wrote the original draft of the manuscript. SC acquired funding for the study. All authors were involved in data analysis, reviewing, and approving the final version of the manuscript.

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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.2021.684487/full#supplementary-material

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REVIEW
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Epidemiological and Clinicopathological Features of *Anaplasma phagocytophilum* Infection in Dogs: A Systematic Review

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Anaplasma phagocytophilum is a worldwide emerging zoonotic tick-borne pathogen transmitted by *Ixodid* ticks and naturally maintained in complex and incompletely assessed enzootic cycles. Several studies have demonstrated an extensive genetic variability with variable host tropisms and pathogenicity. However, the relationship between genetic diversity and modified pathogenicity is not yet understood. Because of their proximity to humans, dogs are potential sentinels for the transmission of vector-borne pathogens. Furthermore, the strong molecular similarity between human and canine isolates of *A. phagocytophilum* in Europe and the USA and the positive association in the distribution of human and canine cases in the USA emphasizes the epidemiological role of dogs. *Anaplasma phagocytophilum* infects and survives within neutrophils by disregulating neutrophil functions and evading specific immune responses. Moreover, the complex interaction between the bacterium and the infected host immune system contribute to induce inflammatory injuries. Canine granulocytic anaplasmosis is an acute febrile illness characterized by lethargy, inappetence, weight loss and musculoskeletal pain. Hematological and biochemistry profile modifications associated with this disease are unspecific and include thrombocytopenia, anemia, morulae within neutrophils and increased liver enzymes activity. Coinfections with other tick-borne pathogens (TBPs) may occur, especially with *Borrelia burgdorferi*, complicating the clinical presentation, diagnosis and response to treatment. Although clinical studies have been published in dogs, it remains unclear if several clinical signs and clinicopathological abnormalities can be related to this infection.

Keywords: canine granulocytic anaplasmosis, *Anaplasma phagocytophilum*, dogs, epidemiology, tick-borne disease, zoonosis

INTRODUCTION

Canine granulocytic anaplasmosis (CGA) is an emerging zoonotic tick-borne disease that is distributed worldwide. The causative agent, *Anaplasma phagocytophilum*, is an obligate intracellular gram-negative alpha-proteobacterium that develops within granulocytic cells. It is usually transmitted by ticks belonging to the genus *Ixodes* and it causes disease in several mammalian species (1, 2). In the USA, both canine and human exposures have progressively increased from 2008 to 2010 with the number of reported human cases increasing by 53% during this period (3, 4). Data from the USA Center for Disease Control and Prevention (4) and Morbidity and Mortality Weekly Report (MMWR) reported 36,342 human cases between 2010 and 2018 and almost a 12-fold increase during this same period (4). Currently, human granulocytic anaplasmosis (HGA), is considered amongst the three most important vector-borne disease (VBD) in the USA with Lyme borreliosis and Zika virus (5, 6) and is increasingly being diagnosed in several European and Asian countries (7, 8).

The focus on canine VBDs has increased the past decade as they represent an important threat to both canine and human health (9). Because of their proximity to humans, dogs may serve as reservoirs of vector-borne pathogens, a source of infection for vectors, mechanical transporters of infected vectors, and as sentinel indicators of regional infection risk (2, 3, 10–15). Furthermore, the strong molecular similarity between human and canine isolates of *A. phagocytophilum* in Europe and the USA (16–21) and the positive association in the distributions of human and canine cases in the USA emphasizes the use for dogs as sentinels in epidemiological studies (3, 4, 9, 15, 22, 23).

The lack of specific clinicopathological signs, the frequent rapid evolution and positive prognosis even without treatment, the prompt response to a commonly used antibiotic and the possibility of coinfections (24–28) all make the diagnosis of CGA challenging for veterinarians. Description of signs and laboratory abnormalities associated with *A. phagocytophilum* infection in dogs is mostly available from Europe and North America. Although some studies have described the most common manifestations of CGA (13, 24–35), it remains unclear if some clinical signs and clinicopathological abnormalities are related to this infection. In this paper, we provide an overview of the current knowledge on the worldwide epidemiological features of *A. phagocytophilum* focusing on dogs, and describe the clinicopathological aspects of CGA with an emphasis on missing data.

DESCRIPTION OF *ANAPLASMA PHAGOCYTOPHILUM*

Classification

Anaplasma phagocytophilum is a bacterium belonging to the family of Anaplasmataceae in the order of Rickettsiales (36). The phylogenetic molecular analysis based on the 16S rRNA and the groEL genes sequencing in addition to morphologic and phenotypic characteristics have led to the reorganization of the family of Anaplasmataceae and the reclassification of some agents. Consequently, the name *A. phagocytophilum* was given in 2001 to three previously distinct agents, i.e., the agent that causes equine granulocytic anaplasmosis (*Ehrlichia equi*), the agent that causes tick-borne fever or pasture fever in sheep and cattle (*Ehrlichia phagocytophila*) and the agent that causes HGA [formerly human granulocytic ehrlichiosis (HGE)] (1). The renaming of these three agents as *A. phagocytophilum* has been controversial because of differences in their host tropism and cell target from other *Anaplasma* species, such as *Anaplasma marginale* (37). Additionally, although these three agents share genetic, antigenic and biological characteristics (1), they are considered phenotypic variants due to differences in their distribution, prevalence, virulence and target host species (38, 39).

Morphology and Genome

Anaplasma phagocytophilum typically exhibits coccoid to ellipsoid shapes measuring ∼0.2–2.0 μm in diameter. The bacteria infect myeloid cells primarily neutrophils (and occasionally eosinophils), forming intracytoplasmic inclusions derived from the host cell membrane measuring 1.5–2.5 μm, called "morula" (from Latin "morum": mulberry) (1, 40).

The *A. phagocytophilum* genome is composed of a single circular double-stranded chromosome. The complete genomic sequence is estimated at 1.47 megabases (Mb) and was published on GenBank in 2006 (NC007797) (19, 36). Despite its apparently simple genome, *A. phagocytophilum* exhibits an extensive genomic diversity (19, 41, 42). More than 500 partial *A. phagocytophilum* pseudogene sequences derived from human, ticks and animals from several US, European and Asian regions are available in GenBank (19). Moreover, twenty complete *A. phagocytophilum* genomes have been sequenced including 16 American and four European strains. However, genomes from only a few different strains per host species are available (aside from humans), underscoring the lack of information on strain diversity within different host species (19, 36).

Genetic Variability

Genetic variability between strains may explain the ecological complexity, the host tropism diversity, the differences in incidence and clinical presentation, severity and evolution of the disease documented in different countries (42–47). Many studies demonstrated different virulence and hosts tropism of specific *A. phagocytophilum* strains (17, 41–50). However, the host specificity of strains seems to be restricted and multiple infections with different strains are often observed. Farm and large wild animals, small mammals and ticks were especially prone to carrying multiple genetic variants. In humans and domestic animals double infections are not so frequent (51). The 16S rRNA gene nucleotide sequences analysis discriminated 15 worldwide variants differing in a variable fragment located near the 5' end of the gene. Among them, two are pathogenic for human and abundant all over the world (52). In the USA, several variants have been identified based on the sequencing of the 16S rRNA and the only pathogenic variant to humans (AP-ha) is also able to induce the disease in dogs, horses and mice but not in cattle. In Europe, other variants have been identified in

humans and the AP-ha variant was also detected in wild ruminant species (41–43, 48, 49). Strains infecting domestic ruminants in Europe and white-tailed deer in the USA seem to genetically differ from those infecting humans, horses and dogs (44, 50). In Washington State, five different 16S rRNA variants (named WA1–5) that differed at four nucleotide positions were identified from dogs displaying clinical signs consistent with CGA. All WA variants were distinct from those identified in sheep in Norway and llama-associated ticks but one was identical to equine and human variants (24). In another European study, seven different 16S rRNA variants were identified from dogs, with the two most common variants showing statistically significant differences in the frequency of clinical signs and hematological abnormalities, which suggests possible differences in strain pathogenicity (45). Finally, a recent study showed that dogs can be naturally infected concurrently with *A. phagocytophilum* variant 1, variant 4, and HGE agent (53). The pathogenic role of the classic sheep variant, *A. phagocytophilum* variant 1, in the canine species is uncertain. Previous studies showed that the "HGA agent" appears to be more pathogenic for dogs than other variants (45).

The 16S rRNA gene was considered too conserved for use in the phylogenetic analysis of different strains of *A. phagocytophilum*. It has a poor resolution and failed to discriminate between ecotypes circulating in wild ruminants compared to other animals. Furthermore, the 16S rRNA sequence analysis could not categorize human-infective isolates in order to detect virulent strains and was unable to distinguish variants according to their geographic origin (43, 54–56). As such, other genes have been proposed to study the genetic variability of *A. phagocytophilum* including msp4, ankA, groEL operon, msp2/p44, pfam01617 superfamily, and drhm genes (19–21, 50, 56–59). Sequencing different genes revealed similarities between human and canine isolates, suggesting that dogs and humans may be infected by the same strains (16–21, 24, 45, 53, 60–62).

VECTORS

Although several transmission modes have been reported (mostly in humans) (63–66), *A. phagocytophilum* is commonly transmitted to people and domestic animals through tick bites (67). It is naturally maintained in complex and poorly understood enzootic tick-wild animal cycles (55, 59, 68) and is transmitted most frequently by ticks of the *Ixodes persulcatus* complex. These ticks are commonly found in the northern hemisphere and their occurrence depends on climatic conditions (between 10 and 30°C, and >80% relative humidity) and the availability of hosts (49, 69).

In the USA, several ixodid ticks transmit this pathogen, depending on the geographic location. The main vector in the humid forests of the upper midwestern, north central and northeastern regions is *Ixodes scapularis* whereas *Ixodes pacificus* is located in shrub forests and deserts of the western USA (70–72). The prevalence of *A. phagocytophilum* DNA among ticks varies from <1% up to 50% throughout the country (73–76). Other tick species have been reported to be infected with *A. phagocytophilum*, such as *Amblyomma americanum* and *Dermacentor* spp., and *Ixodes spinipalpis* and *Ixodes dentatus* are recognized as competent vectors (77–81). Other *Ixodes* species including *Ixodes angustus*, *Ixodes ochotonae*, and *Ixodes woodi* are suggested to act as vectors for the bacterium (82, 83). In central and southern America, very few studies are published on the prevalence of *A. phagocytophilum* among ticks. However, among the three available studies, none have detected the DNA of this bacterium in *Ixodes* spp. ticks. In contrast, its DNA has been amplified from *Rhipicephalus sanguineus*, *Amblyomma cajennense*, *Amblyomma dissimile*, *Amblyomma maculatum*, *Dermacentor variabilis* (84–86). *Amblyomma* spp. and *D. variabilis* were positively correlated with *A. phagocytophilum* infection in Brazil and Mexico (84, 86).

In Europe, the most common vector is *Ixodes ricinus* (69), which is widely distributed from western Europe to central Asia. This tick lives mostly in humid wooded habitats and pastures and is rarely encountered in the Mediterranean region or in mixed or deciduous forests except at high altitudes (67). The prevalence of *A. phagocytophilum* DNA among *I. ricinus* ticks in Europe varies from <1 to 76.7% (87, 88). Other *Ixodes* spp. ticks seem to be involved in epidemiological cycles that are distinct from those involving *I. ricinus* (55, 89, 90). In addition, the DNA of this bacterium has been detected in several other tick species in Europe including *Dermacentor reticulatus, Haemaphysalis concinna, Hyalomma marginatum, Ixodes ventaloi,* and *Ixodes trianguliceps* (58, 91–95). *Rhipicephalus* species were also infected by *A. phagocytophilum* and could act as competent vectors in the eastern Mediterranean area (96–99). *Ixodes persulcatus* is another competent vector of *A. phagocytophilum* in eastern Europe and Asia, with rates of DNA detection up to 16.7 and 21.6%, respectively (100, 101).

Although *I. persulcatus* is considered the primary vector in Asia, *A. phagocytophilum* DNA has been detected in several other tick species including *Ixodes nipponensis, Ixodes ovatus, Rhipicephalus turanicus, Rhipicephalus haemaphysaloides, H. marginatum*, *Boophilus kohlsi, Dermacentor silvarum*, and several *Haemaphysalis* species (96, 102–106). Molecular investigations indicated that *I. ovatus*, *Dermacentor silvarum*, *Hae. concinna*, *Haemaphysalis longicornis*, *Rhipicephalus microplus*, *R. sanguineus*, and *Dermacentor nuttalli* might be involved in the transmission of *A. phagocytophilum* in China (8, 107–109).

In North Africa, one study in Morocco and Tunisia detected *A. phagocytophilum* DNA in 1 and 3% of *I. ricinus* and *Hyalomma detritum,* respectively (110). Two separate studies detected DNA in *R. sanguineus* from free-roaming dogs in Egypt and *H. marginatum* from horses in Tunisia with prevalence rates of 13.7 and 2.3%, respectively (111, 112). These studies indicate that *A. phagocytophilum* is likely to circulate in a wide variety of ticks, but their involvement in transmitting the bacterium to host has yet to be established (112).

DISTRIBUTION AND PREVALENCE OF *ANAPLASMA PHAGOCYTOPHILUM*

Anaplasma phagocytophilum has a worldwide distribution and endemic areas include some regions of the USA (northeastern and mid-Atlantic, Upper Midwest, and Pacific Northwest states),

Europe and Asia (China, Siberian Russia, and Korea). These regions correspond to occurrence areas of *I. persulcatus* group ticks (12, 13, 24, 29, 113). Several prevalence studies in dogs have been conducted in various American, European, Asian and African countries (**Table 1**). However, data are lacking in large parts of Asia, Africa, South America and Australia. The geographic variation in tick exposure, the differences in inclusion criteria to select dog populations, and the use of different serologic tests [i.e., immunofluorescent antibody test (IFAT), enzyme-linked immunosorbent assay (ELISA) or Western blot] make comparison between studies difficult (234, 235). In addition, cross-reactivity with the most important other *Anaplasma* species infecting dogs, i.e., *Anaplasma platys,* is reported to occur for both IFA and ELISA (1, 9, 120, 121, 236–241). Therefore, in regions where both pathogens could be present (southern USA states, southern Europe, South America, Asia, and Africa), seropositivity may not necessarily reflect exposure to *A. phagocytophilum* and potential overestimation of the true prevalence and distribution can occur (9, 162, 189, 198, 234, 236, 238, 241). As a result, PCR-based assays are necessary to determine which of the two agents is responsible for positive serologic test results in regions where both bacteria are present (241). In areas where the *Ixodes* tick vector is less prevalent or absent, a positive *Anaplasma* spp. serologic result could be the result of *A. platys* exposure (164). Less frequent and minor serological cross-reactions were described at low titers between *A. phagocytophilm* and *Ehrlichia* species (i.e., *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, and *Neorickettsis sennetsu formerly Ehrlichia sonnetsu*), especially with hyperimmune sera, when using IFA and immunoblot assay (1, 29, 39, 121, 127, 242, 243). However, it is not clear whether the cross-reactivity with *E. canis* was attributable, in part, to antibodies against *A. platys* because dogs are sometimes exposed to both *E. canis* and *A. platys* (164, 240). In contrast, no cross-reactivity has been documented between *Anaplasma* spp. and *Ehrlichia* spp. when using the point-of-care dot ELISA (234, 240).

The first CGA cases in the USA were detected in California; then, the exposure of dogs to this organism has been recorded in more than 39 USA states and highest rates were noted in the upper Midwestern, northeastern and western states. Serologic surveys revealed prevalence values of *Anaplasma* spp. antibodies ranging from 0.0 to 40.0% (3, 9, 10, 12, 13, 15, 23, 119, 120, 122–126, 236, 242, 244, 245). Five countrywide serologic studies showed an overall prevalence of *Anaplasma* spp. of 1.9 to 4.8% with the highest rates recorded in northeastern regions (3, 9, 15, 23, 119). The study that found a prevalence rate of 1.9%, used species-specific peptides to detect canine antibodies to *A. phagocytophilum* (3). In addition, cases confirmed by PCR were diagnosed in several USA states (12, 13, 24, 26, 27, 29, 124, 245–247). In the USA, over 100,000 and 220,000 dogs were seropositive to *Anaplasma* spp. in 2015 and 2019, respectively (248, 249). Two recent studies analyzing regional trends of *Anaplasma* spp. exposure in dogs showed that seroprevalence increased broadly in the northeastern, upper midwestern states, northern California, mid-atlantic coast and southern Oregon (249, 250). In Canada, six serologic surveys on *Anaplasma* spp. are available (**Table 1**) (3, 114–118), and six cases of CGA from Vancouver Island (251), Saskatoon (252) and Montreal (253) were confirmed by DNA detection. In Latin America and the Caribbean, the seroprevalence of *Anaplasma* spp. ranges from 1.0 to 53.2% (**Table 1**) (133, 134, 254). In addition, two studies and a case report have detected the DNA of *A. phagocytophilum* in Brazil (**Table 1**) (129, 255).

In Europe, *Anaplasma* spp. seroprevalence has been reported in almost all countries with rates ranging from 1.1 to 56.5% (143, 148, 150, 183, 190). The detection of *A. phagocytophilum* DNA has also been reported mostly from central and northern countries (**Table 1**) with prevalence rates up to 14.2% (174). Additionally, several cases of CGA have been described (25, 28, 30–32, 34, 256–262).

In Asia, *Anaplasma* spp. seroprevalence is available from China, Korea, Malaysia, Taiwan and Israel and range from 1.2 to 24.7% (**Table 1**) (212, 214). *Anaplasma phagocytophilum* DNA has also been detected in dogs with prevalence rates up to 39.5 and 57.3% in Jordan and Iran, respectively (219, 222).

In Africa, only a few prevalence studies have been published on *Anaplasma* spp. in dogs (**Table 1**). Seroprevalence rates recorded in African countries range from 11.8 to 47.7% (**Table 1**) (225, 231). Similarly, very limited studies have investigated *A. phagocytophilum* infection in dogs in this continent. The DNA of this bacterium has been detected in Tunisia, Nigeria, Cape Verde and South Africa (**Table 1**) (224, 228, 229, 232) but not in Algeria and Morocco (225, 227). In addition, an *Anaplasma* species closely related to *A. phagocytophilum* was detected in blood samples from South African dogs based on 16S rRNA gene sequencing (263) whereas all dogs from Algeria, Ghana and Maio Island tested negative by PCR (**Table 1**) (225, 231, 233).

EPIDEMIOLOGICAL ROLE OF DOGS

Several wild and domestic animals are receptive to *A. phagocytophilum* infection. However, the disease has been reported only in a few species including domestic ruminants, horses, cats, dogs and humans (22, 24, 63, 264–269). Although dogs are susceptible to *A. phagocytophilum* infection, they are mostly recognized as incidental hosts and their role as potential reservoirs is still controversial (24, 270). As *A. phagocytophilum* is an obligate intracellular bacterium, its reservoirs should be animal hosts permitting its survival, particularly outside the activity period of its vectors (271). To be considered as a host reservoir, a host must be fed on by an infected vector tick at least occasionally, take up a critical number of the infectious agent during the bite by an infected tick, allow the pathogen to multiply and survive for a period in at least some parts of the body, and allow the pathogen to find its way into other feeding ticks (272, 273). Therefore, the detection of pathogens or their DNA in animal hosts is not enough to consider them as reservoir hosts (274).

Dogs are considered unlikely reservoir hosts due to the probable short duration of bacteremia (<28 days) and uncertainty regarding their ability to host enough nymphal tick stages to contribute to the spread of the bacterium (2, 67). In Austria, no significant difference in the seroprevalence of

TABLE 1 | Prevalence of *Anaplasma* spp. (*A. phagocytophilum* and *A. platys*) antibodies and/or DNA detection of *A. phagocytophilum* in blood samples from dogs in several countries.

Countries	Number of dogs	Type of dog population	Prevalence (%)	Method	References
AMERICA					
Canada	86,251	Sick and healthy dogs from 238 practices	0.2	ELISA	(114)
	115,636	Not stated	0.29	ELISA	(115)
	753,468	Not stated	0.4	ELISA	(116)
7 provinces	285	Not stated	1.1	ELISA	(3)
South Ontario, Quebec	53	Suspected to have TBD	0.0	IFA	(117)
Saskatchewan	515	Sick and healthy client-owned dogs	0.6	ELISA	(118)
USA	3,950,852	Not stated	3.8	ELISA	(15)
	3,588,477	Sick and healthy dogs tested for VBD	4.4	ELISA	(23)
	479,640	Dogs suspected to have a VBD	4.8	ELISA	(9)
	14,496	Dogs suspected to have a VBD	1.9	ELISA	(119)
	6,268	Dogs suspected to have a VBD	1.5–3.5	ELISA	(3)
Oregon, California	2,431	Clinically healthy dogs	2.4	ELISA	(120)
North Carolina, Virginia	1,845	Dogs admitted regardless of the reason for examination to the NCSU-VTH	1.1	IFA	(121)
Maine	1,087	Dogs tested for heartworm or undergoing surgery	7.1	ELISA	(122)
California	1,385	Non-ehrlichial related illnesses or well-animal care	8.7	IFA	(10)
	184	Rural dogs with or without clinical signs	40.0 7.6	IFA PCR	(12)
Minnesota	731	Sick and healthy pet dogs	29.0	ELISA	(13)
	273		9.5	PCR	
Oklahoma	259	Dogs suspected to have a VBD	33.0	IFA	(123)
Northern Arizona	233	Pet and stray dogs	11.6 0.0	ELISA PCR	(124)
New Jersey	202	Healthy dogs	9.4	ELISA	(125)
North Carolina	118	Clinically healthy dogs	0.0	ELISA	(126)
Connecticut, New York	106	Sick client-owned dogs living	9.4	IFA, WB	(127)
Cumberland Gap Region	232	Shelter dogs	0.9	ELISA	(128)
Brazil					
Rio de Janeiro	398	Not stated	6.0	PCR	(84)
	253	Not stated	7.1	PCR	(129)
Southeastern	198	Dogs suspected to have TBD	0.0	PCR	(130)
Southern	196	Companion dogs	9.7	ELISA	(131)
Central-northern Parana	138	Rural and urban dogs	13.8	ELISA	(132)
Puerto Rico	629	Dogs from shelters and a veterinary clinic	1.0	ELISA	(133)
Colombia	498	Not stated (abstract only)	33.0	ELISA	(133)
	218	Working, shelter and client-owned dogs	53.2	ELISA	(134)
Haiti	210	Owned dogs	17.6 0.0	ELISA PCR	(135)
West Indies	157	Not stated	10.8	ICG	(136)
Caribbean region	29	Not stated	10.0	ELISA	(3)
Mexico	1,706	Healthy dogs and dogs with clinical signs compatible with VBD	9.9	ELISA	(137)
Costa Rica	408	Apparently healthy dogs	2.7	IFA	(138)
	374		0.3	PCR	
Ecuador Galapagos Islands	58	Without consideration of the patients' presenting complaint	12.1	ELISA	(139)
Nicaragua	329	Dogs presented at veterinary clinics	28.6 2.2	ELISA PCR	(140)
Chile	905	Urban and rural dogs	44.0	IFA	(141)
EUROPE					
Germany	5,881	Sick dogs suspected to have anaplasmosis	21.5	ELISA	(142)

(Continued)

TABLE 1 | Continued

Countries	Number of dogs	Type of dog population	Prevalence (%)	Method	References
	1,124	Dogs suspected to have anaplasmosis	50.1	IFA	(143)
	522	Healthy dogs and dogs suspected of CGA	43.0 5.7	IFA PCR	(144)
	111	Healthy dogs and dogs suspected of CGA	43.2 6.3	IFA PCR	(31)
	1,862	Traveling dogs to Germany	17.8	IFA	(145)
	792	Retrospective analysis of serum sample	41.9	IFA	(146)
Munich	448	Healthy and sick dogs	19.4	ELISA	(147)
	171	Healthy Bernese Mountain Dogs	50.3	IFA	(148)
	57	Healthy dogs from other breeds	24.6		
Brandenburg	1,023	Blood samples from veterinary clinics or a commercial diagnostic laboratory	1.5	PCR	(149)
Russia					
European part	440	Urban dogs with a history of tick bites	1.1	ELISA	(150)
Voronezh Reserve	82	Dogs owned by Voronezh Reserve staff	34.1	ELISA	
Hungary	1,305	Healthy pet dogs	7.9	ELISA	(151)
Southern Hungary	126	Shepherd, hunting and stray dogs	11.0	PCR	(152)
Slovakia	87	Dogs suspected to have babesiosis	8.0	PCR	(153)
		Dogs randomly selected	11.7	ELISA	(154)
Bulgaria					
Central-southern	167	Dogs presented for various clinical reasons	19.2	IFA	(155)
Austria	1,470		56.5	IFA	(156)
United Kingdom	120	Dogs suspected to have TBD	0.8	PCR	(157)
Sweden	611	Dogs not clinically suspected to be infected by *Ehrlichia* spp or *B. burgdorferi* sensu lato	17.7	IFA	(158)
	100	Not stated	17.0	IFA	(159)
Finland	340	Pet dogs with or without clinical signs of illness	5.3	ELISA	(160)
	50	Healthy hunting dogs	4.0		
Albania	30	Clinically healthy semi-domesticated dogs	40	IFA	(161)
Tirana	602	Client-owned dogs	24.1 1.0	IFA PCR	(162)
Latvia	470	Healthy dogs and dogs suspected to have borreliosis and/or anaplasmosis	11.4	ELISA	(163)
Romania	1,146	Guard, pet, shelter, stray and hunting dogs	5.5	ELISA	(164)
	29	Pet and stray dogs from Romania	7.4	IFA	(165)
	109	Dogs imported from Romania to Germany	2.2	PCR	
Eight counties	357	Not stated	5.3	PCR	(166)
Southeastern	257	Not stated	6.2	PCR	(167)
South Central	149	Asymptomatic shelter dogs	3.3	ELISA	(168)
Serbia					
Vojvodina province	84	Randomly selected dogs	15.5	IFA	(169)
Belgrade municipalities	111	Shelter, free-roaming and hunting dogs	28.8 0.0	ELISA PCR	(170)
Poland	3,094	Healthy dogs with a history of tick bite	12.3	ELISA	(171)
Eastern	400	Healthy dogs	8.0 2.8	ELISA PCR	(172) (173)
Northwestern	192	Dogs from endemic regions of borreliosis	1.0	PCR	(174)
	100	Healthy dogs from a shelter	4.0	PCR	
	92	Dogs suspected to have Lyme disease	14.0	PCR	(175)
	50	Dogs diagnosed with babesiosis	0.0		
	79	Apparently healthy sled dogs	1.3		

(Continued)

TABLE 1 | Continued

Countries	Number of dogs	Type of dog population	Prevalence (%)	Method	References
Czech Republic	296	Healthy dos and dogs suspected to have TBD	3.4 26.0	PCR IFA	(176)
Italy					
Stretto di Messina	249	Outdoor-kennel dogs Not stated	38.0	IFA	(177)
	5,881		32.8	IFA	
Central Italy	1,965	Urban and rural dogs without signs of TBD	4.7	IFA	(178)
	1,232	Not stated	8.8	IFA	(179)
	215	Hunting dogs	14.8 0.9	IFA PCR	(180)
	1,026	Owned dogs	3.3	IFA	(181)
Sicily	344	Pet, pound and hunting dogs	0.0	PCR	(182)
	87	Not stated	44.8 0.0	IFA PCR	(183)
	372	Not stated	4.8	PCR	(184)
Southern	165	Dogs with febrile illness and healthy controls	37.8 0.0	IFA PCR	(185)
Northeastern	488	Privately-owned canine blood donors and free-roaming dogs	3.3 0.0	IFA PCR	(186)
Sardinia	50	Dogs suspected of tick bite–related fever	6.0	PCR	(187)
Portugal	1,185	Healthy dogs and dogs suspected to have VBD	4.5	ELISA	(188)
	55	Dogs suspected to have TBD	54.5 0.0	IFA PCR	(189)
	55	Dogs suspected to have TBD	55.0	IFA	(190)
	100	Apparently healthy military dogs	16.0	IFA	(191)
France	919	Not stated	2.7	ELISA	(192)
Spain	466	Sick and healthy dogs	11.5	IFA	(11)
Nothwestern	1,100	Dogs presented to veterinary clinics	3.1	ELISA	(193)
	479		5.0	IFA	(194)
Northern	556	Healthy dogs and dogs with signs compatible with VBDs	1.26	ELISA	(195)
Central	131	Shelter dogs	19.0	ELISA	(196)
Turkey	757	Stray, shelter and pet dogs	0.5	PCR	(197)
Thrace region	400	Healthy shelter dogs	6.0	PCR	(198)
Croatia	1,080	Apparently healthy dogs	0.3	PCR	(199)
	435	Apparently healthy owned and shelter dogs	6.21	ELISA	(200)
Greece	200	Owned and shelter dogs	1.0 0.5	ELISA PCR	(201)
ASIA					
Japan	154	Sick and healthy dogs	0.0	PCR	(202)
	332	Dogs presented at 6 private veterinary clinics in Ibaraki Prefecture	2.1 0.3	IFA PCR	(203)
China	600	Companion, working and shelter dogs	0.5	ELISA	(204)
	234	Stray and pet dogs	13.2	PCR	(205)
	219	Dogs from rural areas	10.0 10.9	IFA PCR	(107)
	26	Dogs from rural areas	7.7 50	ELISA IFA	(206)
	562	Dogs presented for reasons unrelated to suspicion of VBD	2.7	ELISA	(207)
	637	Apparently healthy indoor and breeding dogs	1.4	ELISA	(208)
	201	Apparently healthy stray dogs	11.9	PCR	(209)
Korea	1,058	Shelter dogs	0.1	PCR	(210)
	532	Outdoor dogs	15.6 2.3	ELISA PCR	(211)
	418	Shelter dogs	1.2	ELISA	(212)

(Continued)

TABLE 1 | Continued

Countries	Number of dogs	Type of dog population	Prevalence (%)	Method	References
	229	Urban shelter dogs and rural hunting dogs	18.8	ELISA	(213)
	245	Blood samples from military dogs	4.4 0.0	IFA PCR	(214)
Malaysia	48	Stray dogs	9.3 4.3	ELISA PCR	(215)
India	191	Pets, stray and working dogs	4.7	ELISA	(216)
	230	stray dogs in Tamil Nadu	0.4	PCR	(217)
Israel	195	Healthy pet dogs, stray and shelter dogs	9.0	IFA	(218)
Jordan	38	Stray dogs	39.5	PCR	(219)
	161	Stray, police, or breeding with tick infestation	9.9	ELISA	(220)
Taiwan	175	Asymptomatic dogs	21.1 0.0	ELISA PCR	(221)
Iran	103	Apparently healthy rural dogs	57.3	PCR	(222)
	150	Owned and stray dogs from Tehran	2.0	PCR	(223)
AFRICA					
Tunisia	286	Healthy and sick pet, kenneled dogs	25.2 0.9	IFA PCR	(224)
Algeria					
Algiers	150	Owned dogs admitted for surgery or vaccination	47.7	IFA	(225)
	63	Stray dogs from a shelter	0.0	PCR	
Morocco	217	Owned urban, rural and military healthy dogs or displaying signs of VBD	16.6	ELISA	(226)
Northwestern	425	Owned urban, rural and military healthy dogs or displaying signs of TBD	21.9 0.0	ELISA PCR	(227)
Nigeria	245	Healthy and sick dogs	0.8	PCR	(228)
South Africa	141	Apparently healthy owned and free roaming dogs	2.1	PCR	(229)
	56	Apparently healthy domestic dogs	0.3	PCR	(230)
Ghana	17	Client-owned dogs presented for a variety of complaints or for vaccination	11.8 0.0	ELISA PCR	(231)
Cape Verde					
Priai	57		1.8	PCR	(232)
Mayo Island	153	Apparently healthy dogs	0.0	PCR	(233)

TBD, tick-borne disease; VBD, vector-borne disease; IFA, immunofluorescence assay; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction, WB, western blot; ICG, immunochromatography.

A. phagocytophilum among owners of seropositive pets and owners without pets was observed, suggesting that pets are not a source of infection for humans (275). However, wild and domestic carnivores are considered the primary source of tick-borne zoonotic agents to humans (276) and contact with pet cats and dogs has been proposed as a risk factor for tick exposure and tick-borne disease among humans (277, 278). Moreover, according to some authors, almost all studies investigating the role of dogs in the transmission of tick-borne diseases (TBDs) focused on companion dogs. These animals are usually treated for ectoparasites, have limited free access to the outdoors and host reservoir habitats, and are less exposed to ticks compared with hunting, stray or shelter dogs. Therefore, these studies may not accurately reflect the public health risk associated with dogs in endemic areas (152). Others suggested that domestic animals including dogs could be considered as reservoir hosts of *A. phagocytophilum* in Europe especially in urban areas (18, 270, 279–282). In a study from Hungary, the prevalence of *A. phagocytophilum* DNA in stray dogs was higher than in several studies from other European countries (152). In addition, two studies reported high prevalence rates of *A. phagocytophilum* DNA in dogs suspected to have Lyme disease and rural dogs from Poland and China, respectively (107, 174). *Anaplasma phagocytophilum* was also the most frequently detected bacterium by PCR in stray dogs that lived in close contact with domestic animals and humans in rural and peri-urban areas of the Mediterranean zone of Jordan (219). In addition, high prevalence rates of *A. phagocytophilum* DNA was found in *I. ricinus* collected from dogs in Belgium and Poland, and *R. sanguineus* (adult and nymphs) from free-roaming dogs in Egypt (111, 280, 283). Moreover, *A. phagocytophilum* DNA was detected in experimentally infected dogs during 60 days without immunosuppressive drug, and the canine immune response seems to have evolved to only partially control

infection, suggesting a longer bacteremia possibly allowing timely transmission to the vector (18, 284). Based on these results, dogs could act as potential reservoir for the bacterium at least in some regions, but further studies are needed.

The geographical distribution of canine infection seems to parallel the distribution of HGA in the USA with a positive association of human and canine cases in many states (3, 23). Indeed, several studies found the highest prevalence rates of *A. phagocytophilum* antibodies in dogs from the upper midwest, northeast, and mid-atlantic, which correlate with areas where the highest incidence of human anaplasmosis were reported (3, 4, 9, 15, 22, 23). In addition, the estimated regression coefficient for the endemic risk factor in the contiguous USA model was positive and significant. This implies a higher prevalence among dogs living in areas where HGA is endemic (15). Furthermore, a study has evaluated regional and local temporal trends of canine *Anaplasma* spp. (*A. phagocytophilum* and *A. platys*) exposure using a Bayesian spatio-temporal binomial regression model for analyzing serologic test results. In this study, similarity was found between temporal trends in canine *Anaplasma* spp. seroprevalence and the reported incidence rate of HGA (249). Finally, human and canine strains of *A. phagocytophilum* were similar according to several gene sequencing studies, and human isolates have been reported to induce clinical disease in dogs in both Europe and the USA (16–21). Therefore, in addition to the possible role of dogs as potential reservoir hosts, the prevalence data of *A. phagocytophilum* infection in dogs provides important information on the incidence, risk factors, exposure sources, and real-time risk of exposure for human infection (3). More generally, several studies have documented the utility of using dogs as sentinels for human vector-borne diseases (VBDs) (14, 17, 18).

PATHOGENESIS OF *ANAPLASMA PHAGOCYTOPHILUM* INFECTION

Anaplasma phagocytophilum is transmitted by ticks to their hosts within 24–48 h of feeding time (285–288) but establishment of infections in dogs is apparently dependent on a minimum inoculation dose (288). Bacteremia, however, develops 4–7 days after the tick bite during natural infection or 3–4 days after experimental blood inoculation, suggesting that the bacterium remains at undetectable levels in the blood or replicates in other cells in the early stages of infection (69). Cell surface analysis suggested that the endothelial cells of the microvasculature provide an excellent site for *A. phagocytophilum* dissemination to peripheral blood granulocytes. Endothelial cells may play a crucial role in the development of persistent infections and are stimulated to express surface molecules and cytokines in a dose-dependent manner that may lead to inflammatory responses at the site of infection (289). After inoculation, *A. phagocytophilum* exhibits a biphasic developmental cycle in which the infectious small dense-cored cells bind to host cellular targets and enter the cytoplasm of neutrophils by endocytosis. After, the non-infectious reticulate cells multiply by binary fission within phagosomes until forming morulae. After 28–32 h, replication ceases and reticulate cells re-transition to dense-cored cells that are released after cell lysis to initiate the next wave of infection and possibly spread to multiple organs (40, 290, 291).

Anaplasma phagocytophilum has several strategies to dysregulate the bactericidal functions of neutrophils and ensure its survival and replication. This bacterium regulates host defense and antimicrobial mechanisms by a direct interaction with specific gene regulatory regions in the nucleus of the neutrophil, decreasing endothelial adherence, mobility, transmigration, phagocytic activity, and degranulation. It can also alter the respiratory and oxidative burst mechanism of neutrophils, delay apoptosis and increase the inflammatory recruitment of new neutrophils (289, 292–297). In addition, the antigenic variation of the immunodominant surface proteins msp2/p44 enables the bacterium to evade the specific immune response and to subvert the adaptive immune response (297). Neutrophils circulate for 10–12 h before they enter tissues and undergo apoptosis, which may lead to the destruction of the pathogens. Therefore, the decreased endothelial adherence and delayed apoptosis both enhance the bacterial survival and the replication to form morulae in a normally short-lived, terminally differentiated granulocytic cell. Furthermore, the impaired neutrophil function can result in an immune deficiency, predisposing patients to opportunistic infections (293–295). *Anaplasma phagocytophilum* was suggested to possibly manipulate the host endoplasmic reticulum stress signals to facilitate intracellular proliferation and infection of surrounding cells before or after host cell apoptosis (298).

The immune response induced by *A. phagocytophilum* is thought to play an important role in the initial control of the disease but may also induce inflammatory injuries associated with granulocytic anaplasmosis. Indeed, the absence of *A. phagocytophilum* control induces a clear rise in inflammatory lesions, which is considered the major pathogenic effect in humans and murine models (299–301). In dogs, the hematological modifications associated with *A. phagocytophilum* infection are similar to those induced by other members of *Ehrlichia* or *Anaplasma* genera, although they infect different blood cells, suggesting that the major mechanism of cytological injuries is related to an immunological response or to substances secreted from the bacteria (302, 303). *Anaplasma phagocytophilum* induces an upregulation of chemokine and pro-inflammatory cytokine [IL-8, macrophage inflammatory protein (MIP)-1a, MIP-1b, monocyte chemoattractant protein (MCP)-1] expression *in vitro*, which attracts leukocytes and inhibits hematopoiesis leading to myelosuppression (304, 305). In mice, several leukocyte populations expand during infection including NK and NKT cells followed later by CD4 and CD8 T lymphocytes and the immune response proceeds mostly through production of interferon gamma (IFN-γ), commonly produced by T lymphocytes (301–303, 306). In humans, the manifestation of severe disease is associated with hypercytokinemia and macrophage activation or hemophagocytic syndromes (MAS/HPS). The underlying pathogenesis of MAS/HPS is poorly understood; however, it is frequently associated with a defective function or depletion of cytotoxic cells and is driven mostly by the persistent stimulation

of cytokine production, especially the macrophage-activating IFN-γ (307–309). The clear role of IFN-γ in the pathogenesis of the disease is demonstrated by the observation that the lack of this molecule in *A. phagocytophilum*-infected mice resolves inflammatory tissue injury (300).

Several studies have confirmed the role of the IFN-γ in mediating both the pathology and early control of bacteria, although it is not essential for bacterial clearance. Other protective mechanisms might be involved in the control of *A. phagocytophilum* infection, as some infected mice lacking IFN-γ are able to survive (292, 300–303, 306). Data suggest that the humoral immunity may also play an important role in the clearance of ehrlichial infections, as passive immunization has a moderately protective effect. Moreover, severely immunocompromised mice that lack both B and T cells remained persistently infected, as opposed to mice lacking only T cells, which were able to control the infection (301, 310). Experimentally infected dogs develop serologic responses (immunoglobulin G) 7 days after inoculation. However, positive *A. phagocytophilum* PCR assay results persist up to 42 days despite the high antibody response suggesting that the humoral response is not sufficient to clear the infection (284). It appears that the innate immune mediators used to activate phagocytes to kill other intracellular bacteria (reactive nitrogen intermediates, Toll-like receptor 2 and 4, MyD88, phagocyte NADPH oxidase) do not play a crucial role in *A. phagocytophilum* clearance and may contribute to the observed pathology (301, 303, 311).

CANINE GRANULOCYTIC ANAPLASMOSIS

Clinical Signs

The discrepancy between the high seroprevalence and the relatively low number of sick dogs in endemic areas suggests that most infected dogs remain apparently healthy or develop a mild self-limiting illness (9, 10, 13, 25). The severity of the disease varies from mild subclinical to severe acute forms (24, 34), with severe clinical presentation often associated with co-infections, the immune response of the host and the variability of strains pathogenicity (13, 18, 33, 45).

CGA is a multi-systemic unspecific acute illness characterized by many clinicopathological modifications due to the possible involvement of several body systems. After an incubation period of 1–2 weeks, the most frequently observed clinical signs include fever, lethargy, inappetence or anorexia, weight loss and musculoskeletal pain or discomfort (**Table 2**) (24, 25, 29, 30, 35, 53, 60, 312, 314). More than 75% of dogs display lethargy and inappetence or anorexia (24, 26, 29, 30, 33, 35). Lethargy has been reported in almost all infected dogs (13, 24–26, 29–31, 33, 35, 45) and was the most frequent clinical signs in several studies (24–27, 29, 33, 35). It was also reported to be disproportionately severe in comparison with the lack of other clinical abnormalities in a case report (247). Fever is both inconstant and variable with frequencies ranging from 46 to 100% (24, 33, 35) and values from 39.2 to 41.5°C (25–27, 30, 35, 246, 251, 252, 257, 258, 261, 314). Fever generally coincides with the peak of bacteremia and lasts less than a week (33). Musculoskeletal pain or discomfort has been described in more than 50% of dogs and manifests in reluctance to move, weakness, stiffness, lameness, and myalgia. However, <10% of dogs have overt joint pain (29, 30). Lameness and joint swelling were reported in 11–34% (25, 34, 35) and 6–62% cases (24, 26, 35) respectively. They are more likely related to neutrophilic inflammation (25, 26, 244, 314, 315), but immune-mediated mechanisms also might be involved (244, 315). In a retrospective study, polyarthropathy (50%) was more frequently observed than monoarthropathy (5%) (27). In a report from California investigating the prevalence of tick-borne infections in dogs with polyarthritis and/or thrombocytopenia, *A. phagocytophilum* was the most frequently detected pathogen (244). Lymphadenopathy, splenomegaly and hepatomegaly were frequent findings in CGA (25, 26, 29, 35, 53, 314, 315). Splenomegaly was reported in 12–100% of naturally infected dogs (25, 26, 35). In canine and murine models of *A. phagocytophilum* infection, lymphadenopathy and splenomegaly are due to reactive lymphoid hyperplasia, with concurrent extra-medullary hematopoiesis in the spleen, enlarged activated lymph nodes and increased numbers of macrophages and plasma cells in the red pulp (284, 285, 314). In experimentally infected dogs, non-specific reactive hepatitis and mild periportal inflammatory lesions were also described (284, 314) and lesions tended to be more pronounced in dogs euthanized in the acute stage (314).

Other clinical signs include gastro-intestinal signs, polyuria, polydipsia, respiratory signs, pale mucous membranes, bleeding disorders, uveitis, scleral congestion, polymyositis, and neurological signs (**Table 2**) (13, 24, 26, 29, 30, 32, 33, 35, 53, 60, 256, 257, 312, 314, 316). Gastrointestinal signs include diarrhea, nausea, vomiting, and abdominal pain (25–27, 33–35, 312), but their origin is still unknown. In two cases of CGA displaying gastrointestinal signs, associated pancreatitis was suspected based on biochemistry and abdominal ultrasound abnormalities (246, 252). Respiratory signs include dyspnea, tachypnea, and coughing, which is usually infrequent, soft and non-productive (35, 238, 314). One patient displayed coughing and presented interstitial patterns on thoracic radiographs associated with focal alveolar patterns, and showed morulae within neutrophils upon microscopic examination of tracheal lavage specimen (238). Bleeding disorders including petechiae, gingival bleeding, melena, fresh blood in feces, epistaxis, pulmonary hemorrhage, vaginal hemorrhage or hematoma (35) are infrequent in dogs infected with *A. phagocytophilum*, unlike other rickettsial infections, such as *E. canis*, *A. platys*, and *Rickettsia rickettsii* infections or other infectious diseases, such as aspergillosis, bartonellosis, and leishmaniasis. Indeed, only 3–11% of CGA cases displayed epistaxis (25, 316). In two separate reports, dogs with CGA that presented with epistaxis had mild to moderate thrombocytopenia that could not explain the bleeding disorder. In addition, these dogs were seronegative to *B. burgdorferi*, *E. canis*, and *Dirofilaria immitis*, but other concurrent diseases were not ruled out. Therefore, other factors than thrombocytopenia may cause epistaxis, such as an infection-induced vasculitis (27, 33). Similarly, another report described two dogs with bleeding disorders associated with *A. phagocytophilum* infection that displayed platelet counts within the reference range (35). Although neurological signs were reported to occur

TABLE 2 | Clinical signs associated with canine granulocytic anaplasmosis after natural infection and corresponding frequency recorded in several studies.

Clinical sign	Frequency (%)	Number of dogs included in the study	References
Fever	84	32	(26)
	47	49	(31)
	61	18	(25)
	46	26	(33)
	89	18	(27)
	52	60	(45)
	100	8	(24)
	57	28	(13)
	67	6	(312)
	60	15	(60)
	67	61	(35)
Fever, lethargy	88	17	(29)
Fever, lethargy, depression	93	14	(30)
Fever, lethargy, anorexia	51	107	(34)
Lethargy/depression	94	18	(25)
	88	17	(29)
	74	34	(26)
	72	18	(27)
	67	49	(31)
	81	26	(33)
	67	60	(45)
	26	28	(13)
	50	6	(312)
	83	63	(35)
	73	15	(60)
Inappetence/anorexia	62	34	(26)
	33	49	(31)
	58	26	(33)
	55	60	(45)
	29	51	(13)
	87	8	(24)
	88	17	(29)
	50	6	(312)
	63	63	(35)
	67	15	(60)
Weight loss	25	8	(24)
Pale mucous membrane	28	18	(25)
	12	8	(24)
	50	6	(312)
Dehydratation	37	8	(24)
Musculoskeletal Signs			
Lameness	32	34	(26)
	16	49	(31)
	11	18	(25)
	50	26	(33)
	23	60	(45)
	34	107	(34)
	16	28	(13)
	16	63	(35)
	27	15	(60)
Joint swelling	62	8	(24)
	6	34	(26)
	19	26	(33)
	55	18	(27)
	14	28	(13)
	33	6	(312)
Digestive signs	23	107	(34)
	13	15	(60)
Vomiting	24	34	(26)
	11	18	(25)
	15	26	(33)
	6	18	(27)
	50	6	(312)
	8	63	(35)
Diarrhea	9	34	(26)
	17	18	(25)
	50	6	(312)
	14	63	(35)
Abdominal pain	9	34	(26)
Tense abdomen	28	18	(25)
	40	63	(35)
Lymphadenopathy	32	34	(26)
	19	26	(33)
	6	18	(27)
	13	17	(29)
	6	63	(35)
Splenomegaly	12	34	(26)
	40	60	(45)
	13	17	(29)
	100	18	(25)
	17	6	(312)
	84	57	(35)
Hepathomegaly	8	26	(33)
	33	6	(312)
Hepatosplenomegaly	7	17	(29)
	12	57	(35)
RESPIRATORY SIGNS			
High respiratory rate	29	34	(26)
	2	63	(35)
Cough	8	26	(33)
	37	8	(24)
	3	63	(35)
Respiratory or urinary tract disease	7	91	(34)
Bleeding disorders	12	66	(34)
	13	63	(35)
Petechiae	11	18	(25)
	3	63	(35)
Epistaxis	6	18	(25)
	8	26	(33)
	6	18	(27)
	4	28	(13)
	2	63	(35)
Melena	6	18	(25)
	17	6	(312)
Gingival bleeding, hematoma, fresh blood in feces, pulmonary and vaginal hemorrage	2	63	(35)
Neurological signs	7	28	(13)
Left cerebral dysfunction	6	18	(27)

TABLE 2 | Continued

Clinical sign	Frequency (%)	Number of dogs included in the study	References
Cervical pain	6	18	(27)
	2	63	(35)
Proprioceptifon deficit	7	17	(29)
Seizures	15	40	(34)
	7	17	(29)
	2	63	(35)
Ataxia	67	6	(312)
	7	15	(60)
Skin disease	10	61	(34)
	12	33	(313)

in CGA (35), no studies investigating this association have confirmed the infection by PCR. Moreover, two studies failed to demonstrate an association between *A. phagocytophilum* infection and neurological signs (317, 318). Consequently, *A. phagocytophilum* seems to be a rare cause of neurological disease in dogs and other potential etiologies or concurrent diseases should be ruled out before a final diagnosis of CGA. *Anaplasma phagocytophilum* infection is also suspected to induce skin lesions in dogs (34, 313). In one study that investigated skin-associated lesions in seropositive dogs, four of 12 showed positive DNA amplification from skin lesions. The most frequent lesions identified in these dogs included erythema, papules and plaques that resolved after doxycycline therapy (239). Cutaneous lesions were also present in seropositive but PCR-negative dogs (313). In a previous case report, one dog positive to *A. phagocytophilum* by serologic tests, PCR from blood and post-mortem spleen samples, was presented first for skin problem including pruritus, hair loss and seborrhea in association with regenerative anemia, leukocytosis and thrombocytopenia. *Ehrlichia canis* and *E. chaffeensis* exposure were serologically excluded (256). The lack of typical clinical signs and thrombocytopenia in dogs with PCR-positive skin lesions could be suggestive of a persistent infection as reported in studies in sheep, suggesting that skin could be a site of persistence of *A. phagocytophilum* (313).

Evolution of the Disease

CGA is currently considered to be an acute disease. Clinical signs usually develop during the bacteremic phase (24, 25, 29, 30) and the duration of the disease is variable. In a retrospective study, the duration of illness ranged from 1 to 14 days with a median duration of 3 days (27). Two studies demonstrated that the majority of dogs were sick for <7 days prior to diagnosis (26, 35). However, the duration of clinical signs ranged from 1 day to 2 months (26). In another report, the duration of illness ranged from 1 to 8 days, but one dog remained infected for a month before the diagnosis was established (30).

Chronic or persistent *A. phagocytophilum* infection has not been demonstrated in naturally infected dogs and is still controversial (2, 24, 53, 319). In contrast, experimental studies showed a persistent infection in dogs for more than several months to almost a year (18, 284, 320–324). These studies support the findings of another report that demonstrated that dogs could have long-lasting infections with acute flare-up (30) whereas another one failed to demonstrate a chronic infection in experimentally infected dogs (324). The results of the latter study differ from those of three other reports in which repeated amplification of *A. phagocytophilum* DNA occurred in some dogs probably because of the differences in the way of inoculation. Indeed, in contrast to the other experimental studies in which the bacterium had been inoculated intravenously to the dogs (18, 284, 320–322), in Contreras et al. (324), dogs were infected through tick bites after *Ixodes* spp. infestation. A 1 year persistence of *A. phagocytophilum* infection has been described in a naturally infected Rhodesian ridgeback dog (53). In addition, some authors consider the possibility of a chronic phase characterized by more localized clinicopathological signs (such as lameness and proteinuria) that could be associated with immune-mediated mechanisms secondary to persistent antigen stimulation (34). Studies on *E. canis* infection in dogs showed that the spleen is probably the organ that harbors bacteria for the longest period and is the best source for the diagnosis of carrier state by PCR (325). Similarly, the spleen remained PCR-positive in monkeys and mice experimentally infected with human strains of *A. phagocytophilum* (299, 326).

The prognosis of the disease in dogs is usually favorable with a rapid remission after doxycycline therapy (24, 26–28, 35, 324). However, some fatal cases have been reported (33, 35, 256, 257). Among the 12 fatality cases reported, five died of immune-mediated hemolytic anemia (IMHA) complicated by disseminated intravascular coagulation (DIC) (33, 256, 257). Two of these dogs were seropositive for *Neorickettsia risticii, R. rickettsii*, and *B. burgdorferi* (33). One was euthanized after 14 days because of IMHA and another one died because of epileptic seizures after 3 days (35).

Coinfections

Coinfection with multiple VBPs in dogs appears more frequent in endemic areas (9, 13). In a large retrospective serologic study carried out in North America and the Caribbean, exposure to up to five vector-borne pathogens (VBPs) was detected in the same dogs (3). In a kennel of North Carolina, 40% of dogs had serologic evidence of exposure at the same time with *Anaplasma* spp., *Babesia canis*, *Babesia vinsonii*, *E. canis*, or *R. rickettsii* (236). In another study, 16.5% of USA dog samples were found to be seropositive for more than one pathogen (119). Two serologic surveys showed that 1.32 and 14.3% of dogs had antibodies against two pathogens in Italy and Morocco, respectively (178, 226). In Tunisia, 22.4% of dogs were seropositive for *E. canis* and *A. phagocytophilum* (224). In Algeria, coinfections by *A. phagocytophilum* and 1–3 other pathogens were higher in stray than client-owned dogs (225). Two studies investigated the association between co-infections with several VBPs and the occurrence on clinical canine leishmaniosis (327, 328) and one reported a statistical association between dogs with clinical leishmaniosis stages III and IV and the seroreactivity to *A. phagocytophilum* in Spain (327). Coinfection with *B. burgdorferi* and *A. phagocytophilum*

is frequently described in dogs, probably because pathogens are transmitted by Ixodid ticks and maintained in sylvatic cycles with the same rodent reservoir (13, 33, 225, 329–331). In the USA, almost 22% of *A. phagocytophilum*-seropositive samples were also seropositive for *B. burgdorferi* (240). The prevalence of seropositive dogs to both pathogens was as high as 45% (12, 33). The ability of co-infected *I. scapularis* ticks to transmit *B. burgdorferi* and *A. phagocytophilum* was lower compared with transmission of either agent by singly infected ticks (331).

Experimental studies in mouse and human case reports of *A. phagocytophilum* and *B. burgdorferi* coinfection have described an enhanced severity and complexity of clinical signs along with an increased likelihood of disease compared with single infections (13, 329, 330, 332). Similarly, dogs seropositive for both agents (43%) were more likely to display clinical signs than those seroreactive to either *A. phagocytophilum* (25%) or *B. burgdorferi* (9%) (13). Experimental studies in rodents have demonstrated that coinfection modulates the host immune response to *A. phagocytophilum* and the production of interleukins (ILs), decreases IFN-γ levels and the number of $CD8^+$ T cells which leads to more severe clinical signs, increases pathogen burdens in blood and tissues, and induces more persistent infections (13, 329, 330, 333). Furthermore, the interaction of both pathogens at the blood-endothelial cell interface seems to be a critical point in pathogenesis (332). Two *in vitro* studies on human blood-brain barrier models showed that *A. phagocytophilum*-infected neutrophils enhanced *B. burgdorferi* migration across both systemic and brain microvascular endothelial cells. Several mechanisms are thought to be involved including impaired phagocytic neutrophil function caused by *A. phagocytophilum*, increased production of vasoactive and pro-inflammatory molecules (IL-6, IL-8, IL-10, tumor necrosis factor alpha, and macrophage inflammatory protein 1α) and the release of matrix metalloproteinases (329, 332). These factors lead to enhanced vascular permeability and inflammatory response in tissues and promote *B. burgdorferi* migration, which results in worsened clinical manifestations (329, 330, 332, 333).

LABORATORY ABNORMALITIES

Hematological Modifications

Hematological modifications associated with CGA include thrombocytopenia, anemia, leukopenia, and lymphopenia, although variable white blood cells count (WBC) modifications have been described (**Table 3**) (13, 24–27, 29–33, 35, 45). In experimentally infected dogs, hematological changes usually occurred during the acute stage of infection and normalized a few days after morulae disappeared from blood (302, 314). Suggested mechanisms of cytopenia include cytokine myelosuppression, autoantibodies formation, infection of hematopoietic precursors, and blood cell consumption (especially platelets) (25, 304, 334). Bone marrow aspirates of infected dogs were hyper- or normocellular, with normal, increased, or decreased iron storage, a slight increase in immature erythroid cells, and megakaryocyte and myeloid hyperplasia associated with relative shift toward immature myeloid cells, suggesting impaired myelopoiesis (312, 314).

Thrombocytopenia is the most common disorder associated with CGA. It has been described in 16.7–95% of natural (13, 24–26, 29, 30, 35) and 100% of experimental infections (302, 314). According to some authors, thrombocytopenia reflects an ongoing immunological response in dogs even when associated with low antibody titers against *A. phagocytophilum* (34). A recent study showed a significant association between thrombocytopenia and high concentrations of circulating immune complexes (CIC), low albumin to globulin (A/G) ratios and an acute phase protein concentration. The importance of thrombocytopenia was emphasized as an indicator of acute anaplasmosis, regardless of antibody titer (28). Therefore, thrombocytopenia is considered the most relevant abnormality in the diagnosis of CGA after morulae detection (13, 24–26, 29, 30). The severity of thrombocytopenia varies from mild to severe and the platelet count has been reported to range from 5,000 to 164,000 cells/μl (24, 25, 29, 30, 35, 314). However, in a report, none of the 12 dogs seropositive to *A. phagocytophilum* had platelet counts lower than 105,000 cells/ml and dogs that were also seropositive to *B. burgdorferi* had a lower median platelet count of 51,000 cells/μl (33). In another study, five of the six CGA cases with significant thrombocytopenia had concurrent diseases (lymphoma and systemic lupus erythematosus) or were serologically positive to *B. burgdorferi* or *E. canis* (29). A prospective study aiming to investigate the presence of bacteria belonging to the genera *Anaplasma* and *Ehrlichia* in 159 blood samples from thrombocytopenic dogs, detected only two *A. phagocytophilum*-PCR positive dogs (335). As it has been described for a wide range of *Ehrlichia* species, CGA-associated thrombocytopenia may be related to platelet consumption due to DIC, immunological destruction, spleen sequestration or production of inhibitory factors (336–338). The organism seems to be able to enter megakaryocytes lineage but without impairment of their ability to produce platelets (339). The mechanism inducing thrombocytopenia seems to be more associated with an inflammatory process rather than with the direct action of *A. phagocytophilum* (34). Destruction of platelets has been suggested as a probable mechanism because of the increased number of both mature and immature megakaryocytes in the bone marrow (302). On the other hand, anti-platelet antibodies have been detected in both human and canine cases, with up to 60 and 80% of patients with CGA and HGA displaying anti-platelet antibodies, respectively (25, 35, 257, 336–338). However, thrombocytopenia usually occurs during the early stages of infection, before antibody detection and has also been described in severely immunocompromised mice due to B or T cell suppression, suggesting that mechanisms other than decreased hematopoietic production or immune-mediated destruction are involved. Increased platelet consumption is also suspected to play an important role (340, 341). *In vitro*, increased production of monocyte tissue pro-coagulant activity in peripheral blood mononuclear cells has been observed, supporting the platelet consumption hypothesis (27, 340).

Anemia is an inconstant hematological finding (34) described in 3–82% of dogs with clinical signs compatible with CGA either

TABLE 3 | Hematological abormalities associated with canine granulocytic anaplasmosis after natural infection and corresponding frequency recorded in several studies.

Hematological abnormalities	Frequency (%)	Number of dogs included in the study	References
Thrombocytes count modifications			
Thrombocytopenia	95	22	(26)
	71	–	(32)
	69	49	(31)
	89	18	(25)
	56	25	(33)
	94	18	(27)
	65	60	(45)
	87	8	(24)
	86	7	(29)
	86	7	(30)
	57	28	(13)
	17	6	(312)
	86	63	(35)
Erythrocytes modifications			
Anemia	47	34	(26)
	17	–	(32)
	57	49	(31)
	82	11	(25)
	24	25	(33)
	42	60	(45)
	3	15	(29)
	24	14	(30)
	70	63	(35)
Non regenerative anemia	67	18	(27)
	37	8	(25)
	50	6	(312)
Regenerative anemia	27	11	(25)
IMHA	12	25	(33)
	24	17	(35)
Leukocytes modifications			
Leukopenia	9	31	(26)
	9	–	(32)
	18	49	(31)
	55	18	(27)
	10	60	(45)
	62	8	(24)
	7	14	(30)
	14	63	(35)
Leukocytosis	19	31	(26)
	21	–	(32)
	61	49	(31)
	28	25	(33)
	7	15	(29)
	7	14	(30)
	33	6	(312)
	27	63	(35)
Lymphopenia	65	31	(26)
	12	25	(33)
	39	18	(27)
	33	60	(45)
	100	8	(24)
	67	15	(29)
	44	63	(35)
Eosinopenia	10	49	(31)
	50	18	(27)
	9	63	(35)
Neutropenia	37	8	(24)
	7	15	(29)
Neutrophilia	19	31	(26)
	33	6	(312)
	51	63	(35)
Left shift	28	26	(33)
	20	15	(29)
	33	6	(312)
Monocytosis	45	49	(31)
	6	18	(27)
	33	60	(45)
	43	63	(35)
Morulae			
Morulae detection	36		(29)
	56		(24)
	4	49	(31)
	56	18	(25)
	94	18	(27)
Percentage of neutrophils with morulae	10–24	–	(29)
	7–24	5	(30)
	9–32	8	(24)
	1–5	6	(312)
	0–11	35	(316)

(Continued)

seropositive (33), PCR-positive (25, 27, 35, 45), displaying *A. phagocytophilum*-like morulae on fresh blood smear examination (24, 26) or being positive to two (29, 30) or three (31) aforementioned diagnostic methods (**Table 3**). In a retrospective study, no dogs were anemic, even during the bacteremic phase, but the mean values of hematocrit, hemoglobin concentration and red blood cell counts were significantly lower than in the control group (34). In contrast, three different studies described 63, 67, and 70% of anemic dogs (27, 35, 224). CGA-associated anemia is usually mild to moderate non-regenerative normocytic normochromic resembling anemia of inflammation (24, 27, 29, 31, 302, 312, 314). In nine dogs experimentally infected with *A. phagocytophilum* that developed mild normocytic normochromic anemia, decreased serum iron and total iron-binding capacity were recorded during bacteremia, but levels returned to reference ranges 1 week after the disappearance of morulae (302). In a report, most dogs had mild to moderate anemia with hematocrits ranging from 19 to 39%, but two had severe anemia with hematocrit levels <20% and three had signs of regeneration. Five were suspected to have hemolytic anemia based on increased serum levels of bilirubin but all had negative Coombs tests (25). Regenerative anemia has been less frequently reported, and severe IMHA is an unusual disorder associated with CGA (25, 33, 257, 258). One retrospective duty aiming to investigate infectious causes of lethal immune-mediated anemia in Croatian dogs, only two dogs were found positive to *A. phagocytophilum* DNA and one of these two dogs was also co-infected with *B. canis* (342). Six cases of IMHA in dogs with CGA have been reported in the UK, the USA and Denmark (33, 35, 257, 315). Authors

from Germany described the possible occurrence of IMHA in a small number of dogs (25). Others from Belgium described IMHA in a dog with a positive titer to *A. phagocytophilum* and without other concomitant diseases (262). A previous case report described a dog with *A. phagocytophilum* infection (confirmed by positive PCR from blood and post-mortem spleen samples) with regenerative anemia, severe bilirubinuria, and positive test for osmotic resistance of red blood cells. This dog was serologically negative for babesiosis, leptospirosis, *E. canis* and *E. chaffeensis* infections (256). More recently, four dogs had a positive Coombs test among 17 ones that underwent this analysis in a case series on CGA (35). Only one case series has evaluated the prevalence of IMHA associated with CGA. In this study, three dogs had IMHA based on spherocytes in blood smears and/or positive Coombs test, without evidence of abdominal or thoracic neoplasia. However, two dogs had positive antibodies for at least one other TBP including *Neorickettsia risticii* (formerly *Ehrlichia risticii)*, *B. burgdorferi*, and *Rickettsia rickettsii*. The authors emphasized that both *R. rickettsii* and *B. burgdorferi* are not commonly associated with IMHA and *N. risticii* is not yet associated with clinical disease in dogs as suggested by experimental studies (33). In addition, anti-erythrocyte antibodies have been detected in three dogs with CGA in the USA (312). Even if CGA has not yet been proven to be a common cause of IMHA, *A. phagocytophilum* should be included in the differential diagnosis, especially in endemic area (33).

The most diagnostically relevant hematological abnormality in CGA is the identification of *A. phagocytophilum* inclusions within neutrophils during blood smear examinations. Morulae appear classically as basophilic inclusions detectable by light microscopy of peripheral blood smears (41, 234). They are usually present transiently during the bacteremic phase (4–14 days after inoculation) and persist for 4–8 days in experimentally infected dogs (302, 314). Morulae can also be identified from cytocentrifuged synovial fluid, bone marrow aspirates, and they were also present in the abdominal fluid of an unusual CGA case and in the tracheal wash from a dog with respiratory signs (40, 238, 247, 302, 312, 315). The proportion of neutrophils containing morulae in blood smears varies from <1 to 34% (24, 29, 30, 312, 314, 316). In an experimentally study, the most severely affected dogs were those with higher percentage of neutrophils containing morulae and the lowest proportion was recorded in non-febrile dogs (314). In endemic areas, 38% of dogs displaying clinical signs compatible with CGA had morulae within neutrophils (13). Three studies reported that 56%, 94% (25, 27), and 88 to 93% (33) of dogs presented morulae while other reports failed to identify these inclusions (246, 257). It is important to mention that *A. phagocytophilum* morulae cannot be distinguished from those of *E. ewingii*, which can lead to misdiagnosis in the regions where both pathogens are present. Therefore, other methods, such as PCR are needed to confirm the diagnosis (2, 24, 302).

Experimentally infected dogs developed moderate leucopenia (314), but WBC count modifications in naturally infected dogs are considered non-specific and variable, and both decreased and increased WBC counts have been reported (24–27, 29–35, 45). Therefore, the use of the WBC count as a marker of the course of the disease is controversial (34). Lymphopenia is the most frequently reported WBC count abnormality in CGA (24–26, 29, 302, 314). Other reported modifications include leukocytosis, leukopenia, lymphocytosis, eosinopenia, monocytosis, monocytopenia and mild to moderate neutropenia or neutrophilia (24–27, 29–31, 35, 45, 53, 302, 312, 314). Left shift of neutrophils and toxic changes have also been reported to occur with *A. phagocytophilum* infection in dogs (26, 29, 33, 252, 257, 312).

Serum Biochemistry Profile Modification

Serum biochemistry profile modifications documented in CGA include increased liver enzyme activity, hyperbilirubinemia, hypophosphatemia, hyperproteinemia, hyperglobulinemia, and hypoalbuminemia (**Table 4**) (24–27, 29–31, 33, 35, 45, 314, 316). A moderate increase in alkaline phosphatase (ALP) was reported in 7–100% of CGA cases and mild to moderate hypoalbuminemia was present in 17–66% (25, 26, 29, 33). In a retrospective study, 30% of dogs displayed a slightly increased alanine aminotransferase (ALT) activity but concurrent diseases had not been ruled out (26). In another report, the most frequent findings in dogs with CGA were increased in liver enzymes and hyperbilirubinemia (35). According to some authors, hypoalbuminemia and hyperglobulinemia might be due to a decreased production of albumin in the liver associated with a rise in α- and β-globulin production (304). In a study investigating serum protein profiles of seropositive and PCR-positive dogs, the major modification was a low A/G ratio (84.4%), mostly in groups with antibody titers higher than 1:1,024. Hyperglobulinemia was due to an increase in the acute phase proteins (α2-, β1-, and β-2 globulin). In the same study, 62 and 71.8% of dogs in the group with lower A/G ratios had thrombocytopenia and clinical signs compatible with CGA, respectively, suggesting an acute infectious process. However, other diseases had not been excluded; hence dysproteinemia could possibly be the result of concurrent diseases (28). Others reported hypergammaglobulinemia as a prominent modification associated with CGA but without exclusion of concurrent diseases (316). Decreased serum levels of urea and hypokalemia have been recorded in 27% of dogs (25) and 27–37% of dogs were reported to have hyperbilirubinemia (25, 26, 35). An increase in serum amylase activity was described in 50% of CGA cases (29). Two case reports described dogs diagnosed with CGA with suspected pancreatitis on the basis of increased serum level of amylase and lipase and clinical signs suggesting pancreatitis (abdominal pain in the pancreatic region of one dog and abdominal ultrasound modifications in the pancreatic region of the other dog) (246, 252). In another previous report, two of seven dogs had increased serum lipase concentrations (24).

Prolonged prothrombin time (PT) and activated partial thromboplastin time (aPTT), along with increased fibrin-degradation product concentration and fibrinogen concentration have been reported in some CGA cases (**Table 4**) (25, 35, 252, 257, 314). DIC was suspected or diagnosed in four dogs; two of which had IMHA (25, 33, 257). Elevated aPTT was also described in one dog with SIRS secondary to *A. phagocytophilum* infection (26). In a recent study on portal vein thrombosis, four of 29

TABLE 4 | Serum biochemistry abormalities associated with canine granulocytic anaplasmosis after natural infection and corresponding frequencies recorded in several studies.

Serum biochemistry abnormalities	Frequency (%)	Number of dogs	References
Hyperproteinemia	12	49	(31)
	43	62	(35)
Hypoproteinemia	10	49	(31)
	20	11	(29)
	2	62	(35)
Hypoalbuminemia	44	27	(26)
	29	49	(31)
	17	23	(33)
	28	60	(45)
	44	9	(29)
	55	18	(25)
	50	6	(312)
	62	61	(35)
Hyperglobulinemia	50	6	(312)
	38	61	(35)
Serum protein electrophoresis A/G ratio<0.8	21	145	(28)
CIC	80	204	(28)
Hypophosphatemia	62	8	(24)
Increased ALP	52	27	(26)
	59	49	(31)
	26	23	(33)
	43	60	(45)
	75	8	(24)
	100	9	(29)
	7	14	(30)
	67	61	(35)
Increased ALT	30	27	(26)
	35	49	(31)
	18	62	(35)
Increased bilirubin	37	27	(26)
	31	49	(31)
	34	61	(35)
Azotemia	27	49	(31)
	3	62	(35)
Increased aPTT	60	10	(25)
	55	29	(35)
Increased PT	30	10	(25)
	34	29	(35)

A/G ratio, albumin to globulins ratio; ALP, alkaline phosphatase; ALT, alanine aminotransferase; apt, activated partial thromboplastin time; CIC, circulating immune complexes.

TABLE 5 | Urinary abormalities associated with granulocytic anaplasmosis after natural infection and corresponding frequency recorded in several studies.

Urinary abnormalities	Frequency (%)	Number of dogs	References
Hyposthenuria	12	49	(26)
Proteinuria	15	13	(26)
	87	8	(25)
	27	23	(33)
	38	8	(29)
	50	6	(30)
	58	58	(28)
	40	5	(312)
Glucosuria	12	8	(25)
Bilirubinuria	50	8	(25)
	25	8	(29)
	50	5	(312)
Hematuria	87.5	8	(25)
	40	5	(312)
Hemoglobinuria	60	6	(31)
Urinary sediment			
Casts	50	8	(25)
Epithelial cells	75	8	(25)
Protein electrophoresis			(28)
LMWP	42	36	
MMWP and HMWP	30	36	

LMWP, low molecular weight proteins (<66 kDa); MNWP, middle molecular weight proteins (66–76 kDa); HMWP, high molecular weight proteins (>76 kDa).

dogs had infectious diseases and one had *A. phagocytophilum* infection (343).

Urinalysis Modifications

Acute renal failure (ARF) is a complication described in some HGA cases (344, 345). In a recent study, 30.6% of human patients with confirmed *A. phagocytophilum* infection by PCR had abnormalities on urinalysis including hemoglobinuria or myoglobinuria (not distinguished by further analysis). Hemoglobinuria/myoglobinuria could be the precursor of ARF described in severe human cases (346). Experimental studies revealed evidence of *A. phagocytophilum* DNA in the kidneys of three persistently infected lambs and lesions of vasculitis and thrombosis in the kidney of a horse (347, 348). Similarly, one study amplified *A. phagocytophilum* DNA in the kidney of one dog after necropsy (342). CGA is suspected to induce immune-mediated glomerulonephritis (IMGN) likely by vasculitis (349). In contrast to blood modification, urinary abnormalities have not been fully assessed in dogs and only a few reports have described abnormalities in urinalysis (**Table 5**) (25, 26, 28–30, 33, 312). One such study described the presence of mild to moderate proteinuria, glucosuria, bilirubinuria, hematuria, and epithelial cells in urine sediments. In the same report, only three of eight dogs in which urinalysis was performed were also measured for urine protein to creatinine (UPC) ratios, and one displayed a mild increase (0.88) (25). Another report showed a significant difference in proteinuria between *A. phagocytophilum* seropositive and seronegative dogs (34). In a retrospective study, two dogs displayed proteinuria with UPC ratios of 1.5 and 2.2 (26) and 17% of dogs had proteinuria in another report. In this study the only dog with a UPC ratio higher than one had antibodies against both *A. phagocytophilum* and *B. burgdorferi* (33). More recently, 3% of CGA cases included retrospectively displayed signs of azotemia (35), however other concurrent diseases causing azotemia have not been ruled out. In most studies on CGA, proteinuric dogs were identified mainly on the basis of dipstick and only a few of them underwent UPC measurement. Moreover, urinary tract infection (UTI) was not excluded in all dogs. However, another study demonstrated that

38% of dogs had proteinuria without signs of UTI, which could be compatible with kidney injury (29). Proteinuria due to middle and high molecular weight proteins was found exclusively in 30.5% of *A. phagocytophilum*-seropositive dogs. The authors indicated that proteinuria might be the result of chronic antigenic stimulation and suggested that persistent infection can lead to the development of IMGN (34). In one case of CGA, a persistent proteinuria after 28 days of doxycycline therapy was reported. The dog remained asymptomatic during a 305-day follow up; however, mild proteinuria was still present even with a renin-angiotensin-aldosterone system inhibitor (261). More recently, a case report described a dog with IMGN complicated with systemic hypertension and chronic kidney disease without any identified etiology except an active *A. phagocytophilum* infection on the basis of a very high antibody (1:20,480) titer at first consultation and more than a 4-fold decrease in antibody titer several weeks after (262). Finally, the consensus statement of the American College of Veterinary Internal Medicine (ACVIM) for dogs with suspected glomerular disease recommends serologic screening for anaplasmosis of patients with renal proteinuria in addition to other infectious diseases known to induce proteinuria (350).

CONCLUSION AND FUTURES PERSPECTIVES

Understanding granulocytic anaplasmosis is important due to its zoonotic aspect, potential severe outcomes in both dogs and humans, and the possibility of using epidemiological data in canine species as a good estimation of risk for human exposure. The aims of this review were to summarize the wide epidemiological data published on *A. phagocytophilum* in canine species and to describe the clinicopathological aspects of CGA that are available in the few case series and reports. In this manuscript, the authors wanted to gather together all data on *A. phagocytophilum* in dogs that can be valuable for researchers and to highlight the fields where important information is still missing and toward which future research should be focused. Indeed, information regarding the prevalence of *A. phagocytophilum* in some parts of the world, the potential role of dogs as competent reservoir hosts, the possibility of tick species other than *Ixodes* spp. acting as vectors of *A. phagocytophilum* and the implication of the genetic variability in the pathogenesis of the disease with some strains being potentially more virulent for humans is still incomplete or lacking. The pathogenesis of CGA is not fully elucidated too. Finally, some publications on CGA discussed the possibility of a chronic evolution and the association of this disease with serious clinicopathological manifestations with a crucial impact on the prognosis and management, such as immune-mediated hemolytic anemia, glomerulonephritis, and neurological signs that are still incomplete and thus need further investigations.

AUTHOR CONTRIBUTIONS

SE wrote the manuscript. SD, LD, LE, MK, and HS drafted and revised the manuscript. All authors have made a substantial, intellectual contribution to the work, and read and approved the final manuscript.

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MINI REVIEW
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Advances in the Study of the Tick Cattle Microbiota and the Influence on Vectorial Capacity

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The information from the tick cattle microbiota suggests that the microbial populations may modulate a successful infection process of the tick-borne pathogens. Therefore, there is a need to know the microbial population and their interactions. In this mini-review, we present several examples of how microbiota regulates the survival of pathogens inside the tick and contributes to fitness, adaptation, and tick immunity, among others. The communication between the tick microbiota and the host microbiota is vital to understanding the pathogen transmission process. As part of the tick microbiota, the pathogen interacts with different microbial populations, including the microorganisms of the host microbiota. These interactions comprise a microsystem that regulates the vectorial capacity involved in tick-borne diseases. The knowledge we have about the vectorial capacity contributes to a better understanding of tick-borne pathogens. Additionally, using approaches based on multi-omics strategies applied to studying the microbiota and its microbiome allows the development of strategies to control ticks. The results derived from those studies reveal the dynamics of the microbiota and potential targets for anti-tick vaccine development. In this context, the anti-microbiota vaccines have emerged as an alternative with a good prognosis. Some strategies developed to control other arthropods vectors, such as paratransgenesis, could control ticks and tick-borne diseases.

Keywords: bovine host, vector of transmission, metagenomics, microbiota, microbiome, vectorial capacity, ticks

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INTRODUCTION

Infectious diseases have been one of the main restrictions worldwide for animal production improvement, with significant economic losses. The significant risk that endangers animal health is the arthropod vector and the pathogens they transmit and those that have been controlled or eradicated, and after a while, they reemerge. Ticks are hematophagous ectoparasites, the main biological vectors of numerous infectious diseases (tick-borne diseases) (1–3) (**Figure 1**).

Although the study of the tick microbiota and the biological processes in which it participates are still in progress to date, there is not enough information about its role in the vectorial capacity, infection, and pathogen transmission. This study opens up a research field to study and elucidate new targets for developing drugs/vaccines to prevent diseases that affect animals, including humans. The development of anti-tick vaccines based on the microbiota represents a

promising approach to control tick infestations. In this regard, the study of the tick microbiota contributes to elucidate the interactions that may be influencing vital processes and then avoiding the transmission of pathogens (4, 5). However, there is much work to obtain an effective drug or vaccine to control tick-borne diseases and the pathogens that cause them.

A BRIEF OVERVIEW OF TICK-BORNE DISEASES IN CATTLE

During the last years, the number of tick-borne diseases caused by bacteria *Anaplasma*, *Ehrlichia*, and *Coxiella* has increased, affecting livestock productivity (6). *Anaplasma marginale* causes bovine anaplasmosis resulting in economic losses to the cattle industry due to a significant reduction of beef and dairy production (7). More recently, some coinfections with piroplasmas and other *Anaplasmataceae* reported in cattle and buffaloes show the circulation of diverse genotypes of *A. marginale* worldwide (8).

So far, there exist more than 20 genomes and draft genomes of *A. marginale*, whose content could contribute to the identification and participation of the genes involved in the microbiota–vector–host interaction (9–11).

Ehrlichia ruminantium is transmitted by *Amblyomma* and causes *Heartwater*, the most important livestock disease in Africa and the Caribbean, while the emerging *Ehrlichia minasensis* also infects cattle (12–14). As an example of the intrinsic interaction between pathogen and the host cell, molecular studies reveal that the genome of *Ehrlichia* sp. has lost genes associated with metabolism whose activities are covered by the host cell (15). In the genomic context, only a few genomes are available for *Ehrlichia* species (16, 17).

In European livestock, coxiellosis, caused by *Coxiella burnetii* and transmitted by *Hyalomma* spp. and *Rhipicephalus* spp. has a significant prevalence in the Mediterranean countries (18).

At the moment, there exist more than 75 *Coxiella* genomes reported that contain a large number of genes participating in adhesion, invasion, intracellular trafficking, host-cell modulation, and detoxification (19).

In Africa, Ben Said et al. (20) interestingly identified the spirochaetes, *Borrelia burgdorferi*, in goats, sheep, camels, and cattle, transmitted by *Rhipicephalus* and *Hyalomma*. The recent molecular detection of *Rickettsia* spp. and *C. burnetii* in cattle and water buffalo in Luzon Island of the Philippines reveals the potential zoonotic transmission mediated by *Rhipicephalus microplus* (21).

Hemoplasmosis is not strictly considered a tick-borne disease; however, *Mycoplasma wenyonii* and *Candidatus* Mycoplasma haemobos reported in cattle are probably transmitted by ticks (22–25).

CATTLE TICK MICROBIOTA INTERACTIONS AND VECTORIAL CAPACITY

The microbial communities that comprise the tick microbiota include pathogens, symbionts, and commensals acting as a dynamic and integrative microecosystem, changing in time and scale, that interact into a macrosystem that includes the host (26, 27). Additionally, external factors modulate the diversity of the microbiota: temperature, humidity, geographic location, sex and species, blood intake from the vertebrate host, or even the physical location inside the organs of the tick (gut, ovaries, salivary glands) (28, 29).

Interestingly, this microbiota also contributes to fitness, nutritional adaptation, development, and reproduction and is also involved in establishing pathogens inside the tick (30, 31).

Currently, there exists a close relationship between pathogens and the tick microbiota, which affects their vectorial capacity. Also, colonization, replication, or maturation of an infective form of a pathogen depends mainly on the microbiota composition.

During the blood intake, the hematophagous vector can acquire pathogens from an infected host that transmit to a new host. This is known as vector competence, the ability of a vector to maintain the pathogen development until its transmission to a new host. Vector competence is a component of vectorial capacity that comprises the interactions between vector–pathogen and vector–host, influenced by behavioral and environmental factors such as vector density, longevity, host preference, and feeding habits (32).

In tick cattle, the study of interaction microbiota–vector is still scarce. However, the results reported in other species could be applied as alternatives to identify new targets to control ticks. In addition, after blood intake, some complex interactions are carried out by the triad microbiota–vector–host involved in the pathogen transmission from one host to another (**Figure 2**). In this regard, the first interaction occurs between the tick and the microbiota of the bovine skin, in which several families of bacteria are present, including *Corynebacteriaceae* and *Staphylococcaceae* (teat skin), and *Firmicutes*, *Spirochaetae*, *Bacteroidetes*, and *Actinobacteria* (interdigital skin) (33, 34) (**Figure 2**). The microorganisms penetrate from the surface of the skin host deeper into the dermis and might be inducing local immunomodulation (35). For instance, the bacteria *Prevotella* sp. and *Neisseria* sp., usually found in the skin and mucosal surface of the host, respectively, have been identified in the midgut of *I. ricinus* blood-fed females, regardless of the time point of the blood feeding course (36, 37). In contrast, Miranda-Miranda et al. (38, 39) found that bacteria *Staphylococcus saprophyticus* and *Staphylococcus xylosus*, located in the skin of bovine, can produce a lethal infection in fully engorged female ticks that lose the ability to oviposit and lead them eventually to death. These findings show that the composition of the microbiota does not always favor tick infestations and pathogen transmission.

A second interaction occurs when the microbiota of the salivary glands is in contact with the pathogen that enters the tick. Here, the microbiota has an essential interaction with the pathogens, as in *Amblyomma americanum*, where the *Coxiella*-related symbionts in the salivary glands impair the transmission of *Ehrlichia chaffeensis* (40). These studies illustrate the pathogen interactions occurring in the salivary glands and highlight the role of the tick microbiota that regulates pathogen growth. Alternatively, during the pathogen inoculation, the microbiota of the saliva interacts with the host's components regulating the pathogen passage from the salivary glands to a new bite site.

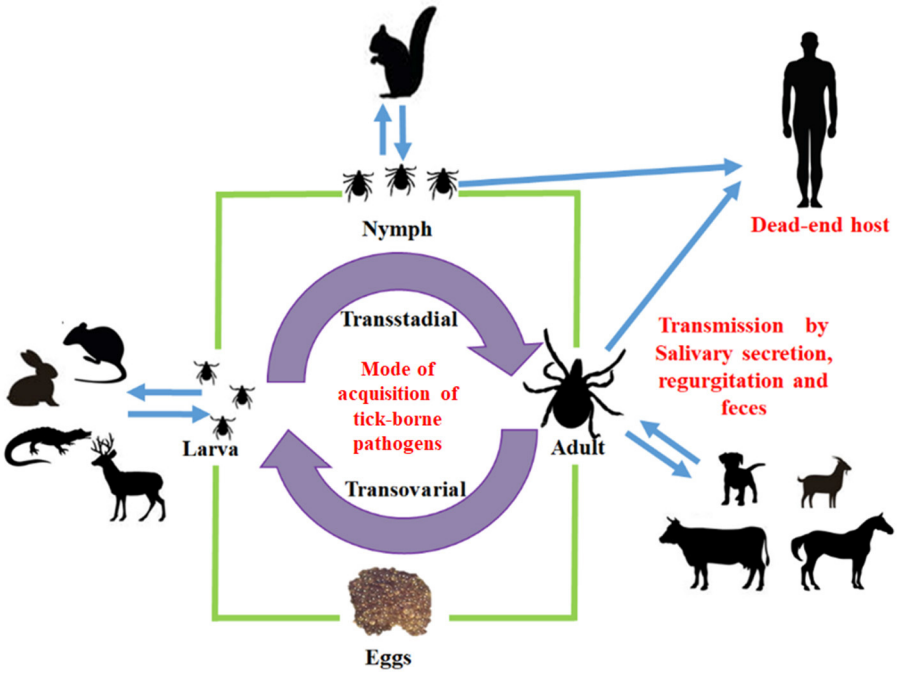

FIGURE 1 | Mode of acquisition of tick-borne pathogens. The different stages of tick (larva, nymph, and adult) may transmit bacteria to intermediate or final host.

The tick gut microbiota possesses different microorganisms that participate in metabolic and digestive processes. In this tissue, the blood and the pathogens ingested interact and alter the microbiota, and a third interaction occurs. In such a way, the altered microbiota, which usually provides cofactors, vitamins B, and folate (e.g., *Coxiella*, *Francisella*, *Rickettsia*, respectively), may affect the tick's development, as occurs in *Ornithodoros moubata*, where antibiotic-based elimination of *Francisella* endosymbionts compromise the nutritional status of vitamin B, which results in some anomalies in tick development and hampers nymph growth and molting to adults (41). Another role of the microbiota is to regulate the pathogen colonization of the tick gut. For example, in *Ixodes scapularis* a gut microbiota composed of a high abundance of *Rickettsia*, *Thioclava*, and *Delftia*, and a low abundance of *Aquabacterium*, *Brevibacterium*, and *Novosphingobium* may influence *B. burgdorferi* colonization negatively (42).

Once established, the pathogens cross the peritrophic membrane and the gut barrier to disseminating to other tissues through the hemolymph. Here, the presence of hemocytes and effector molecules of the tick immune response represents a hostile environment for the pathogen; however, the information about the role of the microbiota of the hemolymph and its interaction is scarce except for the finding of *Staphylococcus aureus* in *Rhipicephalus decoloratus* and *Rhipicephalus geigy*, which were isolated from the adult females hemolymph (43).

Finally, the pathogen could likely take two routes. The first, pathogens redirect to the salivary glands and transmit to a new host, and the second, pathogens transmit by transovarial transmission. In both cases, the pathogen would be interacting with the microbiota of the tissue.

Although the information about the effect of tick-borne pathogens and microbiota is scarce, the report of co-occurences in epidemiological studies has suggested the impact of pathogens on the tick and vice versa (27). For example, the microbial community in the tick gut is related to the virulence and proliferation of *Anaplasma* sp. and *Babesia* sp. (27). Adegoke et al. (44) reported that microbial diversity and composition of *R. microplus* decreases when infected with *Theileria* sp.; this phenomenon is called pathogen-induced dysbiosis. A similar effect occurs in *D. andersoni* where endosymbiont *Francisella* sp. increases the successful pathogenic colonization of *Francisella novicida* and *A. marginale* (45, 46).

To address the potential role of the *I. scapularis* gut bacteria in modulating *B. burgdorferi*, (42) compared dysbiosed larvae and larvae fed on gentamicin-treated mice. They found that both types of larvae significantly increased engorgement weights and decreased *B. burgdorferi* colonization, suggesting that tick-altered gut microbiota impairs pathogen colonization.

On the other hand, the induction of *I. scapularis* antifreeze glycoprotein (IAFGP) occurs in the presence of *A. phagocytophilum*, which sequesters IAFGP to alter its accumulation in the tick midgut to inhibit the development of biofilms, thus allowing the colonization and establishment of the vector (47, 48).

Understanding how microbial diversity of ticks changes in the presence of pathogens could help propose better strategies

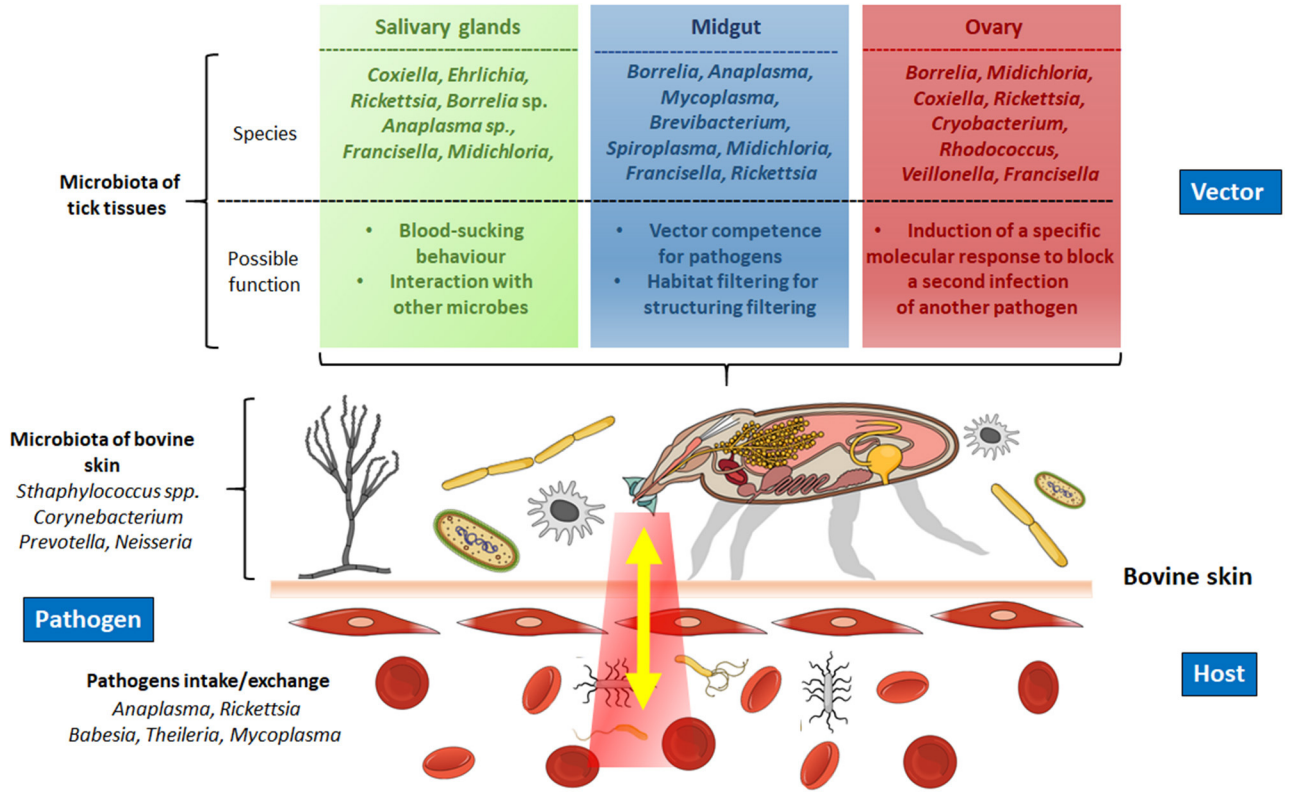

FIGURE 2 | Modulation of the interaction microbiota–pathogen–tick–host during blood intake. Upper panel, the microbiota of the tick interacts with the pathogens ingested during blood intake in different organs of the tick (salivary glands, gut, ovary). The microbiota of these organs promotes the establishment and reproduction of the pathogens and, in some cases, in the vectorial capacity to transmit the definitive host. Lower panel, interaction of the microbiota of the bovine blood with the microbiota of the tick. The microbiota of the bovine's skin also interacts with the tick. Created in the Mind the Graph platform.

to prevent colonization and establishment in the vector (49). Also, the interaction of the tick microbiota with the host microbiota affects vector competence; therefore, the elucidation of the mechanisms involved in these interactions allows the identification of molecular drivers for tick-borne disease (50).

METAGENOMIC APPROACHES TO STUDY PATHOGENS TRANSMITTED BY TICKS

So far, we have known that ticks have a great capacity to transmit various pathogens that cause different diseases in cattle. However, the function of the microbiota within the tick life cycle is still unclear due to the significant limitation of their isolation, cultivation, and propagation in culture media and the difficulty of infesting animals with ticks under controlled experimental conditions (30, 51). Because of these drawbacks, the use of next-generation sequencing, molecular techniques, and or multi-omics technologies represents valuable tools in the study of the tick microbiota and its microbiome.

In this regard, the use of 16S rRNA gene-targeted metagenomics provides new insights into tick-borne pathogens (52). The use of this technique has allowed the identification of bacteria in ticks such as *R. microplus*, *Hyalomma anatolicum*, and *Haemaphysalis montgomery* from cows, where *Ralstonia*, *Staphylococcus*, and *Francisella* were some genera detected (53). Metagenomics studies show that the microbiota of *R. microplus* captured in a rural area in Peru has 147 bacterial genera (54). In contrast, the sequencing of internal tissue and salivary glands from unfed larvae and female ticks of *I. ricinus* revealed commensal bacteria, endosymbionts, and several pathogenic microorganisms (54, 55). Also, metagenomic analysis of *Ixodes persulcatus* and *Dermacentor nuttalli* revealed 10 predominant genera of cattle pathogens and also coinfections (56).

A deeper metagenomic study elucidated the taxonomic and functional profiles of the microbiome of female and male ticks, *Ixodes ovatus*, *I. persulcatus*, and *Amblyomma variegatum* (57). The results of this study showed significant differences in the abundance of genes involved in metabolic pathways between female and male ticks of the same species, suggesting their different lifestyles exert and sex-specific evolutionary pressure independently of the phenomes and microbiomes of the tick gut microbiota.

Complementary information to metagenomics studies in microbiota derives from other multi-omics strategies such as metabolomics, transcriptomics, and proteomics that contribute

to advancing knowledge of pathogens and their interactions with the host and vector (58), as in the case of the studies that show diverse components of tick saliva are capable of modulating host immune response through the binding to cell receptors and regulating the secretion of cytokines, chemokines, and interleukins (35). However, we propose that tick microbiota requires an interdisciplinary approach, where the metagenomics study combined with other multi-omics tools complements the results to give a complete vision of the microbiota–vector–host interactions.

PARATRANSGENESIS AS A STRATEGY TO CONTROL TICKS

Based on the progress of metagenomics study applied to identify microbiota in other arthropods vectors, such as *Aedes*, *Culex*, and *Anopheles*, strategies such as paratransgenesis could control tick infestations. This strategy is an innovative method with a good prognosis in the vectors control. This strategy requires knowledge of the tick microbiota. After the symbiont microorganisms' identification, they are isolated and genetically transformed to generate bacteria capable of expressing the specific inhibitory molecules and then reintroduced to obstruct vital biological processes of the vector. In this regard, some reports suggest that the interference of crucial processes such as ovogenesis and vitellogenesis decreases the fecundity, development, and hatching rates, which results in the inhibition of the vector population growth (59). Similarly, another target is the transformation of bacteria that interfere with the digestive capacity, leading to reduced vector reproduction and preventing pathogen dissemination (59). Adopting strategies based on metagenomic studies allows identifying symbiont bacteria in the tick microbiota that can be genetically modified and cultivated with higher efficiency, which overcomes the need to produce genetically modified vectors (60, 61).

MICROBIAL TARGETS: ANTI-TICK VACCINES

In response to the presence of acaricide-resistant populations, several vaccine proposals were developed in the last years, including the use of antigens such as Gavac, a vaccine based on protein Bm86 (62). The formulation of cocktail anti-tick vaccines has been reported using combinations of antigens, like Bm91 (*R. microplus*); subolesin 4E6 (*I. scapularis*); serpins rRAS-2 and rRAS-4 (*R. appendiculatus*); and glutathione S-transferase rGST-Av (*A. variegatum*), among others (63).

Currently, the use of experimental anti-tick vaccines targeting the microbiota reveals that modifications in the microbial populations of the gut could alter essential processes in a tick. For instance, the use of this experimental anti-tick vaccine developed after a functional metagenomic analysis shows that immunization of α-1,3-galactosyltransferase-deficient mice with *Escherichia coli* BL21 induces the production of anti-*E. coli* and anti-α-Gal IgM and IgG associated with mortality of *I. ricinus* nymphs during feeding, which concurred with the abundance of α-1,3-galactosyltransferase genes and possibly α-Gal identified by tick microbiome metagenomics analysis (5).

Finally, the search for new vaccine targets should encompass those proteins of the tick immune response involved in the tolerance of microbial populations (tick microbiota), like different proteins identified by immunoinformatic analysis (64).

CONCLUSIONS

Here, we show a general view of different approaches for studying the tick microbiota and other arthropod vectors, intending to integrate the current knowledge and present new alternatives for tick control. From our perspective, in this mini-review, we include recent results that show that the more knowledge and manipulation we have of the microbiota, we get closer to new vaccine development.

The study of the tick microbiota based on metagenomics approaches allows identifying microorganisms and can elucidate the genes that shape the microbiome.

Although the microbiota participates in biological processes like adaptation, development, reproduction, defense against environmental stress, and immunity, information about the interactions and mechanisms involved in vectorial capacity is still scarce and, at the same time, is a field with potential for the identification of vaccine targets.

Considering that many biological interactions function as holobionts (host organism and its associated microbial community) and that, in turn, holobionts make up a more extensive consortium, it is impossible to think that the tick microbiota and tick-borne pathogens are isolated processes.

Undoubtedly, deciphering the interaction of the tick and host–microbiota and how they communicate will provide invaluable information to develop novel strategies for controlling ticks and vector-borne pathogens, like those anti-microbiota and anti-tick vaccine candidates that could benefit animal health and provide acaricide-free environments.

AUTHOR CONTRIBUTIONS

REQ-C and HA-D: conceptualization. REQ-C, HA-D, ES-E, MC-C, and IA-E: investigation and original draft preparation and writing and draft preparation. REQ-C and HA-D: review and editing. All the authors have read and agreed to the published version of the manuscript.

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Ehrlichia chaffeensis and Four *Anaplasma* Species With Veterinary and Public Health Significance Identified in Tibetan Sheep (*Ovis aries*) and Yaks (*Bos grunniens*) in Qinghai, China

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Tick-borne diseases (TBDs) can cause serious economic losses and are very important to animal and public health. To date, research on TBDs has been limited in Qinghai-Tibet Plateau, China. This epidemiological investigation was conducted to evaluate the distribution and risk factors of *Anaplasma* spp. and *Ehrlichia chaffeensis* in livestock in Qinghai. A total of 566 blood samples, including 330 yaks (*Bos grunniens*) and 236 Tibetan sheep (*Ovis aries*) were screened. Results showed that *A. bovis* (33.3%, 110/330) and *A. phagocytophilum* (29.4%, 97/330) were most prevalent in yaks, followed by *A. ovis* (1.2%, 4/330), *A. capra* (0.6%, 2/330), and *E. chaffeensis* (0.6%, 2/330). While *A. ovis* (80.9%, 191/236) and *A. bovis* (5.1%, 12/236) infection was identified in Tibetan sheep. To our knowledge, it is the first time that *A. capra* and *E. chaffeensis* have been detected in yaks in China. Apart from that, we also found that co-infection of *A. bovis* and *A. phagocytophilum* is common in yaks (28.2%, 93/330). For triple co-infection, two yaks were infected with *A. bovis*, *A. phagocytophilum*, and *A. capra*, and two yaks were infected with *A. bovis*, *A. phagocytophilum*, and *E. chaffeensis*. Risk analysis shows that infection with *A. bovis*, *A. phagocytophilum*, and *A. ovis* was related to region and altitude. This study provides new data on the prevalence of *Anaplasma* spp. and *E. chaffeensis* in Qinghai, China, which may help to develop new strategies for active responding to these pathogens.

Keywords: tick-borne disease, *Anaplasma capra*, *Ehrlichia chaffeensis*, Tibetan sheep, yak, Qinghai

INTRODUCTION

Anaplasmosis and ehrlichiosis are important diseases caused by tick-borne pathogens, which result in additional economic losses to livestock (1, 2). To date, seven *Anaplasma* species have been identified, including *A. bovis*, *A. phagocytophilum*, *A. centrale*, *A. platys*, *A. marginale*, *A. ovis*, and *A. capra* (3, 4). *A. bovis* parasitizes monocytes and macrophages of ruminants and small mammals (5). *A. phagocytophilum* infects neutrophils of humans and animals, and causing human granulocytic anaplasmosis (HGA), tick-borne fever in ruminants, and canine and equine granulocytic anaplasmosis (5). *A. centrale* and *A. marginale* mainly infect erythrocytes of cattle, while *A. ovis* primarily infect small ruminant animals such as sheep and goats. (6). *A. platys* mainly infect canine platelets and cause cyclic thrombocytopenia in dogs (6). *A. capra* is an emerging pathogen, which can infect ruminants and humans (7). In addition, as a member of the *Ehrlichia* family, *Ehrlichia chaffeensis* can cause human monocytic ehrlichiosis (HME) (8), and ehrlichiosis in animals (9).

Over the past several decades, the *Anaplasma* and *Ehrlichia* infections are very common in many countries (3, 10–12). *A. bovis* is mainly distributed in Africa, Asia, and South America, and cattle are considered the primary hosts (6). Similarly, *A. ovis* is the leading cause of anaplasmosis in small ruminants, which is widely distributed around the world (13). Recently, *A. phagocytophilum*, *A. capra*, and *E. chaffeensis* have received much attention for their potential threats to public health (7, 14). *A. phagocytophilum* has been detected in sheep, cattle, *Capreolus pygargus*, goats, and humans in different areas of China (15–18). *E. chaffeensis* infections are very common in the United States, with an annual rate of 4.46 cases/1,000,000 population (19). For *A. capra*, it was initially isolated from goats and humans in China (7). Subsequently, it was found in many countries (20, 21).

Qinghai is the source of the Yangtze River, the Yellow River, and the Lancang River, located in the northeast of Qinghai-Tibet Plateau and northwest of China with an average altitude of more than 3,000 meters. The complicated topographic features and changeable climate bless the region with advantageous conditions of rich natural resources. Tibetan sheep (*Ovis aries*) and yaks (*Bos grunniens*) are the main domestic animals in Qinghai and an important source of life and income for herders. Ixodid tick infestation in livestock is a common and severe problem, and more than 25 tick species in six genera have been reported in Qinghai (22, 23). However, information about tick-borne diseases (TBDs) in the region has been limited. Therefore, to better understand the situation of TBDs in Qinghai, China, a molecular epidemiologic study was conducted investigating exposure to *Anaplasma* spp. and *E. chaffeensis* in domestic animals across the area.

MATERIALS AND METHODS

Blood Sample Collection of Yaks and Tibetan Sheep

A total of 566 blood samples of yaks ($n = 330$) and Tibetan sheep ($n = 236$) were collected using random sampling from six sampling sites in Maqin (35°2′38″N, 99°12′5″E; altitude 3,877 m), Dari (33°43′4″N, 99°38′2″E; altitude 4,130 m), and Banma (32°43′24″N, 100°42′41″E; altitude 3,864 m) of Guoluo Tibetan Autonomous Prefecture (GL), and Yushu (32°51′18″N, 96°48′57″E; altitude 4,317 m), Zhiduo (33°37′5″N, 95°58′51″E; altitude 4,177 m) and Qumalai (34°10′15″N, 95°49′57″E; altitude 4,279 m) of Yushu Tibetan Autonomous Prefecture (YS) during June 2020 in Qinghai, China (**Figure 1**). GL and YS are similar in altitude and climate, and both belong to the continental climate of the plateau. Except for about 400 Tibetan sheep in Maqin, the number of yaks and Tibetan sheep in other sampling sites is between 100 and 200. All animals adopt a free grazing system. Ticks and *Melophagus ovinus* and their bites can be seen in Tibetan sheep, while ticks are rarely found on yaks.

Extraction and Quantification of DNA

According to the manufacturer's operation manual, genomic DNA was extracted from 200 uL whole blood samples by the TIANamp Genomic DNA kit (TIANGEN biotech, Beijing). The concentration of the extracted DNA was detected by NanoDrop 2,000 (Thermo Fisher Scientific, USA) and then stored at −20°C for pathogens detection.

Detection of *Anaplasma* spp. and *E. chaffeensis*

Conventional PCR or nested PCR was used to screen for *Anaplasma* spp. and *E. chaffeensis* in extracted DNA. Nested PCRs were employed to detect *A. bovis*, *A. phagocytophilum*, *A. centrale*, *A. platys*, and *E. chaffeensis* based on *16S* rRNA gene. Conventional PCR based on the *msp4* genes was employed to detect *A. marginale* and *A. ovis*, while *16S* rRNA gene for detection of *A. capra*. PCR primers and cycling conditions used in this study, as shown in **Table 1**. The DNAs extracted from the whole blood of Tibetan sheep and yaks infected with *A. bovis*, *A. phagocytophilum*, *A. ovis*, *A. capra*, and *E. chaffeensis* that had been verified by sequencing, were used as a positive control for corresponding PCR reactions; double-distilled water was used as a negative control. The PCR products were detected by 1.5% agarose gel electrophoresis with M5 Hipure Next III Gelred (Mei5 Biotechnology Co., Ltd., Beijing, China) stained.

Sequencing and Phylogenetic Analysis

PCR products of all positive samples for *Anaplasma* spp. and *E. chaffeensis* randomly selected from each sampling site were sequenced by BGI (Beijing, China). The sequence obtained by BGI sequencing was submitted to NCBI for BLASTn search (https://blast.ncbi.nlm.nih.gov/Blast.cgi), and sequence alignment and analysis. The representative nucleotide sequences of this study have been deposited in the GenBank database. Phylogenetic trees were constructed using the neighbor-joining method executed with the p-distance model in MEGA X. Bootstrap values were assessed with 1,000 bootstrap replicates (28, 29).

Statistical Analysis

The data were grouped into four variables according to animal species, gender, sampling sites, and the altitude of sampling

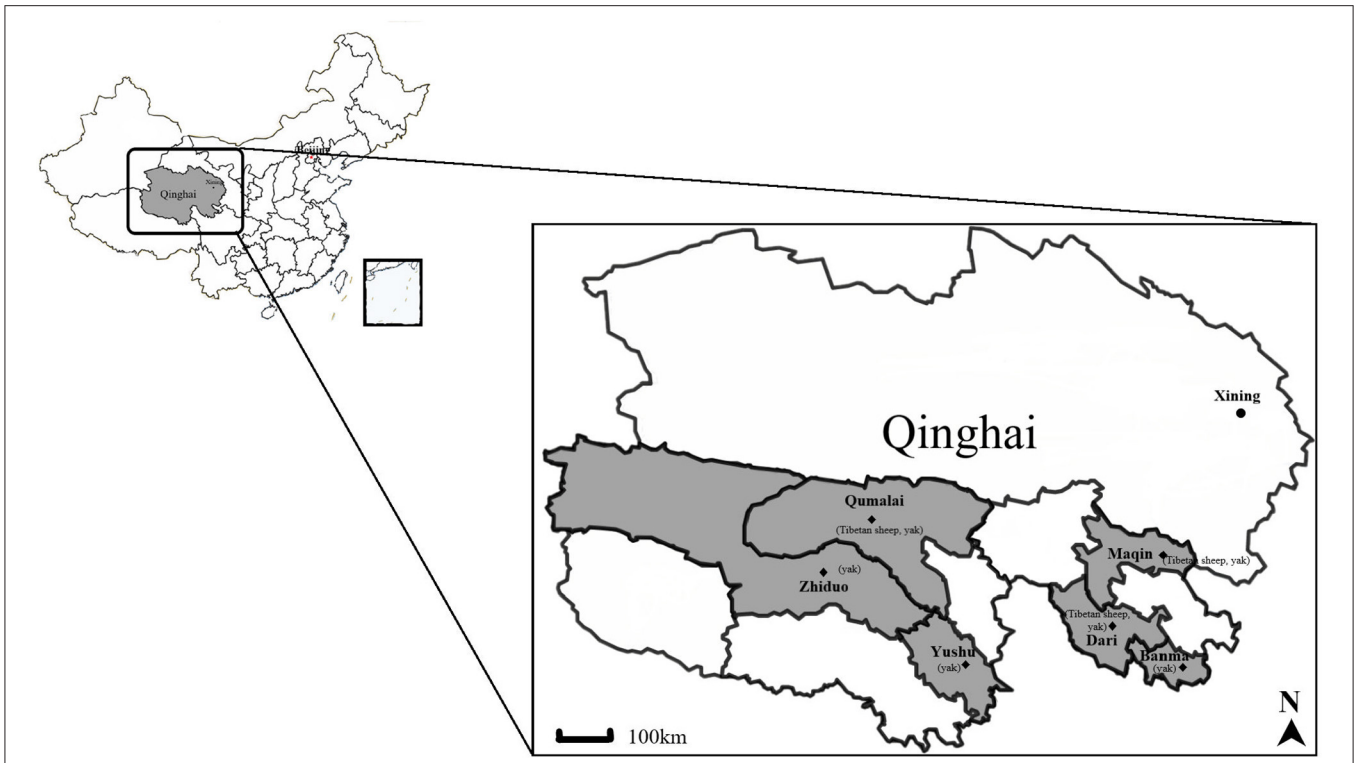

FIGURE 1 | Map of the Qinghai Province, showing the sampling sites and animals included. The yak and Tibetan sheep samples were collected at six sites indicated by ♦. The figure was generated and modified using DITUHUI (https://g.dituhui.com).

sites. The chi-square test was used to calculate the difference of infection rate in SPSS 25.0 software in each group. When $p < 0.05$, the difference was significant.

RESULTS

Prevalence of *Anaplasma* spp. and *E. chaffeensis* in Tibetan Sheep and Yaks

This study identified four *Anaplasma* species and *E. chaffeensis* from Tibetan sheep and yaks (**Table 2**). Of the 566 samples tested, 50% (283/566) were positive for at least one pathogen. The infection rates of *A. bovis* and *A. ovis* were 33.3% and 1.2% in yaks, 5.1% and 80.9% in Tibetan sheep. The infection rates of *A. phagocytophilum*, *A. capra*, and *E. chaffeensis* were 29.4%, 0.6%, and 0.6% in yaks, respectively. This is the first time that *A. capra* and *E. chaffeensis* have been detected in yaks in China. Interestingly, we noticed *A. ovis* infection in yaks and *A. bovis* in Tibetan sheep. The most common co-infection was *A. bovis* and *A. phagocytophilum*, with an infection rate of 28.2% (93/330) in yaks. For co-infection with three pathogens, the infection rate of *A. bovis*, *A. phagocytophilum*, and *A. capra* was 0.6% (2/330), and the infection rate of *A. bovis*, *A. phagocytophilum*, and *E. chaffeensis* was 0.6% (2/330) (**Table 2**). No co-infections by two or more pathogens were detected in Tibetan sheep.

Sequencing and Phylogenetic Analysis

In the current study, 15 representative sequences were obtained and submitted to GenBank (**Table 3**). We compared and analyzed the partial *16S* rRNA gene sequences of *A. bovis*, *A. phagocytophilum*, *A. capra*, and *E. chaffeensis* obtained from blood samples of Tibetan sheep and yaks. BLASTn analysis of the *16S* rRNA gene showed that the *Anaplasma* spp. obtained in this study had 99.04–100% identities to either of *A. bovis*, *A. phagocytophilum*, *A. capra*, and *E. chaffeensis* sequences, respectively. The *E. chaffeensis* sequences (MW048788, MW048789) from yaks were 99.44–100% identical to *E. chaffeensis* isolated from goats (KX505292) in China. Phylogenetic analysis of *16S* rRNA gene sequences confirmed *A. bovis*, *A. phagocytophilum*, *A. capra*, and *E. chaffeensis* in this study (**Figures 2A,B**, **3A,B**). Additionally, we analyzed the *msp4* genomic region of three *A. ovis* (MZ231113-MZ231115) obtained in this study. The results showed that the three sequences were consistent with the homology of the Iranian *A. ovis* (MH790273). *A. ovis* were classified as *A. ovis msp4* Genotypes II based on $T^{366}C^{470}$ (25). Phylogenetic analysis of *msp4* gene sequences confirmed the identity of *A. ovis* in this study (**Figure 4**).

Risk Factors of Tibetan Sheep and Yaks Infected With *Anaplasma* spp. and *E. chaffeensis*

These factors include animal species, gender, sampling sites, and altitude of sampling sites, which were used as variables for statistical analysis of the infection patterns of *Anaplasma* spp. and *E. chaffeensis*. The results indicate that the prevalence of *Anaplasma* spp. and *E. chaffeensis* in female animals was similar

TABLE 1 | Primers used in this study to detect *Anaplasma* spp. and *E. chaffeensis* in Tibetan sheep and yaks in Qinghai, China.

Pathogens	Target gene		Primers (5′ → 3′)	Product (bp)	Annealing temperature (°C)	Reference
A. bovis	*16S* rRNA	EE1	TCCTGGCTCAGAACGAACGCTGGCG	1,430	55	(24)
		EE2	AGTCACTGACCCAACCTTAAATGGCTG			
		AB1f	CTCGTAGCTTGCTATGAGAAC	551	55	(12)
		AB1r	TCTCCCGGACTCCAGTCTG			
A. phagocytophilum	*16S* rRNA	EE1	TCCTGGCTCAGAACGAACGCTGGCG	1,430	55	(24)
		EE2	AGTCACTGACCCAACCTTAAATGGCTG			
		SP2f	GCTGAATGTGGGGATAATTTAT	641	55	(12)
		SP2r	ATGGCTGCTTCCTTTCGGTTA			
A. centrale	*16S* rRNA	EE1	TCCTGGCTCAGAACGAACGCTGGCG	1,430	55	(24)
		EE2	AGTCACTGACCCAACCTTAAATGGCTG			
		AC1f	CTGCTTTTAATACTGCAGGACTA	426	60	(17)
		AC1r	ATGCAGCACCTGTGTGAGGT			
A. platys	*16S* rRNA	EE1	TCCTGGCTCAGAACGAACGCTGGCG	1,430	55	(24)
		EE2	AGTCACTGACCCAACCTTAAATGGCTG			
		Apf	TCCTGGCTCAGAACGAACGCTGGCGGC	506	60	(17)
		APr	AGTCACTGACCCAACCTTAAATGGCTG			
A. marginale/ A. ovis	*msp4*	MSP45	GGGAGCTCCTATGAATTACAGAGAATTGTTTAC	870	60	(25)
		MSP43	CCGGATCCTTAGCTGAACAGGAATCTTGC			
A. capra	*16S* rRNA	Capra-F	GCAAGTCGAACGGACCAAATCTGT	1,261	58	(26)
		Capra-R	CCACGATTACTAGCGATTCCGACTTC			
E. chaffeensis	*16S* rRNA	ECB	CGTATTACCGCGGCTGCTGGCA	450	60	(27)
		ECC	AGAACGAACGCTGGCGGCAAGCC			
		HE1	CAATTGCTTATAACCTTTTGGTTATAAAT	3,90	55	(27)
		HE3	TATAGGTACCGTCATTATCTTCCCTAT			

TABLE 2 | The prevalence of *Anaplasma* spp. and *E. chaffeensis* in Tibetan sheep and yaks in Qinghai, China.

Species	Pathogens	GL*				YS*			
		No. infected/(%)				No. infected/(%)			
		Maqin	Dari	Banma	Total	Yushu	Qumalai	Zhiduo	Total
Yak	No. tested	95	35	84	**214**	56	30	30	**116**
	A. bovis	1 (1.1)	0	84 (100)	**85 (39.7)**	21 (37.5)	0	4 (13.3)	**25 (21.6)**
	*A. phago**	0	0	74 (88.1)	**74 (34.6)**	19 (33.9)	1 (3.3)	3 (10)	**23 (19.8)**
	A. ovis	0	0	0	**0**	0	4 (13.3)	0	**4 (3.5)**
	A. capra	0	0	2 (2.4)	**2 (0.9)**	0	0	0	**0**
	E. chaffeensis	0	0	1 (1.2)	**1 (0.5)**	1 (1.8)	0	0	**1 (0.9)**
	A. bovis + *A. phago*	0	0	74 (88.1)	**74 (34.6)**	16 (28.6)	0	3 (10)	**19 (16.4)**
	A. bovis + *A. phago* + *A. capra*	0	0	2 (2.4)	**2 (0.9)**	0	0	0	**0**
	A. bovis + *A. phago* + *E. chaffeensis*	0	0	1 (1.2)	**1 (0.5)**	1 (1.8)	0	0	**1 (0.9)**
Tibetan sheep	No. tested	143	51	0	**194**	0	42	0	**42**
	A. bovis	12 (8.4)	0	0	**12 (61.9)**	0	0	0	**0**
	A. ovis	109 (76.2)	48 (94.1)	0	**157 (80.9)**	0	34 (81)	0	**34 (81)**

**A. phago = A. phagocytophilum, GL: Guoluo Tibetan Autonomous Prefecture, YS: Yushu Tibetan Autonomous Prefecture.*

to that of male animals ($P > 0.05$). The infection rates of *A. bovis*, *A. phagocytophilum*, and *A. ovis* in yaks in GL and YS were 39.7 and 21.6% ($P = 0.001$), 34.6 and 19.8% ($P = 0.006$), 0 and 3.5% ($P = 0.005$), respectively. In addition, the infection rate of *A. bovis* and *A. phagocytophilum* below 4,000 m was significantly higher than those above 4,000 m ($P = 0.000$). In Tibetan sheep,

TABLE 3 | The DNA sequences submitted to the gene bank in this study.

	Obtained sequences				Reference sequences from GenBank	
Pathogen	**Host**	**Target gene**	**Accession number**	**Length (bp)**	**Identity (%)**	**Accession number (host, country)**
A. bovis	yak	*16S* rRNA	MW048790	516	99.61	MT036513 (sheep, Russia)
	yak	*16S* rRNA	MW048791	525	99.04	MT036513 (sheep, Russia)
	Tibetan sheep	*16S rRNA*	MW048792	524	99.42	MT036513 (sheep, Russia)
	yak	*16S rRNA*	MZ231111	524	99.61	MT036513 (sheep, Russia)
	yak	*16S rRNA*	MZ231112	525	99.81	MN213735 (giraffe, Pakistan)
A. phago	yak	*16S rRNA*	MW048793	620	99.34	MW142385 (*M. ovinus*, China)
	yak	*16S rRNA*	MW048794	617	99.67	MW142385 (*M. ovinus*, China)
	yak	*16S rRNA*	MZ231109	618	99.83	MW142385 (*M. ovinus*, China)
	yak	*16S rRNA*	MZ231110	617	99.67	MW142385 (*M. ovinus*, China)
A. capra	yak	*16S rRNA*	MW577114	1106	100	MF066918 (sheep, Gansu)
A. ovis	Tibetan sheep	*msp4*	MZ231113	826	100	MH790273 (sheep,Iran)
	Tibetan sheep	*msp4*	MZ231114	824	100	MH790273 (sheep,Iran)
	Tibetan sheep	*msp4*	MZ231115	824	100	MH790273 (sheep,Iran)
E. chaffeensis	yak	*16S* rRNA	MW048788	360	100	KX505292 (goat, China)
	yak	*16S* rRNA	MW048789	362	99.44	KX505292 (goat, China)

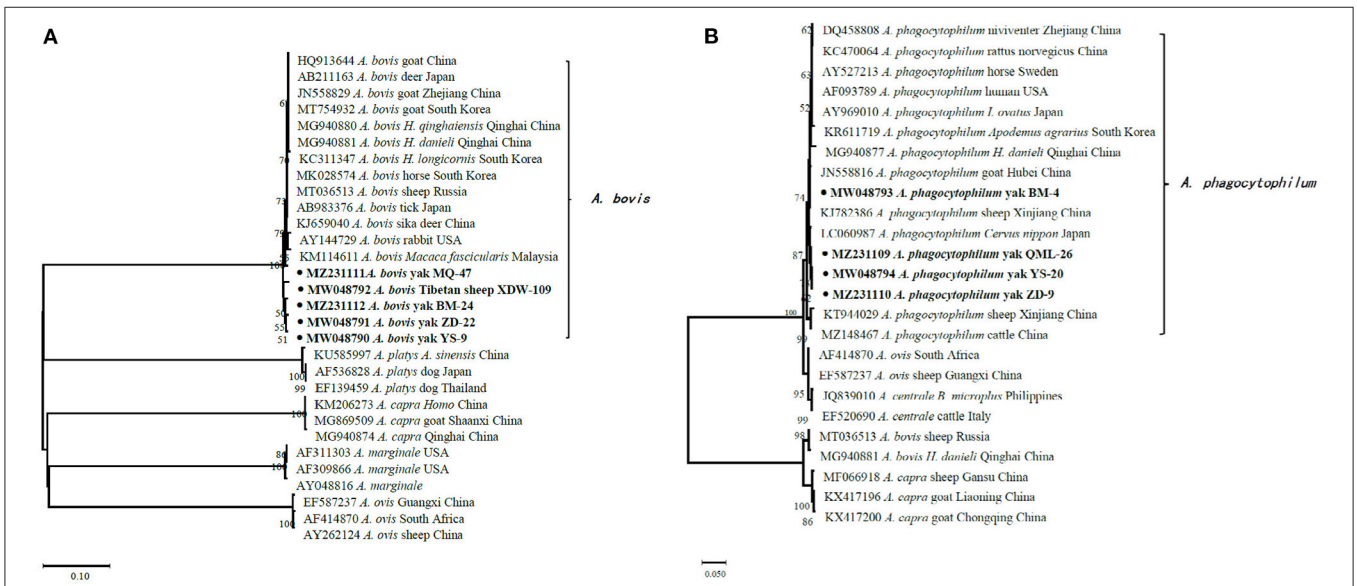

FIGURE 2 | Phylogenetic trees were constructed based on partial sequences of the *16S* rRNA genes of *A. bovis* **(A)** and *A. phagocytophilum* **(B)**. Phylogenetic trees were con-structed by the neighbor-joining method with the p-distance model using the MEGA X software, and the bootstrap test was assessed with 1,000 replicates. The species identified in this study are indicated by • and highlighted in bold.

the infection rate of *A. ovis* above 4,000 m was higher than that below 4,000 m ($P = 0.022$) (**Table 4**).

DISCUSSION

In the present study, *Anaplasma* spp. and *E. chaffeensis* were investigated in domestic animals in Qinghai, China. Four *Anaplasma* species (*A. bovis*, *A. phagocytophilum*, *A. ovis*, and *A. capra*) and *E. chaffeensis* were identified in Tibetan sheep and yaks. Among them, *E. chaffeensis* and *A. capra* were detected in yaks for the first time in China.

The genus *Anaplasma* are widely distributed in domestic animals, wild animals, ticks, and other vectors (23, 30–32). This study found relatively high *A. ovis* infection rates of 76.2, 94.1, and 81.3% in Tibetan sheep in three sampling sites, Maqin, Dari, and Qumalai, respectively, which is higher than in sheep

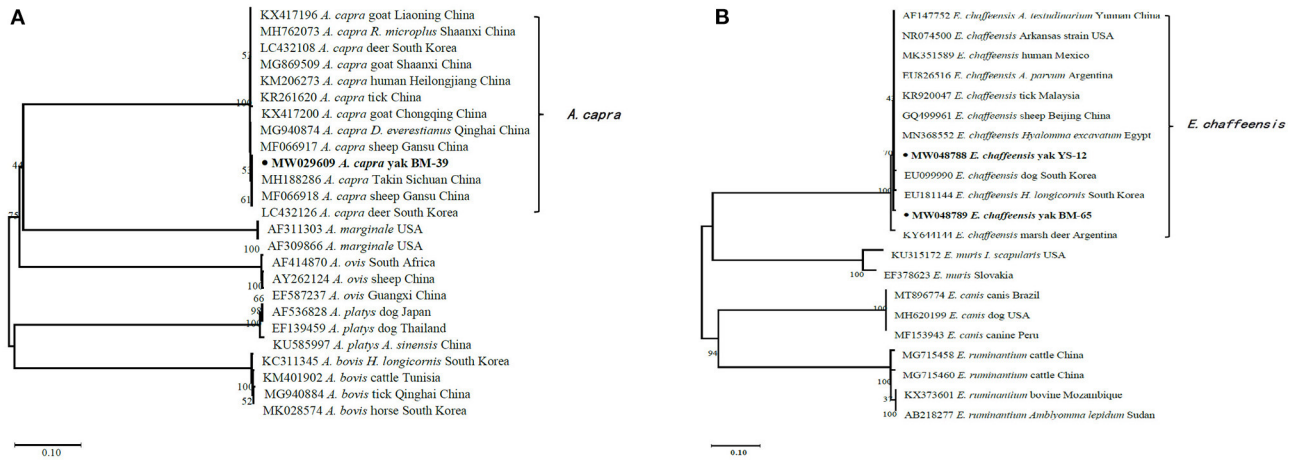

FIGURE 3 | Phylogenetic trees were constructed based on partial sequences of the 16S rRNA genes of *A. capra* **(A)** and *E. chaffeensis* **(B)**. Phylogenetic trees were constructed by the neighbor-joining method with the p-distance model using the MEGA X software, and the bootstrap test was assessed with 1,000 replicates. The species identified in this study are indicated by • and highlighted in bold.

in Xinjiang (40.5%) (16) and Gansu (5.7%) (33), and Tibetan sheep in northeast Qinghai (58%) (34). An explanation for higher infection rates of *A. ovis* in this area could be the bites of ticks and other arthropods. Ticks and *M. ovinus* were found in Tibetan sheep in Maqin and Dari, and data on that *M. ovinus* carried *A. ovis* has been reported in our previous study (31). In addition, we carried out the comparative analysis and phylogenetic analysis of the *msp4* gene sequence of *A. ovis* (25). The results showed that the *A. ovis* strains isolated from Tibetan sheep were identical to those isolated in *M. ovinus* in our previous study (31). Whereas, the *A. ovis* isolated from *Dermacentor nuttalli* in Qinghai by Han et al. (23) belongs to genotypes III, which is in the same clade as those obtained from sheep in Italy (**Figure 4**) (25). Genotypes II and III were also isolated from *M. ovinus* in Xinjiang by Zhao et al. (35). Remarkably, an *A. ovis* variant was reported in humans (36), indicating that this agent has zoonotic potential. Taken together, there are two *A. ovis* genotypes prevalent in domestic animals in northwest China, and arthropods (including *M. ovinus* and ticks) may be the main vectors of *A. ovis*.

A. phagocytophilum and *A. bovis* are frequently detected in ruminants around the world. This study confirms that both *A. phagocytophilum* and *A. bovis* can infect yaks. The infection rate of *A. phagocytophilum* in yaks (29.4%) in this study was higher than that reported in sheep (9.9%), dairy cattle (12%), and white yaks (5.3%) in other areas of China (1, 13, 37), and lower than that in *C. pygargus* (33.3%) from Heilongjiang China (17). Since the first case of HGA, caused by *A. phagocytophilum*, was reported in Anhui, China (38), HGA has been reported in the USA, Europe, Africa, and Asia (11, 39, 40). For *A. bovis*, the infection rate in yaks (33.3%) was higher than that in cattle (4.8%) and white yaks (6.2%) from China (16, 37), cattle (1.0%) from South Korea (20). Recent studies have shown that climate, altitude, longitude, latitude, season, tick bites, contact with wild animals, and feeding methods are important factors affecting *Anaplasma* infection (41). Previous reports have shown that *Haemaphysalis qinghaiensis*, *Dermacentor abaensis*, *D. nuttalli*, and *Dermacentor silvarum* are common ectoparasites among grazing livestock in high altitude areas (2,800 to 4,300 m), and the risk of tick bites with *Anaplasma* spp. was related to altitude and tick species (23). Our results also showed that the risk of infection with *Anaplasma* spp. in Tibetan sheep and yaks is mainly related to altitude and sampling sites. Furthermore, all animals in this study adopted a free grazing system, which increased the risk of domestic animals being exposed to ticks.

A. capra is a novel *Anaplasma* species that emerged in recent years. The novel species was first found in goats and then in sheep (30), *C. pygargus* (17), dogs (42), and ticks (23) in China. In addition, *A. capra* has also been detected in goats, cattle, and *Hydropotes inermis argyropus* in South Korea (32, 43), cattle in Malaysia (10), and *Cervus elaphus* and *Rucervus duvaucelii* in France (21). In 2015, it was isolated from the blood samples of patients with a history of tick bites in northeastern China (7). Subsequently, Peng et al. (44) confirmed the ability of *A. capra* to infect human erythrocytes, HL-60 and TF-1, and further confirmed its zoonotic characteristics. In this study, we detected *A. capra* DNA in yaks in China for the first time. In Qinghai, *H. qinghaiensis* is the most dominant tick species infected with *A. capra*, followed by *D. abaensis* and *D. nuttalli* (23). The above evidence suggests that *A. capra* is widely distributed and could infect a wide range of hosts.

Ehrlichia species include *E. chaffeensis*, *E. canis*, *E. ewingii*, *E. equi*, *E. muris*, and *E. ruminantium*. These species have been detected in many ticks in China, for instance, *Amblyomma testudinarium*, *Haemaphysalis yeni*, *Haemaphysalis longicornis*, *Ixodes sinensis*, *D. silvarum*, *Rhipicephalus sanguineus*, and *Rhipicephalus microplus* (45–48). In previous studies, *E. canis*

FIGURE 4 | Phylogenetic trees were constructed based on the msp4 genes of *A. ovis*. Phylogenetic trees were constructed by the neighbor-joining method with the p-distance model using the MEGA X software, and the bootstrap test was assessed with 1,000 replicates. The species identified in this study are indicated by • and highlighted in bold.

infection was detected in *Cervus nippon* in Gansu (49), and high infection rates of *E. canis* and *E. chaffeensis* were reported in dogs, cattle, sheep, goats, donkeys, and humans in Xinjiang (9, 18, 50). *Ehrlichia* species were also detected in birds and small mammals in other parts of China (51, 52). In the current study, the prevalence rate of *E. chaffeensis* was 0.61%. We present the first report of *Ehrlichia* infection caused by *E. chaffeensis* in yaks in China. However, it is unclear which ticks are responsible for the pathogen. Therefore, further study is needed to determine the vector or reservoir host for this pathogen.

Moreover, mixed-infection is also an important issue that would need to be considered in livestock. The present study results illustrate that mixed infection of *A. phagocytophilum* and *A. bovis* are very common in yaks in Qinghai. Co-infection involving three *Anaplasma* species of *A. bovis*, *A. phagocytophilum,* and *A. capra* was also observed in two yaks in this study. In addition, we found that two yaks were co-infected with *A. bovis*, *A. phagocytophilum* and *E. chaffeensis*. Currently, *A. phagocytophilum*, *A. capra*, and *E. chaffeensis* have been recognized as causative agents of human infection. Mixed-infection of tick-borne pathogens has also been observed in animals in other countries and regions (1, 30, 34, 53). Above all, co-infection of tick-borne pathogens emphasizes the need for differential diagnosis of these pathogens in animal hosts and humans to improve the prevention and control of TBDs.

Notably, all pathogens were detected from apparently healthy animals in this study, consistent with other studies (54–56). This indicates that the appearance of clinical symptoms is mainly dependent on the pathogenicity of these pathogens strains and the breed or species of the infected animals (54). Alternatively, these animals have previously been infected with these pathogens and developed immunity against these pathogens (56). Further research is necessary to confirm these speculations.

TABLE 4 | Patterns of *Anaplssasma* spp. and *E. chaffeensis* prevalence in the yaks and Tibetan sheep, grouped by animal species, gender, sampling sites, and the altitude of sampling sites.

Parameter		Yak							Tibetan sheep				
		No. infected/(%)							No. infected/(%)				
		A. bovis	*p*-value	*A. phago*	*p*-value	*A. ovis*	*p*-value	*A. capra*	*p*-value	*E. chaffeensis*	*p*-value	*A. bovis*	*p*-value	*A. ovis*	*p*-value
Gender	Female	69 (33.8)	0.810	58 (28.4)	0.625	3 (1.5)	0.585	0	0.071	2 (1)	0.265	10 (4.8)	0.597	168 (80.8)	0.862
	Male	41 (32.5)		39 (31)		1 (0.8)		2 (1.6)		0		2 (7.1)		23 (82.1)	
Sampling sites	GL	85 (39.7)	**0.001***	74 (34.6)	**0.006**	0	**0.005**	2 (0.9)	0.296	1 (0.5)	0.659	12 (61.9)	0.098	157 (80.9)	0.997
	YS	25 (21.6)		23 (19.8)		4 (3.5)		0		1 (0.9)		0		34 (81)	
Altitude	3,500–4,000 m	85 (47.5)	**0.000**	74 (41.3)	**0.000**	0	**0.028**	2 (1.1)	0.193	1 (0.6)	0.904	12 (8.4)	**0.04**	109 (76.2)	**0.022**
	>4000 m	25 (16.6)		23 (15.2)		4 (2.7)		0		1 (0.7)		0		82 (88.2)	

*, *Bold indicates significant difference.*

In conclusion, we investigated the epidemic situation of the TBDs in yaks and Tibetan sheep in Qinghai province, China, and confirmed that Tibetan sheep and yaks could be infected with *A. bovis*, *A. phagocytophilum*, *A. ovis*, *A. capra*, and *E. chaffeensis*. This is the first report of *A. capra* and *E. chaffeensis* infection in yaks in China. These pathogens could pose a significant threat to livestock and human health. Thus, future studies should focus more on systematically assessing these pathogens' threats to veterinary and public health.

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 Ethics Committee of the Institute of Zoology, Chinese Academy of Sciences approved the procedures of collecting blood samples from Tibetan sheep and yaks, and obtained the livestock owner's consent. Written informed consent for participation was not obtained from the owners because all the samples in this study were collected by local veterinarians during the daily epidemic surveillance.

AUTHOR CONTRIBUTIONS

YW: investigation, conceptualization, methodology, data curation, visualization, writing—original draft, and writing—review & editing. QZ: investigation, methodology, visualization, writing—original draft, and writing—review & editing. YL: investigation, methodology, data curation, and funding acquisition. SH: investigation, methodology, and writing—original draft. BW, GY, PZ, ZY, and HZ: investigation and methodology. YS, XH, and JC: investigation. HH: investigation, visualization, supervision, validation, writing—review & editing, and funding acquisition. All authors contributed to the article and approved the submitted version.

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Molecular Prevalence of *Anaplasma marginale* and *Ehrlichia* in Domestic Large Ruminants and *Rhipicephalus* (*Boophilus*) *microplus* Ticks From Southern Luzon, Philippines

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Anaplasmosis and ehrlichiosis are tick-borne rickettsial diseases that cause significant economic losses in the livestock industry worldwide. Although bovine anaplasmosis is known to be endemic in the Philippines, epidemiological data is fragmented. Moreover, little is known about bovine ehrlichiosis in the country. In this study, the prevalence of *Anaplasma marginale* and *Ehrlichia* in cattle and water buffalo from provinces in the southern part of Luzon, Philippines, was investigated through PCR. Blood samples from 620 animals comprised of 512 cattle and 108 water buffalo and 195 tick samples were subjected to nested PCR targeting the *groESL* gene of Anaplasmataceae. Positive samples were further subjected to another nested PCR and conventional PCR to amplify the *A. marginale groEL* gene and the *Ehrlichia dsbA* gene, respectively. Selected *A. marginale*-positive samples were also subjected to nested PCR targeting the *msp5* gene. Regardless of the animal host, the overall prevalence in blood samples obtained was 51.9% for Anaplasmataceae, 43% for *A. marginale*, and 1.1% for *Ehrlichia*. No water buffalo were positive for *Ehrlichia.* Meanwhile, 15.9, 6.7, and 2% of the tick samples, all morphologically identified as *Rhipicephalus* (*Boophilus*) *microplus*, were positive for Anaplasmataceae, *A. marginale*, and *Ehrlichia*, respectively. Sequence analysis of selected *A. marginale msp5* amplicons showed that the isolates from the region share 94–98% identity to reported *A. marginale* from other countries. The phylogenetic tree showed clustering of isolates in the region and a close relationship with *A. marginale* isolates from other countries. Sequences of *Ehrlichia* amplicons from cattle and ticks were

97–100% similar to reported *Ehrlichia minasensis* isolates. This study showed the high prevalence of *A. marginale* in Luzon, Philippines, and provided the first molecular evidence of *E. minasensis* in the country.

Keywords: *Anaplasma marginale*, *Ehrlichia*, cattle, *Rhipicephalus (Boophilus) microplus*, tick- borne rickettsiae, water buffalo

INTRODUCTION

Tick-borne diseases (TBDs) cause significant economic losses to the livestock industry worldwide, particularly in tropical and subtropical regions (1). The global annual losses in the cattle industry attributed to ticks and TBDs have been estimated to be between US$ 14 to US$ 19 billion (2). Anaplasmosis and ehrlichiosis are rickettsial TBDs caused by members of the family Anaplasmataceae. Bovine anaplasmosis due to *Anaplasma marginale* is considered the most prevalent TBD of cattle. Hard ticks under the genus *Rhipicephalus* (*Boophilus*) primarily transmit *A. marginale* (3), but it can also be transmitted by blood-sucking flies and sucking lice, as well as contaminated fomites (4). Meanwhile, bovine ehrlichiosis is caused by several species of *Ehrlichia,* also mainly transmitted by several hard ticks. The common species affecting large ruminants are *Ehrlichia bovis*, *E. ondiri*, *E. chaffeensis*, and *E. ruminantium* (5). These pathogens are distributed worldwide but more widely distributed in European countries, India, and South Africa. Additionally, a new species of *Ehrlichia* was isolated from the hemolymph of *R. microplus* and was named *E. mineirensis* (6). Using PCR, another genotype, UFMT-BV, was later detected in naturally infected cattle from Brazil. Through the genetic characterization of 16S ribosomal RNA (*16s rRNA*) and thio-disulfide oxidoreductase (*dsb*) genes, it was later found that the two genotypes represented a single species phylogenetically close to *E. canis* that was named *E. minasensis* (7).

The cattle industry is a developing agricultural sector in the Philippines. In 2020, a population of 2.63 million with a gross value of about US$582 million was reported in the country (8). Water buffalo, which are raised for draft power, meat, and milk, had a reported population of 2.83 million with a gross value of about US$260 million in 2020 (9). Despite continued efforts to boost the large ruminant industry, particularly the production of dairy cattle and water buffalo, health problems, including tick infestation, still hamper high productivity. The tropical climate of the Philippines highly favors the life cycle of the cattle tick *R. microplus*. The wide distribution of this tick in the country is accompanied by the occurrence of TBDs, such as anaplasmosis (10). Several studies provided molecular evidence of the occurrence of *A. marginale* in cattle (11–13) and water buffalo (14, 15) in the Philippines. High genetic diversity of *A. marginale* was also reported based on the analysis of the *msp1a* gene (16). Nevertheless, epidemiological data in the country remains fragmented. Aside from its presence in the blood, we previously reported the detection of *A. marginale* in the milk of dairy cattle (17). A study previously reported the detection of *Ehrlichia* in 35 of 246 bovine blood samples through PCR, but the species was not identified (18). Here, we investigated the epidemiology of *A. marginale* and *Ehrlichia* in cattle, water buffalo, and *R. microplus* ticks from CALABARZON (Region IV-A) in Luzon, Philippines—a top cattle-producing region, also with a large population of water buffalo.

MATERIALS AND METHODS

Blood and Tick Samples

A total of 620 blood samples (512 cattle and 108 water buffalo) and 195 tick samples comprised of various developmental stages were tested in this study by PCR for the presence of Anaplasmataceae bacteria, particularly *A. marginale* and *Ehrlichia*. The number of blood samples sufficient to determine the prevalence in animals was calculated based on the animal population data in the study area at 95% confidence interval using an online software (https://www.openepi.com). The samples were collected between March 2016 and October 2019 from selected commercial farms and smallholder raisers in 44 municipalities/cities throughout the five provinces (Cavite, Laguna, Batangas, Rizal, and Quezon) of the CALABARZON region (Region 4A) in Luzon, Philippines as previously described (19). The animals were of various breed type, age and sex, and most of which are not showing any signs of severe disease at the time of sample collection. All ticks were collected directly from the animals and were morphologically identified as *R. microplus*. DNA was extracted from blood and tick samples using commercial extraction kits (innuPREP® DNA/RNA Mini Kit for blood and blackPREP® Tick DNA/RNA Kit, Analytik Jena, Jena, Germany).

PCR Detection of *Anaplasma* and *Ehrlichia*

Sample screening was initially done through nested PCR amplification of the *groESL* gene of Anaplasmataceae using the primers described by Tabara et al. (20). Samples that showed positive bands were subjected to nested PCR targeting the heat-shock operon (*groEL*) gene for *A. marginale* (12) and conventional PCR targeting the *dsb* gene of *Ehrlichia* ((34)). Selected samples positive for *A. marginale* were also subjected to nested PCR targeting the *msp5* gene ((35)). All primers used in this study are listed in **Supplementary Table 1**. PCR mixtures with a total volume of 10 μl were prepared using Tks Gflex™ DNA Polymerase (TaKaRa, Shiga, Japan), together with 10 pmol each of forward and reverse primers, nuclease-free water, and template (1 μl DNA or 1st PCR product). The PCR conditions are shown in **Supplementary Table 2**. In each PCR batch, a negative control containing nuclease-free water was included. PCR products were subjected to electrophoresis in 2% agarose gel in 1x TAE buffer. After staining the gel with ethidium bromide in

TABLE 1 | Prevalence of *A. marginale* and *Ehrlichia* in cattle and water buffalo from the CALABARZON region in Luzon, Philippines, based on PCR detection in blood samples.

Province	Cattle				Water buffaloes			
	N	No. (%) positive for Anaplasma-taceae	No. (%) positive for *A. marginale*	No. (%) positive for *Ehrlichia*	*n*	No. (%) positive for Anaplasma-taceae	No. (%) positive for *A. marginale*	No. (%) positive for *Ehrlichia*
Cavite	100	73 (73)	66 (66)	1 (1)	0	–	–	–
Laguna	111	68 (61.3)	61 (54.9)	1(0.9)	11	1 (9)	0	0
Batangas	120	85(70.8)	71 (59.2.)	0	8	7 (87.5)	1 (12.5)	0
Rizal	87	32 (35.63)	27 (31.03)	0	0	–	–	–
Quezon	94	51 (54.3)	41 (43.6)	5 (5.32)	89	5 (5.6)	0	0
Total	512	309 (60.3)	266 (51.9)	7 (1.4)	108	13 (12)	1 (0.9)	0

1x TAE, the bands were visualized through a gel documentation system (Bio-Print, Vilber, Lourmat, France).

Sequence and Data Analysis

Selected *A. marginale msp5*- and *Ehrlichia dsbA*-positive samples were subjected to PCR at 50 μl mixtures for sequence reading. The amplicons were purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Leicestershire, England) following the manufacturer's protocol. Capillary sequencing was accomplished by a third-party laboratory using the forward primer for nested PCR. The sequences for each gene were aligned using Clustal Omega software (https://www.ebi.ac.uk), and the percent identity was determined. Sequence readings were compared to sequences of reported isolates using the Basic Local Alignment Search Tool or BLAST® (https://blast.ncbi.nlm.nih.gov/Blast.cgi). A maximum likelihood phylogenetic tree was constructed using MEGA v.7 software, with bootstrap values estimated using 1,000 replicates based on Kimura's two-parameter substitution model (K2P distance). The prevalence of each pathogen in animals was calculated by dividing the number of positive samples by the total number of blood samples and per animal, expressed as a percentage. The positivity rate in ticks was also calculated and expressed as percentage. The occurrence of *A. marginale* based on host attributes including species, purpose, and sex was also calculated, and chi-square analysis at a 95% confidence interval ($\alpha = 0.05$) was performed using the online software WinEpi® to determine the presence of association.

RESULTS

PCR Detection

Table 1 shows the results of PCR detection of *A. marginale* and *Ehrlichia* in blood samples from cattle and water buffalo. Initial screening for Anaplasmataceae through nested PCR targeting the *groESL* gene was performed, which revealed 322 (51.9%) positive samples, of which 309 (60.3%) were from cattle and 13 (12%) were from water buffalo. These positive samples were then subjected to nested PCR for the *A. marginale groEL* gene, which revealed an overall prevalence of 43%. According to the host, 266 cattle were positive, with a prevalence of 51.9%, while only one water buffalo (0.9%) was *A. marginale* positive. Regarding the type of animal, a higher prevalence of 63.8% was observed among dairy animals than beef-type animals that mainly were native Philippine cattle. Meanwhile, the prevalence of *A. marginale* among males and females was almost equal. Chi-square analysis showed significant association ($p < 0.0001$) of *A. marginale* infection with host species and type (**Table 2**).

TABLE 2 | Occurrence of *A. marginale* with regard to host attributes.

Host attribute	*n*	Number (%) positive for *A. marginale*	*p*-value
Species			
Cattle	512	266 (51.9)	<0.0001*
Water buffalo	108	1 (12.5)	
Type			
Dairy	268	171 (63.8)	
Beef	284	94 (33.1)	<0.0001*
Draft	68	1 (1.5)	
Sex			
Male	110	42 (38.2)	
Female	510	207 (40.6)	0.6405

Chi-square analysis was done to determine the presence of association, and p-values are shown. Asterisk indicates statistical significance ($p < 0.05$).

Meanwhile, only seven blood samples, all from cattle, were found positive through conventional PCR for the *Ehrlichia dsbA* gene, resulting in an overall prevalence of 1.1%. PCR detection in tick samples showed that 31 of 195 (15.9%) were positive for Anaplasmataceae (**Table 3**). Further PCR analyses revealed that 13 (6.7%) and 4 (2%) samples were positive for *A. marginale* and *Ehrlichia*, respectively. Almost all of the positive samples were female ticks.

Sequence Analysis

Ten *A. marginale msp5* amplicons from blood samples (two from each province) were subjected to sequence analysis. After multiple sequence alignment of the 160-bp fragment, it was found that the amplicons per province were 98–100% similar, and the amplicons from all provinces shared 97–100% identity. The reported isolate from another island of the Philippines, Cebu, had a homology with the amplicons ranging from 95–98%. BLAST analysis showed the amplicons share 96–99%

TABLE 3 | Positivity rate (%) of *A. marginale* and *Ehrlichia* in ticks from the CALABARZON region in Luzon, Philippines.

Province	*N*	No. (%) positive for Anaplasmataceae	No. (%) positive for *A. marginale*	No. (%) positive for *Ehrlichia*
Cavite	89	0	–	–
Laguna	18	3 (16.7)	2 (11.1)	0
Batangas	50	18(36)	6 (0.12)	0
Rizal	0	–	–	–
Quezon	38	10 (26.3)	5 (13.2)	4 (10.5)
Total	195	31 (15.9)	13 (6.7)	4 (2)

identity with reported *A. marginale* isolates from Australia (MN517223.1), India (MK834272.1), Thailand (MK188829.1), Sri Lanka (LC467711.1), South Africa (MK481012.1), and Brazil (MN517223.1). The phylogenetic tree showed the clustering of amplicons from this study in a single branch (**Figure 1A**). The tree also showed a close relationship with but branching separate from other reported isolates, including that from Cebu, Philippines. The sequences of four *A. marginale msp5* amplicons were deposited in the DNA Data Bank of Japan (accession numbers LC641906–LC641909).

Meanwhile, four *Ehrlichia* amplicons (two blood samples and two tick samples) were sequenced. The alignment of multiple nucleotide sequences of the 350-bp fragment showed that the identity was 99–100%. Based on BLAST analysis, the amplicons were 99% identical to *Ehrlichia* isolated from *R. microplus* ticks, designated as *E. minasensis* strain UFMG-EV (JX629808.1), and share 97–100% identity with reported *E. minasensis* isolates from Brazil (KF621012.1), Colombia (KM015219.1), and China (MN480809.1). A single sequence was deposited in the DNA Data Bank of Japan (accession number LC641910). The phylogenetic tree showed the clustering of amplicons with the abovementioned *E. minasensis* isolates in a single branch (**Figure 1B**).

DISCUSSION

Anaplasmosis and ehrlichiosis are tick-borne rickettsial diseases that can have a detrimental impact on the health and productivity of livestock worldwide. Previous studies in the Philippines showed the widespread distribution of *A. marginale* in cattle and water buffalo, yet epidemiological data is still fragmented. Meanwhile, the occurrence of *Ehrlichia* in cattle, water buffalo, and *R. microplus* ticks in the country has not been established. Thus, this study was conducted to determine the occurrence of *A. marginale* and *Ehrlichia* in cattle, water buffalo, and *R. microplus* ticks in the CALABARZON region, which ranks third and ninth in terms of cattle and water buffalo populations, respectively (PSA 2020).

A high prevalence of *A. marginale* was observed in cattle, but it was lower than the prevalence previously reported in a study in Luzon involving two dairy farms (13) and the detection rate in our study in the same region that only included milking dairy cattle (17). The current study included dairy cattle, most of which are Holstein Friesian or Holstein-Sahiwal crosses, as well as the tropical native Philippine cattle commonly raised for beef. The latter breed is known to be tick-resistant. However, our results show that the native cattle can still harbor the pathogen and become an important source of infection under field conditions. A similar prevalence of *A. marginale* in beef cattle was also reported in Thailand (21). The high occurrence in dairy cattle agrees with the results of the above-mentioned studies. It was noted during field collection that *R. microplus* ticks are more commonly encountered in dairy cattle than in beef cattle. Interestingly, no significant clinical signs were observed from infected cattle, except for a few that were emaciated. It is also possible that most of these *A. marginale*-positive animals are already in the carrier state (22). These carrier animals can, however, pose a problem when introducing naïve animals to the farm, with either breed being at risk of severe disease if exposed to virulent *A. marginale* (23).

Water buffalo, which are mostly utilized as draft animals, had a remarkably lower prevalence of *A. marginale* infection than cattle. Although there was a large difference in the number of samples in this study, this finding may also be explained by the innate resistance of water buffalo to tick infestation due to their thicker skin and habit of submerging in wetlands, which prevents tick attachment (24). While the cattle tick *R. microplus* is also listed as one of the ectoparasites of water buffalo (32), this was rarely observed in water buffalo during our field collection. An experimental study confirmed the potential of water buffalo as hosts of *R. microplus* ticks (25). Thus, water buffalo may act as important reservoirs for the cattle tick and the pathogens that it transmits. Moreover, other blood-sucking arthropods, such as the louse *Haematopinus tuberculatus* which was commonly observed among the water buffalo during sample collection, can transmit *A. marginale* (24, 26). Meanwhile, the obtained prevalence in this study was notably lower than that of previous reports in water buffalo from Luzon (14) and Bohol, another island in the Philippines (15).

Moreover, a significantly higher number of females were infected with *A. marginale* as compared to males. This might be due to their greater susceptibility to infection caused by hormonal disturbances during pregnancy, parturition, and lactation, which causes stress and immunosuppression, especially in high-producing cows. Furthermore, imbalances in progesterone, estrogen, and cortisol serum levels contribute to the impairment of the immune function of females (27). It is important to note that most of the animals included in the study

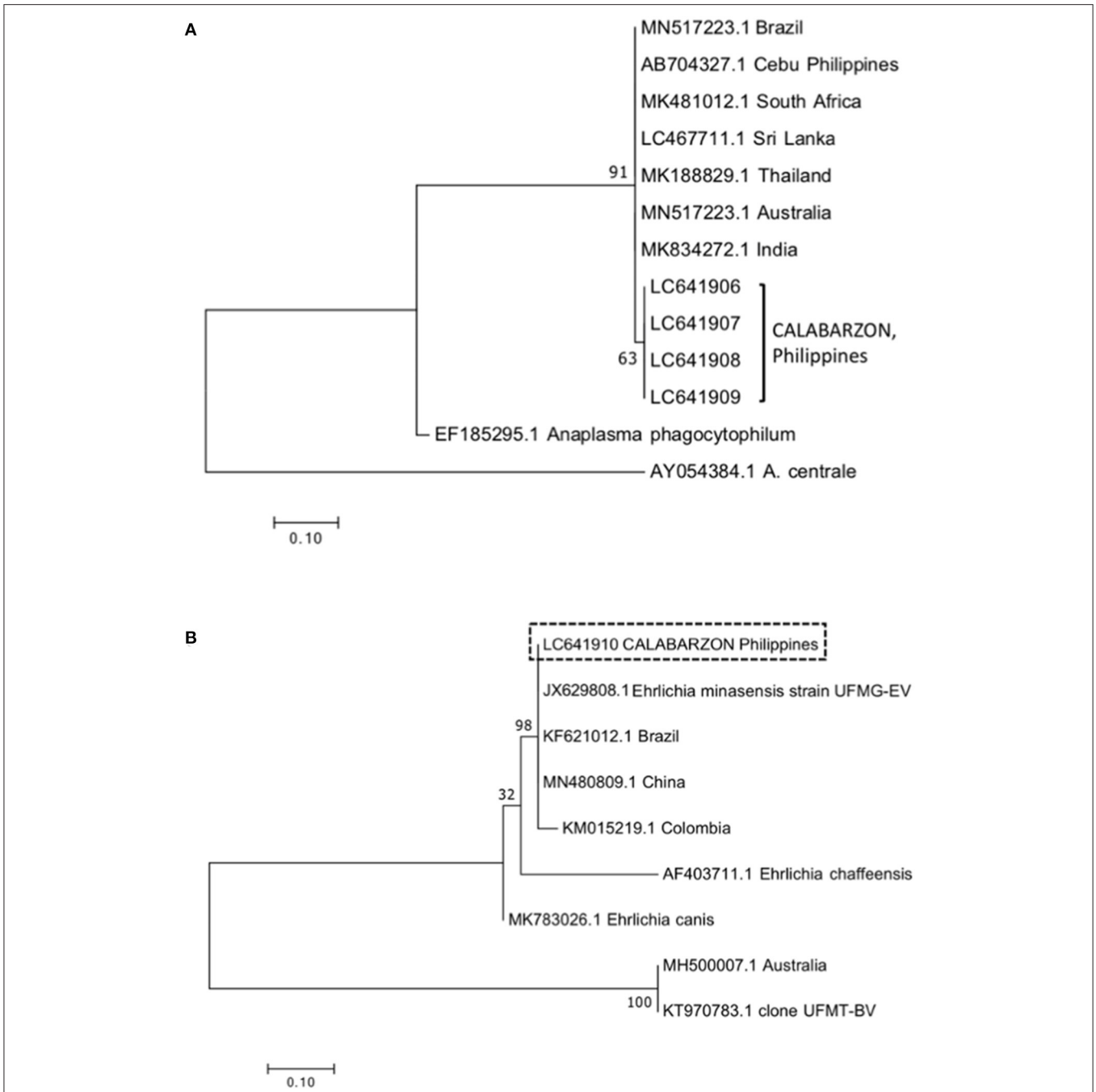

FIGURE 1 | Phylogenetic trees for *Anaplasma marginale msp5* **(A)** and *Ehrlichia dsbA* **(B)** constructed using the maximum likelihood method. Numbers in the nodes indicate support values based on Kimura's two-parameter substitution model (K2P distance). Isolates from this study are indicated within brackets **(A)** or enclosed within a box **(B)**. The bar represents 0.10 substitutions per site.

were lactating dairy animals. The apparent carrier state of dairy females, suggested by the absence of apparent clinical signs at the time of blood collection, still poses a risk for clinical disease since relapse is possible following immunosuppression (28).

On the other hand, *Ehrlichia* was detected in some blood samples and ticks from cattle. Most of the cattle found positive for *Ehrlichia* were female dairy cattle, none showing any clinical signs. Interestingly, none of the positive animals were observed to have ticks at the time of blood collection, suggesting possible chronic or carrier status. Based on sequence analysis, the species detected was *Ehrlichia minasensis*. Similar to the detection rate obtained in this study, ((33)) also reported a low detection rate of *E. minasensis* (previously designated as UFMT-BV) among Brazilian cattle, mostly dairy cattle. Additionally, a low detection

rate was also reported among cattle in Ethiopia (29). Phylogenetic analysis showed that the isolate found in this study was closely related to reported isolates of *E. minasensis* from other countries, suggesting the widespread occurrence of this rickettsial organism.

A great majority of the animals included in this study did not have ticks during blood collection, hence, the discrepancy in the number of blood and tick samples. Most commercial farms included in this study are using chemical acaricides to control tick infestation. The positivity rate in ticks was also lower than in blood, similar to a previous report in Malaysia (30). There were ticks collected from animals whose blood tested positive for *A. marginale* or *Ehrlichia*, suggesting that the tick did not yet acquire the pathogen or the bacterial load in the ticks was below the detection limit of the PCR assays.

Analysis of the *msp5* gene confirmed the close relationship of *A. marginale* isolates in this study with isolates from another island of the Philippines, Cebu, and other countries. A high diversity of *A. marginale* was observed in a previous study based on the analysis of the *msp1a* gene of samples from several provinces in the Philippines, including Cavite and Batangas, which are covered in this study (16). In contrast, the phylogenetic tree based on the *msp5* gene showed the clustering of isolates from different provinces in this study in a single branch, indicating that the gene is conserved among those isolates. On another note, some animals positive for the *groESL* gene of Anaplasmataceae did not turn positive for either *A. marginale* or *Ehrlichia*, suggesting possible infection with another species, such as *A. centrale*. Furthermore, some commercial farms reported having dairy cattle vaccinated against anaplasmosis, which utilized live *A. centrale* (31). Although we were unable to confirm it from the farm records, there is a possibility that those animals positive for *Anaplasma groESL* but negative for *A. marginale* and *Ehrlichia* were vaccinated with live *A. centrale*. Future studies should investigate the occurrence of other *Anaplasma* species through PCR assays that are species-specific.

To conclude, this study showed the high prevalence of *A. marginale* in cattle in southern Luzon, Philippines, and confirmed the presence of *E. minasensis* in naturally-infected cattle and *R. microplus* ticks in the country. Although a more significant number of dairy cattle were found to be infected with either pathogen, the detection of the pathogens in native beef cattle implies the latter's role in maintenance and transmission in the field. The result of our study adds knowledge regarding the epidemiology of *A. marginale* and the geographical location of *E. minasensis*, which is a potential emerging pathogen of cattle. Further studies on the epidemiology of *E. minasensis* in the Philippines are necessary. The effects of *E. minasensis* in the Philippines are necessary. The effects of *E. minasensis* in the health and productivity of cattle should also be investigated further since clinical manifestations were observed after experimental infection in a calf ((33)). The findings of this study highlight the need for continued surveillance and intensified control programs against rickettsial TBDs in the Philippines.

DATA AVAILABILITY STATEMENT

The original contributions generated for the study are included in the article/**Supplementary Files**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The animal study was reviewed and approved by Institutional Animal Care and Use Committee (IACUC) of the College of Veterinary Medicine, University of the Philippines Los Baños. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

RG, BD, MT, and TT conceptualized the study. RG, CL, MarM, AA, AB, CR, KS, LT, and MaaM collected the samples and conducted the laboratory experiments. RG, BD, MT, CA, EM, and NE supervised the work. RG wrote the original draft of the manuscript. RG and TT acquired funding for the study. All authors analyzed the data, reviewed and approved the final version of the manuscript.

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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.2021.746705/full#supplementary-material

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Screening and Analysis of *Anaplasma marginale* Tunisian Isolates Reveal the Diversity of *lipA* Phylogeographic Marker and the Conservation of OmpA Protein Vaccine Candidate

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Bovine anaplasmosis caused by *Anaplasma marginale* is a disease responsible for serious animal health problems and great economic losses all over the world. Thereby, the identification of *A. marginale* isolates from various bioclimatic areas in each country, the phylogeographic analysis of these isolates based on the most informative markers, and the evaluation of the most promising candidate antigens are crucial steps in developing effective vaccines against a wide range of *A. marginale* strains. In order to contribute to this challenge, a total of 791 bovine samples from various bioclimatic areas of Tunisia were tested for the occurrence of *A. marginale* DNA through *msp4* gene fragment amplification. Phylogeographic analysis was performed by using *lipA* and *sucB* gene analyses, and the genetic relationship with previously characterized *A. marginale* isolates and strains was analyzed by applying similarity comparison and phylogenetic analysis. To evaluate the conservation of OmpA protein vaccine candidate, almost complete *ompA* nucleotide sequences were also obtained from Tunisian isolates, and various bioinformatics software were used in order to analyze the physicochemical properties and the secondary and tertiary structures of their deduced proteins and to predict their immunodominant epitopes of B and T cells. *A. marginale* DNA was detected in 19 bovine samples (2.4%). Risk factor analysis shows that cattle derived from subhumid bioclimatic area were more infected than those that originated from other areas. The analysis of *lipA* phylogeographic marker indicated a higher diversity of Tunisian *A. marginale* isolates compared with other available worldwide isolates and strains. Molecular, phylogenetic, and immuno-informatics analyses of the vaccine candidate OmpA protein demonstrated that this antigen and its predicted immunodominant epitopes of B and T cells appear to be highly conserved between Tunisian isolates and compared with isolates from

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other countries, suggesting that the minimal intraspecific modifications will not affect the potential cross-protective capacity of humoral and cell-mediated immune responses against multiple *A. marginale* worldwide strains.

Keywords: *Anaplasma marginale*, *lipA* and *sucB* markers, phylogeographic analysis, OmpA vaccine candidate, conservation assessment, Tunisia

INTRODUCTION

Bovine anaplasmosis caused by *Anaplasma marginale* is a disease responsible for serious animal health problems and great economic losses worldwide (1). *A. marginale*, a species of Gram-negative intracellular obligate bacteria, is the most pathogenic agent responsible for this disease (2). It commonly infects erythrocytes of cattle and wild ruminants and is considered among the most prevalent bacterium transmitted by ticks in the world (3). The major clinical signs related to this disease are hemolytic anemia, jaundice, fever, loss of weight, and decreased milk production. Sometimes, abortion and death of infected animals may be observed in chronic form (3).

Despite the economic impact of this disease, no commercial vaccines against bovine anaplasmosis were developed until now, and only immuno-antigenic elements of the outer membrane preparation including the major surface proteins are studied (1, 4, 5). However, these protein candidates did not confer sufficient immune protection (4, 6, 7). Recently, adhesins were considered as particularly interesting vaccine candidates given that they have an essential role in the survival of obligate intracellular bacteria and are more conserved than other candidate vaccine proteins (8). Among them, OmpA, annotated Am854 in the genome of *A. marginale*, has been classified as an adhesin, which has an important function in the entrance of *A. marginale* in vertebrate host and arthropod vector cells (9). Therefore, this protein could be considered as one of the highly promising vaccine candidates (8).

However, knowledge of the phylogeographic relationships between isolates of *A. marginale* is crucial to better prevent and control infections throughout the world. Indeed, it contributes to the discovery of the mechanisms involved in the difference of pathogenicity of *A. marginale* by passing from one isolate to another (10). Nonetheless, some genes like *msp4* and *msp1a* previously used for *A. marginale* genotyping are relatively efficient for isolate discrimination at the regional level; however, they are not interesting markers for analyzing the phylogeographic relationships between worldwide isolates and strains (11–13).

Therefore, a first multi-locus sequence typing (MLST) scheme for *A. marginale* was developed by Guillemi et al. (10) on 58 isolates and strains from different regions of the world to investigate whether geographically close isolates will have similar sequence types (STs) to each other than will geographically more distant isolates and strains. A total of seven loci (*dnaA*, *ftsZ*, *groEL*, *lipA*, *secY*, *recA*, and *sucB*) were amplified and sequenced, and different STs were obtained on the basis of the nucleotide diversity of the concatenated fragment. However, the authors did not find a clear relationship between geographic regions and STs isolated from various worldwide isolates and strains (10).

More recently, Ben Said et al. (14) examined independently each earlier cited housekeeping gene locus initially employed in the MLST scheme by using the single gene analysis (SGA). This method was performed in order to search a possible phylogeographic resolution at least for one in each of the seven genes. The phylogenetic analysis of each marker revealed that, out of the seven analyzed genes, two (*lipA* and *sucB*) were found to be interesting phylogeographically and allowed the classification of Tunisian isolates and those found in GenBank according to the continents (*lipA*) and according to the New and Old World (*sucB*) (14).

In Tunisia, cross-sectional and longitudinal surveys on bovine anaplasmosis using molecular methods have been conducted in cattle (11, 15, 16). However, these reports were mainly carried out on cattle from the north of the country. In contrast, the epidemiology and risk factors associated, and the heterogeneity of *A. marginale* isolates remain largely unknown and understudied in the other geographic regions.

Therefore, the aims of this study were to evaluate the prevalence of *A. marginale* infection and their potential associated risk factors in cattle from 11 governorates belonging to eight bioclimatic areas from the north to the south of Tunisia and to characterize phylogeographically *A. marginale* isolates using two of the most phylogeographically informative markers (*lipA* and *sucB*). In addition, and considering the potential vaccinal interest of OmpA protein, we intend in our study to assess this protein conservation by comparing Tunisian sequences with those from other countries. In fact, various bioinformatics software were used in order to analyze the physicochemical properties, and the secondary and tertiary structures of their deduced proteins, and to predict their immunodominant epitopes of B and T cells.

MATERIALS AND METHODS

Cattle and Site Description

Between June and September 2019 and 2020, blood was randomly collected from 791 apparently healthy cattle (682 females and 109 males) reared in 165 farms located in 11 Tunisian governorates (Bizerte, Ariana, Manouba, Beja, Jendouba, Siliana, Kairouan, Kasserine, Zaghouan, Sousse, and Gabes) belonging to eight bioclimatic areas (subhumid, upper and lower humid, upper, middle, and lower semiarid, and upper and lower arid) (**Figure 1** and **Table 1**). Visited farms are small, enclosing a mean of 20 bovine heads with traditional and poorly maintained housing facilities. Analyzed cattle were aged between 6 months and 15 years old, and the majority belonged to the Friesian Pie Noire and

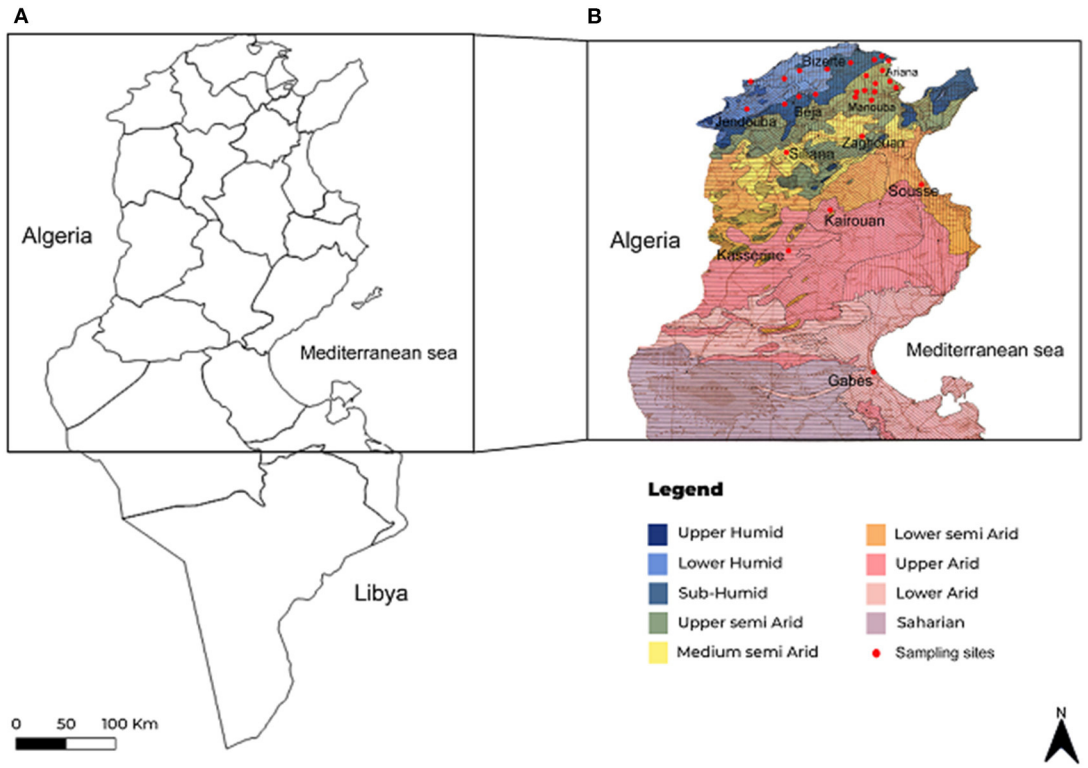

FIGURE 1 | Maps showing geographical position of studied regions in Tunisia **(A)** and sampling sites presented in investigated regions according to bioclimatic stages **(B)**.

Holstein breeds. In spite of the use of acaricide treatment, 42.7% of the surveyed animals were infested with ticks, particularly in the mammary region and the inner surface of the ears (**Table 1**).

Blood Sampling and DNA Extraction

Whole blood samples were collected from the jugular vein of dairy cattle and placed into sterile tubes containing ethylenediaminetetraacetic acid (EDTA). For each animal, gender, age, and presence or absence of ticks were noted. DNA was extracted from 300 µl volume of EDTA-preserved whole blood using the Wizard® Genomic DNA purification kit (Promega, Madison, WI, USA) according to the manufacturer's instructions. DNA quality and quantity were evaluated with a Qubit® dsDNA assays (Thermo Fisher Scientific) and stored at −20°C until use.

Molecular Detection of Anaplasmataceae and *Anaplasma marginale* Bacteria

EHR16SD and EHR16SR primers was used in a single PCR in order to detect all Anaplasmataceae bacteria by amplifying 345 bp of 16S rRNA gene with a thermal cycling profile as mentioned by Parola et al. (17) (**Supplementary Table 1**). Samples positive for Anaplasmataceae bacteria were used for the specific detection of *A. marginale* infection by using a single PCR with Amargmsp4F and Amargmsp4R primers amplifying 344 bp of *msp4* gene [(18); **Supplementary Table 1**]. For *A. marginale* genotyping and phylogeographic analysis, single PCRs were performed on *lipA* (538 bp) and *sucB* (808 bp) partial sequences used in MLST scheme developed earlier by Guillemi et al. (10), which showed better discriminative power than did other loci (14). The amplification profile was as described by Guillemi et al. (10). In order to assess the conservation of the OmpA protein vaccine candidate, the full *ompA* sequence (711 bp) was amplified with a single PCR by using AmOmpAF and AmOmpAR primers (**Supplementary Table 1**). The amplification conditions were as described by Futse et al. (8) (**Supplementary Table 1**). All PCRs were performed in a final volume of 50 µl containing 0.125 U/µl of Taq DNA polymerase (Biobasic Inc., Markham, ON, Canada), 1× PCR buffer, 1.5 mM of $MgCl_2$, 0.2 mM of dNTPs, 2 µl (50–150 ng) of genomic DNA, and 0.5 µM of primers. In this experiment, distilled water and DNA from bovine blood not infected with Anaplasmataceae bacteria, and DNA extracted from *A. marginale* (16) were used as negative and positive controls, respectively. PCR products were observed after electrophoretic migration in 1.5% agarose gels stained with ethidium bromide and under UV transillumination.

Obtaining Sequences and Phylogenetic Analysis

By using the same primers as for the amplification, a selection of PCR products generated from *lipA*, *sucB*, and *ompA* partial sequences were sequenced in both directions after purification

TABLE 1 | Molecular prevalence rates of Anaplasmataceae and *Anaplasma marginale* according to geographic, bioclimatic, and cattle-related risk factors.

Risk factors	Number	Anaplasmataceae		*A. marginale*	
		Positive (% ± CI[a])	*p*-Value	Positive (% ± CI[a])	*p*-Value
Governorates					
Bizerte	189	53 (28.04 ± 0.064)	0.000*	12 (6.35 ± 0.034)	0.006*
Ariana	68	16 (23.53 ± 0.100)		1 (1.47 ± 0.027)	
Manouba	197	49 (24.87 ± 0.060)		1 (0.51 ± 0.009)	
Siliana	35	0 (0)		0 (0)	
Jendouba	62	11 (17.74 ± 0.095)		2 (3.23 ± 0.043)	
Beja	64	24 (37.50 ± 0.118)		2 (3.13 ± 0.042)	
Kairouan	30	4 (13.33 ± 0.121)		0 (0)	
Kasserine	24	2 (8.33 ± 0.110)		1 (4.17 ± 0.079)	
Zaghouan	42	6 (14.29 ± 0.105)		0 (0)	
Sousse	33	1 (3.03 ± 0.058)		0 (0)	
Gabes	47	0 (0)		0 (0)	
Bioclimatic area					
Subhumid	142	65 (45.77 ± 0.081)	0.000*	13 (9.15 ± 0.047)	0.000*
Upper humid	59	11 (18.64 ± 0.099)		2 (3.39 ± 0.045)	
Lower humid	35	8 (22.86 ± 0.138)		0	
Upper semiarid	344	69 (20.05 ± 0.042)		3 (0.87 ± 0.009)	
Medium semiarid	77	6 (7.79 ± 0.059)		0	
Lower semiarid	33	1 (3.03 ± 0.058)		0	
Upper arid	54	6 (11.11 ± 0.083)		1 (1.85 ± 0.035)	
Lower arid	47	0		0	
Total	791	166 (20.99 ± 0.028)		19 (2.40 ± 0.010)	
Gender					
Male	109	14 (12.84 ± 0.062)	0.024*	5 (4.59 ± 0.038)	0.108
Female	682	152 (22.29 ± 0.031)		14 (2.05 ± 0.010)	
Age					
≤2 years	212	33 (15.57 ± 0.048)	0.060	8 (3.77 ± 0.025)	0.214
>2 and ≤5 years	241	52 (21.58 ± 0.051)		3 (1.24 ± 0.013)	
>5 years	338	81 (23.96 ± 0.045)		8 (2.37 ± 0.015)	
Breed					
Friesian pie noire	287	34 (11.85 ± 0.037)	0.000*	7 (2.44 ± 0.017)	0.138
Holstein	257	74 (28.79 ± 0.055)		2 (0.78 ± 0.010)	
Brown Swiss	109	27 (24.77 ± 0.080)		4 (3.67 ± 0.034)	
Cross	95	20 (21.05 ± 0.081)		3 (3.16 ± 0.034)	
Local	20	5 (25 ± 0.189)		1 (5 ± 0.095)	
Other breeds[b]	23	6 (26.08 ± 0.180)		2 (8.69 ± 0.115)	
Tick infestation					
Infested	338	90 (26.63 ± 0.047)	0.000*	13 (3.85 ± 0.020)	0.022*
Not infested	453	76 (16.78 ± 0.034)		6 (1.32 ± 0.010)	
Total	791	166 (20.99 ± 0.028)		19 (2.40 ± 0.010)	

[a]*CI: 95% confidence interval.*

[b]*Other breeds are Tarentaise (n = 12), Montbeliarde (n = 9), Charolais (n = 1), and Belgian Blue White (n = 1).*

**Statistically significant test.*

(**Supplementary Table 1**). The reaction was carried out by using a conventional Big Dye Terminator cycle sequencing ready reaction kit (Perkin Elmer, Applied Biosystems, Foster City, CA, USA) and an ABI3730XL automated DNA sequencer. Chromatograms were edited with Chromas Lite v 2.01. The DNAMAN program (Version 5.2.2; Lynnon Biosoft, Quebec, QC, Canada) was used to perform multiple sequence alignment and to translate nucleotide to amino acid sequences. BLAST analysis (http://blast.ncbi.nlm.nih.gov) was carried out to search nucleotide similarity (19). DNAMAN program was also used to estimate genetic distances calculated by using the maximum composite likelihood method (20). Phylogenetic trees were built by neighbor-joining method integrated in the same software (21). Robustness rates of the internal branches' nodes were

calculated based on a statistical support of 1,000 reiterations. Indeed, a total of 64, 76, and 42 *A. marginale* partial sequences, respectively, of *lipA*, *sucB*, and *ompA* genes from GenBank were included in this analysis. Obtained partial sequences from *A. marginale* Tunisian isolates were deposited under GenBank under accession numbers MZ221566 to MZ221579, MZ221580 to MZ221586, and MZ221587 to MZ221597 for *lipA*, *sucB*, and *ompA* genes, respectively.

Statistical Analysis

Exact confidence intervals (CIs) for prevalence rates at the 95% level were calculated. χ^2 and Fisher's exact tests integrated in the Epi Info 6.01 software (CDC, Atlanta) were used to perform a comparison of Anaplasmataceae and *A. marginale* prevalence rates among different categories for each risk factor and among different governorates and bioclimatic areas. Observed differences were considered to be statistically significant at a 0.05 threshold value. A chi square Mantel–Haenszel test was carried out in order to take into consideration any confusion factor.

OmpA Protein Conservation Assessment

Analysis of OmpA Protein Properties

ProtParam, a freely accessed online server (http://web.expasy.org/protparam/), was used to determine protein properties such as molecular weight, amino acid composition, isoelectric point (pI), stability index, grand average of hydropathicity (GRAVY), and estimated half-life. By using default parameters, the secondary structure of the protein was predicted from primary protein sequence by SOPMA online server (https://npsa-prabi.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_sopma.html).

Prediction of N-Glycosylation Sites, Transmembrane Topology, and Disulfide Bonds

N-Glycosylation sites in protein were predicted using NetNGlyc1.0 server (http://www.cbs.dtu.dk/services/NetNGlyc/). Transmembrane helices were predicted using TMHMM server 2.0 [http://www.cbs.dtu.dk/services/TMHMM/; (22)]. Disulfide bonds in protein were determined using DiANNA 1.1 web server [http://clavius.bc.edu/$\sim$clotelab/DiANNA/; (23)].

Antigenicity and Allergenicity Prediction

Antigenicity of OmpA protein was predicted by using VaxiJen 2.0 server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html) with default threshold of 0.5 for the determination of the antigenic protein (24). This server is used in order to predict the most antigenic proteins serving to be protective antigens or subunit vaccines.

AllerCatPro, a freely accessed online server, was used to determine the allergenicity of our proposed protein for vaccine development (25). This server predicts the allergenic potential of proteins based on similarity of their 3D protein structure and their amino acid sequence compared with those of known protein allergens (25).

B-Cell Epitope Identification

The search for potentially immunogenic epitopes in the analyzed protein sequence is generally carried out in order to identify epitopes essential for the creation of an effective vaccine. This approach considerably reduces the experiments leading to the design of vaccines and to the creation of an immunodiagnostic method. The aim of this prediction is to select the epitopes in a given antigen that would interact with B lymphocytes and initiate an immune response (26).

Linear B-cell epitopes were predicted using ABCpred [http://www.imtech.res.in/raghava/abcpred/; (27)] and Bepipred [http://tools.immuneepitope.org/bcell/; (28)] servers, and the epitopes in common were selected. For determining conformational epitopes, the OmpA protein sequences were submitted to the CBTope [http://www.imtech.res.in/raghava/cbtope/; (29)] and BepiPred 2.0 (30); web servers and the common epitopes that predict using these two programs were considered. The default settings were applied to all the tools used.

The predicted linear and conformational B-cell epitopes were further analyzed using the following tools: Emini et al. surface accessibility prediction (31), Fasman and Chou beta-turn prediction (32), Karplus and Schulz flexibility prediction (33), Parker et al. hydrophilicity prediction (34), and Kolaskar and Tongaonkar antigenicity (35). All these tools are available at IEDB analysis resource (http://tools.immuneepitope.org/bcell/), and the default settings were applied to all the used tools.

T-Cell Epitope Identification

The identification of the major histocompatibility complex class I (MHC class I) T-cell epitopes was performed by using the NetCTL 1.2 server (36). The selection method is based on the peptide MHC I binding, proteasomal C terminal cleavage, and transporter associated with antigen processing (TAP) transport efficiency. Epitope prediction was limited to 12 MHC-I supertypes. MHC-I binding and proteasomal cleavage were obtained via artificial neural networks, and the weight matrix was employed for the efficiency of TAP transport. The parameter used for this analysis was set at the threshold of 0.5 in order to obtain a sensitivity and specificity of 0.89 and 0.94, respectively, allowing the prediction of more epitopes for further analysis. A combined algorithm of MHC-I binding, TAP transport efficiency, and proteasomal cleavage efficiency was used to predict overall scores (36).

By using the IEDB server (http://tools.immuneepitope.org/mhcii/), MHC class II T-cell epitopes were predicted. The selection IEDB Recommended uses the Consensus approach (37), combining NN-align (38, 39), SMM-align (40), and CombLib and Sturniolo et al. (41) if any corresponding predictor is available for the molecule; otherwise, NetMHCIIpan is used (39, 42). The Consensus approach considers a combination of any three of the four methods, if available, where Sturniolo is a final choice. The predictive performances are based on large-scale evaluations of the performance of the MHC class II binding predictions (37, 43, 44).

The antigenicity of the predicted T-cell epitopes was assessed using the Kolaskar and Tongaonkar antigenicity (35) belonging to the IEDB analysis resource (http://tools.

immuneepitope.org/bcell/) using the standard threshold value of 1.030.

Epitope Conservancy Analysis

The conservation of predicted epitopes among different isolates was analyzed using IEDB epitope conservancy analysis tool [http://tools.immuneepitope.org/tools/conservancy/iedb input; (45)].

Three-Dimensional Structure Prediction, Refinement, and Validation of OmpA Protein

The three-dimensional structure prediction of OmpA protein was performed by using I-TASSER at http://zhanglab.ccmb.med.umich.edu/I-TASSER/ (46). I-TASSER uses a hierarchical approach to predict protein structure and function. A confidence score (C-score) in the range of (−5, 2) was used to evaluate the quality of the modeled structures, and a high C-score confirms a high confidence model. Pymol 1.7 was used to visualize the tertiary structures after modeling (47). After selecting the best three-dimensional models according to the C-score, the ModRefiner server (http://zhanglab.ccmb.med.umich.edu/ModRefiner/) was used to refine the selected structures (48). The quality of the refined structures was assessed by Ramachandran plot, by using the PROCHECK function (49) in PDBSUM tool (https://www.ebi.ac.uk/thorntonsrv/databases/pdbsum/Generate.html). The backbone conformation of the protein structures was verified by analyzing φ (Phi) and ψ (Psi) dihedral angles for each residue and finally classifies the residues into favorable, allowed, and outlier regions well-visualized in the Ramachandran plot (50). The selected B and T epitopes were schematically showed in their protein region after three-dimensional prediction using Pymol 1.7 (47).

TABLE 2 | Designation and information about sequencing of *Anaplasma marginale* genotypes identified in this study.

Gene	Isolate	Governorate (district)	GenBank[a]	Genotype	BLAST analysis
lipA	TunBvBz105	Bizerte (Utique)	MZ221566	lipATunGv1	100% MG807984
	TunBvBz106	Bizerte (Utique)	MZ221567		
	TunBvBz107	Bizerte (Utique)	MZ221568		
	TunBvAr312	Ariana (Raoued)	MZ221569		
	TunBvBz346	Bizerte (El Alia)	MZ221570		
	TunBvBz271	Bizerte (Ras Jebel)	MZ221571		
	TunBvBz362	Bizerte (Ghar El Melh)	MZ221572		
	TunBvBz108	Bizerte (Utique)	MZ221573	lipATunGv2	99.8% MG807984
	TunBvBz113	Bizerte (Utique)	MZ221574	lipATunGv3	99.4% MG807984
	TunBvBj489	Beja (Amdoun)	MZ221575	lipATunGv4	100% MG807970
	TunBvBj488	Beja (Amdoun)	MZ221576		
	TunBvBz274	Bizerte (Ras Jebel)	MZ221577		
	TunBvJa724	Jendouba (Tabarka)	MZ221578	lipATunGv5	100% MG807982
	TunBvJa742	Jendouba (Tabarka)	MZ221579		
sucB	TunBvBz107	Bizerte (Utique)	MZ221580	sucBTunGv1	100% MG808018
	TunBvAr312	Ariana (Raoued)	MZ221581		
	TunBvBz346	Bizerte (El Alia)	MZ221582		
	TunBvBz362	Bizerte (Ghar El Melh)	MZ221583		
	TunBvJa724	Jendouba (Tabarka)	MZ221584		
	TunBvJa742	Jendouba (Tabarka)	MZ221585		
	TunBvBz105	Bizerte (Utique)	MZ221586		
ompA	TunBvBz105	Bizerte (Utique)	MZ221587	ompATunGv1	100% CP000030
	TunBvBz107	Bizerte (Utique)	MZ221588		
	TunBvBz108	Bizerte (Utique)	MZ221589		
	TunBvBz113	Bizerte (Utique)	MZ221590		
	TunBvBj488	Beja (Amdoun)	MZ221591		
	TunBvBz346	Bizerte (El Alia)	MZ221592		
	TunBvAr312	Ariana (Hessiene)	MZ221593		
	TunBvBz362	Bizerte (Aousseja)	MZ221594		
	TunBvBz361	Bizerte (Aousseja)	MZ221595		
	TunBvKa639	Kasserine (Sebitla)	MZ221596		
	TunBvBz106	Bizerte (Utique)	MZ221597	ompATunGv2	99.9% CP000030

MG807984 is the GenBank accession number of the isolate "TunBv74/1" infecting cattle from Tunisia, and CP000030 is the GenBank accession number of the "St. Maries" strain infecting cattle in the United States. [a]GenBank accession number.

RESULTS

Molecular Prevalences of Anaplasmataceae and *Anaplasma marginale*

The overall prevalence rates of Anaplasmataceae and *A. marginale* were 20.99 and 2.40%, respectively. The statistically highest molecular prevalence of Anaplasmataceae is observed in cattle belonging to the governorate of Beja (37.50%, 24/64), while those belonging to the governorate of Sousse are the least infected (3.03%, 1/33) ($p < 0.001$). In addition, no cattle belonging to Siliana and Gabes governorates were infected with Anaplasmataceae bacteria. The highest molecular prevalence of *A. marginale* is recorded in cattle belonging to the governorate of Bizerte (6.35%, 28/189), while those from Manouba are the least infected (0.51%, 1/197). In addition, cattle belonging to the governorates of Siliana, Kairouan, Zaghouan, Sousse, and Gabes were not infected with this bacterial species ($p = 0.006$; **Table 1**).

The statistically highest molecular prevalence rates of Anaplasmataceae and *A. marginale* are recorded in cattle from the subhumid bioclimatic area estimated, respectively, at 45.77% (65/142) and 9.15% (13/142) ($p < 0.001$). Furthermore, females (22.29%, 152/682) are more infected with Anaplasmataceae than males (12.84%, 14/109) ($p = 0.022$). Holstein cattle (28.79%, 74/257) were significantly more infected with Anaplasmataceae ($p < 0.001$) than other breeds (**Table 1**). Cattle infested by ticks were statistically more infected with Anaplasmataceae (26.63%, 90/338) and *A. marginale* (3.85%, 13/338) than were those free

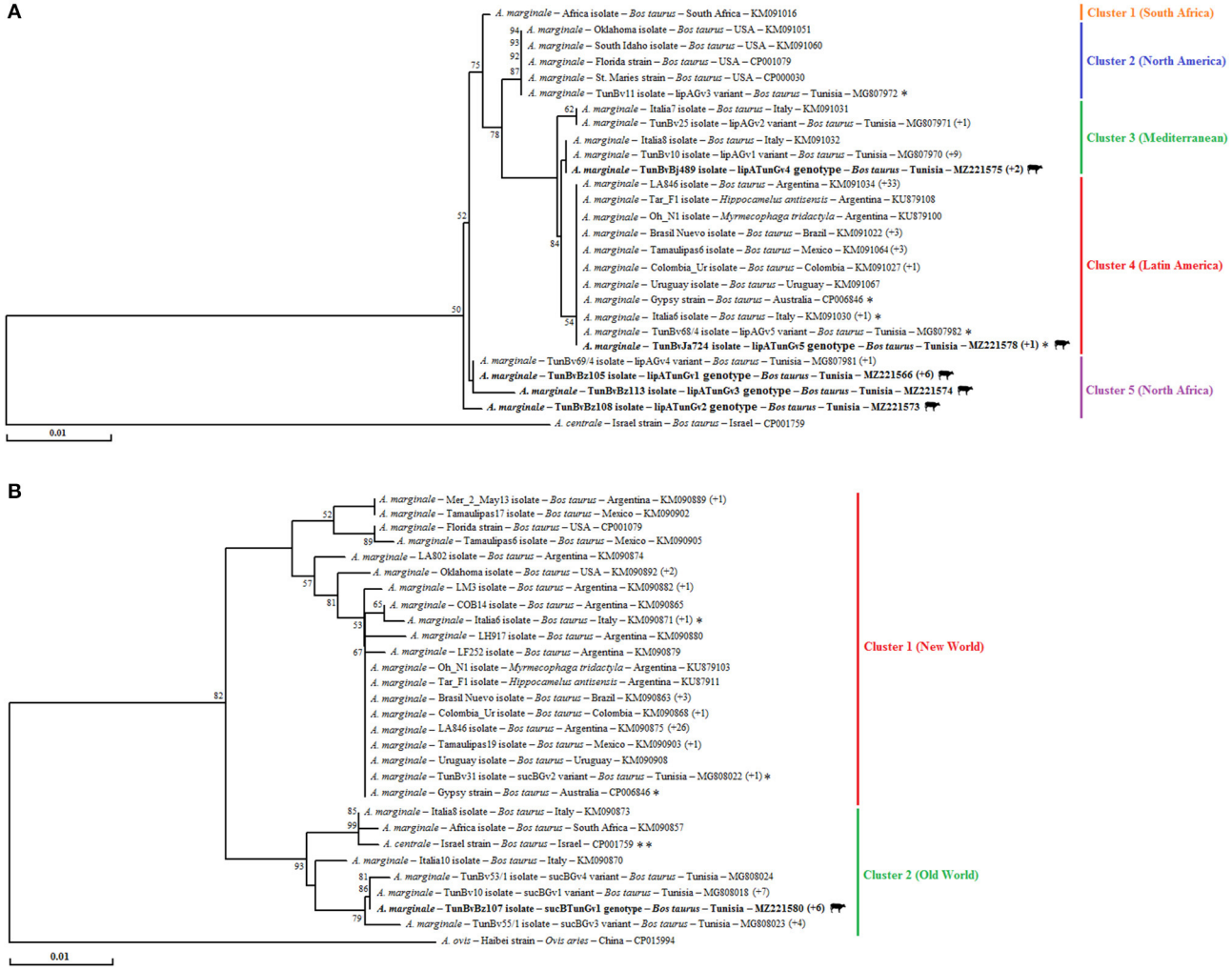

FIGURE 2 | Phylogenetic tree showing all *Anaplasma marginale* genetic variants based on multiple alignment of *lipA* **(A)** and *sucB* **(B)** partial nucleotide sequences (501 and 681 bp, respectively) using the "neighbor-joining" method. The numbers related to the nodes represent robustness rates over 1,000 iterations supporting the nodes (only rates >50% are shown). The host, strain or isolate, country of origin, and GenBank accession number are indicated. The sequences of *A. marginale* newly obtained in the present study are in bold and marked with a bovine picture. Sequences that are not classified into their appropriate geographic regions represented by clusters are indicated with an asterisk. The numbers that are in parentheses at the end of some sequences represent isolates or strains that are represented by an identical sequence from the isolate or strain present in the tree.

of ticks [16.78% (76/453) and 1.32% (6/453), respectively] ($p < 0.001$ and $p = 0.022$, respectively; **Table 1**).

Genotyping and Diversity Analysis

Anaplasma marginale LipA Partial Sequences

A total of five distinct genotypes (lipATunGv1 to lipATunGv5), which differ in nine nucleotide positions, were identified after the alignment of *lipA* partial nucleotidic sequences (501 bp) of 14 Tunisian isolates (**Table 2** and **Supplementary Tables 2**, **3**). Two genotypes (lipATunGv2 and lipATunGv3) showed nucleotide diversity as compared with all sequences presented in GenBank and are recognized to be new genetic variants (**Figure 2A** and **Table 2**). Nucleotide sequence homology rates between *lipA* genotypes obtained in this study were 98.4–99.8% (100% at the protein level) (**Supplementary Table 2**). Additionally, Tunisian genotypes were 98.4–100% similar to all *A. marginale* sequences analyzed in the phylogenetic tree (**Figure 2A** and **Supplementary Table 2**). Nucleotide homology rates decreased (86.2–87.2%) when the Tunisian variants are compared with the *Anaplasma centrale* reference sequence (CP001759) published in GenBank.

Multiple alignments of the five Tunisian genotypes, representing the 14 sequenced isolates, with the 73 *A. marginale* partial sequences obtained from GenBank, generated a phylogenetic tree made up of five main clusters (**Figure 2A**). The first cluster is formed exclusively by the South African strain. The second cluster consists mainly of isolates from North America (represented exclusively by the United States). The third cluster is formed by Mediterranean isolates from Italy and Tunisia. The fourth cluster mainly contains strains from Latin America like Mexico, Brazil, Colombia, Uruguay, and Argentina. The last cluster includes isolates infecting cattle located in North Africa represented until this study by Tunisia. The last cluster includes isolates exclusively infecting Tunisian cattle (**Figure 2A**).

Our isolates were assigned to the last three clusters (one genotype in the third and fourth clusters and three in the fifth). Particularly, the genotypes lipATunGv1 to lipATunGv3 grouped together in the fifth cluster with other isolates reported earlier in Tunisia. Within the third cluster, the lipATunGv4 genotype is included with other Mediterranean isolates from Tunisia and Italy. In the fourth cluster, the lipATunGv5 genotype was identical to numerous isolates mainly originating from Latin America such as Argentina, Brazil, and Mexico (**Figure 2A**).

Anaplasma marginale SucB Partial Sequences

Seven *sucB* partial sequences (681 bp) of the *A. marginale* Tunisian isolates were aligned, allowing the identification of a single genotype (sucBTunGv1) (**Table 2**). The sucBTunGv1 genotype was 97.5–100% identical to all *A. marginale* sequences analyzed in the phylogenetic tree giving homology rates between 96.9 and 100% at the protein level (**Supplementary Table 4**). In fact, 27 single-nucleotide polymorphisms (SNPs) were observed giving eight amino acid substitutions (**Supplementary Figure 1**). By comparing the revealed genotype to *A. centrale* reference sequence (CP001759), the identity rate was 98.7%.

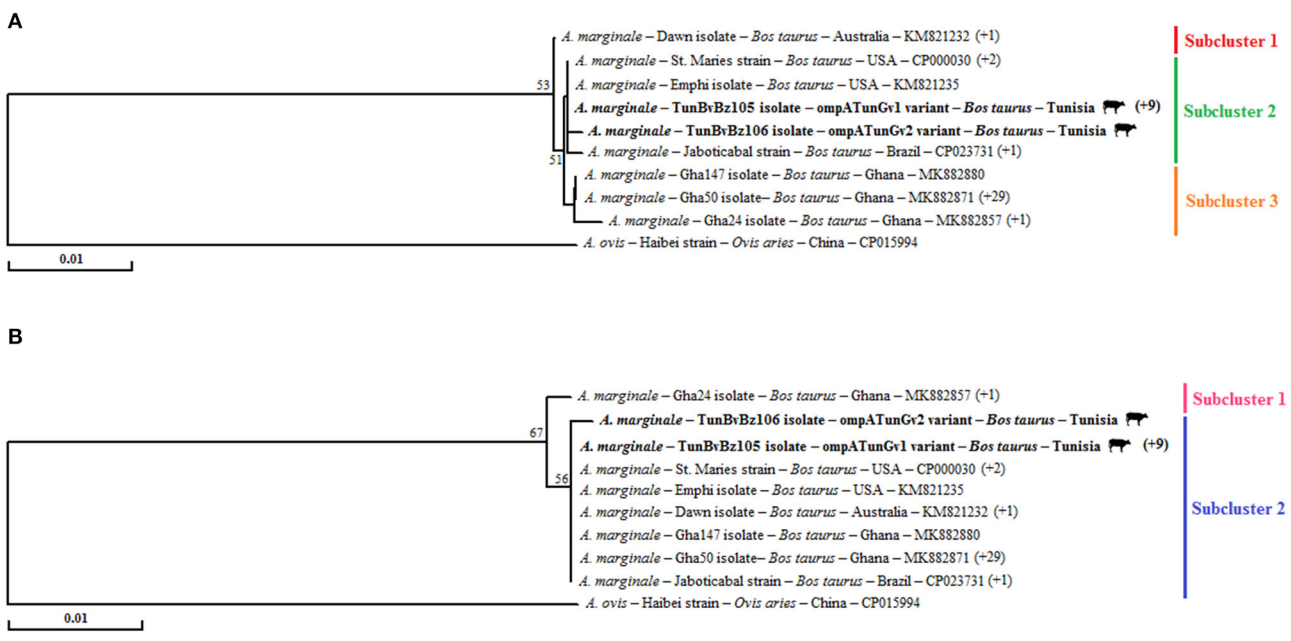

FIGURE 3 | Phylogenetic analysis of all available genetic variants of *Anaplasma marginale* based on the multiple alignment of partial nucleotide sequences (679 bp) of *ompA* gene **(A)** and their deduced amino acid sequences (236 AA) of the OmpA protein **(B)** using the "neighbor-joining" method. The numbers related to the nodes represent the robustness rates over 1,000 iterations (only the rates >50% are represented). The host, strain or isolate, country of origin, and GenBank accession number are listed. The sequences of *A. marginale* newly obtained in this study are in bold and marked with a bovine picture. The numbers that are in parentheses at the end of some sequences represent isolates or strains that are represented by an identical sequence from the isolate or strain present in the tree.

The phylogenetic tree based on the alignment of our genotype with all *sucB* partial sequences of *A. marginale* found in GenBank and one *A. centrale* reference sequence added as an outgroup shows the occurrence of two main clusters (**Figure 2B**). Except the two Italian isolates (Italia6 and Italia7), the Tunisian TunBv31 isolate and the Australian strain (Gypsy), the first cluster includes all isolates from New World countries (i.e., United States, Mexico, Brazil, Colombia, Uruguay, and Argentina). The second cluster contains several isolates from Old World countries like Italy (isolates Italia8 and Italia10) and South Africa (isolate Africa) in addition to the Israeli *A. centrale* strain (**Figure 2B**). The sucBTunGv1 genotype representing the seven Tunisian revealed that isolates were assigned to the second cluster (**Figure 2B**). Within this cluster, sucBTunGv1 forms, with other previously revealed Tunisian isolates, a distinct subcluster relatively distant from the *A. centrale* reference strain grouped with other isolates from the Old World countries only represented by Italy (isolates Italia8 and Italia10) and South Africa (isolate Africa) (**Figure 2B**).

Anaplasma marginale OmpA Partial Sequences

Partial *ompA* sequences (679 bp) of the 11 Tunisian *A. marginale* isolates revealed in this study were aligned, allowing the identification of two different genotypes (ompATunGv1 and ompATunGv2) (**Table 2**). The ompATunGv1 genotype was found to be identical to the "St. Maries" strain (GenBank accession number CP000030) infecting cattle in United States, and the ompATunGv2 genotype showed a degree of nucleotide diversity compared with all *ompA* sequences published in GenBank and was considered as a novel genetic variant (**Table 2**). Nucleotide homology rates between the two genotypes obtained in this study and all the other available genetic variants were from 99.1 to 99.9% (98.2–100% at the amino acid sequence) (**Supplementary Table 5**). In fact, nine SNPs were observed representing five non-synonymous substitutions (**Supplementary Table 6**). By comparing our genotypes ompATunGv1 and ompATunGv2 with those belonging to the species closest to *A. marginale*, sequence identity was more important with *Anaplasma ovis* reference sequence (CP015994) (85.0 and 85.2%) compared with that of *A. centrale* (CP001759) (81.5 and 81.3%, respectively).

For this gene, phylogenetic trees based on the alignment nucleotide and amino acid sequences of our *A. marginale* isolates with those found in GenBank show the presence of a single cluster (**Figures 3A,B**). This finding was confirmed by calculating the rates of concerned nodes, which did not exceed 53 and 67%, respectively, based on nucleotide and amino acid sequences (**Figures 3A,B**). In tree based on nucleotide sequences, this unique cluster is formed by three subclusters. The first includes one isolate and one strain from Australia. The second is formed with all isolates and strains from New World countries like the United States and Brazil. The third subcluster contains all the Ghanaian isolates (**Figure 3A**). Tunisian isolates were assigned to the second subcluster. In particular, the ompATunGv1 genotype is grouped with all strains originating from the United States, while the ompATunGv2 genotype is classified separately in this same subcluster (**Figure 3A**). In the tree based on amino acid sequences, the unique cluster is composed by two subclusters. The first formed with only one Ghanaian isolate (Gha24, MK882857). The second is formed with all isolates from Tunisia, isolates and strains from New World countries like the United States and Brazil, and the remaining Ghanaian isolates (**Figure 3B**).

OmpA Protein Conservation Assessment

OmpA Protein Properties

By taking the amino acid sequence deduced from the genetic variant V1 (MK882880) as a reference, the primary OmpA amino acid sequence was given as input for analysis by using ExPASy ProtParam tool. OmpA protein consisted of 236 amino acids with 13.1% of positively charged amino acids (arginine and lysine) and 16.1% of negatively charged amino acids (aspartate and glutamate). The molecular weight of the protein was estimated to be about 25,686 Da. The theoretical isoelectric point (pI) was about 5.32, predicting that protein is negatively charged at neutral pH. The extinction coefficient of the protein was estimated considering water as solvent at 280 nm. The extinction coefficients were computed to be 17,210 and 16,960 M^{-1} cm^{-1} when assuming all pairs of cysteines form cystines and when considering all pairs of cysteines are reduced, respectively.

The estimated half-lives of the protein were 30, >20, and >10 h in mammalian reticulocytes (*in vitro*), yeast (*in vivo*), and *Escherichia coli* (*in vivo*), respectively. The instability index was predicted to be 55.55, classifying it to be an unstable protein. The aliphatic index was estimated to be 79.75, and GRAVY was calculated to be −0.317.

SOPMA secondary structure prediction method was used to analyze the secondary structure of OmpA protein by giving the primary sequence of the protein as input. Other parameters were studied by setting the default threshold values. The protein was predicted to be made of 30.51% of alpha-helix (Hh), 22.03% of extended strand (Ee), 7.20% of beta-turn (Tt), and 40.25% of random coil (Cc) (**Figure 4**).

Predicted Disulfide Bonds in OmpA Protein

DIANNA 1.0 web server was employed to predict the disulfide bonds in protein. This server predicted two disulfide bonds by giving primary amino acid sequence as input. The server algorithm consists of five steps. In the first step, the submitted input sequence was executed in PSIBLAST. In step 2, the secondary structure of the protein was predicted using PSIPRED. In step 3, the oxidation state of the disulfide was estimated. In step 4, the disulfide bonds were computed using a diresidue neural network. In the end, the predicted disulfide bonds were weighted by Ed Rothberg's implementation of the Edmonds–Gabow maximum weight matching algorithm. Disulfide bonds were to be formed between positions 9–155 and 19–109.

Transmembrane Helices and N-Glycosylation Sites in OmpA Protein

Transmembrane helices were predicted by using the TMHMM server. Indeed, an absence of transmembrane helices was noted in OmpA protein. Asparagine in NXS/T sequence, where N is asparagine, X is any amino acid, S is serine, and T is threonine,

A

Variant	V1		V2		V3		V4		GV1		GV2		GV3		OmpATnGv1		OmpATnGv2	
Sequence length :	236	100.00%	236	100.00%	236	100.00%	236	100.00%	236	100.00%	236	100.00%	236	100.00%	225	100.00%	225	100.00%
Alpha helix (Hh) :	72	30.51%	72	30.51%	70	29.66%	72	30.51%	70	29.66%	86	36.44%	69	29.24%	69	30.67%	67	29.78%
3_{10} helix (Gg) :	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Pi helix (Ii) :	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Beta bridge (Bb) :	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Extended strand (Ee) :	52	22.03%	52	22.03%	53	22.46%	52	22.03%	53	22.46%	46	19.49%	53	22.46%	46	20.44%	50	22.22%
Beta turn (Tt) :	17	7.20%	17	7.20%	16	6.78%	17	7.20%	16	6.78%	18	7.63%	13	5.51%	14	6.22%	10	4.44%
Bend region (Ss) :	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Random coil (Cc) :	95	40.25%	95	40.25%	97	41.10%	95	40.25%	97	41.10%	86	36.44%	101	42.80%	96	42.67%	98	43.56%
Ambiguous states (?) :	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%
Other states :	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%	0	0.00%

B

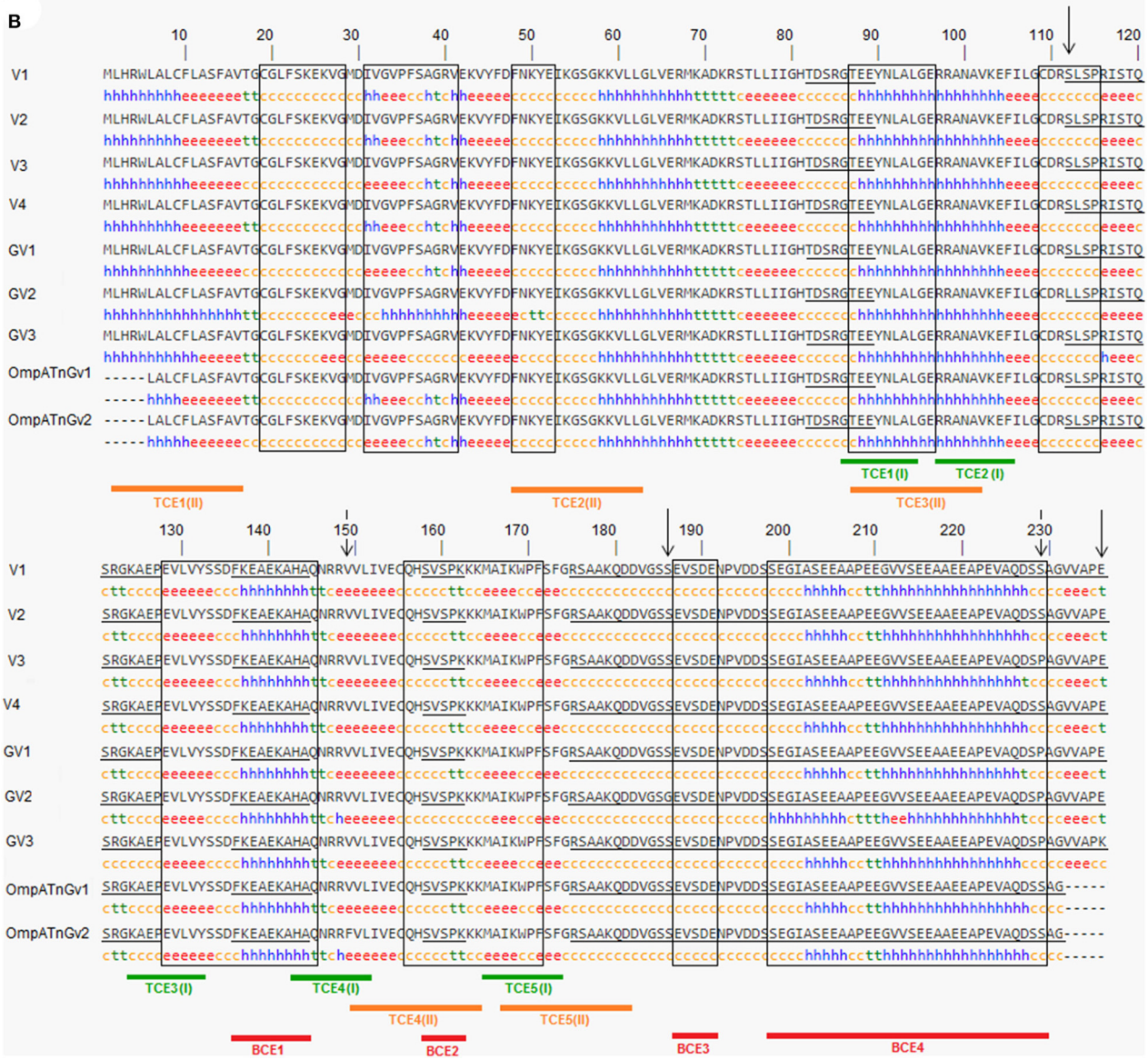

FIGURE 4 | Information on the secondary structure composition of OmpA proteins deduced from the nine genetic variants representing all *Anaplasma marginale* strains and isolates available in GenBank **(A)** and multiple alignment of predicted amino acid sequences and secondary structures of the OmpA proteins representing all *A. marginale* strains and isolates available in GenBank **(B)**. Sequential and conformational epitopes linked to B cells are, respectively, underlined and boxed. Red lines represent epitopes (BCE1–BCE4), which are in common between sequential and conformational epitopes. The green and orange lines represent the epitopes [TCE1(I)–TCE1(I)] linked to MHC class I T cells and the epitopes [TCE1(II)–TCE1(II)] binding to MHC class II T cells. The positions where there are amino acid substitutions between the analyzed protein sequences are represented by descending arrows.

is generally glycosylated in proteins produced by eukaryotes, archaea, and very rarely bacteria. In addition, a prediction of *N*-glycosylation sites in OmpA protein was performed by the NetNGlyc 1.0 server. A default potential threshold of 0.5 was considered for this analysis, which showed a total absence of glycosylation sites in all OmpA proteins deduced from all available genetic variants.

Antigenicity and Allergenicity of OmpA Whole Protein

Estimation of antigenicity and allergenicity are crucial in the selection of the candidate protein, which can be used in a vaccine using unilamellar liposomes. The antigenicity of the OmpA protein was predicted by using the VaxiJen v2.0 server by adding the primary sequence of the protein as an additional input at a threshold of 0.5 and with the selection of bacteria as model. The server predicted that OmpA protein can be an antigen with an overall antigenicity prediction score of 0.7675.

Allergenicity of OmpA protein was obtained from AllerCatPro online server by giving the primary sequence of the protein as an entry. A weak evidence of allergenicity for this protein was predicted with a rate of 100%.

Tertiary Structure Prediction, Refinement, and Validation of the OmpA Protein

Five 3-dimensional structures were predicted for the OmpA protein sequence by using the I-TASSER server. Of these, the

TABLE 3 | Linear epitopes of the OmpA protein of *Anaplasma marginale* predicted using BepiPred and ABCpred and epitope conservancy result.

Number	Name	Start	End	Epitope sequences	Length	Epitope conservancy (conserved sequence/total)
1	SE1	82	89	TDSRGTEE	8	100% (9/9)
2	SE2	113	128	S(L)LSPRISTQSRGKAEP	16	88.9% (8/9)
3	SE3	136	144	FKEAEKAHA	9	100% (9/9)
4	SE4	158	162	SVSPK	5	100% (9/9)
5	SE5	175	192	RSAAKQDDVGSS(G)EVSDEN	18	88.9% (8/9)
6	SE6	193	210	PVDDSSEGIASEEAAPEE	18	100% (9/9)
7	SE7	211	228	GVVSEEAAEEAPEVAQDS	18	100% (9/9)
8	SE8	229	236	S(P)AGVVAPE(K)	8	55.6% (5/9)

The reference sequence considered in this analysis is that deduced from the genetic variant V1 (GenBank accession number MK882880). The letters found in parentheses represent the amino acids substituted in at least one of the proteins deduced from genetic variants other than the reference variant V1.

TABLE 4 | B-cell conformational epitopes of the OmpA protein of *Anaplasma marginale* predicted using BepiPred 2.0 and CBTope and epitope conservancy result.

Number	Name	Start	End	Epitope sequences	Length	Epitope conservancy (conserved sequence/total)
1	CE1	19	28	CGLFSKEKVG	10	100% (9/9)
2	CE2	31	41	IVGVPFSAGRV	11	100% (9/9)
3	CE3	48	52	FNKYE	5	100% (9/9)
4	CE4	87	96	TEEYNLALGE	10	100% (9/9)
5	CE5	109	115	CDRS(L)LSP	7	88.9% (8/9)
6	CE6	128	145	EVLVYSSDFKEAEKAHAQ	18	100% (9/9)
7	CE7	156	171	QHSVSPKKKMAIKWPF	16	100% (9/9)
8	CE8	187	191	EVSDE	5	100% (9/9)
9	CE9	198	229	SEGIASEEAAPEEGVVSEEAAEEAPEVAQDSS(P)	32	55.6% (5/9)

The reference sequence considered in this analysis is that deduced from the genetic variant V1 (GenBank accession number MK882880). The letters found in parentheses represent the amino acids substituted in at least one of the proteins deduced from genetic variants other than the reference variant V1.

TABLE 5 | Common epitopes between B-cell linear and conformational peptides obtained from *Anaplasma marginale* OmpA protein by using BepiPred, ABCpred, BepiPred 2.0 and CBTope, and epitope conservancy result.

Number	Name	Start	End	Epitope sequences	Length	Epitope conservancy (conserved sequence/total)
1	BCE1	136	144	FKEAEKAHA	9	100% (9/9)
2	BCE2	158	162	SVSPK	5	100% (9/9)
8	BCE3	187	191	EVSDE	5	100% (9/9)
9	BCE4	198	229	SEGIASEEAAPEEGVVSEEAAEEAPEVAQDSS(P)	32	55.6% (5/9)

The reference sequence considered in this analysis is that deduced from the genetic variant V1 (GenBank accession number MK882880). The letters found in parentheses represent the amino acids substituted in at least one of the proteins deduced from genetic variants other than the reference variant V1.

best structure was selected according to the highest C-score. The resulting structure was refined by using the ModRefiner server. The best refined model was selected based on the results of the Ramachandran plot (**Supplementary Figure 2B**). In fact, 48.5, 36.4, 11.1, and 4.0% of the residuals in the initial model were, respectively, in main, authorized, generous, and non-authorized regions. However, in the best refined model, 74.2, 21.7, 2, and 2% of the tailings were, respectively, in the main, authorized, generous, and prohibited regions (**Supplementary Figure 2B**).

Predicted B-Cell Epitopes of OmpA Protein

Based on ABCpred and BepiPred servers, eight common linear epitopes (SE1–SE8) are selected (**Table 3**). In addition, nine conformational B-cell epitopes (CE1–CE8) of OmpA protein were predicted using Bepipred 2.0 and CBTope programs (**Table 4**). By combining the results from the four servers, four epitopes (BCE1–BCE4) common between B-cell linear and conformational peptides were obtained from *A. marginale* OmpA protein (**Table 5**). By analyzing the secondary structures

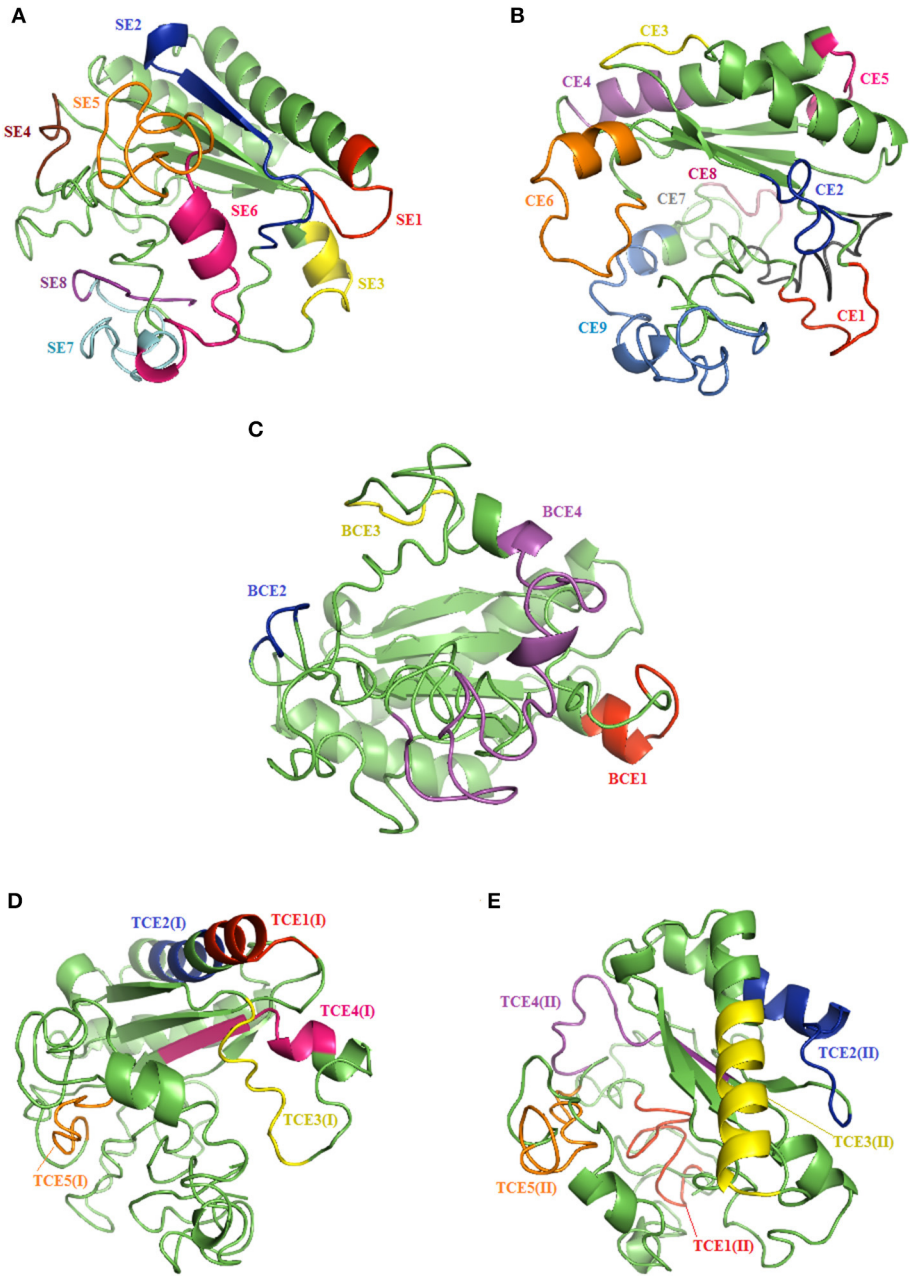

FIGURE 5 | Results of the tertiary structure prediction of the reference OmpA protein (V1) showing linear **(A)**, conformational **(B)**, and common **(C)** epitopes bound to B cells, and MHC class I **(D)** and II **(E)** T-cell epitopes.

of the proteins deduced from the nine genetic variants, all the sequential and/or conformational epitopes selected except SE3, SE7, and CE4 contain high proportions of extended strands and random coils (**Figure 4**).

All of these epitopes were analyzed for a set of factors that determine the potentiality for B-cell epitopes (e.g., surface accessibility, hydrophilicity, secondary structures, flexibility, and antigenicity). Combining the results of hydrophilicity (**Supplementary Figure 3A**), beta-turn (**Supplementary Figure 3B**), surface accessibility (**Supplementary Figure 3C**), and flexibility (**Supplementary Figure 3D**), we found that all revealed sequential and/or conformational epitopes could be potential B-cell epitopes. By analyzing the antigenicity results from Kolaskar and Tongaonkar, we found that all epitopes are potentially antigenic except for the CE3 and BCE1 epitopes (**Supplementary Figure 4**).

The IEDB conservancy analysis tool analyzed the conservancy of the predicted B-cell epitopes, which are presented in **Tables 3**–**5**. The results showed that the epitope conservation rate was 100% for most epitopes, allowing their total preservation within the different *A. marginale* isolates, while, for the rest of the epitopes for which the conservation rates are 88.9 and 55.6%, the change of a few amino acids did not remove or modify either sequential or conformational epitopes (**Figure 5** and **Tables 3**–**5**).

Predicted T-Cell Epitopes of OmpA Protein

NetCTL server of the IEDB analysis resource was used to predict MHC class I T-cell epitopes. We gave the primary protein sequence as input and left the values of 0.15 and 0.05 by default, relative to weights for C-terminal cleavage and TAP transport efficiency, respectively. All serotypes ($n = 12$) were selected for the study. A value of 0.5 was given as an input, allowing the screening of epitopes at a sensitivity of 89% and a specificity of 94%. Among several epitopes predicted by the NetCTL server, we only selected the best five [TCE1(I)–TCE5(I)] based on the high combinatorial score of NetCTL. **Table 6** shows MHC class I T-cell epitopes predicted by NetCTL, which are related to the A1, B27, A1, B8, and B58 supertypes, respectively. The epitopes are ordered according to the decreasing order of the NetCTL score.

In order to filter the antigenic epitopes from the non-antigenic epitopes, we again used the Kolaskar and Tongaonkar antigenicity prediction tool at the IEDB analysis resource; however, this time, the window length was set to 9. From Kolaskar and Tongaonkar antigenicity prediction (**Supplementary Figure 4**), we could conclude that all selected epitopes (based on NetCTL score) were antigenic noting that the epitopes TCE2(I), TCE3(I), and TCE4(I) are more antigenic than TCE1(I) and TCE5(I). In addition, all predicted epitopes were 100% conserved in all *A. marginale* isolates except the TCE4(I) epitope, which contains a single mutation at position 149 in a single variant, which did not cause deletion or modification of this epitope (**Figure 4** and **Table 6**).

For predicting the epitopes binding to MHC class II alleles, we used IEDB online server. The server predicts the MHC class II binding regions from the primary amino acid sequence using uses the Consensus approach, combining NN-align, SMM-align, CombLib, and Sturniolo. The primary amino acid sequence of OmpA protein (V1, MK882880) was given as input in FASTA format. Of the several epitopes predicted by the IEDB server, we considered only the top five epitopes [TCE1(II)–TCE5(II)] based on the low adjusted rank score, which means high MHC class II binders. **Table 7** lists the predicted MHC class II T-cell epitopes by NetCTL, which are binding to MHC class II alleles HLA-DRB1*15:01, HLA-DRB5*01:01, HLA-DRB5*01:01, HLA-DRB5*01:01, and HLA-DRB5*01:01. The epitopes are ordered based on the ascending order of the adjusted rank score. To filter antigenic epitopes from non-antigenic epitopes, we again used Kolaskar and Tongaonkar antigenicity prediction tool at IEDB analysis resource, and the window length was set to 9. From Kolaskar and Tongaonkar antigenicity prediction (**Supplementary Figure 4**), we could conclude that all selected epitopes were antigenic noting that the epitopes TCE1(II), TCE2(II), and TCE4(II) are more antigenic than TCE3(I) and TCE5(I). In addition, all predicted epitopes binding to MHC class II alleles were 100% conserved in all *A. marginale* isolates (**Figure 4** and **Table 7**).

In order to assess the antigenic features of the epitopes binding to MHC class I and II alleles, we predicted their secondary structure using SOPMA Server software. A greater proportion of extended strands and random coils present in the structure of all predicted epitopes except TCE1(I), TCE2(I), and TCE3(II) corresponded with an increased likelihood of these peptides forming antigenic epitopes. The predicted secondary structure results for the potential epitopes binding to MHC class I and II alleles are demonstrated in **Figure 5**.

DISCUSSION

The present study is the first molecular–epidemiological report on *A. marginale* infection in cattle covering several geographical and bioclimatic areas extending from the north to the south of Tunisia. Compared with other surveys carried out in Tunisia and other countries, the overall infection rate (2%) estimated in our study is similar to that observed in Turkey (2.3%) (51), Egypt (3.7%) (52), Sudan (6.1%) (53), Kenya (7.8%) (54), and Mongolia (8.7%) (55). However, this rate is lower than that reported in Algeria (11.1%) (56), Pakistan (16.3%) (57), Morocco (21.9%) (58), and other regions from Tunisia (31.5%) (11). Compared with other studies from African countries, *A. marginale* prevalence rates were significantly higher in cattle from Zambia (47.9%) (59), Kenya (31–96.2%) (60, 61), South Africa (60%) (62), Nigeria (75.9%) (63), and Madagascar (89.7%) (64). Indeed, these variations in *A. marginale* prevalence rate between the different countries and regions of the same country could be due to several factors, in particular the sampling seasons and bioclimatic zones, the type of used molecular assays and associated genes, the variation of incriminated arthropod vectors, the susceptibility of animal breeds, and/or other risk factors related to herd management (2, 15).

In this study, a significant discrepancy in *A. marginale* infection prevalence was revealed among bioclimatic areas. In

TABLE 6 | The selected best five potential epitopes of the OmpA protein of *Anaplasma marginale* binding to MHC class I alleles, on the basis of their overall score predicted by the NetCTL server, and epitope conservancy result.

Number	Name	Start position	End position	Peptide sequence (9-mer)	Overall score (nM)	Supertypes	Epitope conservancy (conserved sequences/total)
1	TCE1(I)	86	95	GTEEYNLAL	1.5946	A1	100% (9/9)
2	TCE2(I)	97	106	RRANAVKEF	1.8235	B27	100% (9/9)
3	TCE3(I)	124	133	KAEPEVLVY	2.0026	A1	100% (9/9)
4	TCE4(I)	143	152	HAQNRRV(F)VL	2.0276	B8	88.9% (8/9)
5	TCE5(I)	165	174	MAIKWPFSF	1.9799	B58	100% (9/9)

The reference sequence considered in this analysis is that deduced from the genetic variant V1 (GenBank accession number MK882880). The letters found in parentheses represent the amino acids substituted in at least one of the proteins deduced from genetic variants other than the reference variant V1.

TABLE 7 | The selected best five potential epitopes binding to MHC class II alleles, on the basis of their adjusted rank predicted by the IEDB server and epitope conservancy result.

Number	Name	Start	End	Peptide sequence (15-mer)	Adjusted rank	Allele/haplotype	Epitope conservancy (conserved sequences/total)
1	TCE1(II)	2	16	LHRWLALCFLASFAV	1.90	HLA-DRB1*15:01	100% (9/9)
2	TCE2(II)	48	62	FNKYEIKGSGKKVLL	1.60	HLA-DRB5*01:01	100% (9/9)
3	TCE3(II)	87	101	TEEYNLALGERRANA	2.40	HLA-DRB5*01:01	100% (9/9)
4	TCE4(II)	150	164	VLIVECQHSVSPKKK	3.60	HLA-DRB5*01:01	100% (9/9)
5	TCE5(II)	167	181	IKWPFSFGRSAAKQD	2.60	HLA-DRB5*01:01	100% (9/9)

The reference sequence considered in this analysis is that deduced from the genetic variant V1 (GenBank accession number MK882880). The letters found in parentheses represent the amino acids substituted in at least one of the proteins deduced from genetic variants other than the reference variant V1.

fact, cattle from subhumid area were more infected than those from other bioclimatic areas. This difference, which was also reported in cross-sectional and longitudinal surveys performed on *A. marginale* infection in cattle from Tunisia (11, 15, 16) and Morocco (58), is probably caused by the effect of bioclimatic conditions on the phenology and the distribution of tick vectors and biting flies. Additionally, overall, *A. marginale* prevalence rate differed statistically among geographic regions. This discrepancy may be mainly due to differences in husbandry practices, farm management, tick control programs, and/or wildlife reservoirs (2, 58) but also to bias in sample recruitment. However, in spite of this, our study has confirmed that cattle infested by ticks were statistically more infected by *A. marginale* than those free of ticks. Our results are in agreement with other Tunisian studies that reported that cattle and small ruminants infested with ticks were statistically more infected with *Anaplasma* spp. than those free of ticks (2, 11, 65).

During the last two decades, several methods and markers have been developed to study the phylogeography of different worldwide *A. marginale* isolates. Most of the phylogenetic analyses of *A. marginale* strains were performed using partial sequences of genes that encode merozoite surface proteins (MSPs), mainly *msp4* and *msp1α* (11, 66, 67). However, due to the high degree of sequence diversity in endemic areas, *msp4* gene did not provide phylogeographic information on a global scale, but this gene could be useful in regional-level strain comparisons and could provide important data on host–pathogen coevolution and vector–pathogen relationships (11, 13, 68, 69). On the other hand, *msp1α* partial sequences did not provide a high phylogeographic resolution given that this gene is subjected to a positive selection pressure and appears to be rapidly evolving (67).

Recently, an MLST approach was used in order to gain more complete knowledge about the evolution and the genetic diversity of *A. marginale* strains. This typing method theoretically constitutes an interesting alternative, mainly by its multi-locus power, and offers the advantage that the selection of target loci does not require a complete knowledge of the whole genome but only by using generally seven housekeeping genes sequenced from studied isolates. Thereby, Guillemi et al. (10) developed and applied this method on *A. marginale*; however, by studying these concatenary sequences, they did not find an evident association between the geographic regions and the genetic variants isolated from different isolates from several regions of the world. For this reason, Ben Said et al. (14) examined each of these seven markers independently using the single locus analysis method. Phylogenetic analysis of each locus revealed that out of the seven analyzed genes, two (*lipA* and *sucB*) appear to be more interesting phylogeographically compared with other genes (14).

Therefore, in this study, we choose to assess the phylogeography of our *A. marginale* isolates by the genetic analysis of *lipA* and *sucB* markers. The molecular study based on *lipA* partial sequence revealed that, out of the 14 analyzed isolates, five different genotypes, two of which were novel, were identified (**Table 2**). This relatively high heterogeneity in relation to the number of infected cattle probably reflects movements of cattle between different countries and regions of the same country. Based on the same gene, similar results were observed by analyzing *A. marginale* isolates from Italy (10) and Tunisia (14).

Phylogenetic analysis based on *lipA* gene allowed the classification of our isolates according to five main clusters strongly supported by bootstrap values ≥75% with some minor exceptions [only six (6.87%) sequences out of 87 are not classified in their appropriate geographic regions]. In particular, this marker clearly differentiates between strains from South African (cluster 1), North American (cluster 2), Mediterranean (cluster 3), Latin American (cluster 4), and North African (cluster 5) countries (**Figure 2A**). Most of our Tunisian isolates (10/12) were assigned to the third and fifth clusters, confirming the discriminative power of *lipA* gene and a greater heterogeneity of our isolates compared with those found in other countries as previously suggested by Ben Said et al. (14). This finding could be due to the importance of cattle mobility through commercial exchanges between Tunisian regions and with other neighboring countries.

Moreover, *sucB* partial sequence also allows classifying strains according to geographical regions. However, this classification is not according to all or part of continents as for *lipA* gene but according to the New (cluster 1) and the Old World (cluster 2) (**Figure 2B**) as recently suggested by Ben Said et al. (14). This finding was strongly supported by a high bootstrap value estimated at 82% and the classification of 76 (93.82%) *sucB* partial sequences out of 81 in their appropriate geographic regions (**Figure 2B**). Otherwise, sequence analysis showed that *sucB* partial sequence was conserved in Tunisian *A. marginale* isolates. Indeed, only one genotype (sucBTunGv1) was revealed in seven sequenced samples. This is in agreement with the results of Guillemi et al. (10), which showed low diversity given that only one genotype was recorded in 39 Latin American isolates. In addition to sucBGv2, sucBGv3, and sucBGv4 genotypes earlier reported by Ben Said et al. (14), a genotype (sucBTunGv1) previously described in Tunisia (sucBGv1) was also detected in seven infected cattle from five different studied regions and, therefore, appears to be predominant in our country (**Figure 2B** and **Table 2**).

One of the main limitations to the development of an effective vaccine against *A. marginale* is the diversity of strains but also the inability to produce cross-protection against various isolates with a single vaccine. Therefore, the search for potential vaccine antigen candidates has focused on identifying outer membrane proteins that are widely conserved in remote geographic areas (8). Recently, the protein OmpA, annotated Am854 in the *A. marginale* genome, has been identified as an adhesin playing an essential role in the entry of *A. marginale* bacteria in mammalian and tick cells (9). Therefore, this protein could serve as a highly relevant vaccine antigen candidate (8). In fact, the conservation of the OmpA protein in different regions of the world is essential for the development of an international commercial vaccine. In Tunisia, where potentially pathogenic *A. marginale* strains can be ubiquitous in our farms (14), there is an almost complete lack of information regarding the extent of genetic variation in vaccine candidate proteins like OmpA protein.

In this study, a molecular and phylogenetic study followed by a physicochemical characteristics analysis and a prediction of B- and T-cell epitopes, and secondary and tertiary structures of the deduced OmpA proteins were performed from all available protein variants. Subsequently, an evaluation of the conservation of this protein in its linear, secondary, and tertiary forms and of the predicted epitopes was established between OmpA variants deduced from Tunisian isolates and those published in GenBank.

Genetic analysis of nucleotide and amino acid sequences and phylogenetic study based on *ompA* gene and its deduced protein demonstrated that the OmpA antigen appears to be highly conserved between isolates from geographically diverse worldwide regions. Indeed, a maximum of five amino acid differences were recorded between all OmpA protein variants available in GenBank and the Tunisian variants revealed in this study with amino acid sequence homology >98.2% (**Supplementary Tables 5**, **6**). Our result agrees with that of Futse et al. (8), who demonstrated a high conservation of the OmpA protein in its linear form in Ghanaian *A. marginale* strains by comparing them to the reference variant V1 representing St. Maries, Virginia, Kansas 6DE, Colville C51 and C52, and Nayarit (MX) N3574 strains.

The creation of epitope-based vaccines is a difficult and highly specific technology, requiring the use of molecular biology and immunology techniques. Obtaining the necessary information on the epitopes of the candidate antigen is one of the crucial steps in vaccine design. During this decade, the evolution of bioinformatics tools allowed the improvement of the quality of epitope prediction.

Indeed, many factors influence the vaccine efficacy as the physicochemical parameters, the structure, and the location of protein candidates (70). In addition, the secondary structure of the protein is involved in the distribution of epitopes. Moreover, antigenicity and hydrophilicity are the main factors involved in epitope formation, although interrelated factors, such as flexibility, exposed area, and conformation of secondary and tertiary structure, are also essential.

In the secondary structure of proteins, alpha helices and beta sheets are very regular components, which do not easily deform owing to the presence of hydrogen bonds, which act to maintain a certain structural stability. However, alpha helices and beta sheets do not allow easy ligand binding since they are usually located inside the protein. In contrast, beta-turns and random coil regions are located on the protein surface, thus ensuring the functional needs of the protein. Therefore, these structures are suitable for binding ligands and, consequently, have a high possibility of forming epitopes.

OmpA protein, analyzed by the SOPMA software, potentially consists of 30.51% alpha helix (Hh), 22.03% extended strand (Ee), 7.20% beta-turn (Tt), and 40.25% random coil (Cc). Using this tool, we found that random coil and beta-turn are the most represented types of secondary structure in the OmpA protein. Therefore, we assume that this protein has the required characteristics to be an interesting vaccine candidate. In addition, we predicted the tertiary structures of the OmpA protein from the deduced amino acid sequences of all available genetic variants using I-TASSER, which were refined by ModRefiner server and then validated using the Ramachandran plot, which also confirmed the structural stability of this protein. Therefore, we assume that OmpA protein has the required characteristics to be an interesting

vaccine candidate and that the minimal differences found between OmpA amino acid sequences will probably not affect protein binding.

An effective vaccine candidate must not only have a stable protein structure but also be able to induce a powerful immune response. The ideal is to find a vaccine that triggers both humoral and cellular immunity (71). As the combined effects of T and B cells are necessary for antigen removal, it was also important to analyze the T- and B-cell epitopes of the OmpA antigen.

In this study, we identified B-cell epitopes (linear, conformational, and common epitopes) on the basis of several different characteristics such as the antigenicity, the accessibility, the hydrophilicity, the flexibility, and the secondary structure, by using the IEDB, BepiPred, ABCpred, BepiPred 2.0, and CBTope servers. The results of the predicted sites and conformations of B-cell epitopes showed that all predicted epitopes were accessible, flexible, hydrophilic, and found in the beta-turn (Tt) and/or the random coils (Cc) regions (**Supplementary Figure 3**). In addition, most of these epitopes were antigenic (8/9, 7/8, and 3/4, respectively, for linear, conformational, and common epitopes) (**Supplementary Figure 3**).

Moreover, T-cell epitopes have to transform into peptides, which bind to the corresponding MHC and are then recognized by the T-cell receptor (TCR) (71). T-cell epitopes are presented by MHC class I and MHC class II molecules, which are recognized by long-term culture (LTc) and T helper (Th) cells, respectively. In this study, the five best selected potential epitopes binding to MHC class I alleles were predicted by the NetCTL server, and the five best selected potential epitopes binding to MHC class II alleles were selected by the IEDB server. The results of the predicted T-cell epitope sites and conformations demonstrated that all of the predicted T-cell epitopes were antigenic (**Supplementary Figure 4**) and almost were mainly found in the beta-turn (Tt) and/or the random coil (Cc) regions (4/5 for both types of epitopes binding to MHC class I and II alleles) (**Figure 4**).

After the selection of the epitopes of the B and T cells present in the OmpA protein, we confirmed that the amino acid differences between all available OmpA protein variants so far have neither removed nor modified the epitopes of B and T cells (**Figure 4** and **Tables 3**–**7**). These results suggest that the minimal differences found between the OmpA sequences will not affect the bindings to B-cell antibodies and to MHC class I and class II T cells when using this antigen in a recombinant or multi-epitope vaccine.

CONCLUSION

In this study, the analysis of *lipA* phylogeographic marker indicated a higher diversity of Tunisian *A. marginale* isolates compared with other available worldwide isolates and strains, probably due to multiple introductions from infected cattle from different origins. Despite this heterogeneity, the analysis of the vaccine candidate OmpA protein demonstrated that this antigen appears to be highly conserved, suggesting that the minimal intraspecific modifications will not affect the potential cross-protective capacity of humoral and cell-mediated immune responses against multiple *A. marginale* strains. However, experimental and immunologic studies are needed to confirm this assumption.

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 reviewed and approved by the Ethics Committee of the National School of Veterinary Medicine of Sidi Thabet, University of Manouba. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

MBS, LM, and HB conceived the idea. MBA, RA, RS, and MM carried out the blood sampling. HB, RA, RS, MK, and MBA performed the experiments. HB, SZ, MBA, RA, and MBS performed risk factor analysis and bioinformatics study. HB and MBS wrote the manuscript. HB, RS, MD, LM, and MBS finalized it. All authors have read and approved the final version.

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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.2021.731200/full#supplementary-material

Supplementary Figure 1 | Nucleotide **(A)** and amino-acid **(B)** alignments showing differences between all *sucB* genetic variants available until this study.

Supplementary Figure 2 | Graph representing the best refined model of the OmpA sequence of the 3D structure visualized by PyMol software **(A)** and the Ramachandran plot of the initial model (1) and the best refined model (2) of the candidate developed vaccine **(B)**. The best refined model represents the variant protein OmpATunGv2. The coloring/shading on the Ramachandran plot represents the different regions: the darkest areas (in red) correspond to the "main" regions representing the most favorable combinations of phi-psi values. According to the results, the distribution of the residuals in the refined model was refined by 25.7, 14.7, 9.1, and 2% in the main, authorized, generous, and non-authorized regions, respectively, compared to the initial model.

Supplementary Figure 3 | Results of the prediction of the hydrophilicity of Parker **(A)**, the accessibility to Emini surface **(B)**, the flexibility by Karplus and Schulz **(C)**, and the antigenicity of Kolaskar and Tongaonkar **(D)** within the OmpA protein showing the location of the predicted B cell-bound epitopes. The x and y axes represent, respectively, the position of the sequence and the score relating to each analyzed factor. The cut-off values are 2.098, 1.000, 1.005, and 1.030, respectively, for hydrophilicity, accessibility, flexibility, and antigenicity. Regions above the threshold are hydrophilic, contain the "Beta turn" structure type, accessible, flexible, and antigenic, and are shown in yellow. The black lines represent the sequential (SE1–SE8) and conformational (CE1–CE9) epitopes. The rectangles colored in red represent the epitopes selected in common between the sequential and conformational epitopes.

Supplementary Figure 4 | Result of the prediction of the antigenicity of Kolaskar and Tongaonkar within the OmpA protein of *A. marginale* showing the location of predicted T cell epitopes. The x and y axes represent, respectively, the position of the sequence and the score for the antigenicity of the protein. The threshold value is 1.030. Regions above the threshold are potentially considered antigenic and are shown in yellow. The rectangles colored in green and orange represent the epitopes [TCE1(I)-TCE5(I)] binding to MHC class I T cells and the epitopes [TCE1(II)–TCE5(II)] binding to MHC class T cells II.

Supplementary Table 1 | Single PCR primers used for the identification and/or the genetic characterization of *Anaplasmataceae* and *Anaplasma marginale* infecting cattle from Tunisia.

Supplementary Table 2 | Nucleotide **(Bottom)** and amino-acid **(Top)** homology rates between all available genetic variants based on the analyzed *lipA* partial sequence. [a]Name of the variant, genotype, or isolate. Isolate "LA846" is found in Argentinean cattle and represented by GenBank accession number KM091034. The isolates "Italia7" and "Italia8" are isolated from cattle located in Italy and represented by GenBank accession numbers KM091031 and KM091032, respectively. The strain "St. Maries" is isolated from cattle in USA and represented by GenBank accession number CP000030. The "Africa" isolate is isolated from South African cattle and represented by GenBank accession number KM091016.

Supplementary Table 3 | Nucleotidic and amino-acid differences between different *lipA* genetic variants available until this study. [a]Name of the genetic variant, genotype, or isolate. [b]The numbers represent the nucleotide positions relative to the *A. marginale lipA* sequence of the St. Maries strain from USA (GenBank accession number CP000030). The conserved nucleotidic positions relative to the first sequence are indicated by asterisks. Amino acid changes, if they exist, are shown in parentheses with a single letter code.

Supplementary Table 4 | Nucleotide **(Bottom)** and amino-acid **(Top)** homology rates between all available genetic variants based on the analyzed *sucB* partial sequence. [a]Name of the genetic variant, genotype, or isolate.

Supplementary Table 5 | Nucleotide **(Bottom)** and amino-acid **(Top)** homology rates between all available genetic variants based on the studied *ompA* partial sequence. [a]Name of the genetic variant or genotype. V1 represents the strain "St. Maries" (GenBank accession number MK882880) infecting cattle in USA and other strains with the same sequence. V2 represents strain "Dawn" isolated from Australian cattle (GenBank accession number KM821232) and other strains with the same sequence. V3 represents the isolate "Emphi" from infected cattle in USA (GenBank accession number KM821235). GV1 represents the isolate "Gha147" (GenBank accession number MK882880) isolated from a Ghanaian cattle and other Ghanaian isolates with the same sequence. GV2 represents the isolate "Gha24" (GenBank accession number MK882857) isolated from a Ghanaian cattle and other Ghanaian isolates with the same sequence. GV3 represents the isolate "Gha50" (GenBank accession number MK882871) isolated from a Ghanaian cattle and other Ghanaian isolates with the same sequence.

Supplementary Table 6 | Nucleotide and amino-acid differences between different *ompA* genetic variants available until this study. [a]Name of the variant, genotype, or isolate. [b]Numbers represent nucleotide positions relative to *A. marginale ompA* sequence of St. Maries strain from the USA (GenBank accession number CP000030). The conserved nucleotide positions with respect to the first sequence are indicated with asterisks. Positions where sequencing has not been performed are represented by dashes. Amino acid changes are shown in parentheses with a single letter code. Amino-acids: S, serine; L, leucine; V, valine; F, phenylalanine; G, glycine; P, proline; E, glutamic acid; K, lysine. Nucleotides: T, thymine; C, cytosine; G, guanine; A, adenine.

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The Bacterial Community in Questing Ticks From Khao Yai National Park in Thailand

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Ticks are known vectors for a variety of pathogens including bacteria, viruses, fungi, and parasites. In this study, bacterial communities were investigated in active life stages of three tick genera (*Haemaphysalis*, *Dermacentor*, and *Amblyomma*) collected from Khao Yai National Park in Thailand. Four hundred and thirty-three questing ticks were selected for pathogen detection individually using real-time PCR assays, and 58 of these were subjected to further metagenomics analysis. A total of 62 ticks were found to be infected with pathogenic bacteria, for a 14.3% prevalence rate, with *Amblyomma* spp. exhibiting the highest infection rate (20.5%), followed by *Haemaphysalis* spp. (14.5%) and *Dermacentor* spp. (8.6%). *Rickettsia* spp. were the most prevalent bacteria (7.9%) found, followed by *Ehrlichia* spp. (3.2%), and *Anaplasma* spp. and *Borrelia* spp. each with a similar prevalence of 1.6%. Co-infection between pathogenic bacteria was only detected in three *Haemaphysalis* females, and all co-infections were between *Rickettsia* spp. and Anaplasmataceae (*Ehrlichia* spp. or *Anaplasma* spp.), accounting for 4.6% of infected ticks or 0.7% of all examined questing ticks. The prevalence of the *Coxiella*-like endosymbiont was also investigated. Of ticks tested, 65.8% were positive for the *Coxiella*-like endosymbiont, with the highest infection rate in nymphs (86.7%), followed by females (83.4%). Among tick genera, *Haemaphysalis* exhibited the highest prevalence of infection with the *Coxiella*-like endosymbiont. Ticks harboring the *Coxiella*-like endosymbiont were more likely to be infected with *Ehrlichia* spp. or *Rickettsia* spp. than those without, with statistical significance for *Ehrlichia* spp. infection in particular (p-values = 0.003 and 0.917 for *Ehrlichia* spp. and *Rickettsia* spp., respectively). Profiling the bacterial community in ticks using metagenomics revealed distinct, predominant bacterial taxa in tick genera. Alpha and beta diversities analyses showed that the bacterial community diversity and composition in *Haemaphysalis* spp. was significantly different from *Amblyomma* spp. However, when examining bacterial diversity among tick life stages (larva, nymph, and adult) in *Haemaphysalis* spp., no significant difference

among life stages was detected. These results provide valuable information on the bacterial community composition and co-infection rates in questing ticks in Thailand, with implications for animal and human health.

Keywords: co-infection, metagenomics in ticks, *Amblyomma* spp., *Haemaphysalis* spp., *Dermacentor* spp., questing ticks in Thailand

INTRODUCTION

Ticks are recognized as a medically important group of arthropods that transmit a number of diseases to humans (1). Different tick species favor different habitats, which ultimately defines their geographical distribution and thus the risk areas for human or animal infections. Several pathogens are known to be carried by either hard ticks (Ixodidae) or soft ticks (Argasidae), including a wide range of viruses, bacteria, fungi, and protozoa (2–6).

In Southeast Asia, 97 tick species (5 in Argasidae and 93 in Ixodidae) have been described (7). In Thailand, four species within Argasidae (2 *Argas* and 2 *Ornithodoros*) and 58 species within Ixodidae have been recorded. Of the hard ticks in Thailand, there are 25 species within genus *Haemaphysalis*, 10 *Ixodes*, 13 *Amblyomma*, 5 *Dermacentor*, 4 *Rhipicephalus*, and *Nosomma monstrosum*. Among tick species found in Thailand, *Amblyomma* spp. and *Dermacentor* spp. were associated with human otoacariasis, especially *Amblyomma testudinarium* (8) and *Dermacentor steini* (9). However, *Haemaphysalis* spp. and other tick genera are occasionally found on humans as well. *Rickettsia* spp. are the main tick-borne pathogens causing human infections in Thailand (10–12) with scattered reports of other tick-borne disease (TBD) in human and animals such as Q fever (13, 14) and anaplasmosis in companion pets and animals (15–17).

Ticks also harbor many non-pathogenic organisms. Bacterial endosymbionts have been recognized as important microorganisms required for tick fitness, especially with regard to regulating host reproduction and immunity (18, 19). Some studies suggest that these symbionts have a potential role in providing key vitamins absent in the bloodmeal (20, 21). Others reported that they might have an important role in facilitating pathogen colonization in the gut as in the case in *Ixodes scapularis* ticks, in which alteration of the symbiont abundance resulted in decreased *Borrelia burgdorferi* colonization (22). Likewise, the level of *Anaplasma marginale* acquisition was lower in *Dermacentor andersoni* when their microbiome was altered by antibiotic treatment leading to an increase in proportion and quantity of *Rickettsia bellii* in the microbiome (23). Moreover, the prevalence and transovarial transmission of bacterial endosymbionts occurs at a high rate, suggesting that they might have an obligate relationship with the host (24, 25). In addition to the bacterial microbiota in ticks, other microorganisms are as important and abundant. For example, several studies reported that two groups of bunyaviruses (South Bay virus and Phlebovirus) were commonly associated with *Ixodes* spp. ticks in America and Europe (26–28) and were more abundant than bacteria or eukaryote counterparts (29).

It is thought that because ticks harbor a diverse range of microorganisms, co-infection or co-occurrence of bacteria, parasites, and viruses in ticks is possible (30). Co-infection may lead to increased disease severity as it complicates disease diagnosis and treatment (31–34). Co-infections between *B. burgdorferi* and *Anaplasma phagocytophilum* are widely recognized and reported (35). Other co-infections among different types of microorganisms have also been reported, such as between *B. burgdorferi* and South Bay virus (SBV), *Babesia microti*, and *B. burgdorferi*, as well as between a novel filarial worm (*Onchocercidae* sp. ex. *Ixodes scapularis*) and *Wolbachia* spp (29).

In this study, we used both conventional and high-throughput sequencing methods to study bacterial pathogen co-infections and pathogen association with bacterial endosymbionts in questing ticks collected in Khao Yai National Park. Metagenomics were used to determine the bacterial communities in individual ticks and conventional methods (real-time PCR, PCR, and Sanger sequencing) were used to detect pathogenic bacteria: *Rickettsia* spp., *Anaplasma* spp., *Ehrlichia* spp., *Borrelia* spp., *Coxiella burnetii*, and pathogenic *Francisella* spp. in *Amblyomma* spp., *Dermacentor* spp., and *Haemaphysalis* spp. ticks individually. All positive samples underwent DNA sequencing to identify pathogens to the species level. Co-infections between bacteria and the association between pathogenic bacteria and the *Coxiella*-like endosymbiont were investigated.

MATERIALS AND METHODS

Tick Collection

Questing ticks were collected by dragging a 1-m^2 cotton cloth over vegetation in four tourist attraction sites in Khoa Yai National Park (14°26'19.5"N 101°22'20.1"E). Sampling was conducted by six people at each site for 1 h. Ticks were collected in one trip in November 2020 and immediately preserved in 90% ethanol before transporting to the laboratory for further processing. All sampling procedures and experimental manipulations were reviewed and approved as part of the animal collection protocol entitled "Surveillance of Tick- and Flea-Borne Diseases of Public Health Importance" (PN# 21-01). The project was also approved by Mahidol University – Institute animal care and use committee (MU-IACUC 2019/3). Research was conducted in compliance with the Animal Welfare Act and other federal statutes and regulations related to animals and experiments involving animals, and adhered to principles outlined in the "Guide for

the Care and Use of Laboratory Animals," NRC Publication, 2011 edition.

Tick Surface Sterilization and DNA Extraction

Questing ticks (unfed ticks) were morphologically identified using taxonomic keys (36, 37). Each tick was vortexed for 1 min in 3% sodium hypochlorite and then transferred to a new tube and vortexed for 1 min in 70% alcohol followed by three washes in sterile PBS in the same manner. Ticks were air dried for 10 min on Whatman® filter paper (Sigma-Aldrich, Saint Louis, MO, USA) before DNA extraction. Whole ticks in 250 μl of ATL buffer (lysis buffer, component of the extraction kit) were punctured with a fine tip under a stereomicroscope to release the tissue from the hard chitin exoskeleton prior to adding 2 mg/ml of Proteinase K solution. Samples were then incubated at 55°C overnight. A total volume of 250 μl of homogenized solution was then used for DNA extraction on the QIAsymphony® SP instrument with QIAsymphony® DSP DNA Mini Kit using Tissue LC 200 DSP protocol (Qiagen, Hombrechtikon, Switzerland). The DNA was eluted in 50 μl of ATE buffer (elution buffer, component of extraction kit) and stored at−20°C until use. Ultrapure DNA/RNA-free distilled water as well as PBS buffer used for tick surface sterilization were also included as an extraction control.

Amplification of Bacterial 16S rDNA

Following DNA extraction, the bacterial-specific 16S rDNA (V3–V4, a 550-bp fragment) was amplified as previously described (15). Negative control PCR reactions were included in every experimental run using Ultrapure DNA/RNA-free distilled water in place of DNA template. PCR reactions were also performed with eluates from mock DNA extractions as well as from PBS buffer used for tick surface sterilization. PCR product was cleaned using AMPure magnetic bead-based purification system (Beckman Coulter, UK) following the manufacturer's instructions. Purified PCR products were eluted and quantified using the Qubit dsDNA HS Assay Kit (Invitrogen Life Technologies, MA).

Library Preparation and High-Throughput Sequencing

The library was prepared with dual indices and Illumina sequencing adapters attached to purified PCR products using the Nextera XT Index Kit following the manufacturer's protocol (Illumina Inc., San Diego, CA). For index control reaction, a combination of index primers that were not used with samples was also included with PCR grade water as template. The number of reads recovered from these particular index combinations were used to filter the cross-contaminations between indexed PCR primers and to identify errors in an Illumina sample sheet. Libraries were cleaned using Agencourt AMPure XP beads. The purity of the libraries was checked on the QIAxcel Advanced System (Qiagen) with a QIAxcel DNA High Resolution Cartridge. Purified amplicon libraries were quantified using the Qubit dsDNA HS Assay Kit (Invitrogen). DNA concentration was calculated and normalized to reach 4.0 nM for each library. Five microliters of DNA from each library was pooled for a NGS run. Pooled libraries were denatured and diluted to a final concentration of 8 pM with a 10% PhiX (Illumina) control. Sequencing was performed using the MiSeq Reagent Kit V3 on the Illumina MiSeq System.

Data Analysis for Metagenomics and Diversity Estimates

The sequence reads generated by the 16S rRNA on MiSeq sequencers were processed on CLC Genomics workbench v 12.0.3 (Qiagen, Aarhus A/S, http://www.clcbio.com). High-throughput sequences were imported into CLC Genomics Workbench according to quality scores of Illumina pipeline 1.8. In order to achieve the highest quality sequences for clustering, paired reads were merged in CLC microbial genomics module v 4.8 using default settings (mismatch cost = 1; minimum score = 40; gap cost = 4 and maximum unaligned end mismatch = 5). Primer sequences were trimmed from merged reads using parameters (trim using quality scores = 0.01, trim ambiguous nucleotides = 2, and discard read length shorter than 150 bp). Samples were removed from analysis if the number of reads was <100 or <25% from the median (the median number of reads across all samples). Chimeric sequences were detected and removed. Only filtered and merged sequences were clustered into operational taxonomic units (OTUs) according to a threshold of 97% sequence identity. All such processes were performed using CLC microbial genomics module v 4.8. Reference OTU data used in the present study were downloaded from the Greengenes database V13.8 (38). OTUs with combined abundance <10 reads were removed from the downstream analysis. Alpha diversity estimates (Observed OTUs, Simpson's index, and Shannon entropy) were analyzed on the quality-filtered OTU table at the genus level using CLC Microbial Genomics Module v 4.8. Beta diversity analysis for microbiome compositional difference between groups was calculated using a distance-based non-parametric test, the generalized UniFrac distances (39). The statistical significance of microbiome compositional difference between groups (tick genera, tick developmental stages, *Francisella persica* infection, *Coxiella*-like endosymbiont infection, and *Rickettsia* spp. infection statuses) was then compared using PERMANOVA using 99,999 permutations of the distance values. All analyses mentioned here were performed with CLC Microbial Genomics Module v 4.8.

Bacterial Pathogen Detection by Real-Time PCR and Conventional PCR

Real-time PCR and PCR assays were performed on 433 individual ticks for the detection of bacteria (*Rickettsia* spp., *Borrelia* spp., *Anaplasma* spp., *Ehrlichia* spp., and *Coxiella*-like endosymbiont) and the taxonomic species assignment. Other potential pathogenic bacteria (*Francisella* spp. and *Coxiella* spp.) detected by NGS analysis (read count > 1) were also confirmed by real-time PCR and PCR assays. Detailed methods for assays and target gene(s) for selected pathogens are provided as online Supplementary data (**Supplementary Table 1**). For all real-time PCR, the reaction consisted of 1X Platinum quantitative PCR SuperMix-UDG (Invitrogen) using standard

TABLE 1 | Tick species collected in Khao Yai National Park, Thailand (2020).

Tick species	Larvae	Nymphs	Males	Females	Selected for NGS	Passed quality-filter
Unidentified Larvae	20,796	0	0	0	0	0
Amblyomma spp.	35	2	0	0	7	7
Amblyomma testudinarium	0	0	1	6	4	1
Dermacentor spp.	35	0	0	0	5	5
Dermacentor auratus	0	0	6	14	6	4
Dermacentor steini	0	0	1	2	3	1
Haemaphysalis spp.	50	0	0	0	10	9
Haemaphysalis lagrangei	0	0	4	51	6	3
Haemaphysalis longicornis	0	0	34	17	6	5
Haemaphysalis obesa	0	0	26	30	6	3
Haemaphysalis papuana	0	0	0	3	3	1
Haemaphysalis shimoga	0	0	13	22	6	4
Haemaphysalis spp.	0	81	0	0	20	15
Total	20,916	83	85	145	82	58
Selected for study	120*	83	85	145	82	58

Number of ticks included in this study and for studying bacterial community profile using 16S rRNA gene Next-Generation Sequencing is shown. (*), only larvae belonging to the three tick genera were selected for further analyses.

real-time PCR conditions with primer/probe concentrations and annealing temperatures as indicated in **Supplementary Table 1**. For conventional PCR, the assay was carried out in a 50-μl reaction volume containing 0.5 U of iProof High-Fidelity DNA Polymerase, 200 μM dNTPs, $MgCl_2$, and primer concentration as indicated (**Supplementary Table 1**). The PCR conditions consisted of 98°C for 3 min, followed by 40 cycles of 98°C for 10 s, annealing temperature for 30 s (indicated in **Supplementary Table 1** for each pathogen), and 72°C for 45 s.

DNA Sequencing

PCR amplicons were purified using the QIAquick® PCR Purification Kit (Qiagen) according to the manufacturer's instructions. The PCR products were cycle-sequenced using an ABI BigDye™ Terminator v3.1 Cycle Sequencing Kit, ethanol precipitated, and run on a SeqStudio Genetic Analyzer (Applied Biosystems ThermoFisher, Thailand). Sequences of each sample and pathogen were assembled using Sequencher™ ver. 5.4.6 (GeneCodes Corp., Ann Arbor, MI). The pathogen sequences were aligned with reference sequences retrieved from the GenBank database using the MUSCLE codon alignment algorithm (40). A maximum likelihood phylogenetic tree was then constructed from bacterial target genes (**Supplementary Table 1**) using the best fit model of nucleotide substitution with bootstrapping (1,000 replicates) in MEGA 6 (41).

Statistical Analysis

Differences in alpha diversity indices of the bacterial community composition, based on metagenomics data, were determined by Mann–Whitney *U* test (between two groups) or Kruskal–Wallis test (across all groups) and the critical range ($p < 0.05$) was determined. Statistical analyses (two-way ANOVA tests and mean, 95% confidence interval) and scatter plots were performed using GraphPad Prism version 5.04 for Windows (GraphPad Software, San Diego, CA. www.graphpad.com). Some graphical illustrations presented in this study as well as the Chi-square independence test and Fisher's exact test were performed in the R environment for statistical computing (42, 43). A nucleotide distance matrix was generated using "Compute Pairwise Distance" in MEGA 6 (41).

RESULTS

Tick Species Diversity

A total of 21,229 questing ticks were collected in November 2020 in Khao Yai National Park. The majority of ticks collected were larvae ($n = 20,916$) accounting for 98.5%, followed by females ($n = 145$, 0.7%), males ($n = 85$, 0.4%), and nymphs ($n = 83$, 0.4%). Species identification was done for the adult stage only and *Haemaphysalis lagrangei*, *Haemaphysalis obesa*, and *Haemaphysalis longicornis* were found at the highest rate (22%−24%), followed by *Haemaphysalis shimoga* (15%), *Dermacentor auratus* (9%), *Amblyomma testudinarium* (4%), *Haemaphysalis papuana* (1%), and *Dermacentor steini* (1%). All adult ticks ($n = 230$) and nymphs ($n = 83$) and 0.6% of larvae ($n = 120$) were selected for pathogen detection individually ($n = 433$). Identification of larval stage was performed on 120 selected samples and *Amblyomma* spp. ($n = 35$), *Dermacentor* spp. ($n = 35$), and *Haemaphysalis* spp. ($n = 50$) were found. In total, there were *Haemaphysalis* spp. (331, 76.4%), *Dermacentor* spp. (58, 13.4%), and *Amblyomma* spp. (44, 10.2%) included in this study. Additionally, a subset of ticks ($n = 82$) were selected for studying the bacterial community profile using 16S rRNA Next-Generation Sequencing (**Table 1**). Five to six ticks per species and life stage were selected for NGS, with the exception of immature *Haemaphysalis* spp., where 10 larvae and 20 nymphs were included. The number of ticks selected

TABLE 2 | Prevalence of pathogenic bacteria (*Rickettsia* spp., *Anaplasma* spp., *Ehrlichia* spp., and *Borrelia* spp.) in questing ticks from Khao Yai National Park, by species and stages.

Species	Stages	*N*	No. of positive (% Infection)				
			All pathogens	***Rickettsia* spp.**	***Anaplasma* spp.**	***Ehrlichia* spp.**	***Borrelia* spp.**
Amblyomma spp.	Immature	37	5 (13.5%)	4 (10.8%)	1 (2.7%)	0	0
A. testudinarium	Mature	7	4 (57.1%)	4 (57.1%)	0	0	0
Total	All stages	44	9 (20.5%)	8 (18.2%)	1 (2.3%)	0	0
Dermacentor spp.	Immature	35	4 (11.4%)	0	0	0	4 (11.4%)
All species	Mature	23	1 (4.3%)	1 (4.3%)	0	0	0
D. steini	Mature	3	1 (33.3%)	1 (33.3%)	0	0	0
D. auratus	Mature	20	0	0	0	0	0
Total	All stages	58	5 (8.6%)	1 (1.7%)	0	0	4 (6.9%)
Haemaphysalis spp.	Immature	131	13 (9.9%)	9 (6.9%)	1 (0.8%)	2 (1.5%)	1 (0.8%)
All species	Mature	200	35 (17.5%)	16 (8.0%)	5 (2.5%	12 (6.0%)	2 (1.0%)
H. shimoga	Mature	35	3 (8.6%)	3 (8.6%)	0	0	0
H. longicornis	Mature	51	10 (19.6%)	5 (9.8%)	1 (2.0%)	4 (7.8%)	0
H. lagrangei	Mature	55	14 (25.5%)	8 (14.5%)	1 (1.8%)	5 (9.1%)	0
H. obesa	Mature	56	8 (14.3%)	0	3 (5.4%)	3 (5.4%)	2 (3.6%)
H. papuana	Mature	3	0	0	0	0	0
Total	All stages	331	48 (14.5%)	25 (7.6%)	6 (1.8%)	14 (4.2%)	3 (0.9%)
Grand Total		433	62 (14.3%)	34 (7.9%)	7 (1.6%)	14 (3.2%)	7 (1.6%)

Immature, larva and nymph; Mature, adult female and male.

for NGS and the final number of ticks that passed the quality filter are described in detail in the online Supplementary data (**Supplementary Table 2**).

Pathogen Prevalence in Questing Ticks and Species Identification

Overall, pathogenic bacteria were detected in 62 out of the total number of examined ticks collected from Khao Yai National Park, for a 14.3% prevalence rate. Pathogens were found in 48 of the tested *Haemaphysalis* spp. (14.5%), nine in *Amblyomma* spp. (20.5%), and five in *Dermacentor* spp. (8.6%). *Rickettsia* spp. was the most common pathogenic bacteria (7.9%), followed by *Ehrlichia* spp. (3.2%), and *Anaplasma* spp. and *Borrelia* spp. with a similar rate of 1.6% (**Table 2**). *Haemaphysalis* spp. (n = 331) were infected by all pathogens mentioned with *Rickettsia* spp. at the highest rate (7.6%), followed by *Ehrlichia* spp. (3.2%), *Anaplasma* spp. (1.8%), and *Borrelia* spp. (0.9%). In *Amblyomma* spp. (n = 44) and *Dermacentor* spp. (n = 58), two bacterial pathogens were found in each genus. Both were infected with *Rickettsia* (18.2% and 1.7% prevalence rates, respectively). *Anaplasma* spp. was detected only in *Amblyomma* spp. (2.3%), while *Borrelia* spp. was detected only in *Dermacentor* spp. (6.9%). When examining pathogen prevalence in tick life stages, the infection rate was found to increase from 10.0 to 19.3% from larvae to adults, respectively (**Figure 1**). The trend of increasing pathogen prevalence by life stage is clearly observed for *Rickettsia* spp. and *Ehrlichia* spp.

Sequence and phylogenetic analyses showed that among *Rickettsia* species detected in ticks, *R. montana* (n = 21) was the predominant species found in *Haemaphysalis* spp., while *R. raoultii* (n = 6) was detected mostly in *Amblyomma* spp. (n = 5), and three other *Rickettsia* species (2 = *R. heilongjiangensis*, 3 = *R. monacensis*, and 2 = *Rickettsia* sp.) were detected in *Haemaphysalis* spp. and *Amblyomma* spp. (**Table 3**). *Borrelia theileri* (n = 7) was the only *Borrelia* species found, and was found in nearly equal numbers in *Dermacentor* spp. and *Haemaphysalis* spp. ticks. The majority of Anaplasmataceae bacteria (7 = *Anaplasma* spp. and 14 = *Ehrlichia* spp.) were mostly detected in *Haemaphysalis* spp. with *E. ewingii* (n = 9) being the dominant species found. Phylogenetic analyses for all bacteria can be found in online Supplementary Data (**Supplementary Figure 1**).

Co-infection of Bacterial Pathogens

Co-infection between pathogenic bacteria examined in this study only occurred in a small number of ticks, primarily female *Haemaphysalis* spp. (n = 3). All co-infections occurred with *Rickettsia* spp. and Anaplasmataceae (*Ehrlichia* spp. or *Anaplasma* spp.) (**Figures 2A,B**) and accounted for 4.8% of infected ticks and 0.7% of all ticks examined. All three co-infected ticks were also positive for the *Coxiella*-like endosymbiont.

Bacterial Endosymbionts and Co-occurrence With Pathogenic Bacteria

The *Coxiella*-like endosymbiont was detected in 65.8% of all ticks examined, with the highest infection rate in nymphs (86.7%) and females (83.4%) (**Table 4**). Of all genera, *Haemaphysalis* spp. exhibited the highest rate of infection with the *Coxiella*-like endosymbiont, with an 81.0% infection rate. The majority of *Haemaphysalis* spp. infected were females (95.1%), followed by nymphs (88.9%), males (87.0%), and, to a lesser extent, larvae (24.0%). The second highest infection

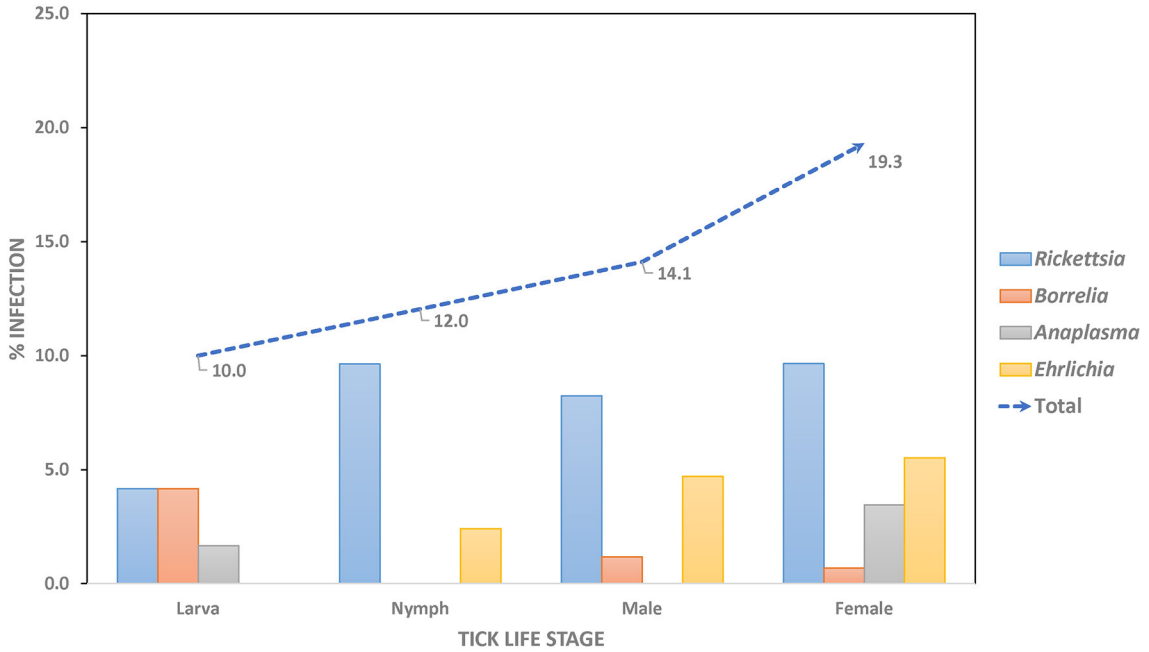

FIGURE 1 | Prevalence rate of pathogenic bacteria in questing ticks, by stages.

TABLE 3 | Bacterial species identification by DNA sequence and phylogenetic analyses.

Pathogen species	No. of positive (% Sequence identity)			
	***Amblyomma* spp.**	***Dermacentor* spp.**	***Haemaphysalis* spp.**	**Total**
Rickettsia sp.	0	0	2 (99.4%)	2
Rickettsia raoultii	6 (99.2–99.4%)	1*	0	7
Rickettsia montana	0	0	21 (98.8–99.0%)	21
Rickettsia monacensis/Rickettsia tamurae	3 (99.0–100%)	0	0	3
Rickettsia heilongjiangensis	0	0	1 (100%)	1
Borrellia theileri	0	4 (99.3–99.8%)	3 (99.8%)	7
Anaplasma sp.	1*	0	4 (99.6–100%)	5
Anaplasma bovis	0	0	2 (100%)	2
Ehrlichia spp. similar to *E. ewingii*#	0	0	4 (99.1–100%)	4
Ehrlichia spp. similar to *Ehrlichia ewingii*	0	0	9 (91.7–94.4%)	9
Ehrlichia sp. similar to *Candidatus* Ehrlichia shimanensis	0	0	1 (96.6%)	1
Total	9	5	48	62

(*), short sequence, species identification was from the most similar reference sequence from BLASTN search, (#), sequence similarity based on 16S rRNA gene.

rate belonged to *Amblyomma* spp. with a 38.6% infection rate and only detected in females (66.7%) and larvae (37.1%). Surprisingly, no *Dermacentor* spp. were positive for the *Coxiella*-like endosymbiont.

Figures 2A,B are scatter plots of individual ticks depicting the pathogenic bacterial infection status compared between two tick populations—those harboring the *Coxiella*-like endosymbiont and those without. There was no significant difference in the number of ticks infected with *Borrelia* spp. or *Anaplasma* spp. between the *Coxiella*-like endosymbiont-positive and -negative groups. However, ticks harboring the *Coxiella*-like endosymbiont had greater rates of *Ehrlichia* spp. or *Rickettsia* spp. infection than those without the *Coxiella*-like endosymbiont. Chi-square and Fisher's exact tests were used to test for significant differences of *Rickettsia* spp. or *Ehrlichia* spp. infection between endosymbiont-positive and -negative ticks. Results show that the proportion of *Rickettsia* spp. infection in ticks harboring the *Coxiella*-like endosymbiont [8.8%, CI (5.5, 12.1%)] was not significantly different from the proportion in ticks without the *Coxiella*-like endosymbiont [6.1%%, CI (2.2, 9.9%)] with statistical values; Chi-square = 0.638, df = 1, p-value = 0.424. Likewise, the proportion of all pathogenic bacterial infection between ticks

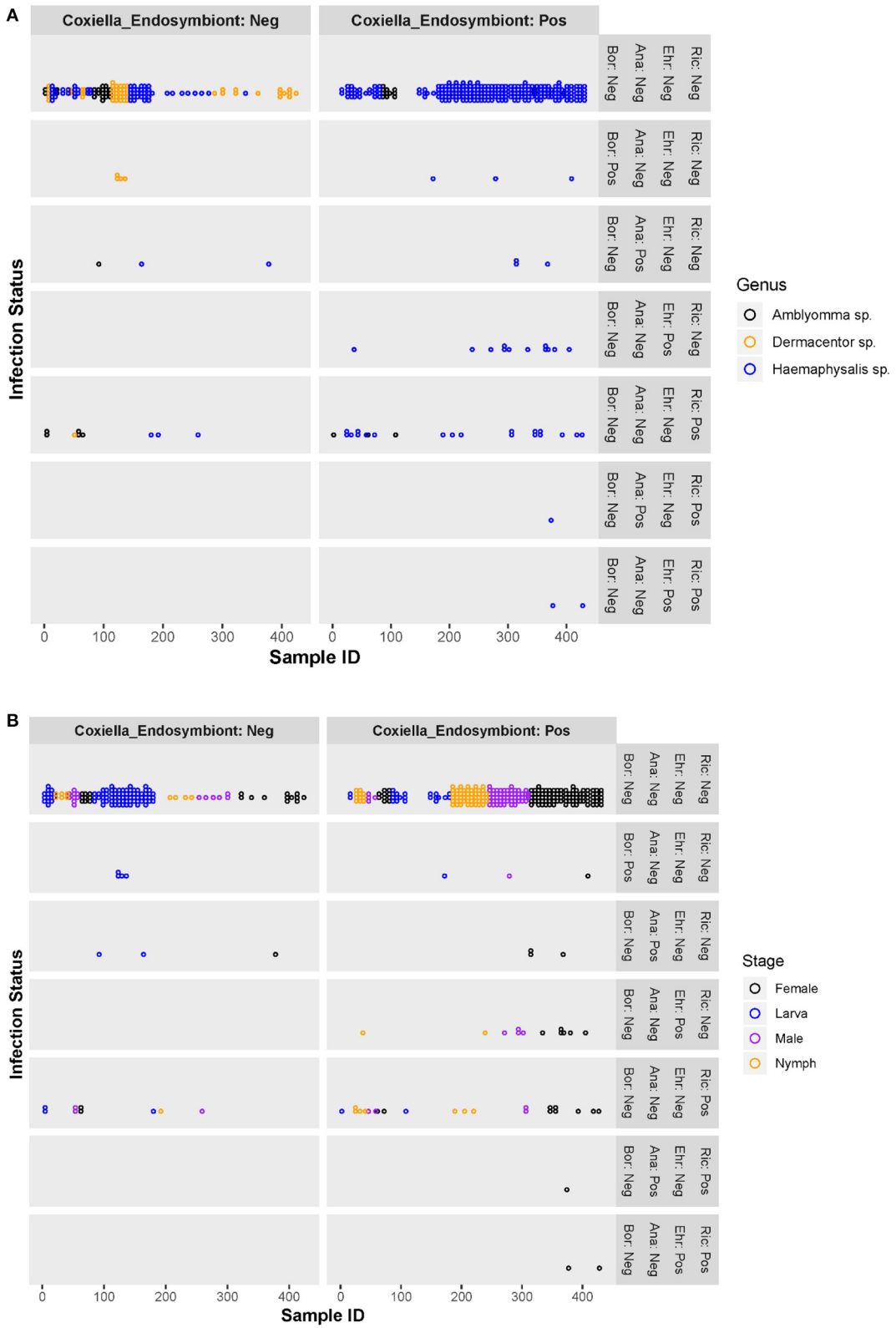

FIGURE 2 | Scatter plots of individual ticks (with or without *Coxiella*-like endosymbiont) and their pathogenic bacterial infection status, by tick genera **(A)** and tick stages **(B)**. Ric, *Rickettsia* spp.; Ehr, *Ehrlichia* spp.; Ana, *Anaplasma* spp.; Bor, *Borrelia* spp.; Coxiella_Endosymbiont, *Coxiella*-like endosymbiont; Pos, positive; Neg, negative.

TABLE 4 | Prevalence of *Coxiella*-like endosymbiont in questing ticks, by tick genera and stages.

Tick species	Total number of tick tested (*N*)	No. of *Coxiella*-like endosymbiont-positive ticks/total no. of tick (% Infection)				
		Larva	Nymph	Male	Female	All stages
Amblyomma spp.	44	13/35 (37.1)	0/2 (0)	0/1 (0)	4/6 (66.7)	17/44 (38.6)
Dermacentor spp.	58	0/35 (0)	0/0 (0)	0/7 (0)	0/16 (0)	0/58 (0)
Haemaphysalis spp.	331	12/50 (24.0)	72/81 (88.9)	67/77 (87.0)	117/123 (95.1)	268/331 (81.0)
Total	433	25/120 (20.8)	72/83 (86.7)	67/85 (78.8)	121/145 (83.4)	285/433 (65.8)

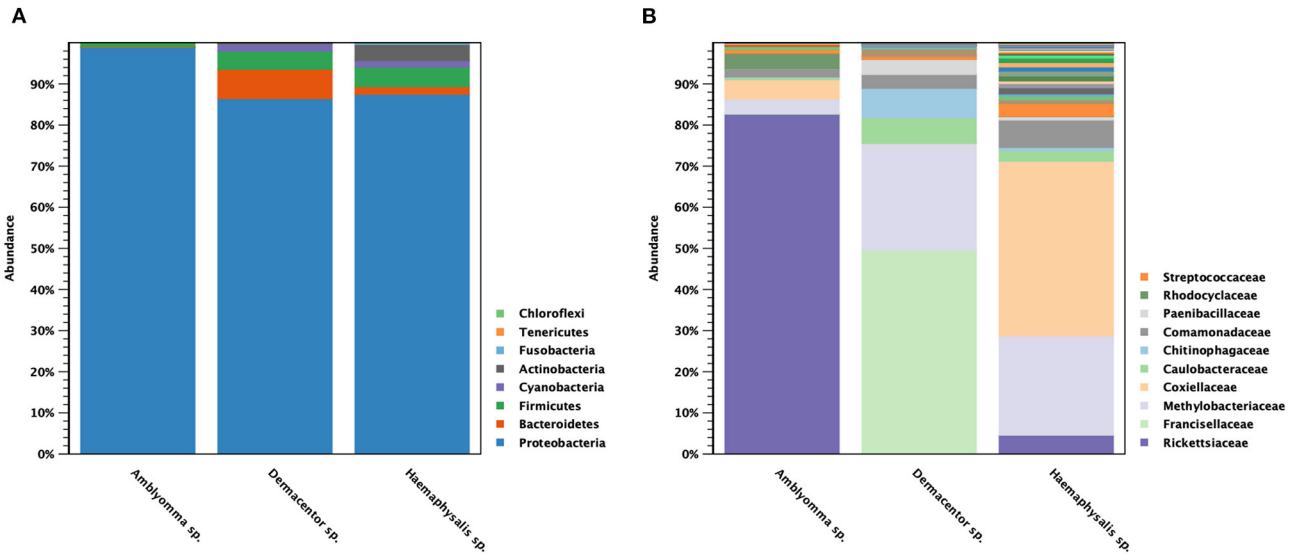

FIGURE 3 | Taxonomic diversity and relative abundance at the phylum **(A)** and family **(B)** level of bacterial community in questing ticks. The percent relative abundances are of the total number of OTUs. Color legend for each phylum **(A)** or family **(B)** was indicated next to the bar graph.

harboring the *Coxiella*-like endosymbiont [16.1%, CI (11.9, 20.4%)] and ticks without *Coxiella*-like endosymbiont [10.8%, CI (5.8, 15.8%)] was not significantly different (Chi-square = 0.7374, df = 1, *p*-value = 0.3905). *Ehrlichia* infection was observed only in ticks harboring *Coxiella*-like endosymbiont with the proportion of 4.9%, CI [2.4%, 7.4%]. Fisher's Exact test was used to determine the association between *Ehrlichia* infection and infection with the *Coxiella*-like endosymbiont. Results indicate that infection by these two bacteria in ticks is dependent (alpha = 0.05, *p*-value = 0.003), suggesting that the *Ehrlichia* infection is significantly associated with ticks harboring the *Coxiella*-like endosymbiont.

Bacterial Profile in Ticks

Of 433 samples tested in this study, 82 samples were selected for metagenomics NGS analysis (**Table 1**). After performing sequence quality filters and removing samples with low reads, only 58 samples (range: 1,085–472,236, mean number of reads ± SD = 37,780 ± 98,225) passed the criteria and were subjected to further OTU clustering and alpha and beta diversity analyses. A total of 2,222,970 reads passed quality filters and 268 OTUs were found across all samples. Comparison of the number of passed-filter reads being used for OTU clustering between tick stages showed that larvae (n = 19; 96,713 ± 157,880) generated more reads than nymphs (n = 17; 10,161 ± 9,845) and adults (n = 22; 8,226 ± 8,603). Eight *Amblyomma* spp., 10 *Dermacentor* spp., and 40 *Haemaphysalis* spp. were included in metagenomics analysis (**Table 1**).

The classification of OTUs from each sample was made against the Greengenes reference database and the similarity threshold was set at 0.97 in the CLC microbial genomics module. There were nine recorded phyla found among all tick samples studied: Proteobacteria (91%), Bacteroidetes (4%), Firmicutes (3%), Cyanobacteria (1%), and Actinobacteria (1%) (**Figure 3A**). There were five major phyla found in controls for DNA extraction, PCR, and Indexing; however, the majority of Proteobacteria phylum (73%) detected was genus *Enterobacteria* (89%), and very small amount of genus *Rickettsia* (0.0092%) (**Supplementary Figure 2**). Only 1% of reads could not be classified using Greengenes reference and were removed before performing downstream process of abundance analysis. **Figure 3B** shows the abundance of bacterial taxa in tick genera at the family level. There were one or two predominant bacterial taxa in each tick genus. Rickettsiaceae (83%) was the predominant bacterial taxon in *Amblyomma* spp., while Francisellaceae (50%) and Methylobacteriaceae (26%) were the

TABLE 5 | Alpha diversity estimates of bacterial communities based on metagenomics.

Categories	Number	Diversity Mean [95% CI]		
		Observed OTUs	Simpson's index	Shannon
Tick genera	58			
Amblyomma	8	9.47 [5.20, 13.74]	0.32 [0.09, 0.55]	1.07 [0.32, 1.82]
Dermacentor	10	9.03 [4.61, 13.45]	0.39 [0.15, 0.62]	1.28 [0.48, 2.09]
Haemaphysalis	40	14.18 [12.62, 15.74]	0.55 [0.48, 0.61]	1.81 [1.58, 2.05]
Tick stages (only *Haemaphysalis* spp.)	36			
Larva	9	12.87 [9.30, 16.44]	0.58 [0.43, 0.72]	1.80 [1.35, 2.26]
Nymph	15	12.99 [10.72, 15.25]	0.58 [0.49, 0.68]	1.85 [1.52, 2.18]
Female	7	18.21 [15.77, 20.64]	0.46 [0.24, 0.67]	1.56 [0.89, 2.23]
Male	5	17.65 [9.71, 25.58]	0.48 [0.07, 0.89]	1.85 [0.28, 3.43]
Francisella persica	58			
Positive	4	3.08 [1.04, 5.11]	0.03 [−0.01, 0.08]	0.13 [−0.01, 0.26]
Negative	54	13.35 [11.97, 14.73]	0.52 [0.46, 0.58]	1.73 [1.52, 1.95]
Coxiella-like endosymbiont	58			
Positive	26	13.63 [11.72, 15.54]	0.50 [0.41, 0.59]	1.65 [1.36, 1.94]
Negative	32	11.84 [9.65, 14.03]	0.48 [0.37, 0.58]	1.60 [1.24, 1.95]
Rickettsia infection	58			
Positive	9	9.58 [5.08, 14.08]	0.37 [0.20, 0.54]	1.10 [0.59, 1.61]
Negative	49	13.20 [11.66, 14.74]	0.51 [0.44, 0.58]	1.72 [1.47, 1.97]
Categories	**Number of group**	**Group comparison (*p*-value)**		
Tick genera*	3	**0.01**	0.08	0.07
Amblyomma vs. *Haemaphysalis***	2	**0.02**	**0.04**	**0.04**
Dermacentor vs. *Haemaphysalis***	2	**0.02**	0.2	0.1
Tick stages*	4	**0.03**	0.6	0.9
Female vs Nymph**	2	**0.004**	0.2	0.5
Female vs. Larva**	2	**0.02**	0.2	0.4
Francisella persica infection**	2	**0.001**	**0.001**	**0.0009**
Coxiella-like endosymbiont**	2	0.3	1.0	0.9
Rickettsia infection**	2	0.06	0.08	**0.03**

Statistical analysis used; (*), Kruskal–Wallis for across all groups comparison; (**), Mann–Whitney U test for two groups comparison numbers in bold indicate statistical significance.

major taxa found in *Dermacentor* spp. *Haemaphysalis* spp. harbored a more diverse bacterial spectrum than the other two tick genera. Coxiellaceae (43%) and Methylobacteriaceae (24%) were the predominant bacterial taxa found, with a lesser abundance of Rickettsiaceae (4.6%). Beta analysis using distance-based non-parametric test indicated that microbiome composition differed significantly across tick genera by the generalized UniFrac distance (df = 2, pseudo-F = 5.812, p-value = 0.00001).

Alpha diversity estimates of bacterial communities in ticks and statistical significance (p-value) for group comparison are summarized in **Table 5**. The alpha diversity analyses showed that the bacterial community of *Haemaphysalis* spp. (n = 40) was significantly different from *Amblyomma* spp. (n = 8) with p-value < 0.05 for all tests (Observed OTUs, Simpson's index, Shannon entropy) when measured at the genus level as shown in **Figure 4A**. While there were few ticks positive for *Francisella persica* (n = 4), alpha diversity estimates indicated that their bacterial profiles were quite different from ticks that were not infected with *F. persica* with statistical significance (p-value < 0.001) (**Figure 4B**).

As three *Haemaphysalis* spp. life stages (Larva = 9, Nymph = 15, Male = 6, Female = 10) were included in NGS, this genus was selected for testing the bacterial community difference among tick life stages (**Figures 5A,B**). Alpha diversity estimates (Simpson's index and Shannon entropy) showed that while the overall bacterial profiles among life stages were not significantly different with regard to bacterial taxa present, the number of observed OTUs among life stages was significantly different (**Table 5**; **Figure 5B**). Likewise, the beta diversity analysis using distance-based non-parametric test showed no significant difference across *Haemaphysalis* spp. life stages by the generalized UniFrac distance (df = 3, pseudo-F = 0.905, p-value = 0.5638). Comparing Coxiellaceae abundance across life stages, this group comprised 57–59% abundance of the total bacteria community in male and female ticks, 40% in nymphs, and 28% in larvae (**Figure 5A**).

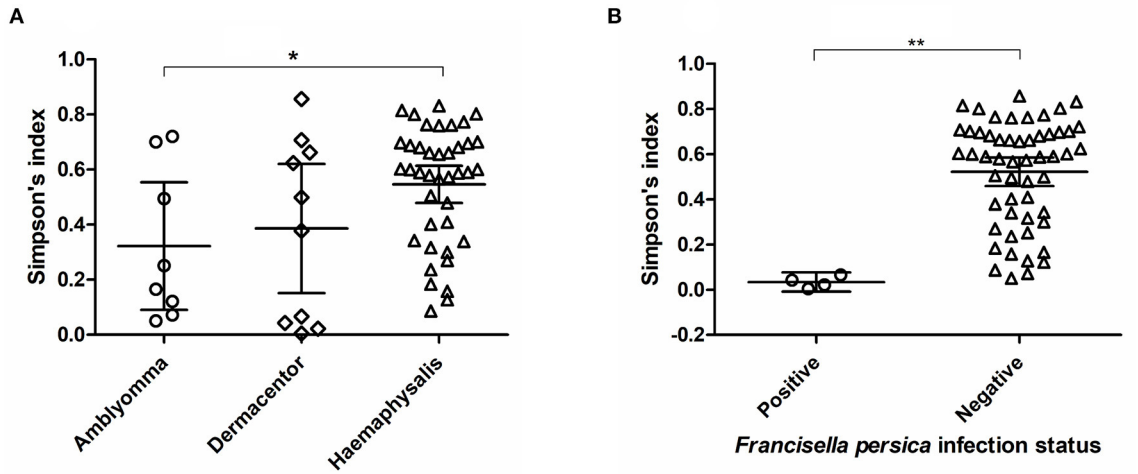

FIGURE 4 | Alpha diversity measures (Simpson's index) based on 16S rRNA gene database for each tick genus **(A)** and *Francisella persica* infection status **(B)**. The statistically significant differences between groups are indicated (*p-value < 0.05, **p-value < 0.001). The solid lines show mean and 95% confidence interval (CI) of alpha diversity for each group. Scatter plots and Mann–Whitney *U* test were created and tested using GraphPad Prism version 5.04.

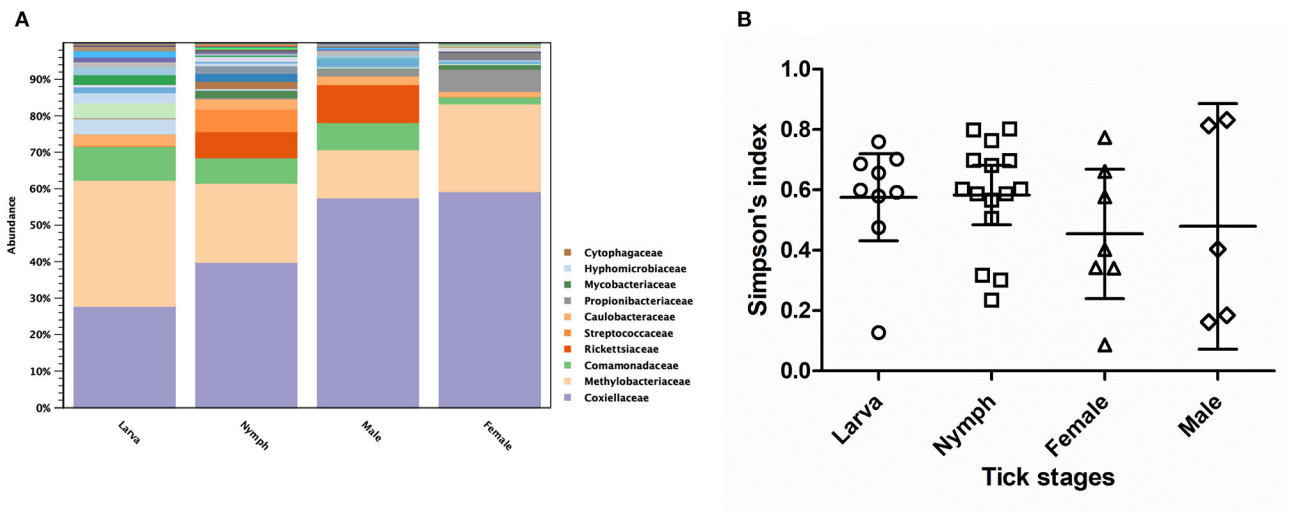

FIGURE 5 | Taxonomic diversity and relative abundance at the family level of bacterial community **(A)** and scatter plots of alpha diversity measure (Simpson's index) **(B)** in three developmental stages of *Haemaphysalis* spp. ticks. The solid lines show mean and 95% Confidence Interval of alpha diversity for each group. Color legend for each family was indicated next to the bar graph.

DISCUSSION

In this study, we examined the bacterial community profile and co-infection of questing ticks collected by dragging from Khao Yai National Park using metagenomics. The majority of ticks collected were in the larval stage; however, adults and nymphs were also collected and identified as *Haemaphysalis* spp., *Amblyomma* spp., and *Dermacentor* spp. The greater number of *Haemaphysalis* spp. found in comparison to other genera may be due to the collection method used (dragging). The prevalence of *Rickettsia* spp. in ticks collected in Khao Yai National Park in this study was lower than in a previous report that found 30% of *Amblyomma testudinarium* positive for *Rickettsia* spp. and approximately 17% positive in *Haemaphysalis* spp. (44). However, they did not detect other bacteria such as *Borrelia* spp., *Francisella* spp., or the common symbiont *Wolbachia* spp. In this study, we detected *Rickettsia* DNA in 18% and 8% in *Amblyomma* spp. (n = 44) and *Haemaphysalis* spp. (n = 331) respectively, along with other bacteria such as *Anaplasma* spp., *Ehrlichia* spp., and *Borrelia* spp. at lower prevalence rates. We did not detect *Wolbachia* spp. in any of the ticks tested but found other

bacterial endosymbionts, namely *Rickettsia* spp., *Francisella* spp., and *Coxiella* spp., using metagenomics and real-time PCR assay. Sequence analyses of *Coxiella* spp. and *Francisella* spp. detected by NGS confirmed the detection of these bacterial endosymbionts in examined ticks (**Supplementary Figure 1**). *Rickettsia* spp. that were detected by NGS but were not confirmed by subsequent qPCR and PCR assays are suspected to be a *Rickettsia* endosymbiont.

Co-infection of pathogenic bacteria occurred in a small number of ticks ($n = 3$), with the majority of co-infections between *Rickettsia* spp. and the Anaplasmataceae family. High prevalence of *Rickettsia* spp. in domestic animals and their ectoparasites, especially fleas, in Thailand has been recognized (15, 16, 45). Less than 10% of *Rickettsia* spp. was found in ticks collected from animals (45). However, some studies found infection rates as high as 24% for *Rickettsia* spp. and 32% for *Anaplasma* spp. in adult ticks collected from under leaves along animal trails across the country (46). The finding of co-infections between *Rickettsia* spp. and other bacteria in female ticks in this study was likely due to the ability of the bacteria to be maintained in ticks through transovarial and transstadial transmission (47–49). As females have already taken two bloodmeals during their development from larva to adult, the probability for *Rickettsia*-infected or Anaplasmataceae-infected ticks acquiring additional bacteria during feeding would be high (50–52). Our study also revealed the increased infection rate in adult ticks compared with larvae or nymphs. Similar findings were reported for an increasing *A. phagocytophilum* infection in adult ticks, especially in females, in Hanover, Germany (53). The study also reported that the co-infection between *A. phagocytophilum* and *Rickettsia* spp. was higher in females (5.2%) than in males (2.4%) or nymphs (1.6%).

A similar study was conducted by Nooroong et al. (46), in which ticks were collected under leaves along animal trails across Thailand were screened for bacterial pathogens. They found co-infections between *Rickettsia* spp. and *Anaplasma* spp. This co-infection was also observed in adult *A. testudinarium* ticks carrying the *Coxiella*-like endosymbiont in Nakhon Nayok, the same province where Khao Yai National Park is located. In Nooroong et al. (46), only adult ticks were collected and a 5.97% co-infection rate between *Rickettsia* spp. and *Anaplasma* spp., as well as a total infection rate of 35.8% for *Rickettsia*, was reported in the location near our study. This higher infection rate is likely due to the fact that Nooroong et al. (46) focused on the adult stage and did not include data from immature ticks. While all tick life stages collected by dragging were included in our study, the majority of ticks collected were larvae. Several previous studies in Thailand have primarily focused on ticks collected from animals; therefore, true co-infection status is inconclusive as pathogens may have been from the host animal. Our study focuses on co-infections in questing ticks actively seeking hosts, which directly represents the human risk of encountering ticks that can transmit multiple pathogens simultaneously. The co-infections investigated in previous studies were mostly among pathogenic bacteria and/or with bacterial endosymbionts (54), or among bacteria and protozoa such as *Coxiella* and *Babesia* spp. in *H. bispinosa* (55), or even co-infection with two to four pathogens of Anaplasmataceae, *Babesia* spp., and *Hepatozoon* spp. in *Rhipicephalus sanguineus* sensu lato collected from dogs in Bangkok, Thailand (56). Co-infection with more than three microorganisms was also reported in questing *Ixodes scapularis* ticks in Wisconsin, USA (29). Additionally, another study reported by Moutailler et al. (57) found up to 45% of questing *I. ricinus* ticks were co-infected with five to eight different pathogens including bacteria (*Borrelia* spp.), parasites, viruses, and endosymbionts. No significant interactions between endosymbionts and pathogens were found but there was a significant association between two *Borrelia* species: *B. garinii* and *B. afzelii*. Our results show that *Ehrlichia* infection in ticks harboring *Coxiella*-like endosymbiont was significantly higher than those without, suggesting that there might be some kind of relationship between *Ehrlichia* spp. and the *Coxiella*-like endosymbiont. However, we found no other association between Anaplasmataceae (*Ehrlichia* spp. and *Anaplasma* spp.) or *Rickettsia* spp. infection with the *Coxiella*-like endosymbiont. The data observed in this study implied that there was no negative or competitive effect of the *Coxiella*-like endosymbiont on the maintenance or existence of other bacteria in tick hosts. Endosymbionts relate to their host and pathogenic bacteria in many ways, such as providing nutrition lacking in blood meal, and sometimes possess an obligate relationship with the host (58, 59). However, other endosymbionts may interfere with the transmission to vertebrate hosts as was shown in *R. rickettsii* and *R. peacockii* in *D. andersoni* (60, 61). Another example of transmission interference can be seen in the salivary glands of *Amblyomma* spp., where a *Coxiella*-related symbiont impairs the transmission of *E. chaffeensis* (62). Alteration of the bacterial microbiome can also interfere with the colonization of pathogenic bacteria as seen in *B. burgdorferi* by modulation of the host immune response (63), which indicates that gut microbiota in ticks also plays a role in pathogen colonization in the gut lumen (22, 63).

Bacterial profiles using metagenomics used in this study showed a few dominant bacterial taxa harbored by questing ticks collected in Khao Yai National Park. Three main bacteria taxa were found in each tick genus—*Coxiella* spp. in *Haemaphysalis* spp., *Rickettsia* spp. in *Amblyomma* spp., and *Francisella* spp. in *Dermacentor* spp., making up the majority of bacterial taxa in ticks with relative abundance ranged from 40 to 80%. Our findings were consistent with other studies reporting that hard tick microbiomes are dominated by a small number of bacterial species, most of which are endosymbionts (20, 64). For example, *Coxiella* spp. was the main taxon found with a relative prevalence of 89.5% in *Rhipicephalus turanicus* (65), 89–100% for *A. americanum* (66–68), 98.2% in the female ovaries of *Rhipicephalus* (*Boophilus*) *microplus* (69), and 39.2% in *Haemaphysalis* spp. collected from animals in Malaysia (64). *Coxiella* spp. is a ubiquitous bacterium found in many tick species and maintained through transovarial transmission, as it was shown to pass on to their eggs and larvae from adult laboratory-reared *R. sanguineus* ticks (65, 70). Other bacterial endosymbionts detected in our study

were *Rickettsia* spp., and depending on the tick species, *Rickettsia* spp. could represent up to 83% of the bacterial community, as observed in *Amblyomma* ticks in our study, while it was found to be less prevalent in the other two genera (*Haemaphysalis* and *Dermacentor*). A *Francisella*-like endosymbiont was another taxon we detected in high prevalence along with *Methylobacteria* in *Dermacentor* spp. The same finding was previously reported by several groups in which two *Dermacentor* species were dominated by three core bacterial taxa: *Francisella*, *Sphinogomonas*, and *Methylobacterium* (61, 71, 72). In addition to *Dermacentor* spp., *A. maculatum* was reported to harbor a *Francisella*-like endosymbiont at high abundance as well (73, 74). Our study also found that bacterial community composition varied significantly among tick genera, especially between *Amblyomma* and *Haemaphysalis* ticks. However, the species richness and diversity slightly decreased during development in *Haemaphysalis* spp. Other studies found that microbiome richness and diversity significantly decreased during development and varied greatly among species (72, 75). In one study, the obvious difference was on core OTUs of endosymbiont bacteria among tick species where *Dermacentor* spp. was dominated by *Francisella* spp., while *H. leporispalustris* and *I. pacificus* had *Coxiella* spp. and *Rickettsia* spp. as dominant bacterial species, respectively (72). Differences in reports might be from the techniques used to study the microbiome, geography, and natural vs. laboratory-reared tick populations (22, 76–78). However, the striking similarity among these studies is the difference in bacterial endosymbionts among tick genera, suggesting that there could be a competition among symbionts within tick genera. As it was reported in *R. turanicus*, *Coxiella* was the primary symbiont and *Rickettsia* was the secondary, having lesser relative abundance (65). Other studies discovered competition between *Rickettsia* species in *Dermacentor* spp. in which one species prevented another from transovarial transmission (79, 80).

Understanding the microbiome composition in ticks of different species, their vector capacities, as well as the role of bacterial endosymbionts on tick physiology, including their influence on pathogen transmission, may provide insight into vector control to prevent human infection and the emergence of tick-borne diseases in the future. This study provides important information on bacterial community composition and co-infection rates in questing ticks in Thailand with implications for animal and human health.

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 below: GenBank accession number(s) OK161258-61, OK161265-68, OK161272-79, OK161279-84, and OK180561-611. The SRA BioProject ID PRJNA766341.

ETHICS STATEMENT

The animal study was reviewed and approved by AFRIMS Institutional Animal Care and Use Committee (IACUC), U.S. Army Medical Directorate-Armed Forces Research Institute of Medical Sciences.

AUTHOR CONTRIBUTIONS

RT and SC conceived and designed the experiments. JS, NC, NY, SP, and BT performed the experiments. SP, BT, NC, WT, KS, and SC conducted field works. RT and JS analyzed the data. RT wrote/revised the manuscript. BP-S and PM reviewed the manuscript. All authors contributed to the article and approved the submitted version.

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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.2021.764763/full#supplementary-material

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Discovering the Potent Inhibitors Against *Babesia bovis in vitro* and *Babesia microti in vivo* by Repurposing the Natural Product Compounds

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In the present study, we screened 502 natural product compounds against the *in vitro* growth of *Babesia* (*B.*) *bovis*. Then, the novel and potent identified compounds were further evaluated for their *in vitro* efficacies using viability and cytotoxicity assays. The *in vivo* inhibitory effects of the selected compounds were evaluated using *B. microti* "rodent strain" in mice model. Three potent compounds, namely, Rottlerin (RL), Narasin (NR), Lasalocid acid (LA), exhibited the lowest IC_{50} (half-maximal inhibitory concentration) as follows: 5.45 ± 1.20 μM for RL, 1.86 ± 0.66 μM for NR, and 3.56 ± 1.41 μM for LA. The viability result revealed the ability of RL and LA to prevent the regrowth of treated parasite at 4 × IC_{50} and 2 × IC_{50}, respectively, while 4 × IC_{50} of NR was sufficient to stop the regrowth of parasite. The hematology parameters of *B. microti in vivo* were different in the NR-treated groups as compared to the infected/untreated group. Interestingly, intraperitoneal administration of NR exhibiting inhibition in the growth of *B. microti* in mice was similar to that observed after administration of the commonly used antibabesial drug, diminazene aceturate (DA) (76.57% for DA, 74.73% for NR). Our findings indicate the richness of natural product compounds by novel potent antibabesial candidates, and the identified potent compounds, especially NR, might be used for the treatment of animal babesiosis.

Keywords: natural product compounds, *Babesia bovis*, *in vitro*, *Babesia microti*, *in vivo*

INTRODUCTION

Babesiosis is an important tick-borne disease (TBD) caused by the protozoa *Babesia* (*B.*) that infects domestic and wild animals, sometimes humans (1). In cattle, the infection is mainly caused by *B. bovis* and *B. bigemina* (2). The disease is typified by high fever, hemolytic anemia, hemoglobinuria, and occasionally death causing huge economic losses in the animal industry worldwide (1, 3).

Generally, control of babesiosis depends on three main strategies: (i) vector control, (ii) vaccine development, and (iii) administration of antibabesial drugs. One of the promising strategies against parasite is to control the receptor-ligand interactions of parasite molecules and their target cells, such as RON-AMA-1 (4). Two novel exported multigene families (mtm) that encode predicted multi-transmembrane, integral membrane proteins were identified in *B. bovis*. One mtm gene was down-regulated, resulting in decreased growth rate, reduced RBC surface ridge numbers, mis-localized VESA1, and abrogated cytoadhesion to endothelial cells (5). Furthermore, Sun et al. revealed that 70% invasion-competent *B. divergens* invading the erythrocyte were <45 s and all invasion-competent parasites achieved invasion within 10 min of contact (6). On the other hand, the commonly used antibabesial drugs, diminazene aceturate (DA) and imidocarb dipropionate (ID), exhibited resistance either from the treated parasites or toxic effect to the host (7–9), subsequently leading to discovery of safer and effective novel antibabesial compounds and becoming an urgent demand. In this regard, a non-biased screening of large libraries of compounds was recently developed to identify novel inhibitors for babesiosis (9–12). Following this pattern, Rottlerin (RL), Narasin (NR), and Lasalocid Acid (LA) are three natural compounds which were repurposing the already approved drugs. RL and NR derivative of *Mallotus philippinensis* and *salinomycin* possess multiple anti-cancer biological activities (13, 14). LA is another *ionophorous* antibiotic which also possesses anti-cancer efficacy (15). Of note, the antiparasitic efficacy of RL, NR, and LA has been reported against the growth of *Plasmodium* spp., *Eimeria* spp., *Toxoplasma gondii*, and *Trypanosoma* spp. (16–19).

RL is a polyphenol with natural anthelminthic activity isolated from *Mallotus philippinensis* in 1964 (20). In addition, RL is a polyphenol with autophagic promoting properties, and potential benefits of this compound have been identified, including anti-inflammatory, antiallergic (21), antibacterial (22), and anticancer compound (23). Furthermore, a lot of research focused on the toxicity and pharmacological mechanism of Rotterin in tumor and cancer. Moreover, RL exhibited its major and recent cytotoxic properties of human amelanotic A375 melanoma cells, that is, growth arrest, apoptosis induction, and translation shutoff. Although the RL is used in a variety of fields especially anticancer (24), as an anti-parasite, only Ietta et al. reported that RL can act against *Toxoplasma gondii*; as a result, this compound is an inducer of autophagy and inhibition of protein synthesis (25). Therefore, in the current study, we screened 502 compounds from the natural product compounds (NPCs) against the *in vitro* growth of *B. bovis* and against the *in vivo* growth of *B. microti*.

MATERIALS AND METHODS

Parasites

Babesia bovis (Texas strain) was cultured in 8% purified bovine red blood cells (RBCs) suspended in GIT medium using 24-well-culture plates in a 37°C incubator with an atmospheric condition of 5% CO_2 and 5% O_2. The medium was replaced every 24 h (12).

Babesia microti (Munich strain) was used for the *in vivo* studies and was recovered from −80°C stock in one 6-week-old female BALB/c mice (CLEA Japan Inc., Tokyo, Japan) (26). The animal experiment was conducted in accordance with The Regulations for Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine, Japan (Accession numbers 18–40).

Chemical Reagents

SYBR Green I (SGI) nucleic acid stain (Lonza, USA; 10,000×) was stored at −30°C, and lysis buffer containing EDTA (10 mM), Tris (130 mM at pH 7.5), saponin (0.016% w/v), and TritonX-100 (1.6% v/v) was prepared and stored at 4°C, as previously described (12). Five hundred and two NPCs (2 mg/ml) (**Supplementary Table 1**) were received from the Cancer Research Institute of Kanazawa University (Ishikawa, Japan) and stored at −30°C until use for *in vitro* screening against *B. bovis*. DA (Novartis, Japan) was used as a positive control drug. RL, NR, and LA (all from Sigma-Aldrich, Japan) were prepared as a 100 mM stock solution and stored at −30°C until use. Cell Counting Kit-8 (CCK-8, Japan) was used for cytotoxicity assay.

In vitro Growth of Initial Inhibitory Assay

The *in vitro* inhibitory efficacies of 502 NPCs were evaluated against the growth of *B. bovis* using fluorescence assay (27). All compounds were initially screened against *B. bovis* using 2.5 μg/ml at 1% parasitemia and 2.5% hematocrit (HCT) for 4 successive days in 96-well-plates. Fluorescence values were evaluated at day 4 after adding 100 μl of lysis buffer mixed with 2× SGI using the fluorescence spectrophotometer (485 and 518 nm, Fluoroskan Ascent, USA). After the initial screening, IC_{50} values were calculated for the compounds that exhibited the highest inhibitory efficacies >60% (RL, NR, LA) with concentrations ranging from 0.10 to 100 μM using the non-linear regression analysis (Curve fit) in GraphPad Prism 7 (GraphPad Software Inc., USA). Non-parasitized RBCs and 0.50% dimethyl sulfoxide (DMSO) were loaded into triplicate wells and used as a blank and negative control, respectively. Each drug concentration was tested in triplicate, and the experiment was repeated three times.

Viability Test and Morphological Changes Determination

The viability changes in drug-treated *B. bovis* were observed as previously described by Tayebwa et al. (28). A 96-well-plate was used, and 10 μl of 1% iRBCs with 90 μl medium at various drug concentrations were suspended in each well. The plate was incubated as aforementioned, and the medium was changed every 24 h for 4 consecutive days and replaced with their respective concentrations of RL, NR, and LA. The various concentrations of RL, NR, and LA used in this experiment were 0.50×, 1×, 2×, and 4× of the IC_{50}, respectively. On day 5, 3 μl of RBCs from treated wells was added to 7 μl of fresh RBCs in a new 96-well plate (no drug) and the medium was replaced daily for the next 6 days. Giemsa-stained thin blood smears (GBS) and fluorescence values were determined in 5 days. Each experiment was performed in triplicates in three separate trials.

Cytotoxicity of RL, NR, and LA on MDCK Cell Line

The drug-exposure viability assay was performed following the recommendation of the Cell Counting Kit-8 (CCK-8, Japan). In brief, 5×10^4 cells/ml of Madin-Darby Bovine Kidney (MDBK) cells were seeded on 100 µl per well in a 96-well-cell culture plate and incubated for 24 h. One hundred microliters of 3-fold drug dilutions were added to each well to a final concentration of 1–100 µM in triplicates. After 24 h, 10 µl of CCK-8 was added to each exposed drug. After 4 h incubation, the absorbance values were determined at 450 nm using MTP-500 microplate reader (Corona Electric, Japan). The wells with only the culture medium were used as blank, while those containing cells in a medium with 0.50% DMSO were used as control (29).

Chemotherapeutic Evaluation of RL, NR and LA in Mice

The mouse model infected with *B. microti* was used to determine the inhibitory effect of the selected compounds in this study as previously described by Rizk et al. (26). Thirty-five 8-week-old female BALB/c mice (CLEA Japan Inc., Tokyo, Japan) were used in the *in vivo* study and divided equally into seven groups. First, second, and third groups were treated with RL, NR, and LA at dose rates 5 mg/kg, 7 mg/kg, and 1 mg/kg, respectively. DA (the commonly used antibabesial drug) was administrated to the mice in the fourth group at a dose rate of 25 mg/kg. Mice in the fifth group were treated with a combination therapy consisting of 3.5 mg/kg NR + 10 mg/kg DA. All drugs were administrated by intraperitoneal (IP) route in all groups. Mice in the sixth and seventh groups were kept as positive (infected and untreated) control and negative (uninfected and untreated) control, respectively. The treatment of compound was initiated and continued for 5 successive days (day 4 to day 8) when parasitemia reached 1% in the infected mice.

Prior to the beginning of the *in vivo* experiments, a *B. microti* positive mouse was prepared according to Nugraha et al. (10). In brief, *B. microti* was recovered and injected into a mouse, and then parasitemia was checked every 2 days by Giemsa-stained blood smear. After the parasitemia reached

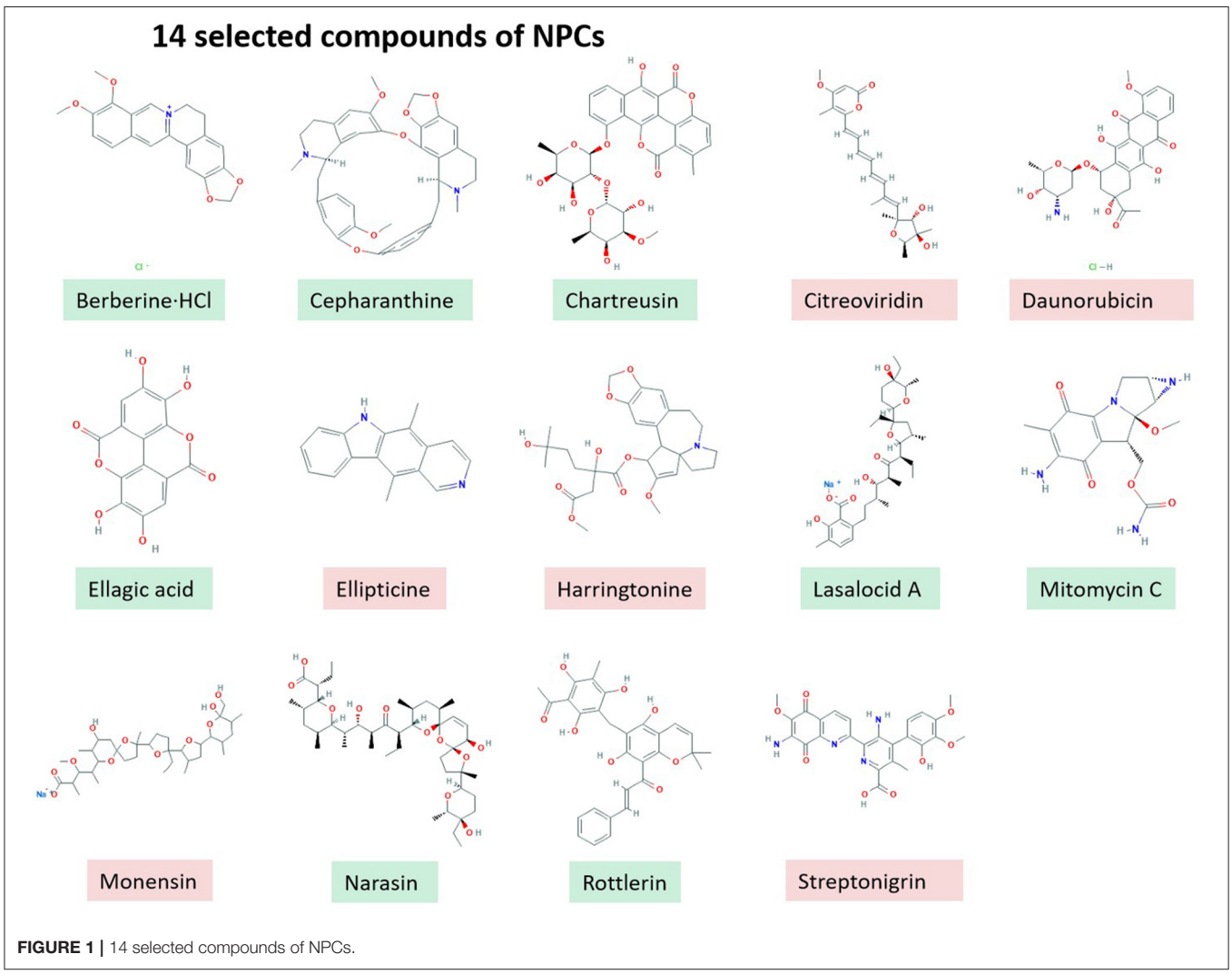

FIGURE 1 | 14 selected compounds of NPCs.

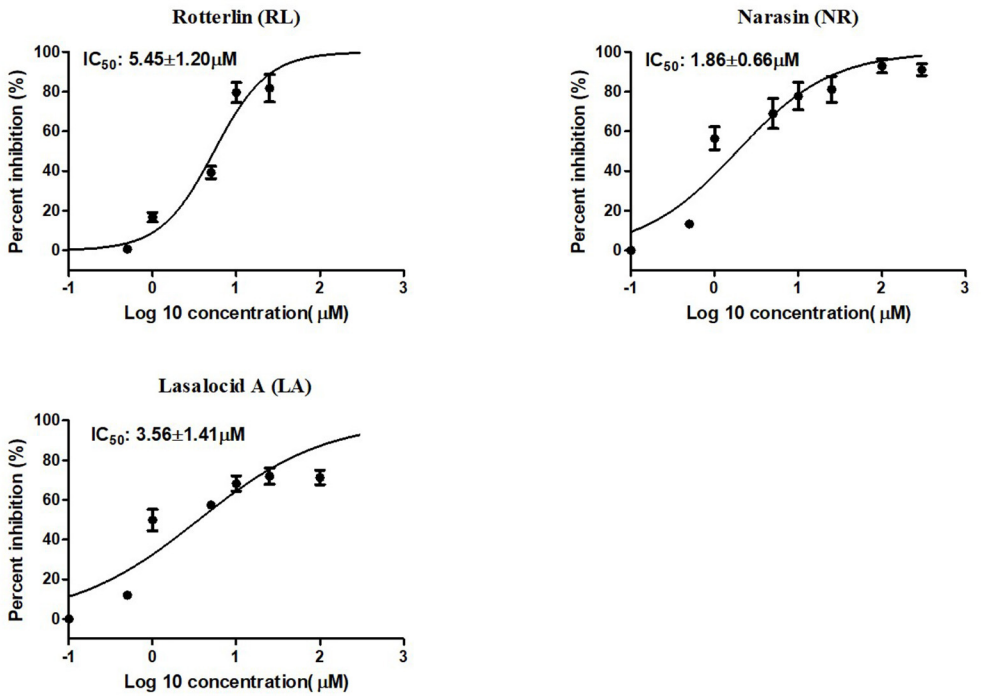

FIGURE 2 | IC50 of Rotterlin, Narasin, Lasalocid A.

30%, the mouse was anesthetized and blood was collected by cardiac puncture. The blood was then diluted by 1 × PBS to acquire 2 × 10^7/ml *B. microti* iRBCs. Except the negative control group, all mice were injected IP with 0.5 ml dilution iRBC to achieve 1 x 10^7/ml iRBCs and parasitemia was monitored every 2 days. Venous tail blood samples (2.5 μl) were collected from each mouse every 2 days until 32 days post-inoculation or the cessation of parasitemia. The blood samples were collected in a 96-well plate (RPMI 1,640 medium 100 μl + lysis buffer 50 μl), and uninfected mice RBCs were used as blank control. Then, 50 μl of lysis buffer containing 2× SGI nucleic acid stain was added directly to each well and gently mixed (26). Next, the plate was incubated in the dark for 1 h and the fluorescence values were determined as described above using a fluorescence spectrophotometer. Meanwhile, 10 μl of blood from the tail was collected every 4 days and used to determine the hematological profiles using an automatic hemocytometer (Celltac α MEK-6,450, NihonKohden, Tokyo, Japan). All parameters were monitored until day 30. All the *in vivo* the experiments were repeated twice.

Statistical Analysis

The IC_{50} values of RL, NR, LA, and DA were determined using the non-linear regression curve fit in GraphPad Prism 6.0 (GraphPad Software Inc., USA). The differences in the fluorescence values of the *in vitro* cultures and among groups for the *in vivo* studies were analyzed with a statistical software program (GraphPad Prism version 5.0 for Windows; GraphPad Software, Inc., San Diego, CA, USA), using an independent Student's *t*-test and one-way ANOVA. A *P*-value of <0.05 was considered statistical difference, and a *P*-value of <0.01 was considered statistically significant difference.

RESULTS

RL, NR, and LA Inhibit the *in vitro* Growth of *B. bovis* and *in vitro* Cytotoxicity

In vitro screening of 502 NPCs against the growth of *B. bovis* at 2.5 μg/ml concentration revealed that 14 compounds including RL, Berberine·HCl, Cepharanthine, Chartreusin, Citreoviridin, Daunorubicin, Ellagic acid, Ellipticine, Harringtonine, LA, Mitomycin C, Monensin, NR, and Streptonigrin exhibited over 60% inhibitory effects (**Figure 1**). However, cytotoxicity assay on MDBK showed that only eight compounds have low toxic effects over 100 μM (Green Color, **Figure 1**) and other screened compounds ($n = 6$) exhibited toxic effects (Red Color, **Figure 1**).

RL, NR, and LA inhibited the growth of *B. bovis* in a dose-dependent manner. The IC_{50} values of RL, NR, and LA were 5.45 ± 1.20 μM, 1.86 ± 0.66 μM, and 3.56 ± 1.41 μM, respectively (**Figure 2**).

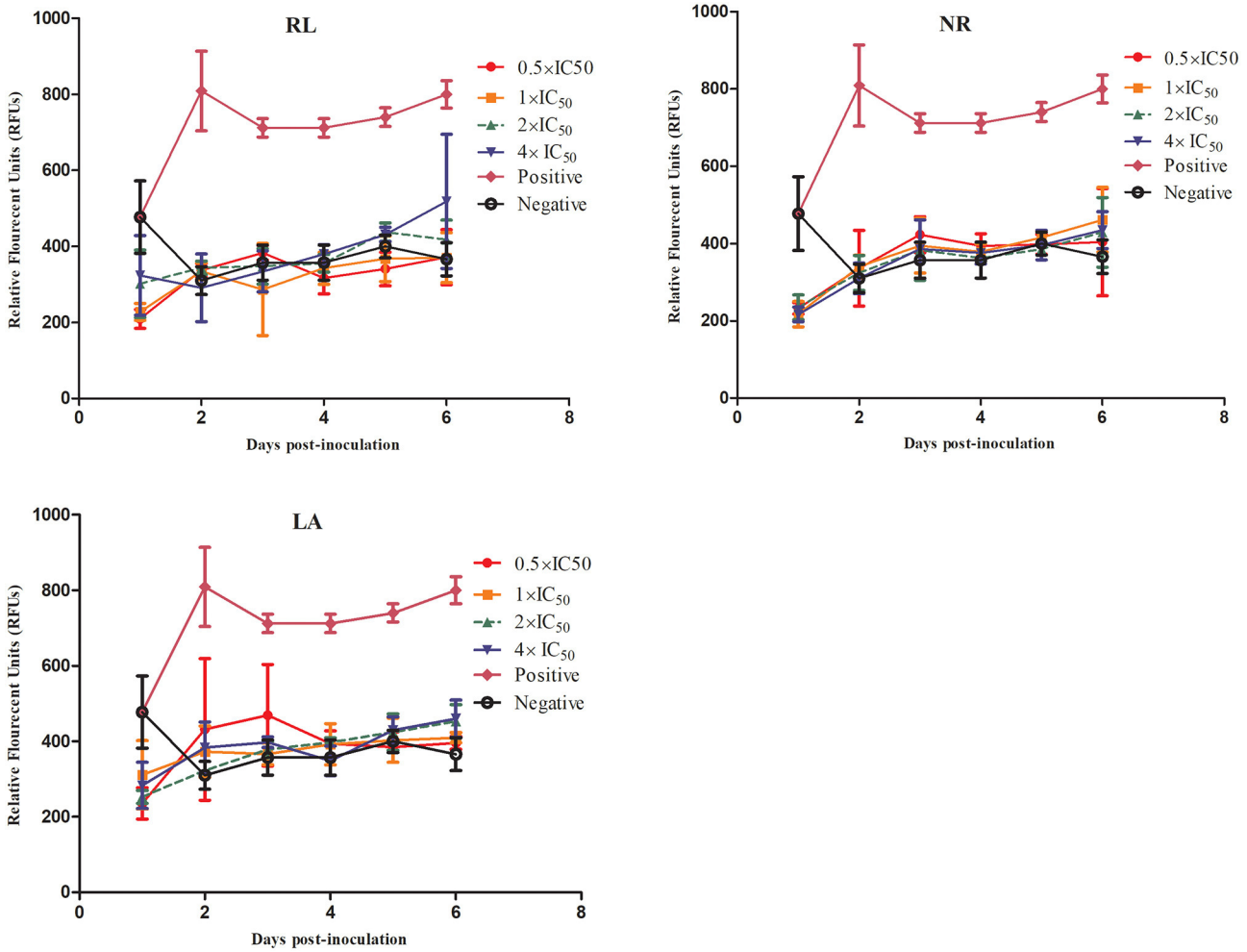

FIGURE 3 | Viability test of RL, NR, and LA.

Viability and Morphological Changes of RL-, NR-, and LA-Treated *B. bovis*

To further validate the potent identified natural product compounds as anti-*B. bovis* compounds, viability test and morphological changes in the treated culture were performed. The results showed that RL-, NR-, and LA-treated *B. bovis* lack the ability to regrow at 1×, 2×, and 4× the IC_{50} values. The concentrations at which *B. bovis* did not regrow were 5.45, 10.90, and 21.80 μM for RL treatment, 1.86, 3.72, and 7.44 μM for NR, and 3.56, 7.12, and 14.24 μM for LA (**Figure 3**).

The parasitemia was examined for all parasites after 24 h, 72 h, and 7 days of incubation with the two crude methanolic extracts in Giemsa-stained blood smears. Morphological observation of *B. bovis* treated with 0.50×, 1×, 2×, RL, NR, and LA identified that the parasites (2 × the IC_{50}) appeared smaller and disintegrated at 24 h in RL, NR, and LA as compared to the control (P3d) (**Supplementary Figures 1–3**). The remnants of the dot parasites within the RBC were observed in micrographs of *B. bovis* in RL, NR, and LA, while the LA-treated parasite faintly appeared at 72 h (**Supplementary Figures 1–3**).

In vitro Cytotoxicity

In vitro treatment by NR exhibited no cytotoxicity until 50 μM, while the RL and LA *in vitro* treatment showed cytotoxicity on MDBK at 5 μM (**Supplementary Figure 4**). Fortunately, the most potent natural product compound identified, NR exhibited a low IC_{50} on MDBK with subsequently very high selectivity index (SI) (**Table 1**). NR was shown the highest SIs (**Table 1**), suggesting their possible promising future use for *in vivo* study.

Chemotherapeutic Effect of RL, NR, and LA on *B. microti* in Mice

The promising efficacy of RL, NR, and LA *in vitro* prompted further research to evaluate the antibabesial effects against *B. microti* in mice. In treated of NR groups, the parasitemia increased at a significantly lower rate than the control group ($P < 0.01$) at days 12, 16, and 20 IP. The relative fluorescent units

TABLE 1 | IC_{50}, CC_{50}, and selectivity indices of potent Natural compounds and diminazene aceturate evaluated against the *in vitro* growth of *B. bovis*.

Drugs	IC_{50} values (μM)[a]	CC_{50} (μM)	Selectivity indices[b]
RL	5.45 ± 1.20	27.22 ± 10.49	4.99 ± 0.68
NR	1.86 ± 0.66	95.02 ± 35.60	45.71 ± 6.12
LA	3.56 ± 0.44	9.85 ± 1.23	2.76 ± 0.11
DA	0.47 ± 0.09	ND	ND

[a] *IC_{50} values for each drug were calculated on the fourth day of the in vitro culture using a fluorescence assay in three separate experiments. Each drug concentration was made in triplicate in each experiment, and the final obtained IC_{50} values were the mean SD of values obtained from three separate experiments. DA, diminazene aceturate; RL, Rottlerin; NR, Narasin; LA, Lasalocid acid; ND, not detected.*

[b] *Selectivity indices (SIs) were calculated based on the ratio CC_{50} (MDBK)/IC_{50} of the compound.*

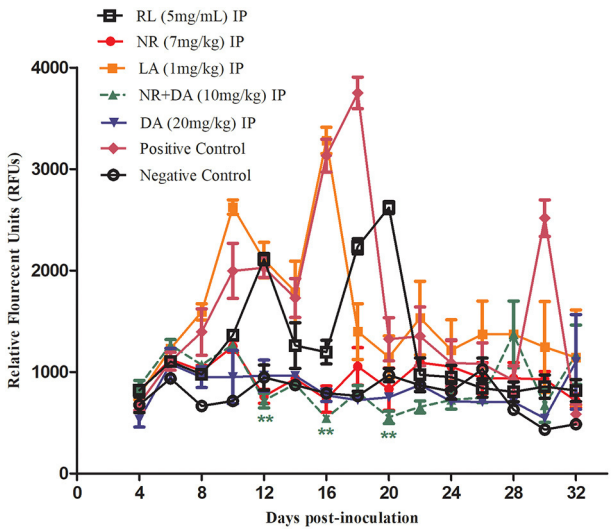

FIGURE 4 | Chemotherapeutic evaluation (RFUs) of RL, NR, and LA in mice.

(RFUs) in treated groups reached 960, 1,200, 3,100, and 890 in 7 mg/kg of NR, 5 mg/kg of RL, 1 mg/kg of LA, and 20 mg/kg of DA, respectively, at 16 days IP, as compared to 3,800 peak RFUs in the control group (**Figure 4**). At day 16 pi, 20 mg/kg DA, 7 mg/kg NR, 5 mg/kg of RL, and 1 mg/kg LA cause 76.57, 74.73, 68.42, and 18.42% inhibition in the growth of *B. microti* in comparison with the untreated mice, respectively. Interestingly, the 5 mg/kg RL showed 68.42% inhibition in the growth of *B. microti* which is similar to those observed after treatment with the commonly used drug, DA.

The hematological parameters that included the RBCs (**Figure 5A**), HGB concentration (**Figure 5B**), and HCT percentage (**Figure 5C**) were significantly different in the NR-treated groups as compared to the infected untreated group. On the other hand, there was no significant reduction ($P > 0.05$) in the number of RBCs, HGB concentration, and HCT percentage in the NR treated groups in comparison to the DA-treated group. The RBC, HCT, and HGB values of NR, NR+DA, and DA groups showed identical dots and line during days 8 to 16, wherein *B. microti* were having active growth. Three lines indicating RBC, HCT, and HGB were almost same as the uninfected mice, while LA did not depict much difference between infected-untreated mice. Interestingly, a decreasing trend was observed in hematological data of RL at days 0–12, but later on, low hematological parameters were shown in mice after RL was given. RL group also exhibited significant difference ($P < 0.01$) for RBCs, HCT, and HGB at day 12.

DISCUSSION

Babesia bovis is one of the most important bovine disease. So far, the vaccines against the protozoa are not very effective, but screening new effective compounds for babesiosis could be the alternative strategy for prevention and treatment of the disease. In this study, the preliminary inhibitory effects and the results of cytotoxicity assay using MDBK showed that three compounds including RL, NR, and NR exhibited the highest inhibitory efficacy against the *in vitro* growth of *B. bovis* and the lowest toxicity effect.

NR is an antibiotic to treat coccidiosis in poultry, which was isolated from *Streptomyces albus*. NR, a derivative of salinomycin (an ionophoric anticoccidial compound), and the anti-inflammatory and antiparasitic (*Plasmodium* spp. and *Eimeria* spp.) actions of NR have been previously documented (16, 18). Furthermore, NR have the inhibitory effect against the growth of *Toxoplasma gondii* (30), exhibiting over 45.56% inhibition rate when used at 1 μg/ml. According to Hickey et al. NR can also significantly reduce viable cell numbers in *Staphylococcus* aureus, and it displayed the least toxicity against mammalian cell lines including Hep G2, HFF-1, MCF-7, and MDBK (IC_{50} 39.52 μg/ml) (31). In this study, the potent inhibitory effect on *B. bovis in vitro* and *B. microti in vivo* for NR was identified. Although, this time, the data only showed the effects of natural compounds against *Babesia* pathogens, the results could be supportive for the signaling pathway found in previous scientific reports. In canine babesiosis, *Babesia rossi* has been recognized as causative agent of low triiodothyronine (T3) syndrome. The concentration of thyrotropin (TSH), total thyroxin (TT4), and free thyroxin (FT4) was found to be low in serum (32). Intriguingly, NR was identified as a small molecule antagonist for the thyroid hormone receptor beta (Thrb) in Norway rat. Thrb mediates the biological activities of thyroid hormone and is also identified in cattle and house mouse [https://pubchem.ncbi.nlm.nih.gov/gene/24831#section=Ensembl-ID, (33)]. The inhibitory effect of NR in *B. microti* (*in vivo*) may be influenced by thyroid hormone pathway. For instance, Gulia-Nuss et al. identified that the signaling pathways that regulate the innate immune response, such as the Toll-like receptors, IMD (Immunodeficiency), and JAK-STAT (Janus Kinase/ Signal Transducers and Activators of Transcription), also occur in ticks (34). Obviously, *Babesia* as one of the tick-borne pathogens can be carried and transmited by vector tick species. Gulia Nuss's investigation may explain the signaling pathway relationship for ticks and babesiosis in one aspect (34). On the other hand, Chen et al. reported that NR inhibited proliferation, migration, and invasion of human metastatic estrogen receptor

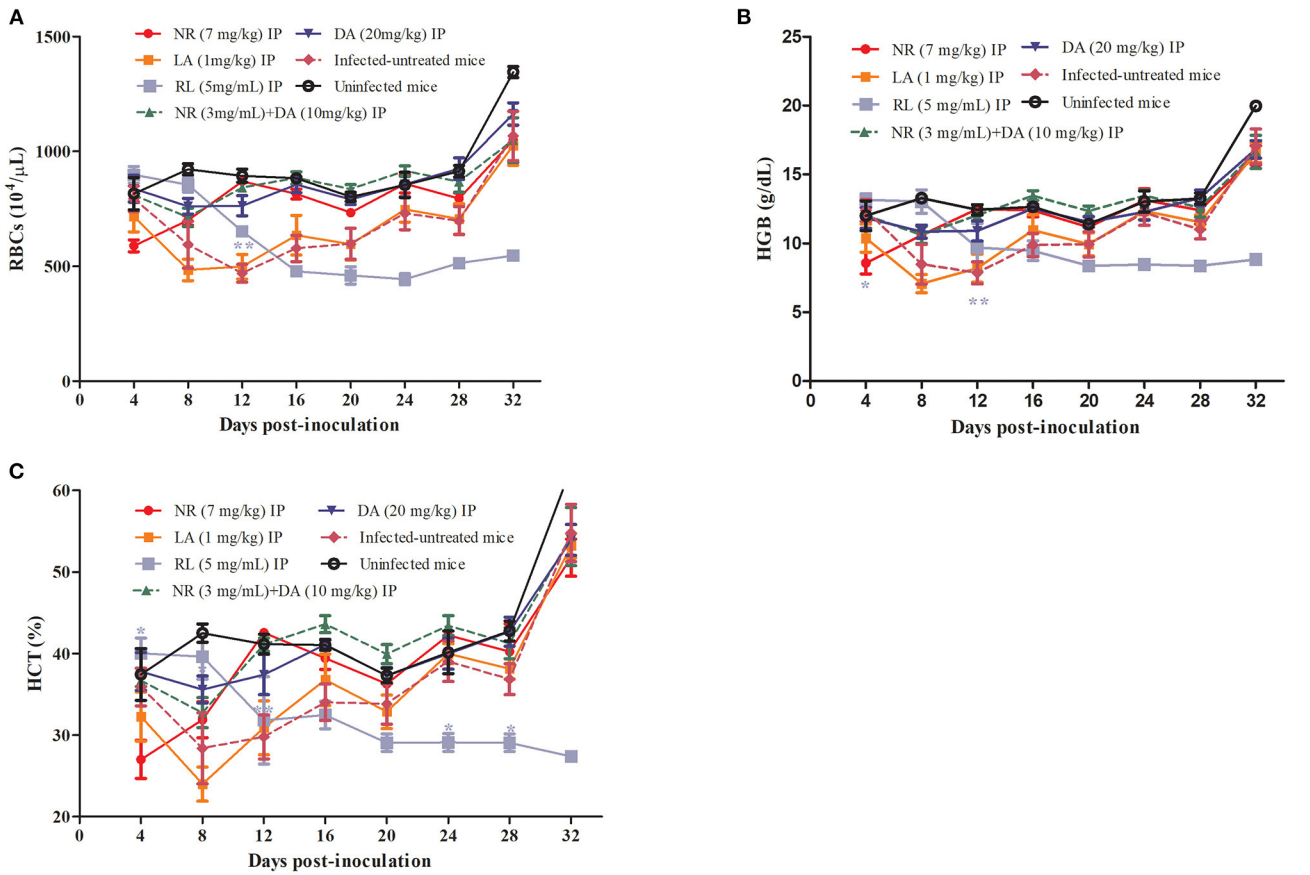

FIGURE 5 | RL, NR, and LA in treatment of anemia associated with Babesia. The hematological parameters of RBCs **(A)**, HGB **(B)**, HCT **(C)**.

positive breast cancer cells by inactivating IL-6/STAT3-mediated EMT signaling pathways *in vitro* and *in vivo* (35).

RL is inhibitor of PKC-δ (Protein kinase C delta) gene which is also identified in human, cattle, and house mouse. PKC-δ is a tumor suppressor as well as positive regulator of cell cycle progression. Moreover, this protein can positively or negatively regulate apoptosis [https://pubchem.ncbi.nlm.nih.gov/gene/5580, (36)]. In this study, RL has the inhibitory effect on *in vitro* of *B. bovis*, but its repressing function is not well pronounced *in vivo* of *B. microti* chemotherapeutic evaluation (RFUs) in BALB/c mice. Interestingly, the identified potent NPCs prevented the development of anemia, as there was no significant reduction in the assessed hematological variables in treated mice on days p.i. The potential inhibitory effects of identified potent NPCs against *B. microti* together with the ability of these compounds to prevent the progress of anemia in the treated mice have added merit to these compounds over previously evaluated antipiroplasm candidates thymoquinone (37), fluroquinolones (38), and Zingiber officinale rhizome (39). However, further research is needed to discover the mechanism by which these compounds protect the treated mice from the hemolytic-macrocytic anemia caused by babesiosis. In some cases, the antitumor action of IFN-α has been shown to involve the induction of apoptosis through the activation of JNK *via* PKC-δ, leading to upregulation of TRAIL and activation of Stat-1. The RL as the inhibitor of PKC-δ will downregulate TRAIL and Stat-1 (40). Fascinatingly, EVs (Extracellular vesicles) from parasite or *Trypanosoma cruzi*-infected macrophages interacting with TLR2 were able to elicit translocation of NF-κB and, as a consequence, to alter the EVs, the gene expression of proinflammatory cytokines, and STAT-1 and STAT-3 signaling pathway (41). In leishmaniasis, the nuclear factor kappa-light-chain enhancer of activated B cells (NF-kB) signaling pathway and pro-apoptotic protein kinase C delta (PKC-δ) were downregulated, while inhibition of caspase-3 activation prevented *L. aethiopica* spreading (42). On the other hand, STAT-5 is also related to promote T cell proliferation and differentiation with immune-related proteins, while spleen continued to initiate immune responses to combat the infection of *B. microti* (43).

LA as a medicated feed additive is a polyether ionophore antibiotic produced by strains of the bacterium *Streptomyces lasaliensis*. This compound is used for the control of coccidiosis in cattle and poultry (44). As far as the parasite is concerned, LA reduced the growth rate of trypanosomes by 50%, and further increasing the concentration of the drug to 1.75 and 10 μM to kill the parasite. LA induced even a rapid swelling in trypanosomes

than salinomycin which was considered as a trypanocidal related ionophore (45). For their anti-malarial activity, only some lasalocid acid and monensin analogs showed potent activity against selected *Plasmodium* species *in vivo* culture (14). The cyclic polyether antibiotic valinomycin and carboxylic ionophore salinomycin have been shown to exhibit potent *in vitro* anti-babesial activity against the related canine pathogen *B. gibsoni*. After using LA, the inhibitory effects of *B. bovis in vitro* were also observed. The reason of effects of lasolocid acid against *B. bovis* is probably familiar with the ionophores directly against under low concentrations of potassium (which completely lack Naþ/Kþ-ATPase activity) (45). The lysis of the red blood cells and, subsequently, the killing of *B. gibsoni* occurred when containing high concentrations of potassium (46). Unfortunately, the result of *B. microti in vivo* in BALB/c mice was not very well. Of note, polyether ionophore antibiotics have been shown to display activity against both tachyzoite and bradyzoite stages of *T. gondii* (47). On the other hand, lasalocid acid did not reduce the rate of abortion and neonatal mortality in sheep infected with *T. gondii* (48).

Although this study evaluated the inhibitory efficacy of potent compounds from the natural product against the *in vivo* growth of *B. microti*, the study neither followed up the presence of parasite remnant in different tissues of the treated mice nor determined the serum chemistry panel data before and after treatment in the mice. Therefore, additional future experiments are required to follow up the efficacy of the identified potent compounds in NPC-treated mice. This study evaluated the *in vitro* inhibitory effect of NPCs against the growth of *B. bovis* without determining the developmental stage in which the inhibition occurs. Therefore, additional future experiments are required to determine which developmental stage of the parasite might be affected by the identified NPCs, the merozoite stage outside the erythrocyte, or the parasite stage inside the erythrocyte, reducing viability or inhibiting the parasite division. In fact, the cellular targets of the identified three potent NPCs are still unclear and future studies are required to address this point.

In conclusion, new chemical compounds from natural product compounds were identified in the previous study, which suppressed the *in vitro* growth of *B. bovis* (9). But the further experiment using *B. microti* in BALB/c mice showed the toxic effects in mice using natural product compounds, and NR from the three selected compounds was found to have good inhibitory effect whether *in vitro* of *B. bovis* and *in vivo* of *B. microti* in BALB/c mice. Our findings indicated that natural product compounds are a precious source for discovering novel antibabesial drugs and the identified potent compounds, especially NR that might be used for the treatment of Babesiosis.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s. And I detected the following words and expressions: Global list: https://pubchem.ncbi.nlm.nih.gov/, ncbi.nlm.nih.gov/, PubChem, pubchem.ncbi.nlm.nih.gov/.

ETHICS STATEMENT

The animal experiment was conducted in accordance with the Regulations for Animal Experiments of Obihiro University of Agriculture and Veterinary Medicine, Japan (Accession No. 18–40).

AUTHOR CONTRIBUTIONS

YL: methodology, validation, statistical analysis of the results, writing—original draft, and writing—review and editing. MR: methodology, validation, statistical analysis of the results, and writing—review and editing. EG, ML, and JL: validation and writing—review and editing. AR, SJ, and IZ: recorded samples' data and writing—review and editing. MT and BB: writing—review and editing. NY and II: methodology. BC: writing—review and editing and funding acquisition. XX: conceptualization, writing—review and editing, and funding acquisition. All authors contributed to the article and approved the submitted version.

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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.2021.762107/full#supplementary-material

Supplementary Table 1 | Natural compounds BML-2865 information.

Supplementary Figure 1 | Morphological changes observed in 0.5×-2× Rotterolin treated *B. bovis*.

Supplementary Figure 2 | Morphological changes observed in 0.5×-2× Narasin treated *B. bovis*.

Supplementary Figure 3 | Morphological changes observed in 0.5×-2× Lasalocid A treated *B. bovis*.

Supplementary Figure 4 | Cytotoxicity of RL, NR, and LA in MDBK.

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The *Ixodes scapularis* Symbiont *Rickettsia buchneri* Inhibits Growth of Pathogenic Rickettsiaceae in Tick Cells: Implications for Vector Competence

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Ixodes scapularis is the primary vector of tick-borne pathogens in North America but notably does not transmit pathogenic *Rickettsia* species. This tick harbors the transovarially transmitted endosymbiont *Rickettsia buchneri*, which is widespread in *I. scapularis* populations, suggesting that it confers a selective advantage for tick survival such as providing essential nutrients. The *R. buchneri* genome includes genes with similarity to those involved in antibiotic synthesis. There are two gene clusters not found in other Rickettsiaceae, raising the possibility that these may be involved in excluding pathogenic bacteria from the tick. This study explored whether the *R. buchneri* antibiotic genes might exert antibiotic effects on pathogens associated with *I. scapularis.* Markedly reduced infectivity and replication of the tick-borne pathogens *Anaplasma phagocytophilum*, *R. monacensis*, and *R. parkeri* were observed in IRE11 tick cells hosting *R. buchneri*. Using a fluorescent plate reader assay to follow infection dynamics revealed that the presence of *R. buchneri* in tick cells, even at low infection rates, inhibited the growth of *R. parkeri* by 86–100% relative to *R. buchneri*-free cells. In contrast, presence of the low-pathogenic species *R. amblyommatis* or the endosymbiont *R. peacockii* only partially reduced the infection and replication of *R. parkeri.* Addition of host-cell free *R. buchneri*, cell lysate of *R. buchneri*-infected IRE11, or supernatant from *R. buchneri*-infected IRE11 cultures had no effect on *R. parkeri* infection and replication in IRE11, nor did these treatments show any antibiotic effect against non-obligate intracellular bacteria *E. coli* and *S. aureus.* However, lysate from *R. buchneri*-infected IRE11 challenged with *R. parkeri* showed some inhibitory effect on *R. parkeri* infection of treated IRE11, suggesting that challenge by pathogenic rickettsiae may induce the antibiotic effect of *R. buchneri*. This research suggests a potential role of the endosymbiont in preventing other rickettsiae from colonizing *I. scapularis* and/or

being transmitted transovarially. The confirmation that the observed inhibition is linked to *R. buchneri*'s antibiotic clusters requires further investigation but could have important implications for our understanding of rickettsial competition and vector competence of *I. scapularis* for rickettsiae.

Keywords: *Ixodes scapularis*, tick, *Rickettsia*, endosymbiont, antibiosis, interference, competition

INTRODUCTION

The blacklegged tick *Ixodes scapularis* Say (Acari: Ixodidae) is the primary vector of zoonotic tick-borne pathogens in North America. It transmits seven pathogens, including those causing Lyme borreliosis (*Borrelia burgdorferi*, *Borrelia mayonii*), human anaplasmosis (*Anaplasma phagocytophilum*), and human babesiosis (*Babesia microti*) (1). Interestingly, in contrast to other major human-biting species of *Ixodes* in different parts of the world, *I. scapularis* does not transmit pathogenic *Rickettsia* species. *Ixodes ricinus* and *I. persulcatus*, the tick vectors primarily responsible for the transmission of *B. burgdorferi*, *A. phagocytophilum*, tick-borne encephalitis virus, and *Babesia* spp. in Europe and Asia, respectively, are commonly infected with *Rickettsia* spp. linked to human disease. Both *R. helvetica* and *R. monacensis* are commonly detected in *I. ricinus* across Europe (2–7), and various other rickettsiae including *R. raoultii* and *R. slovaca*, which are primarily vectored by other tick species, have also been identified in *I. ricinus* (3, 8). Meanwhile, *I. persulcatus* is infected with a wider range of *Rickettsia* spp., with *R. helvetica*, *R. raoultii*, *R. sibirica*, *R. heilongjiangensis*, and "*Candidatus* R. tarasevichiae" often detected in this tick species (3, 4, 9–15). In eastern Australia, *Ixodes holocyclus* is a vector of *R. australis*, the causative agent of Queensland tick typhus (16, 17).

Instead, *I. scapularis* hosts a rickettsial endosymbiont, *R. buchneri* (18), which dominates the tick microbiome, particularly in females where it typically constitutes almost 100% of the microbiome (19–24). These bacteria, formerly known as "rickettsial endosymbiont of *I. scapularis*" (REIS), have been detected in *I. scapularis* populations throughout its range (**Figure 1**) and are often present at high prevalence (18–23, 25–53), suggesting an established relationship between the tick and its endosymbiont. *Rickettsia buchneri* reside primarily in the ovaries of adult female ticks (18), although there is also some evidence of their presence in salivary glands (22, 52). The endosymbiont is transovarially transmitted and can be found in all life stages (22, 26), yet it is still unclear where it resides within adult males and immature stages, or what roles it may play in tick biology. The existence of genes in the endosymbiont encoding complete biosynthetic pathways for biotin and folate (54, 55) suggests that it may aid the tick by supplying essential nutrients lacking in blood. Phylogenetic analyses imply that *R. buchneri* is ancestral to the spotted fever group (SFG) rickettsiae (54), which contains the majority of tick-transmitted *Rickettsia* species; *R. buchneri* is most closely related to *R. monacensis* from *I. ricinus*, and the rickettsial endosymbiont of *Ixodes pacificus*, "*R. monacensis*" strain Humboldt (18, 56). Like other Rickettsiales, the SFG rickettsiae are obligate intracellular Gram-negative bacteria (57).

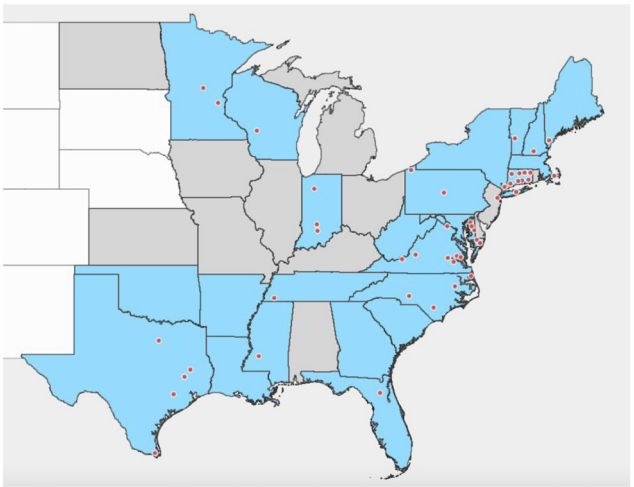

FIGURE 1 | Distribution of detections of *R. buchneri* and "Rickettsial endosymbiont of *Ixodes scapularis*" in *Ixodes scapularis* in the United States of America. Shaded area indicates states with established populations of *Ixodes scapularis* in 2016 based on Eisen and Eisen (1). States shaded blue are those where *R. buchneri*/REIS has been detected in *I. scapularis*; records with county-level data are shown by dots. Based on data from references published 2007–2021 (18–23, 25–53).

Cases of SFG rickettsioses are on the rise in the United States (58). While human cases of severe illness due to infection with *R. rickettsii* (Rocky Mountain spotted fever) appear to be rare, there have been increases in cases of milder spotted fever, thought to be primarily due to infection with other less pathogenic *Rickettsia* species and the geographic expansion of the lone star tick *Amblyomma americanum* (59). This tick commonly bites humans and is a potential vector of both *R. rickettsii* (60, 61) and *R. parkeri* (62), which causes a relatively mild eschar-associated rickettsiosis (63). Additionally, *R. amblyommatis*, originally considered an endosymbiont of *A. americanum* and highly prevalent in this tick, has been linked to mild disease (64–66), so it may also be contributing. The main vector of *R. parkeri*, the Gulf Coast tick *A. maculatum*, is also expanding its distribution (67), and populations are increasingly being found in more northerly US states (68, 69). The distribution of *I. scapularis* overlaps in large parts of the country with those of tick species responsible for the transmission of pathogenic SFG *Rickettsia* spp., particularly *A. americanum* and *Dermacentor variabilis*. These ticks may share similar habitats and hosts, therefore making it possible for *I. scapularis* to come into contact with pathogenic *Rickettsia* species. However, field-collected *I.*

scapularis are very rarely infected with *Rickettsia* species other than *R. buchneri*. This might suggest that the presence of *R. buchneri* plays a role in excluding other *Rickettsia* spp. from its tick host. Evidence of competition (or "interference") between different *Rickettsia* species exists in other ticks; for example, the presence of the endosymbiont *R. peacockii* in the Rocky Mountain wood tick *Dermacentor andersoni* has been associated with reduced transovarial transmission of pathogenic *R. rickettsii* in the tick (70). In addition, infection of *D. variabilis* with either *R. montanensis* or *R. rhipicephali* prevented the transovarial transmission of the competing rickettsia in reciprocal challenge experiments (71), and *A. americanum* infected with *R. amblyommatis* were less likely to acquire *R. parkeri* than uninfected ticks (72). Similarly, while *A. americanum* larvae infected with *R. amblyommatis* were able to acquire *R. rickettsii*, its prevalence was significantly lower compared to that in *R. amblyommatis*-free larvae (73). Furthermore, milder symptoms were observed in guinea pigs infected with *R. rickettsii* by dually infected nymphs than those infected by *R. amblyommatis*-free nymphs (73), suggesting that *R. rickettsii* load was reduced by the presence of the additional *Rickettsia* species. In field studies, a high prevalence of "*Candidatus* Rickettsia andeanae" in *A. maculatum* populations was hypothesized to be linked to the exclusion of *R. parkeri* from these ticks (74). Mechanisms for the competition between *Rickettsia* species, or whether these might differ for endosymbiotic and pathogenic species, have not been elucidated.

Two genome sequences of *R. buchneri* are currently available. The REIS (Wikel) genome was extracted from the genome sequence of *I. scapularis* from the Wikel colony (54), and the *R. buchneri* ISO7^T genome was sequenced from the *R. buchneri*-type strain isolated from the ovaries of a female *I. scapularis* removed from a dog in Minnesota (18). A gene cluster encoding aminoglycoside antibiotic biosynthesis machinery has been identified in *R. buchneri* (54), which is not present in other rickettsiae, and therefore antibiotic production might represent a mechanism by which *R. buchneri* is able to exclude pathogenic *Rickettsia* species from *I. scapularis*. Genes from the cluster were found to be highly similar to those of kanamycin and gentamicin synthesis gene clusters found in members of Actinobacteria and Firmicutes (54), yet to date no experimental studies have examined whether these genes are functional in *R. buchneri*. In this study, we report that an aminoglycoside biosynthesis gene cluster, almost identical to that described by Gillespie et al. (54), is also present in the *R. buchneri* ISO7^T genome, along with a second gene cluster encoding genes similar to those for polyketide and non-ribosomal peptide antibiotic synthesis, which appears to be only partially present in the REIS (Wikel) genome. Additionally, this study shows that genes from these clusters are actively transcribed and examines whether competition exists between *R. buchneri* and rickettsial pathogens associated with ticks, using *in vitro* experiments to provide preliminary evidence that the presence of the endosymbiont in tick cells has an inhibitory effect on the infection and replication of other intracellular bacteria.

MATERIALS AND METHODS

Bioinformatic Analysis of *Rickettsia buchneri* Antibiotic Gene Clusters

Annotation of the sequenced *R. buchneri* ISO7^T genome [GenBank: JFKF01000000.1; (18)] identified the presence of two clusters of genes with similarity to bacterial genes for aminoglycoside, polyketide, and non-ribosomal peptide synthesis. This strain of *R. buchneri* was isolated from the ovaries of an *I. scapularis* female collected from a dog in Minnesota (18). To determine potential functions of proteins in the two putative antibiotic clusters, amino acid sequences from the *R. buchneri* ISO7^T genome were searched against other available sequences using the NCBI protein–protein BLAST algorithm, performed with default parameters. Putative protein function was determined by examining data for each protein in the InterPro database as well as performing literature searches. The gene clusters were also compared to the *R. buchneri* genome derived from the *I. scapularis* genome sequence, REIS (Wikel) [GenBank: CM000770.1; (54)]. Protein sequences obtained from annotated genomes were aligned using ClustalW (75) and MUSCLE (76) in MacVector version 18.1.5.

Cell and Rickettsia Culture

Embryonic tick cell lines ISE6 (77), IRE11 (78), and AAE2 (79), derived from *I. scapularis*, *I. ricinus*, and *A. americanum*, respectively, were maintained at 34°C in L15C300 medium supplemented with 5% heat-inactivated fetal bovine serum (FBS), 5% tryptose phosphate broth (TPB), and 0.1% lipoprotein concentrate (LPC; MP Biomedicals, Irvine, CA, USA), adjusted to pH 7.2–7.5 with 1 M NaOH, as previously described (77). *Rickettsia buchneri* ISO7^T [(18); hereafter referred to as *Rb*-WT] and *R. buchneri* expressing GFPuv from the plasmid pRAM18dRGA [(80); hereafter referred to as *Rb*-GFPuv] were maintained in IRE11 at 28°C in a modified L15C300 medium containing 10% FBS, 5% TPB, 0.06% $NaHCO_3$, 6 mM HEPES, and 0.1% LPC; pH was not adjusted. *Rickettsia parkeri* Tate's Hell expressing mKate from plasmid pRAM18dSFA (*Rp*-mKate) (80, 81), *R. peacockii*-GFPuv [pRAM18dSGK; (80)], *R. monacensis* IrR/Munich with mKate (pRAM18dSFA), and *A. phagocytophilum* HGE1 expressing mCherry from an intergenic Himar1 transposon insertion (82) (*A. phagocytophilum*-mCherry) were grown in ISE6 cells, and *R. amblyommatis* Darkwater (kindly supplied by Chris Paddock, CDC) were grown in AAE2 cells. Infected tick cell cultures were maintained at 34°C in L15C300 with 10% FBS, 5% TPB, 0.1% LPC, 0.25% $NaHCO_3$, and 25 mM HEPES, adjusted to pH 7.5 (83). The infection level of cell cultures was assessed by Giemsa staining. Vero cells (African green monkey kidney) were grown in Gibco RPMI 1640 (Thermo Fisher, Waltham, MA, USA) supplemented with 10% FBS and 2 mM L-glutamine at 34°C following established methods (84). All cultures were grown in 25-cm^2 culture flasks (CELLSTAR, Greiner Bio-One, Monroe, NC, USA).

Host cell-free bacteria were prepared as previously described (83); heavily infected tick cells were added to tubes containing rock tumbler grit (60/90 coarse silicon carbide, Lortone,

Mukilteo, WA, USA), vortexed for 30 s, then passed through a 2 μm filter to remove cellular debris. Cell-free bacteria were then collected by centrifugation at 13,200 × g for 5 min at 4°C.

Reverse Transcriptase PCR

Cell-free *Rb*-WT were prepared from infected IRE11 cultures as described above. Bacterial pellets were washed once in SPG buffer, then resuspended in 1 ml TRI Reagent (Sigma-Aldrich, St Louis, MO, USA), vortexed, and rested at room temperature for 10 min. Samples were centrifuged at 12,000 × g to remove particulates and the supernatant transferred to new tubes and mixed 1:2 with 100% ethanol, followed by vortexing. RNA was purified using a Direct-zol RNA Miniprep Kit (Zymo Research, Irvine, CA, USA). Contaminating DNA was removed by treating three times with Ambion TURBO DNA-free Kit (Thermo Fisher), followed by purification with an RNA Clean & Concentrator Kit (Zymo Research). Reverse transcriptase PCR (RT-PCR) was carried out using the Access RT-PCR system (Promega, Madison, WI, USA) in 25 μl reactions consisting of 5× reaction buffer, 10 μM dNTPs, 10 μM of each primer, 0.5 μl Tf1 DNA polymerase, 14.5 μl nuclease-free water, and 1 μl sample. Nuclease-free water was used as a negative control, and no reverse transcriptase (no RT) controls included water instead of the DNA polymerase to confirm the absence of contaminating DNA. RT-PCR was performed in a Techne TC-312 Thermocycler with the following cycling conditions: 45 min at 45°C; 2 min at 94°C; 40 cycles of 30 s at 94°C, 1 min at 55°C or 58°C, and 2 min at 68°C; final extension 1 min at 68°C. Amplification of *HTH*, *lagD*, *ppsE_1*, *kanC*, and *btrB* was performed with an annealing temperature of 58°C, and amplification of *glycogen synthase*, *homoserine kinase*, and *lgrB* at 55°C. Primers used in this study are shown in **Table 1**. RT-PCR products were visualized on a 1.2% agarose gel stained with GelGreen (Biotium, Fremont, CA, USA).

In vitro Competition Assays

Competition assays were set up in 24-well plates to compare the infection and replication of *R. monacensis*-mKate, *Rp*-mKate, and *A. phagocytophilum*-mCherry in tick cells with and without *Rb*-GFPuv infection. For the *R. monacensis* and *R. parkeri* experiments, three 24-well plates were prepared; the first contained uninfected IRE11, the second contained IRE11 heavily infected with *Rb*-GFPuv, and the third contained a mixture of infected and uninfected cells to give an approximate level of *Rb*-GFPuv infection of 45%. Cells (0.4 ml) were applied to each well, giving 1×10^6 cells/well in the *R. monacensis* experiment or 3×10^5 cells/well in the *R. parkeri* experiment. Plates were incubated in a humidified candle jar at 28°C. After 24 h, plates were infected with 250 μl fresh medium containing host cell-free *R. monacensis*-mKate or *Rp*-mKate, in 10-fold serial dilutions. Each dilution was applied to each plate in triplicate, and the remaining wells were used as negative controls. The number of mKate-positive colonies in the lowest-dilution wells was determined by fluorescent microscopy and used to extrapolate the number of rickettsiae in each dilution. Wells were observed on a Nikon Diaphot fluorescent microscope, and adhesion/invasion and replication were determined for each dilution on each plate by visualization of red fluorescent rickettsiae over 14 days. Additionally, the ability of *Rp*-mKate to infect IRE11 was assessed daily over the 14 days by observing at least 500 cells per well and determining the percentage of cells containing replicating fluorescent *R. parkeri*.

For *A. phagocytophilum* experiments, five 24-well plates were prepared; two contained uninfected IRE11, two contained IRE11 heavily infected with *Rb*-GFPuv, and the fifth contained a mixture of infected and uninfected cells to give an approximate level of *Rb*-GFPuv infection of 25%. Cells (0.5 ml) were applied to each well to give 1×10^6 cells/well. One uninfected IRE11 plate and one infected IRE11 plate were incubated at 27°C in a humidified candle jar; the remaining three plates were incubated at 34°C with 4% CO_2. After 24 h, plates were infected with 250 μl medium containing 10-fold serial dilutions of host cell-free *A. phagocytophilum*-mCherry, added to plates in triplicate. The number of *Anaplasma* in each dilution was estimated by testing each dilution in a sixth 24-well plate seeded with ISE6 cells and counting the number of mCherry-positive colonies in the lowest-dilution wells and then extrapolating to each dilution. Wells were observed on a Nikon Diaphot fluorescent microscope, and adhesion/invasion and replication were determined for each dilution on each plate by visualization of red fluorescent bacteria.

Fluorescent Plate Reader Assays

A fluorescent plate reader assay was used to measure the growth dynamics of fluorescent *Rickettsia* in IRE11 cells. Uninfected IRE11 and IRE11 heavily infected with *Rb*-GFPuv (>95% cells infected) were adjusted to 1×10^5 cells/ml in fresh 10% FBS medium. Heavily infected and uninfected cells were then mixed to create additional populations with 25%, 50%, and 75% cells infected (**Figure 2**). A volume of 200 μl of each cell population was added in triplicate to wells of a clear-bottomed black-sided 96-well plate (Falcon, Corning, NY, USA), with dH_2O added between wells to prevent drying, and incubated at 28°C in a humidified candle jar for 24 h to allow cells to settle to the bottom of wells. Host cell-free *Rp*-mKate were resuspended in 1 ml fresh medium and enumerated on a Petroff–Hausser chamber. The cell-free bacteria were diluted to create 1,000:1, 100:1, and 10:1 multiplicity of infection (MOI) ratios and then added to IRE11 cells in 96-well plates in a volume of 10 μl. The plate was returned to the humidified candle jar and incubated at 28°C. Fluorescence readings were taken 24 h later and then every 24 h up to 14 days postinfection. Readings were taken at room temperature (∼22–25°C) on a BioTek Synergy H1 microplate reader at excitation/emission 395/509 for GFPuv and 588/633 for mKate and adjusted to uninfected IRE11 to account for background fluorescence.

Plate reader experiments for *R. amblyommatis* used a similar protocol to the above, except that only uninfected and 25% and >95% infected cell populations were used, and only 1,000:1 and 10:1 *Rp*-mKate challenges were performed. Additionally, only mKate fluorescence was measured for these experiments. The *R. peacockii* experiments were carried out using the same protocol as for *Rb*-GFPuv. Prior to IRE11 plate experiments, cell-free *R. amblyommatis* and *R. peacockii* (grown in AAE2 and ISE6 cells, respectively) were transferred to IRE11 and cultured in modified L15C300 medium at 28°C for at least 2 weeks to adjust them to

TABLE 1 | Primers used for the amplification of *Rickettsia buchneri* antibiotic cluster genes.

Product name	Forward primer (5′-3′)	Reverse primer (5′-3′)
HTH domain	AGC TGA TTT AGA AAG AAA GGC A	GAG GTA ACA TCA ATA CAG GGA AG
ppsE_1	CCT GGA GGT ATA AGA TCT GCT AAT G	GCT CCT TGT CCT GGG AAT AAA
lgrB	CTA CCG GAC AAC CTA AAG GAA TAG	CGG AAA CCT CGA ACC TTA ACT
btrB (choline dehydrogenase)	CGG GTT AAA TCC TTT CCC TAC TC	AGT AAC GAC AAG TCC CAT GTA AG
kanC	GGA GGA ATC CCA GGA AAC ATA G	CAA TGA GCA TAC CTA ACC CTA CA
lagD	AGT TCG GGT ATT GCC ACA TAT T	TGG TAT GCC ATA GGT AAG GAT TTC
Homoserine kinase	GTT CTA GCG CAA TAC CCT CTT	CGC GCA ATG TCC CAA ATA C
Glycogen synthase	TCC TGG CTA CTC GGT ACA TTA	CTC TGG CAA TAC GAC CAA CA
ppsE_1 (qRT-PCR)	ACG TAC TCC TAT GAA GCT CCG	GCT CCT TGT CCT GGG AAT AAA
lgrE (qRT-PCR)	TTT TCC CTT TCG CAG GTG GG	ACC CCA GAT ATT TTC CAC GTC C
btrB (qRT-PCR)	CGG GTT AAA TCC TTT CCC TAC TC	GCA TTC ATG CCT GCA AAA ATA G
kanC (qRT-PCR)	TAC ATG TCC AAG AGT ATG GCC G	AGC AGA GGC GAT AAA GCT AGT

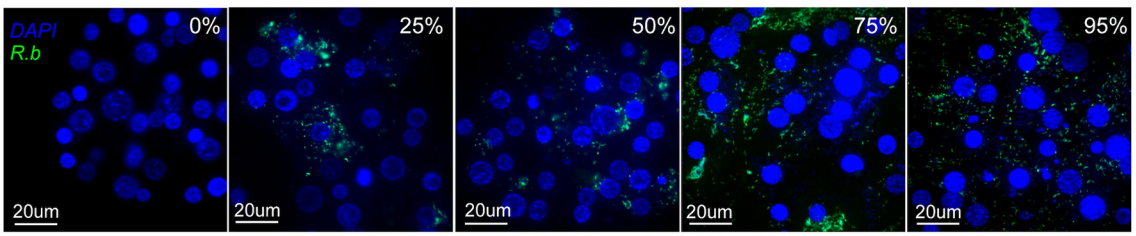

FIGURE 2 | Images of IRE11 cells with different levels of *R. buchneri*-GFPuv infection. Live IRE11 cells were stained with NucBlue Live ReadyProbes Reagent (Hoechst 33342; Invitrogen) and spun onto slides with a Cytospin centrifuge, following Wang et al. (81). Images were captured on an Olympus BX61 DSU Confocal Microscope using a ×60 objective and a double-wavelength filter (DAPI; FITC). DNA shown in blue, GFPuv shown in green.

growing in these conditions. Both *Rickettsia* species were found to grow well in IRE11 cells.

To assess the effect of cell-free *R. buchneri* and lysate of *R. buchneri* on the replication of *Rp*-mKate, a 96-well plate was set up with 200 µl of uninfected IRE11 adjusted to 1×10^5 cells/ml in fresh medium, as above. Cells were treated with either 50 µl medium (negative control), 50 µl of cell-free *Rb*-WT prepared from 2.5×10^5 heavily infected IRE11, 50 µl of a 1:10 dilution of the cell-free *Rb*-WT, 50 µl cell lysate from 2.5×10^5 IRE11 heavily infected with *Rb*-WT, or 50 µl cell lysate from 2.5×10^5 uninfected IRE11. Lysates were prepared by sonicating cells on ice at full power for a total of 1 min (separated into 3 × 20-s bursts, with 20-s intervals resting on ice). After 2 h, cells were challenged with cell-free *Rp*-mKate at 1,000:1, 100:1, or 10:1, and the plate was incubated at 28°C in a humidified candle jar. mKate fluorescence was measured every 24 h for 14 days, as described above, and adjusted to uninfected, untreated IRE11.

To determine if *R. parkeri* could induce *R. buchneri* antibiosis activity, an additional plate reader assay was used to examine whether lysates from *R. buchneri* challenged with *Rp*-mKate for varying lengths of time exhibited inhibitory effects on the growth of *Rp*-mKate in IRE11 cells. Wells of a 6-well plate were seeded with 2 ml IRE11 75% infected with *Rb*-WT, at 1×10^5/ml. One well served as a no challenge control, while to the remaining wells cell-free *Rp*-mKate at a ratio of 100:1 was added. The plate was incubated at 28°C in a candle jar. After 24 h, cells from the control and one of the challenged wells were collected, and rickettsiae were isolated from IRE11 as above. Cell pellets were then frozen at −70°C. At 48, 72, 120, and 168 h after infection, this was repeated for cells from each of the remaining wells. Lysates were prepared from the pellets by four freeze thaw cycles of −70 to 37°C. The rickettsial lysates from each treatment were resuspended in 120 µl medium, and then 10 µl was added to 12 wells of a 96-well plate, each containing 200 µl IRE11 at 1×10^5/ml, prepared 24 h previously. Wells were then challenged with *Rp*-mKate at ratios of 1,000:1, 100:1, and 10:1. Medium without *Rp*-mKate was added to control wells for each treatment. The plate was incubated at 28°C in a humidified candle jar, and mKate fluorescence was measured every 24 h for 14 days, as described above.

Antibiotic Susceptibility Assays

To test the antibiotic activity of *R. buchneri* against extracellular bacteria, antibiotic susceptibility testing was performed against *Escherichia coli* D21 and *Staphylococcus aureus* MN8 using disk diffusion assays. IRE11 infected with *Rb*-WT and uninfected IRE11 was pelleted by centrifugation at 350 × *g* for 6 min at 4°C, and the pellets were frozen at −70°C. Cell-free *Rb*-WT was prepared by vortexing with rock tumbler grit and filtration through a 2-µm filter, as described above, then the bacteria were pelleted by centrifugation at 13,600 × *g* for 7 min at 4°C, and the cell pellet frozen at −70°C. Lysates of the three samples were

prepared by freeze-thawing (four cycles of −70 to 37°C) and then centrifugation at 13,600 × *g* for 5 min at 4°C. Bacterial or cell lysates were then resuspended in 50 μl medium, of which 30 μl was added to 50 μl of absolute methanol. A volume of 20 μl of each sample was then added to separate filter paper disks. Spectinomycin at 10 and 100 μg was added to two additional filter paper disks as positive controls. Disks were air-dried in a biosafety cabinet for 20–30 min and then placed onto Mueller–Hinton agar plates streaked with *E. coli* D21 or *S. aureus* MN8. Plates were incubated for 18 h at 37°C.

The disk diffusion assays were repeated using pellets of live IRE11, *Rb*-WT-infected IRE11 (25%, 50%, and >95% infected), and cell-free *Rb*-WT, as well as supernatant from IRE11 cultures at various levels of infection with *Rb*-WT (25, 50, >95%) or uninfected. Cells were pelleted by centrifugation at 350 × *g* for 6 min at 12°C, supernatant was removed to separate tubes, and then pellets were resuspended in 100 μl medium. A volume of 20 μl of resuspended cell pellets or 20 μl supernatant was added to filter paper disks. Spectinomycin was added to additional disks at 100 μg for positive controls. Disks were air-dried for 20-30 min in a biosafety cabinet, then applied to Mueller–Hinton agar plates streaked with *E. coli* D21 or *S. aureus* MN8. Plates were incubated for 18 h at 37°C.

To further test the antibiotic activity of supernatant from *Rb*-WT-infected IRE11 cultures against *R. parkeri* grown in mammalian cells, Vero cell cultures were grown at 34°C in 2.5 ml RPMI medium supplemented with 2.5 ml supernatant from either uninfected IRE11 or IRE11 heavily infected with *Rb*-WT. Cell-free *Rp*-mKate was added to Vero cultures and flasks checked daily for evidence of infection, which was assessed by the appearance of plaques in the cell layer as well as the timing and size of plaques.

Expression of Antibiotic Genes in Response to *R. parkeri* Challenge

To determine whether the expression of antibiotic genes by *Rb*-WT was induced by challenge with *R. parkeri*, and to determine the time of maximal expression, a time course experiment was set up comparing unchallenged and *Rp*-mKate-challenged *Rb*-WT-infected IRE11. A 6-well plate was prepared with each well containing IRE11 75% infected with *Rb*-WT at 1×10^5/ml in 2 ml modified L15C300 medium. One well served as a no challenge control, while to the remaining wells cell-free *Rp*-mKate at a ratio of 100:1 in 10 μl was added. The plate was incubated at 28°C in a candle jar. After 24 h, cells from the control well were collected and centrifuged for 2 min at 500 × *g*, and the cell pellet was resuspended in 750 μl RNAlater solution (Qiagen, Hilden, Germany) and stored at−20°C. Cells from the challenge wells were collected at 24, 48, 72, 120, and 168 h after infection, resuspended in RNAlater solution, and stored at −20°C. RNA isolation was performed using TRIzol–chloroform extraction. cDNA was then prepared using Takara RT PrimeScript Kit (Takara Bio, Kusatsu, Japan), after a 30-min treatment with a gDNA eraser at room temperature to remove contaminating DNA. Quantitative PCR (qPCR) was performed on an Agilent Mx3005P RT-PCR system using the Agilent Brilliant II SYBR Green Master Mix, using 10-μM primers against various targets from both gene clusters (**Table 1**) and 1 μl cDNA preparation. Since both *R. buchneri* and *R. parkeri* were present in samples, *I. scapularis GAPDH* was used as a reference gene. Nuclease-free water and no reverse transcriptase controls were included on each plate. Each sample was run in triplicate. Cycling conditions were 10 min at 95°C, followed by 40 cycles of 30 s at 95°C, 1 min at 55°C (*glycogen synthase*, *GAPDH*), or 52°C (*btrB*, *kanC*, *lgrE*, *ppsE_1*), 1 min at 72°C. Results were compared to *GAPDH* expression to adjust for total cDNA per sample. To measure primer efficiency, standard curves consisting of cDNA from *R. buchneri*-infected IRE11 were also added to reaction plates. To account for differences in primer efficiency, relative quantification was calculated with a PCR efficiency correction (85).

Statistical Analysis

All statistical analyses were carried out using GraphPad Prism version 9.1.2. For plate reader experiments, the growth of *Rp*-mKate in IRE11 cultures infected with other *Rickettsia* or treated with lysates was analyzed using a two-way ANOVA with Dunnett's multiple-comparison test, using *Rp*-mKate growth in uninfected IRE11 wells as the control. Relative gene expression was analyzed with a two-way ANOVA with Dunnett's multiple-comparison test, comparing the expression in *Rp*-challenged groups to that in the unchallenged control group. Statistical significance was assigned when $p < 0.05$.

RESULTS

Description and Expression of Antibiotic Gene Clusters in *Rickettsia buchneri*

Analysis of the sequenced *R. buchneri* ISO7^T genome revealed that it contains two gene clusters encoding proteins similar to those involved in antibiotic synthesis. Neither gene cluster is present in other members of *Rickettsiaceae*. The first cluster contains eleven genes including those for polyketide and other non-ribosomal peptide synthesis enzymes (**Table 2**; **Figure 3A**). Biosynthesis pathways for these compounds (which include the penicillins, cyclosporin A, vancomycin, and erythromycin) involve large multi-modular enzymes that act as assembly lines for the catalysis of chain elongation and addition of modifications (86). These are usually clustered with additional genes encoding tailoring enzymes that further modify the resulting compound, for example by methylation or cyclization, and/or by releasing it from the assembly line (87, 88). Proteins in the *R. buchneri* polyketide cluster show similarity to sequences from the Gammaproteobacteria *Legionella israelensis*, *Erwinia amylovora*, *Pantoea ananatis*, and *Pectobacterium* spp. as well as Cyanobacteria (**Supplementary Figure S1**; **Supplementary Data S1**). Interestingly, one of the hypothetical proteins in the cluster appears to be a type IV pilin, whose sequence obtained no blastp hits except those from the two *R. buchneri* genomes. Only the first three genes in the cluster are conserved in the REIS (Wikel) genome, while the remaining genes are not present (**Figure 3A**; **Table 2**; **Supplementary Figure S1**). Instead, the region of the REIS

TABLE 2 | Genes in *Rickettsia buchneri* putative polyketide synthesis cluster.

Label	Locus tag	Accession	Length (aa)	Annotation[a]
HTH domain*	REISMN_01150 (REIS_1819)	KDO03565.1	142	**Helix-turn-helix transcriptional regulator**
Hypothetical	REISMN_01155 (REIS_1817)	KDO03566.1	62	**hypothetical protein;** type IV pilin
ppsE_1*	REISMN_01160 (REIS_1816)	KDO03567.1	1448	**Beta-ketoacyl-acyl-carrier-protein synthase I**; type I polyketide synthase; erythronolide synthase; acyltransferase domain-containing protein
ppsE_2	REISMN_01165	KDO03568.1	636	**Beta-ketoacyl-acyl-carrier-protein synthase I**; type I polyketide synthase; SDR family NAD(P)-dependent oxidoreductase
pksL_1	REISMN_01170	KDO03569.1	522	**Polyketide synthase PksL;** SDR family NAD(P)-dependent oxidoreductase; type I polyketide synthase
lgrB*	REISMN_01175	KDO03570.1	630	**Linear gramicidin synthase subunit B;** non-ribosomal peptide synthetase
ppsB	REISMN_01180	KDO03571.1	878	**Plipastatin synthase subunit B;** non-ribosomal peptide synthetase
ppsE_3	REISMN_01185	KDO03572.1	554	**Beta-ketoacyl-acyl-carrier-protein synthase I;** polyketide synthase
lgrE	REISMN_01190	KDO03573.1	239	**Linear gramicidin dehydrogenase LgrE;** thioesterase
Hypothetical	REISMN_01195	KDO03574.1	265	**hypothetical protein;** GNAT family N-acetyltransferase
ysdC	REISMN_01200	KDO03575.1	392	**Putative aminopeptidase ysdC;** M42 family metallopeptidase

Asterisks indicate genes with transcription confirmed by RT-PCR.
Locus tags for corresponding genes in the REIS (Wikel) genome are shown in brackets.
[a]*Bold type indicates annotation recorded in the genome; regular type indicates additional annotations gained through protein BLAST searches.*

genome downstream of *ppsE_1* encodes numerous *tra* genes (REIS_1815 = *traC*, REIS_1814 = *traV*, REIS_1813 = truncated *traB*, REIS_1810 = *traE*). The region upstream of the cluster is homologous in both genomes with a similar arrangement of genes (**Figure 3A**). Interestingly, the genes following *ysdC* in the *Rb* ISO7 genome are homologous to those in a different region of the REIS genome, with the next gene after *ysdC*, REISMN_1205, identical to REIS_1393 (**Figure 3A**). Notably the area upstream of REIS_1393 is heavily populated with transposase sequences, suggesting that a transposition event may have led to the loss of the remaining polyketide cluster genes in the REIS genome.

Gillespie et al. (54) also identified an additional polyketide synthase with a putative frameshift mutation (REIS_0330). This prompted an examination of the *Rb* ISO7 genome for additional polyketide synthase genes. A cluster of three were identified, and these are annotated as *pksL_2* (REISMN_07055), *pksR* (REISMN_07060), and *pksN* (REISMN_07065). Corresponding genes encoding identical proteins are annotated in the REIS (Wikel) RefSeq on GenBank, with corresponding locus tags REIS_RS15050 (hypothetical protein), REIS_RS10770 (methyltransferase), and REIS_RS01405 (KR domain-containing protein), respectively.

The second cluster contains fifteen genes, with eleven showing similarity to genes involved in aminoglycoside antibiotic synthesis, as well as genes coding for putative antibiotic exporters and an antibiotic resistance factor (**Table 3**; **Figure 3B**). This second antibiotic cluster was also identified in the REIS (Wikel) genome (54) and found to be associated with the *Rickettsiales*-amplified genetic element, RAGE-A. The aminoglycoside antibiotics include streptomycin, kanamycin, and gentamicin and are produced through complex biosynthetic pathways involving many enzymatic reactions; few of these pathways have been fully characterized (89–91). Proteins of the *R. buchneri* aminoglycoside cluster show similarity to those from antibiotic synthesis gene clusters from Actinobacteria and Firmicutes (54), while the putative multidrug exporter mde/mdlB, transcriptional regulator LuxR, and ABC transporter lagD show greater similarity to proteins from the Gammaproteobacteria (**Supplementary Figure S2**; **Supplementary Data S2**). Meanwhile, the nucleotide translocase tlcA1/tlc2 is related to those from other *Rickettsia* species (54). This cluster is conserved in both *R. buchneri* genomes (**Figure 3B**; **Table 3**), with the majority of sequences identical (**Supplementary Figure S2**).

To determine whether these genes are actively transcribed by *R. buchneri*, eight genes from the two antibiotic clusters were selected for RT-PCR analysis (**Tables 2**, **3**). Transcripts for all eight genes were detected, as indicated by bands of the expected sizes observed on gel electrophoresis (**Figure 3C**). Negative controls and no RT controls showed no products, except for *HTH* where a less robust band was seen, suggesting some DNA contamination, although there is clearly less amplification than in the RT well. Smaller bands observed in the *btrB*, *lagD*, and *kanC* reactions likely represent nonspecific products.

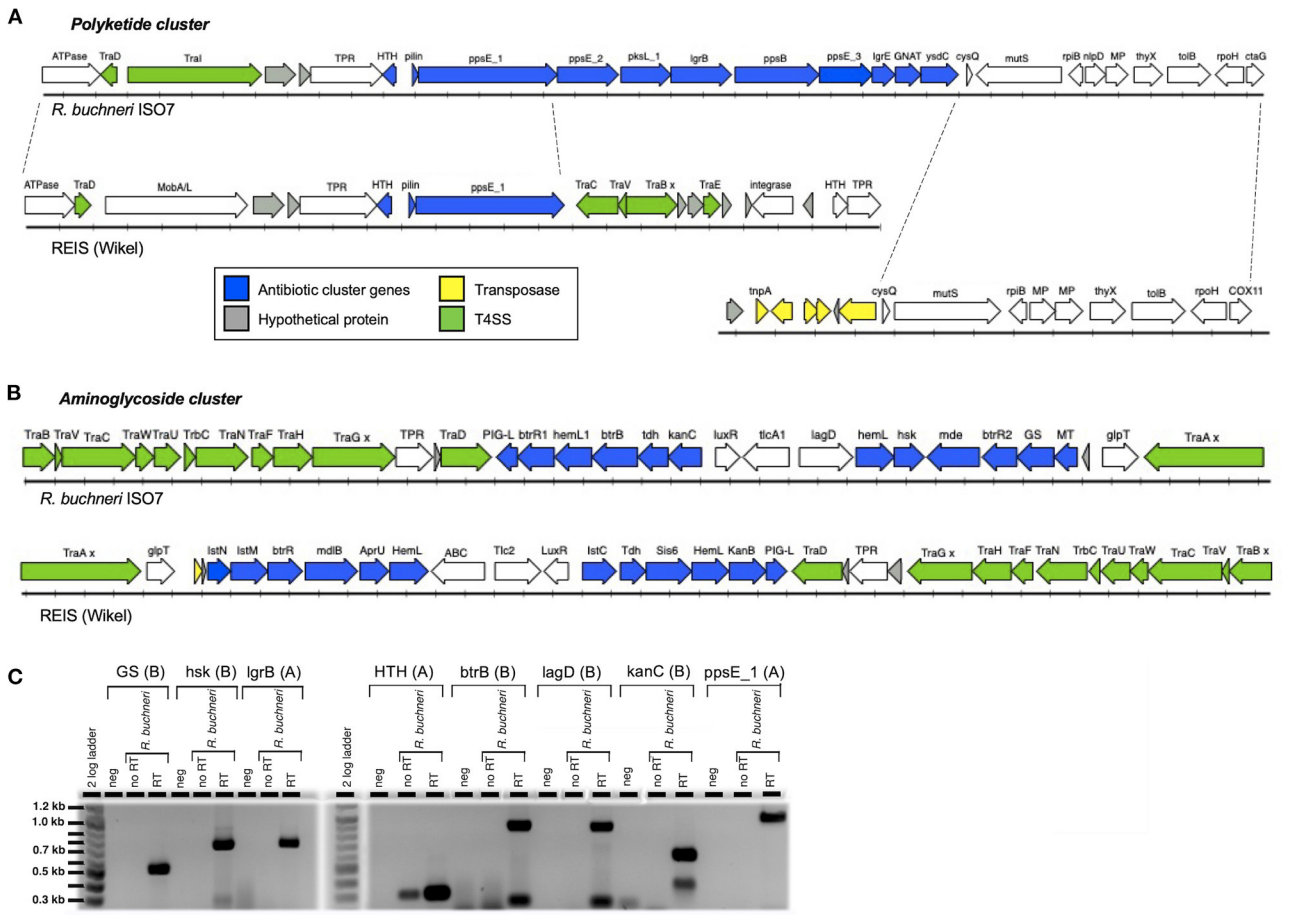

FIGURE 3 | Antibiotic gene clusters in *Rickettsia buchneri.* Diagrams showing the gene arrangement of *R. buchneri* ISO7 antibiotic gene clusters containing polyketide **(A)** and aminoglycoside **(B)** synthesis genes, and neighboring regions of the genome, and their comparison with those in the REIS (Wikel) genome. GS—glycogen synthase; hsk—homoserine kinase; MT—methyltransferase; MP—metallopeptidase; x denotes a mutation resulting in a truncation. **(C)** RT-PCR of *R. buchneri* ISO7 RNA showing the transcription of selected genes from antibiotic clusters. The gene product and antibiotic cluster (A = polyketide, B = aminoglycoside) are shown above the wells of the gel. See **Tables 2**, **3** for additional information.

In vitro Antibiosis Experiments

To determine whether *R. buchneri* might exhibit antibiosis against other bacteria infecting tick cells, cell-free red fluorescent *A. phagocytophilum*, *R. monacensis*, or *R. parkeri* was used to challenge tick cell cultures containing different levels of green fluorescent *R. buchneri.* Infectivity was measured 14 days after inoculating serial dilutions of cell-free rickettsiae/*Anaplasma* into replicated wells of a 24-well tissue culture plate seeded with *R. buchneri*-infected IRE11 cells. All three pathogens showed markedly reduced ability to infect and replicate in tick cell cultures infected with *R. buchneri* (**Figure 4**), and even a low level of infection with *Rb*-GFPuv led to a reduction in pathogen infection and replication. Fluorescent microscopy revealed that *R. monacensis* and *A. phagocytophilum* did not replicate in tick cells infected with *R. buchneri* (**Supplementary Figure S3**). Infectivity of *R. monacensis*, *R. parkeri*, or *A. phagocytophilum* in tick cell cultures infected with *R. buchneri* was reduced by 3–5 orders of magnitude compared to that in cells without *R. buchneri* (**Figure 4**), confirming that the presence of *R. buchneri* was inhibitory to the growth of other intracellular tick-borne bacteria. Furthermore, examining the percentage of infected cells by fluorescent microscopy during *Rp*-mKate infection showed that in IRE11 without *R. buchneri*, cells became completely infected at all dilutions over the 14-day period (**Figure 4D**). In contrast, *Rp*-mKate infection only reached low levels (<10%) in IRE11 cultures containing the endosymbiont in 45% cells, and almost no cells became infected with *Rp*-mKate in cultures with >95% cells harboring *R. buchneri.*

To further investigate the growth dynamics during coinfection, a fluorescent plate reader was used to measure the replication of *Rp*-mKate in IRE11 cells infected with *Rb*-GFPuv at levels of 25, 50, 75, and >95%, in comparison to uninfected IRE11. GFPuv measurement could clearly differentiate the various levels of infection with *Rb*-GFPuv and indicated a steady replication of the endosymbiont over the 14-day experiment (**Figure 5A**). No increases in GFPuv fluorescence

TABLE 3 | Genes in *Rickettsia buchneri* putative aminoglycoside synthesis cluster.

Label	Locus tag	Accession	Length (aa)	Annotation[a]
PIG-L (PIG-L)	REISMN_01820 (REIS_1505)	KDO03398.1	234	**GlcNAc-PI de-N-acetylase;** PIG-L family deacetylase
btrR_1 (KanB)	REISMN_01825 (REIS_1504)	KDO03399.1	421	**L-Glutamine:2-deoxy-scyllo-inosose aminotransferase;** DegT/DnrJ/EryC1/StrS family aminotransferase
hemL1 (HemL)	REISMN_01830 (REIS_1503)	KDO03400.1	420	**Glutamate-1-semialdehyde 2,1-aminomutase 1;** aminotransferase class III-fold pyridoxal phosphate dependent enzyme
btrB* (Sis6)	REISMN_01835 (REIS_1502)	KDO03401.1	518	**Choline dehydrogenase;** GMC family oxidoreductase
tdh (Tdh)	REISMN_01840 (REIS_1501)	KDO03402.1	342	**L-Threonine 3-dehydrogenase**
kanC* (lstC)	REISMN_01845 (REIS_1500)	KDO03403.1	383	**2-Deoxy-scyllo-inosose synthase**
luxR (LuxR)	REISMN_01850 (REIS_1499)	KDO03404.1	275	**luxR family transcriptional regulator**
tlcA1 (Tlc2)	REISMN_01855 (REIS_1498)	KDO03405.1	521	**ADP/ATP translocase 1**
lagD* (ABC)	REISMN_01860 (REIS_1497)	KDO03406.1	605	**Lactococcin-G-processing and transport ATP-binding protein LagD;** ABC transporter ATP-binding protein/permease
hemL (HemL)	REISMN_01865 (REIS_1496)	KDO03407.1	435	**Glutamate-1-semialdehyde 2,1-aminomutase**
Homoserine kinase* (AprU)	REISMN_01870 (REIS_1495)	KDO03408.1	342	**Homoserine kinase**
mde protein (mdlB)	REISMN_01875 (REIS_1494)	KDO03409.1	594	**Putative multidrug export ATP-binding/permease protein**
btrR_2 (btrR)	REISMN_01880 (REIS_1493)	KDO03410.1	392	**L-Glutamine:2-deoxy-scyllo-inosose aminotransferase;** DegT/DnrJ/EryC1/StrS family aminotransferase
Glycogen synthase* (lstM)	REISMN_01885 (REIS_1492)	KDO03411.1	422	**Glycogen synthase**
Methyl transferase (lstN)	REISMN_01890 (REIS_1491)	KDO03412	262	**Hypothetical protein;** class I SAM-dependent methyltransferase

Asterisks indicate genes with transcription confirmed by RT-PCR. Protein labels and locus tags for corresponding genes in the REIS (Wikel) genome are shown in brackets.
[a]*Bold type indicates annotation recorded in the genome; regular type indicates additional annotations gained through protein BLAST searches.*

were seen in uninfected IRE11. With a high (1,000:1) challenge, fluorescence from *Rp*-mKate growth indicated a rapid infection and replication of *Rp*-mKate in uninfected IRE11 from day 3 which then began to level off from day 6 onward (**Figure 5B**). In contrast, there were significantly lower rates of mKate fluorescence increase in IRE11 harboring *Rb*-GFPuv at all levels of infection from day 4 onward ($p < 0.0001$); compared to uninfected IRE11 at day 14, there was an 89% reduction in *Rp*-mKate in IRE11 with >95% *Rb*-GFPuv, 88% reduction in IRE11 with 75% *Rb*-GFPuv, 84% reduction in IRE11 with 50% *Rb*-GFPuv, and 76% reduction in IRE11 with 25% *Rb*-GFPuv. Similarly, 100:1 challenge of uninfected IRE11 with *Rp*-mKate resulted in a rapid increase in fluorescence from days 5 to 6, reaching a peak by day 12, indicating replication and spread of *Rp*-mKate in the cells (**Figure 5C**). However, in IRE11 with *Rb*-GFPuv, significant differences in mKate fluorescence were observed from day 6 ($p < 0.001$), and at day 14 the reduction was 99% in 50%, 75% and >95% infected cells, and 95% in IRE11 with 25% *Rb*-GFPuv. With a low challenge (10:1), mKate fluorescence increased from days 7 to 8 and reached its height at days 13–14 in uninfected IRE11, whereas in *Rb*-GFPuv-infected IRE11 mKate fluorescence was reduced by 99%-100% in 50%, 75%, and >95% infected cells, and by 98% in 25% infected cells (**Figure 5D**), with significant differences seen from day 8 ($p < 0.0001$) compared to IRE11 without *Rb*-GFPuv. No changes in mKate fluorescence were observed in control wells to which *Rp*-mKate was not added (data not shown). Together, these results suggest that the presence of *R. buchneri* in IRE11 has a significant inhibitory effect on the ability of *R. parkeri* to successfully infect and replicate in the culture.

In order to begin to separate whether the observed inhibition of pathogen growth might be due to antibiosis by *R. buchneri* or competitive exclusion as observed previously (70–73) between various species of *Rickettsia* (that do not contain antibiotic synthesis gene clusters), additional plate reader experiments were performed using different *Rickettsia* species in place of *R. buchneri* as the resident bacteria. Firstly, this was assessed using the low-pathogenic species *R. amblyommatis* at infection levels of 25 and >95% in IRE11 cells, and uninfected control IRE11, which were then challenged with *Rp*-mKate at 1,000:1 (high) and 10:1 (low). In contrast to results obtained with *Rb*-GFPuv, the presence of *R. amblyommatis* in tick cells resulted in only partial inhibition of *Rp*-mKate replication. Growth of *Rp*-mKate in *R. amblyommatis*-infected IRE11 was inhibited in a manner that was relative to the level of *R. amblyommatis* infection (**Figures 6A,B**); i.e., at the low level of infection (25%), there was a lower inhibition of *Rp*-mKate (35% inhibition at 10:1 challenge, and 37% at 1,000:1, compared to IRE11 without *R. amblyommatis* at day 14), while at the high level of infection (>95%) there was a higher inhibition of *Rp*-mKate (54% inhibition at 10:1 challenge and 56% at 1,000:1).

To investigate whether endosymbiotic rickettsiae may have greater exclusionary effect on pathogenic bacteria than other pathogenic *Rickettsia* species, as well as to further investigate

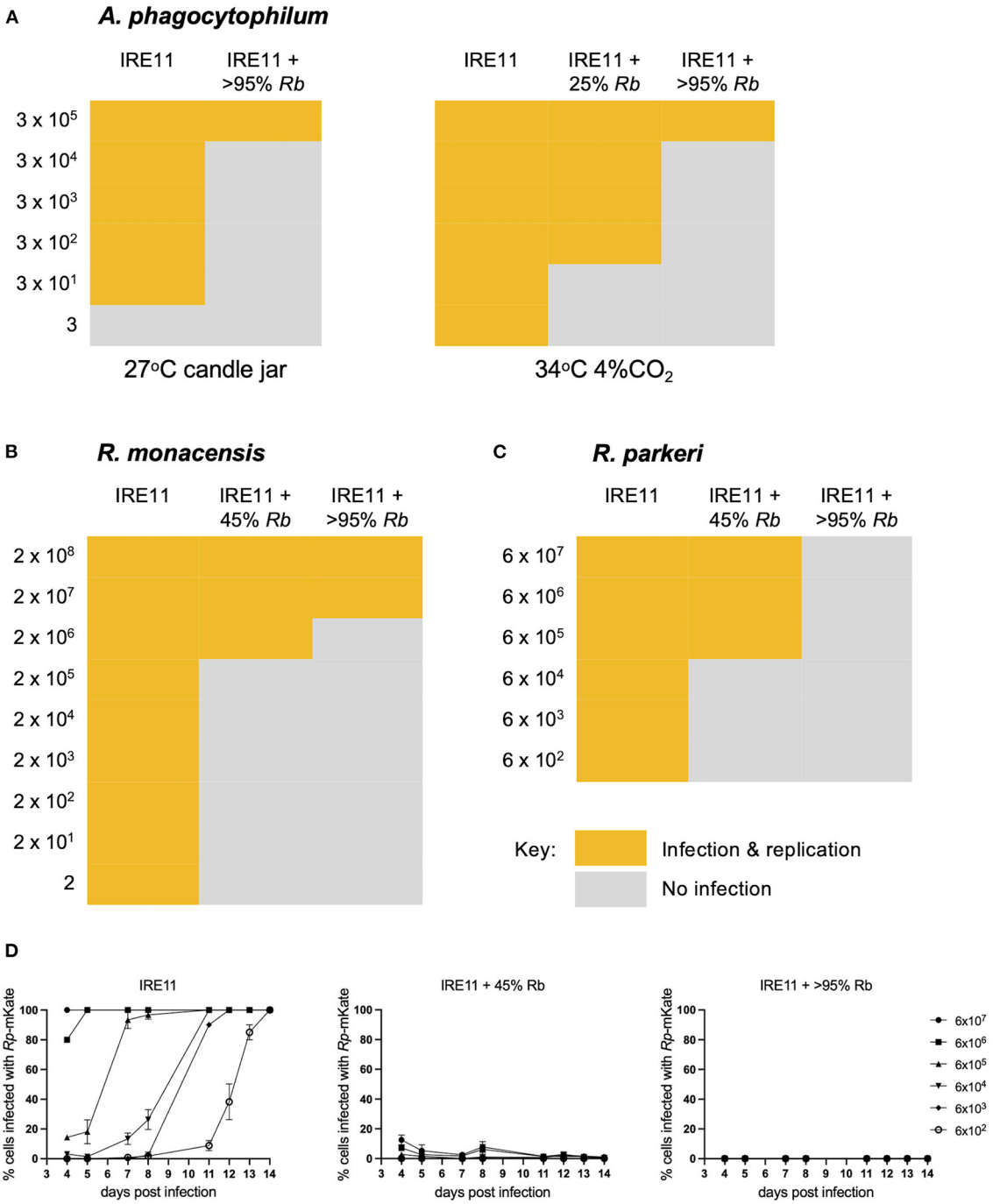

FIGURE 4 | *Rickettsia buchneri* inhibits infection and replication of other tick-borne bacteria in tick cell culture. IRE11 cells infected with different levels of *R. buchneri*-GFPuv were infected with serial dilutions of red fluorescent *A. phagocytophilum* **(A)**, *R. monacensis* **(B)**, or *R. parkeri* **(C)**, and plates were monitored over 14 days for infection and replication, compared to uninfected IRE11 cells. **(D)** The percentage of IRE11 cells infected with *R. parkeri*-mKate was assessed by fluorescent microscopy. Measurements not taken on days 6, 9, and 10.

the contribution of the involvement of potential antibiotic production by *R. buchneri*, additional plate reader competition assays were conducted using *R. peacockii* (an endosymbiont of *D. andersoni*) in place of *Rb*-GFPuv. Wells contained either uninfected IRE11 or IRE11 infected with *R. peacockii*-GFPuv at levels of 25, 50, 75, and >95%. The wells were challenged with *Rp*-mKate at 1,000:1, 100:1, and 10:1. In the high-challenge (1,000:1) wells, mKate fluorescence was significantly lower than

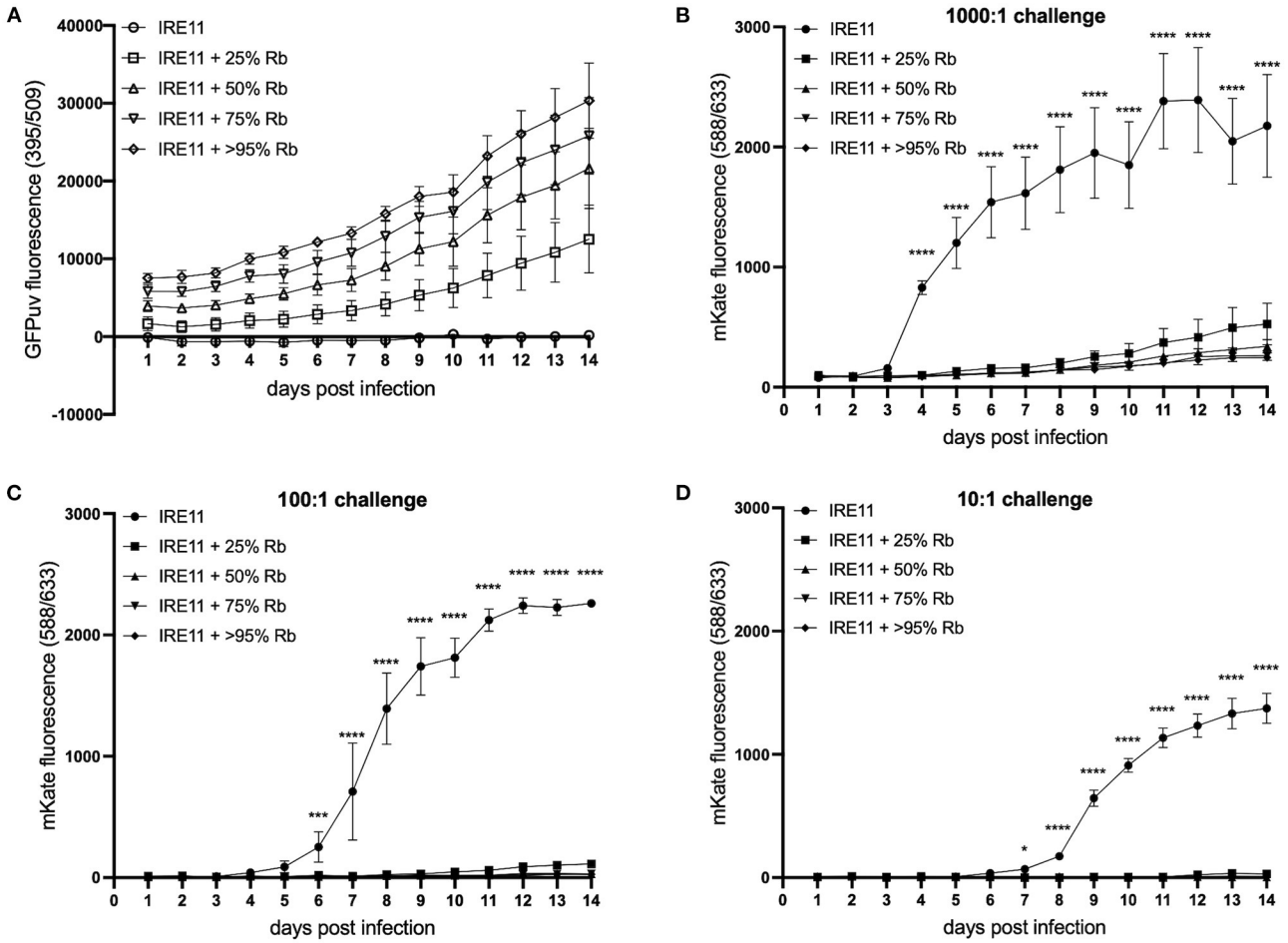

FIGURE 5 | *Rickettsia buchneri* prevents infection and replication of *Rickettsia parkeri* in tick cell culture. Uninfected IRE11 and IRE11 infected with different levels of *R. buchneri*-GFPuv were challenged with different doses of *R. parkeri*-mKate. Rickettsial replication in IRE11 cells was monitored for 14 days by measuring GFPuv and mKate fluorescence on a microplate reader. **(A)** GFPuv fluorescence indicating replication of *R. buchneri*-GFPuv. **(B–D)** mKate fluorescence indicating growth of *R. parkeri*-mKate at challenge doses of 1,000:1 **(B)**, 100:1 **(C)**, and 10:1 **(D)**; lines show mean and error bars standard deviation of three replicate wells. Means were compared to the uninfected control IRE11 using a two-way ANOVA with Dunnett's multiple-comparison test; statistically significant values are marked by asterisks $*p < 0.05$, $***p < 0.001$, $****p < 0.0001$. Data are representative of two independently performed experiments (see **Supplementary Figure S4**).

that in IRE11 without *R. peacockii*-GFPuv at day 4 onward in cells infected with >95% *R. peacockii*-GFPuv (**Figure 6C**). mKate fluorescence in IRE11 with 75% or 50% *R. peacockii*-GFPuv was significantly lower from day 8 and day 10 onward, respectively, while growth of *Rp*-mKate in IRE11 with 25% *R. peacockii*-GFPuv was not significantly different from IRE11 without the endosymbiont (**Figure 6C**). At day 14, compared to that in IRE11 without *R. peacockii*-GFPuv, there was a 31% reduction in mKate fluorescence in IRE11 with 50% *R. peacockii*-GFPuv, a 38% reduction in IRE11 with 75% *R. peacockii*-GFPuv, and a 51% reduction in IRE11 with >95% *R. peacockii*-GFPuv. Similarly, in the 100:1 challenge wells, mKate fluorescence in 50, 75, and >95% *R. peacockii*-GFPuv-infected IRE11 was significantly different from that in IRE11 without *R. peacockii*-GFPuv, from day 5 (>95%) or day 6 (50 and 75%) onward, while mKate fluorescence in 25% *R. peacockii*-GFPuv-infected IRE11 was similar to the control (**Figure 6D**). At day 14, compared to that in IRE11 without *R. peacockii*-GFPuv, there were reductions of 35%, 43%, and 43% in mKate fluorescence measured in IRE11 with 50, 75, and >95% *R. peacockii*-GFPuv, respectively. In the 10:1 challenge experiment, mKate fluorescence was significantly reduced in IRE11 50, 75, and >95% infected with *R. peacockii*-GFPuv relative to IRE11 without *R. peacockii*-GFPuv from day 7 onward (**Figure 6E**). In IRE11 25% infected with *R. peacockii*-GFPuv, mKate fluorescence was significantly lower than the control from days 11 to 14 (**Figure 6E**). At day 14, in comparison to that in IRE11 without *R. peacockii*-GFPuv, mKate fluorescence was reduced by 16, 47, 61, and 55% in IRE11 with 25, 50, 75, and >95% *R. peacockii*-GFPuv, respectively. Measurement of GFPuv fluorescence could differentiate the different populations of IRE11 infected with 25, 50, 75, and >95% *R. peacockii*-GFPuv and implied a steady replication of *R. peacockii*-GFPuv over time (**Figure 6F**). No increase in GFPuv fluorescence was observed in uninfected IRE11; rather, there was a decline, likely due to lysis of

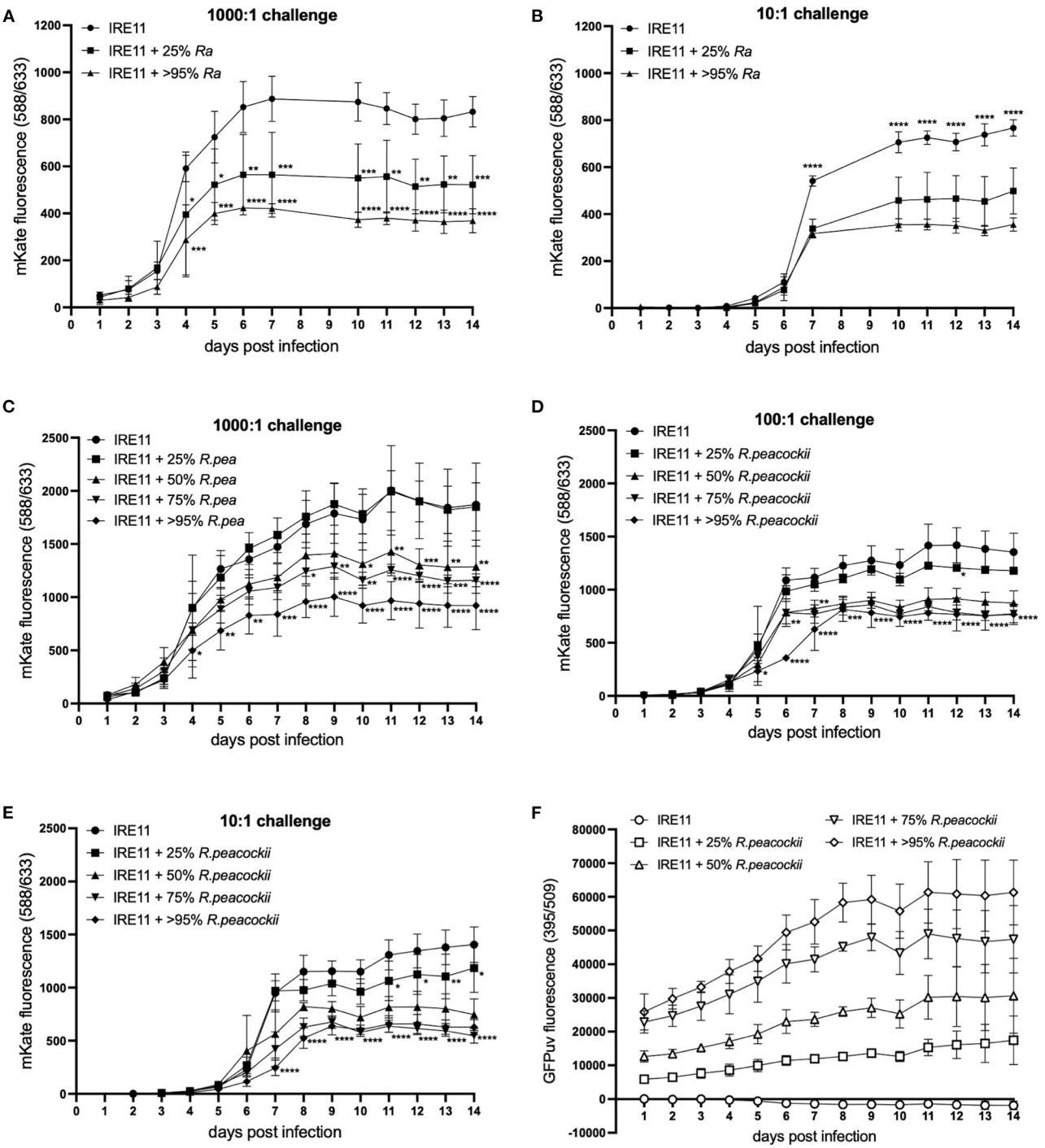

FIGURE 6 | *Rickettsia parkeri* replication in the presence of *R. amblyommatis* or *R. peacockii.* Replication of *R. parkeri*-mKate in tick cells was monitored for 14 days by measuring mKate fluorescence on a microplate reader. **(A,B)** *R. parkeri*-mKate replication in IRE11 cells with or without *R. amblyommatis* at 28°C in a candle jar at challenge doses of 1,000:1 **(A)** and 10:1 **(B)**; readings not taken on day 8 or 9. **(C–E)** *R. parkeri*-mKate replication in IRE11 cells with or without *R. peacockii*-GFPuv at 28°C in a candle jar at challenge doses of 1,000:1 **(C)**, 100:1 **(D)**, and 10:1 **(E)**. **(F)** GFPuv fluorescence indicating replication of *R. peacockii*-GFPuv. Data show mean and error bars standard deviation of three replicate wells. Means were compared to the uninfected control IRE11 using a two-way ANOVA with Dunnett's multiple-comparison test; statistically significant values are marked by asterisks $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$. Data are representative of two independent experiments (see **Supplementary Figure S4**).

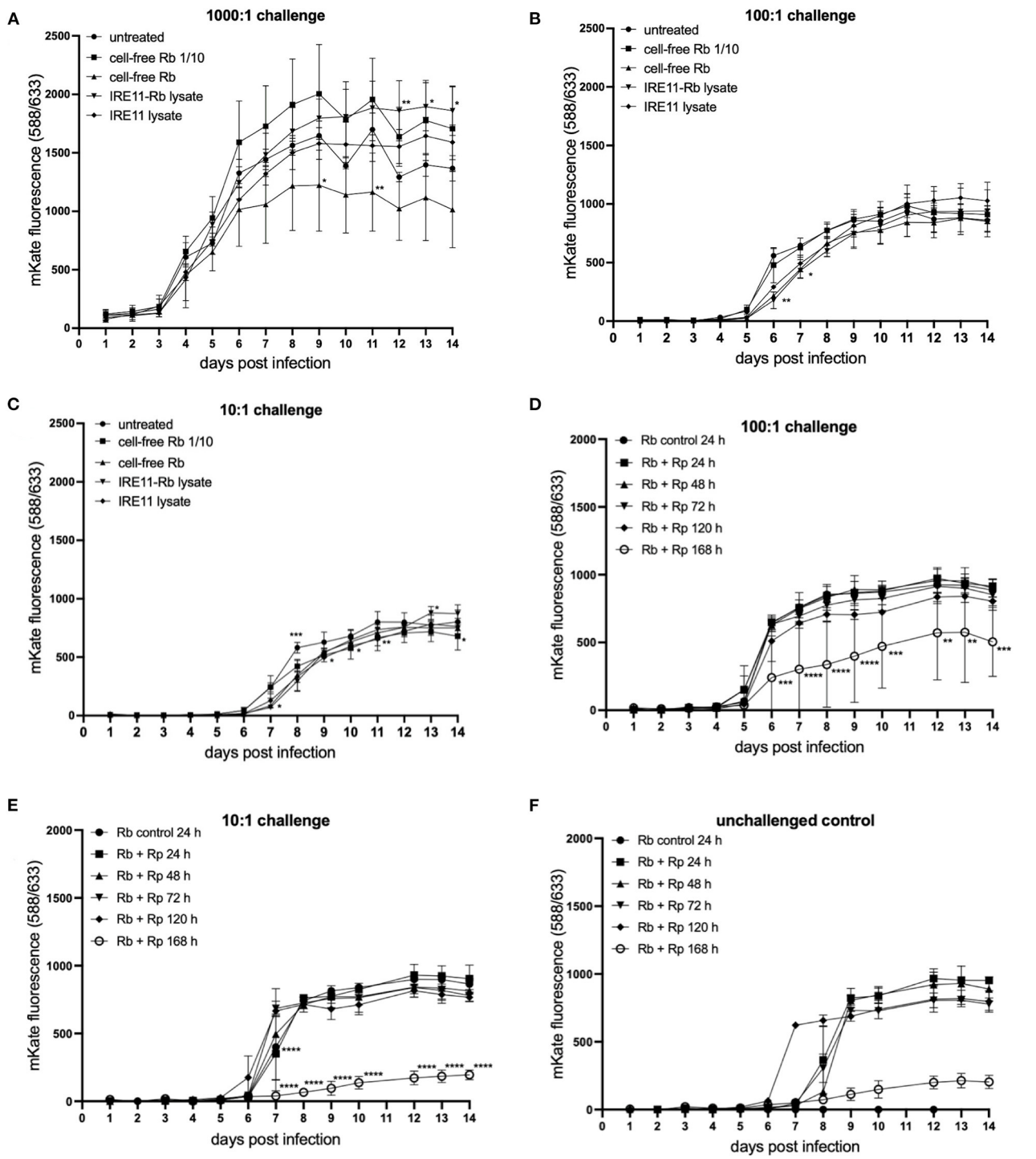

FIGURE 7 | *Rickettsia parkeri* replication in IRE11 treated with cell-free *R. buchneri* or *R. buchneri* lysate. Replication of *R. parkeri*-mKate in tick cells was monitored for 14 days by measuring mKate fluorescence on a microplate reader. **(A–C)** *R. parkeri*-mKate replication in IRE11 at challenge doses of 1,000:1 **(A)**, 100:1 **(B)**, and 10:1 **(C)** in the presence of cell-free *R. buchneri*, lysate from *R. buchneri*-infected IRE11, or lysate from uninfected IRE11. **(D–F)** *R. parkeri*-mKate replication in IRE11 treated with lysates from *R. buchneri* challenged with *Rp*-mKate for 24, 48, 72, 120, or 168 h. Treated cells were challenged with 100:1 **(D)** or 10:1 **(E)** *Rp*-mKate. Unchallenged control indicates that viable *Rp*-mKate are present in cell lysate **(F)**. Data show mean and error bars standard deviation of three replicate wells. Means were compared to the uninfected control IRE11 using a two-way ANOVA with Dunnett's multiple-comparison test; statistically significant values are marked by asterisks $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

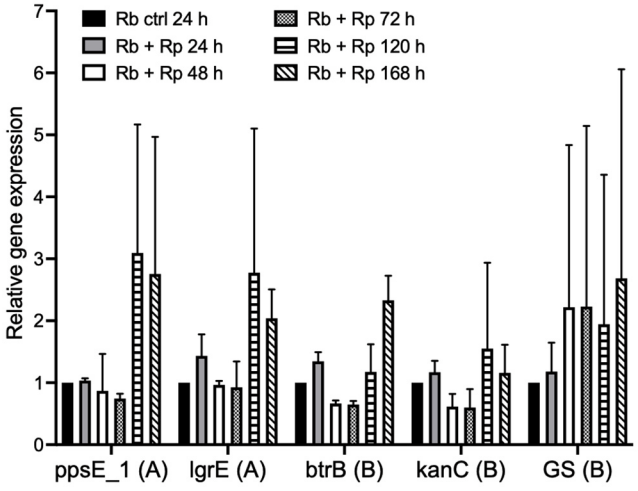

FIGURE 8 | Expression of *R. buchneri* antibiotic cluster genes in response to *Rickettsia parkeri* challenge. Relative expression of *R. buchneri* antibiotic cluster genes in response to *R. parkeri*-mKate challenge over 7 days. Bars show the expression of each gene relative to the control (unchallenged *R. buchneri*) as the mean and standard deviation of two independent experiments for each qRT-PCR assay. Ct values were normalized to *GAPDH* expression in infected IRE11 cells. Genes from the polyketide and aminoglycoside clusters are denoted by (A) and (B), respectively. Means were compared to the unchallenged control using a two-way ANOVA with Dunnett's multiple-comparison test.

IRE11 by *Rp*-mKate in infected cells (**Figure 6F**). Together, these results suggest that neither *R. amblyommatis* nor *R. peacockii* possess the ability to inhibit growth of competing *R. parkeri* to the same extent as *R. buchneri*.

Further experiments were performed to examine whether the inhibitory effect of *R. buchneri* on *Rp*-mKate infection was due to the secretion of antibiotic products. Uninfected IRE11 was added to a 96-well plate then treated with either cell-free *Rb*-WT, a 1:10 dilution of cell-free *Rb*-WT, lysate from IRE11 heavily infected with *Rb*-WT, or lysate from uninfected IRE11. After 2 h, cells were challenged with *Rp*-mKate. Although there were some significant differences in the growth of *Rp*-mKate under some of the treatments, these were inconsistent across the three different challenge doses, suggesting that rather they were due to experimental variations, for example in challenge dose or cell density (**Figures 7A–C**). Taking these data together, there seems to be no obvious effect of any of the treatments on the growth of *R. parkeri* in comparison to untreated control cells. Furthermore, these results suggest that *R. buchneri* need to be intracellular to inhibit *R. parkeri* growth and that there is also no antibiotic effect of extracellular addition of *Rb*-WT lysate.

An additional experiment was performed to determine whether challenging *R. buchneri*-infected IRE11 with *Rp*-mKate would increase the inhibitory activity of lysates against *Rp*-mKate. Lysates were prepared from cell-free *Rb*-WT isolated from IRE11 challenged with *Rp*-mKate for 1–7 days and then used to treat IRE11 cells in a 96-well plate. The wells were then challenged as previously with *Rp*-mKate at challenge doses of 1,000:1, 100:1, and 10:1, and mKate fluorescence was measured over 14 days and compared to mKate fluorescence in cells treated with lysate from unchallenged *Rb*-WT-infected IRE11. At 1,000:1 challenge, there was no difference in mKate fluorescence between the control and any of the treatments (**Supplementary Figure S5**), showing a growth curve similar to that in other plate reader experiments. However, at challenge doses of 100:1 and 10:1, there were significant differences in mKate fluorescence in the wells treated with lysates from *Rb*-WT challenged with *Rp*-mKate for 7 days (168 h) in comparison to the control, with 43 and 77% reductions at day 14, respectively (**Figures 7D,E**). The mKate fluorescence in the other treatment groups was similar to that in the control wells. Unchallenged control wells were also incorporated into the experiment to check the viability of any rickettsiae in the lysate; mKate fluorescence was observed increasing on days 6–8, suggesting that viable *Rp*-mKate were present in the lysate (**Figure 7F**). However, their growth in wells treated with 168-h-challenged lysate was also much lower than in other treatment wells. The fact that the mKate fluorescence in the unchallenged control wells peaked later than in challenged wells suggests that the low level of viable *Rp*-mKate contributed little to the results seen in the *Rp*-mKate-challenged wells. Overall, these results indicate that after 7 days in the presence of *Rp*-mKate, lysates from *Rb*-WT were able to inhibit the replication of *Rp*-mKate in IRE11 cells at lower doses (100:1 and 10:1), which could be due to increased antibiotic activity at this time point.

Expression of Antibiotic Genes in Response to *R. parkeri* Infection

To investigate whether the expression of genes from the putative antibiotic clusters of *R. buchneri* was upregulated in response to the presence of potentially competing bacteria, a time-course experiment was set up and qRT-PCR was used to examine the relative expression of selected genes from each antibiotic cluster in the presence and absence of *Rp*-mKate during infection. Over a 7-day time course, several of the examined genes appeared to be upregulated in response to multiple days' challenge with *Rp*-mKate (**Figure 8**); however, there was wide variation between replicate experiments and the differences were not statistically significant.

Investigation of *R. buchneri* Antibiotic Activity

The antibiotic activity of *R. buchneri* against extracellular bacteria was investigated using antibiotic susceptibility assays. Filter paper disks were treated with cell-free *Rb*-WT, *Rb*-WT-infected IRE11, and supernatant from IRE11 cultures infected with various levels of *Rb*-WT (25%, 50%, and >95%). Spectinomycin (10 and 100 μg)-treated disks were used as positive controls, and uninfected IRE11 cells and IRE11 culture supernatant were used as negative controls. Disks were placed onto Mueller–Hinton agar plates streaked with *E. coli* strain D21 or *S. aureus* strain MN8. None of the IRE11- or *Rb*-WT-derived treatments resulted in any inhibition of *E. coli* or *S. aureus* growth, whereas spectinomycin-treated disks prevented bacterial growth (**Table 4**).

TABLE 4 | Results from antibiotic susceptibility tests (disk diffusion assays) against *E. coli* and *S. aureus*.

	***E. coli* D21**	***S. aureus* MN8**
Experiment 1—cell lysates		
Cell-free Rb	-	-
Rb-infected IRE11	-	-
Uninfected IRE11	-	-
Spectinomycin 10 μg	+	+
Spectinomycin 100 μg	+	+
Experiment 2—live cells and supernatant		
Cell-free Rb	-	-
IRE11 + 25% Rb	-	-
IRE11 + 50% Rb	-	-
IRE11 + >95% Rb	-	-
Uninfected IRE11	-	-
IRE11 + 25% Rb supernatant	-	-
IRE11 + 50% Rb supernatant	-	-
IRE11 + >95% Rb supernatant	-	-
IRE11 supernatant	-	-
Spectinomycin 100 μg	+	+

+ inhibition of bacterial growth; - no inhibition of bacterial growth.

Supernatant from IRE11 heavily infected with *Rb*-WT was also tested for activity against *Rp*-mKate growing in Vero cells. Addition of the supernatant to Vero cultures prior to infection with *Rp*-mKate did not inhibit infection; there were no differences observed in the progression of plaque formation and size of plaques in comparison to a duplicate experiment using supernatant from an uninfected IRE11 culture (**Supplementary Figure S6**).

DISCUSSION

There is growing evidence that competition between endosymbiotic and pathogenic *Rickettsia* species in tick vectors may play an important role in the persistence and transmission of rickettsial pathogens (70–73). This study describes the existence of two putative antibiotic synthesis gene clusters in the genome of the *I. scapularis* endosymbiont *R. buchneri* isolated in Minnesota. Furthermore, *in vitro* experiments show that *R. buchneri* exerts an inhibitory effect on the growth of pathogenic rickettsiae in tick cell culture. Inhibition of *R. parkeri* growth by *R. buchneri* was greater than that exhibited by the other rickettsiae examined in this study, the low pathogenic *R. amblyommatis*, and the endosymbiont *R. peacockii*, which may suggest that this is due to the presence of antibiotic synthesis genes in *R. buchneri* that are lacking in other rickettsiae. Even at low infection rates, the presence of *R. buchneri* resulted in significant perturbation of *R. parkeri* growth, while the presence of either *R. amblyommatis* or *R. peacockii* showed a more direct competition where higher infection rates lead to a greater reduction in the growth of *R. parkeri*. These results correlate with what is known from current field and laboratory data, which suggest reduced horizontal and vertical transmission of *R. parkeri* or *R. rickettsii* by ticks in the presence of coinfecting *R. amblyommatis* or *R. peacockii* (70, 72, 73) and an almost complete absence of any coinfecting *Rickettsia* species in *R. buchneri*-infected *I. scapularis*. While a link between this inhibition and antibiotic synthesis is yet to be proven, this work raises the possibility that antibiotic production by *R. buchneri* may be a mechanism for exclusion of competing intracellular bacteria from its host tick.

Although genes from the clusters are actively transcribed by *R. buchneri*, no evidence of antibiotic activity was found in lysates or supernatants from *R. buchneri*-infected cultures, against either *R. parkeri* or the extracellular bacteria *E. coli* and *S. aureus*. However, when grown in the presence of *R. parkeri* for 7 days, lysates from *R. buchneri*-infected cells showed some inhibitory activity against lower challenge doses (100:1 and 10:1) of *Rp*-mKate, suggesting that *R. parkeri* challenge could be a trigger that might induce antibiotic activity of *R. buchneri*. However, results from qRT-PCR examining the expression of certain genes in the clusters in the presence of *R. parkeri* were highly variable and therefore inconclusive. Further analyses to obtain additional information about the regulation of the antibiotic synthesis clusters are required to understand how they respond to *R. parkeri* challenge. For example, it is unknown whether the clusters are transcribed as operons or in what ratios the different components of the clusters are required for antibiotic production. The antibiotic compounds produced by *R. buchneri* might only act intracellularly to prevent cells inhabited by the endosymbiont being invaded by other bacteria which could compete for resources. However, the fact that even when only a quarter of cells are occupied by *R. buchneri* results in inhibition of *R. parkeri* growth suggests that there must be some effect of *R. buchneri* on neighboring cells, which could potentially be mediated through the delivery of compounds to adjacent cells that are either antimicrobial or make cells refractory to infection. Further in-depth studies are required to elucidate the mechanisms of anti-rickettsial activity as well as how the putative compounds may be transported.

The proteins encoded by the aminoglycoside gene cluster show similarity to those from Actinobacteria, particularly *Streptomyces* spp. and Firmicutes (54), while the polyketide gene cluster encodes proteins with similarity to those of Gammaproteobacteria, particularly *Erwinia amylovora*, *Pantoea ananatis*, and *Legionella* spp. This suggests these gene clusters were likely obtained from environmental bacteria; indeed, members of these phyla have been identified in the *I. scapularis* microbiome (21, 22, 24, 32, 92). The *R. buchneri* genome is known to be highly plastic and contains multiple mobile genetic elements and sections of genetic material from other microorganisms (54). As ticks spend a large portion of their life in the environment in close association with soil, leaf litter, and vegetation, and also come into contact with the skin and blood of animals during host-seeking and feeding, it is likely that they encounter numerous environmental bacteria from which these gene clusters could have been transferred. Further examination of the gene clusters and similar pathways in the bacterial taxa from which these most likely originated may give us a better understanding of the likely antimicrobial

compounds that could be synthesized by *R. buchneri*, and this could lead to future isolation and characterization of these products and determination of their antimicrobial activity. For example, *Legionella* spp. are known to contain multiple gene clusters for synthesis of polyketides/non-ribosomal peptides (93), and polyketide synthases were identified in *Pantoea* and *Erwinia* species that showed antagonistic activity (attributed to antibiosis) against the rice pathogen *Xanthomonas oryzae* (94). Similarly, strains of *P. ananatis* and *P. agglomerans* contain antibiotic biosynthesis clusters that allow them to compete with *E. amylovora* (95, 96). Interestingly, the *Rb* ISO7 polyketide cluster includes a putative type IV pilin; these proteins have diverse functions including adherence, motility, biofilm formation, host cell manipulation, DNA transfer, and protein secretion (97). It will be interesting to further investigate the role of this protein in *R. buchneri*, as many functions of this class of pilins could be related to the antibiotic activity of the cluster. Most genes in the polyketide cluster are absent from the REIS (Wikel) genome, and there may have been some recombination of this region resulting in excision of a large portion of the cluster that is found in *Rb* ISO7. This might suggest that this gene cluster is not essential for the endosymbiont, and the lack of antimicrobial products it synthesizes is compensated for by the presence of the aminoglycoside cluster. The REIS (Wikel) genome is derived from that of a lab colony of *I. scapularis*, whereas the *Rb* ISO7 genome originates from a field-collected tick, so an alternative explanation for the loss of these genes in REIS (Wikel) is that they are not essential for survival in the lab but could be necessary under natural conditions for protection against challenge from environmental microbes, for example. Further experiments to compare *R. buchneri* with REIS (Wikel) might determine whether absence of the polyketide cluster has any effects on survival or competition with other bacteria.

Given the inhibitory effect of *R. buchneri* on the *in vitro* infection and replication of the intracellular pathogens *A. phagocytophilum*, *R. monacensis*, and *R. parkeri*, one role of the endosymbiont may be the exclusion of pathogens from the tick. As *R. buchneri* is primarily restricted to the ovaries of female ticks, in nature it might be involved in preventing the colonization of this organ and subsequent transovarial transmission of intracellular pathogens, such as other *Rickettsia* species. While inhibition of *A. phagocytophilum* was seen in tick cell culture, it is unclear whether *R. buchneri* has any effect on *A. phagocytophilum* infection within the tick vector. Sakamoto *et al.* found that *A. phagocytophilum* levels were higher in male ticks, which have significantly lower titers of *R. buchneri* (19), while Steiner *et al.* found no correlation between *R. buchneri* and *A. phagocytophilum* infection prevalence (48), yet few other studies have examined interactions between these two bacteria in tick populations. Similarly, there is little data on whether *R. buchneri* has any effect on *I. scapularis* infection with the spirochete *B. burgdorferi*. Steiner and colleagues found that *B. burgdorferi* infection rates were significantly higher when *R. buchneri* was not detected, but only for male *I. scapularis* (48). One microbiome study found that *Rickettsia* reads were significantly less abundant in *B. burgdorferi*-positive ticks (98), while another found no differences in bacterial composition between *B. burgdorferi-positive* and negative ticks, for either males or females (20). Neither of these pathogens are transovarially transmitted, with *A. phagocytophilum* localizing to the salivary glands (99) and *B. burgdorferi* residing in the midgut (100). That both pathogens are highly prevalent in *I. scapularis* populations supports the hypothesis that any antibiotic activity *R. buchneri* may have is likely restricted to the ovaries. In this study, *R. buchneri* inhibition of tick-borne pathogens was observed in a tick cell culture system that is likely not directly comparable to life inside the tick. Further studies examining the effect of *R. buchneri* on infection by these pathogens using *ex vivo* ovaries (101) and/or live *I. scapularis* should provide additional insights into the dynamics of this competition. With the ability to remove *R. buchneri* from ticks with the use of ciprofloxacin (26), *in vivo* studies to further examine the consequences for ticks lacking the endosymbiont and whether they are susceptible to infection with pathogenic rickettsiae can be performed in future.

Ixodes pacificus, the main tick vector of *B. burgdorferi* and *A. phagocytophilum* in the western US, also harbors a highly prevalent rickettsial endosymbiont, "*R. monacensis*" strain Humboldt (56, 102, 103), and is not known to vector pathogenic rickettsiae. However, application of genome similarity sequence-threshold criteria indicates that this endosymbiont is a new distinct species closely related to *R. buchneri* and *R. monacensis* (104). In addition, *I. pacificus* is often coinfected with *Rickettsia* phylotype G022 (102, 103). While little is currently known about phylotype G022, it is more closely related to pathogenic SFG rickettsiae than to *R. buchneri* and *Rickettsia* strain Humboldt (103). Cheng et al. suggested that it is likely to be the "Tillamook agent" previously isolated from *I. pacificus* and shown to be mildly pathogenic in guinea pigs (105, 106). However, this agent has recently been characterized and found to be a separate species (*R. tillamookensis* sp. nov.) related to the transitional group of *Rickettsia* (107), meaning that *I. pacificus* is associated with two potentially pathogenic rickettsiae in addition to its endosymbiont. The *Rickettsia* strain Humboldt genome (NZ_LAOP01000001.1) does not appear to contain antibiotic gene clusters similar to those found in *R. buchneri*, which might be one reason that *I. pacificus* can be coinfected with both its endosymbiont and potentially pathogenic species. Interestingly, field-collected *I. scapularis* have occasionally been found containing *R. amblyommatis*, *R. montanensis*, or *R. parkeri* (25, 31, 36, 41, 42, 44, 53, 108), which could potentially occur through "spillover" from host feeding alongside infected *A. americanum* or *D. variabilis*; however, these infected *I. scapularis* appear to be individuals lacking *R. buchneri* since the endosymbiont was not detected in these ticks. Only one coinfection of *I. scapularis* with *R. buchneri* and *R. parkeri* has been reported (44), and this was in a blood-fed tick collected from Louisiana black bears (*Ursus americanus luteolus*) also being fed on by *R. parkeri*-infected *A. maculatum*, making it likely that the pathogen was present in the infected blood meal.

Interference between rickettsiae has been little studied since it was proposed 40 years ago, but existing research shows that infection with a first *Rickettsia* species may reduce transovarial transmission of a second *Rickettsia* (70, 71, 73), reduce acquisition of the second *Rickettsia* from infected

hosts, and/or reduce its replication in the tick (72, 73), all of which could potentially lead to reduced transmission of pathogenic rickettsiae in enzootic cycles. In this study, the presence of either *R. amblyommatis* or *R. peacockii* led to a reduction in the ability of *R. parkeri* to infect and replicate in tick cells, reflecting what has been observed in *in vivo* studies in ticks (70–73). However, the mechanisms by which this interference occurs remain unexplored. One potential mechanism that has been suggested is immune priming (26), in which extracellular *Rickettsia* could stimulate the tick innate immune response, making the vector less susceptible to infection with a second *Rickettsia.* Symbionts have been shown to be important for immune development and protection from pathogens in other arthropods (109–111). It is also possible that occupation of tick cells or tissues by one *Rickettsia* species could physically prevent them being infected by a second *Rickettsia*, reducing their ability to effectively spread and replicate in the tick.

In summary, this research provides evidence that the endosymbiont of *I. scapularis*, *R. buchneri*, exerts an inhibitory effect on the growth of pathogenic tick-borne bacteria in cell culture and possesses two gene clusters encoding putative antibiotic biosynthesis machinery. This might suggest that besides being a potential nutritional endosymbiont, *R. buchneri* could also provide the service of preventing pathogenic *Rickettsia* species from occupying the ovaries, which could be detrimental to the tick's biology as has been shown to be the case for *R. rickettsii* in *D. andersoni* and *D. variabilis* (112, 113). While a correlation between the presence of antibiotic clusters and the ability to inhibit the growth of pathogenic Rickettsiae was found in this study, confirmation that the observed inhibition is directly linked to *R. buchneri*'s antibiotic clusters requires further investigation. Supportive evidence from *in vivo* studies could have important implications for our understanding of rickettsial interference and the vector competence of *I. scapularis* for SFG rickettsiae.

DATA AVAILABILITY STATEMENT

The datasets generated for this study can be found in the Data Repository for the University of Minnesota, https://doi.org/10.13020/ZQXG-JF78.

AUTHOR CONTRIBUTIONS

BC, JO, TK, and UM conceived and designed the experiments. BC, NB, TK, and X-RW performed the experiments. BC and TK analyzed the data. CT developed the fluorescent plate reader assay. BC drafted the manuscript and prepared the figures. All authors contributed to the manuscript revision and approved the submitted version.

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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.2021.748427/full#supplementary-material

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