Evidence of pathogenic zoonotic tick-borne Rickettsia and Borrelia spp. in some communal farms in the Eastern Cape Province, South Africa

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Abstract

The abundance of tick populations in South Africa represents a probable risk for both animal and human health. Rickettsia and Borrelia are well-known significant agents of emerging human tick-borne infectious diseases throughout the world. Nevertheless, the epidemiology of their infections has been underreported in South Africa, Therefore, the aim of this study was to profile for zoonotic Rickettsia and Borrelia species from ticks infesting domesticated animals in the Eastern Cape, South Africa. Morphological and molecular identification techniques were conducted on 1,200 tick samples collected from domestic animals before screening for the target bacterial pathogens. The molecular identification of the tick samples was based on the amplification of the 12S rRNA mitochondrial DNA while those of Rickettsia and Borrelia species were carried out by amplifying fragments of glt A, omp A and omp B genes for Rickettsia and fla B gene for Borrelia spp. Thereafter, the positive ticks, Borrelia and Rickettsia omp B amplicons were sequenced and further analysed. Eight species of ticks belonging to three genera; Rhipicephalus, Amblyomma, and Haemaphysalis were identified. A total of 320 (27%) samples were confirmed positive for Rickettsia out of which 74 (23%) were positive for both omp A and omp B genes. Phylogenetic analysis of omp B revealed a high homology to rickettsial reference strains from GenBank, while there was no positive result for Borrelia. The generated sequences showed 99.1 to 100% homology with R. africae -KX227790 (100%), R. parkeri - KY113111 (99.8%), R .peacock (99.3%) and R. slovaca - KJ675445, JX683122 (99.1%) representative sequences in GenBank. The findings from this study revealed that ticks collected from domesticated animals were parasitized by Rickettsia species with possible zoonotic potential, which is detrimental to human health if bitten by infected ticks.

Introduction

Vector-borne diseases constitute a serious risk to human health causing substantial morbidity and mortality worldwide[1,2]. Ticks are haematophagous ectoparasites of vertebrates that obtain their nutrition by feeding on blood, hence, they have been described as competent vectors of diseases, and over 10% of the currently known population of tick species have been described to be of medical or veterinary significance[3,4].

After mosquitoes, ticks are considered the second most important vectors of human diseases and the major vectors of pathogenic organisms in animals[5], as well as the most important vectors for numerous severe zoonotic infections worldwide[6]. Also, an increase in the range of tick-borne diseases infecting domestic animals and humans has been observed recently, and several significant zoonotic TBDs such as rickettsioses[7], and Lyme borreliosis[8] are on the increase, worldwide. Tick-borne pathogens (TBPs) have been reported to maintain lifecycles that include ticks and animals and sometimes they are transmitted to infested humans who are usually the dead-end hosts[9].

Rickettsia and *Borrelia* spp. are both transmitted by ticks and are among the numerous zoonotic pathogens responsible for febrile illness and sometimes death among humans. Rickettsial diseases, caused by organisms of the genus *Rickettsia* are classified into three biogroups: spotted fever group (SFG) which includes Rocky Mountain spotted fever (RMSF) and rickettsial pox caused by *R. rickettsia and R. akari respectively, as well as other spotted fevers such as Boutonneuse fever (Mediterranean spotted fever, Kenya*

tick-bite fever, Israeli spotted fever, African tick typhus, Marseilles fever, and Indian tick typhus,) caused by several other Rickettsia species.

Second is the typhus group (TG) ricketssiae which are responsible for similar diseases but with a different epidemiology[10] and the etiologic agents are *R. typhi* and *R. prowazekii*, although they have been described to be similar to causative agents for the spotted fever group but are distinct antigenically[11]. Lastly, the scrub typhus biogroup, a single taxonomic name (*Orientia tsutsugamushi) has been described for the rickettsial agents of this biogroup.* Nevertheless, these pathogens represent a diverse collection of pathogens that are extremely different from *Rickettsia* spp. of the typhus and spotted fever groups; hence they have three major serotypes which are Karp, Gilliam, and Kato [12,13].

However, a re-classification pattern for *Rickettsia,* with the inclusion of a fourth family has been proposed, which is known as transitional group (TRG) *Rickettsiae*. It is distinctive from SFG and TG *Rickettsiae* and harbours genes from likely interactions with TG *Rickettsiae* through conjugation [14]. Rickettsial diseases have been reported to be very challenging to diagnose, owing to their similar symptoms and epidemiology shared with several other febrile illnesses. Thus, the overall reported cases of rickettsial diseases are probably inaccurate as they are often times underreported[15,16].

Borrelia, a genus of bacteria belonging to the spirochete phylum [17], a causative agent of borreliosis and a zoonotic infectious disease transmitted by ticks. Over 50 species of *Borrelia* have been categorized into two groups, the first group comprises of about 21 species transmitted by the hard ticks, within *Borrelia burgdorferi* sensu lato complex, and is related with Lyme borreliosis group, while 19 species are described to be mainly transmitted by soft ticks associated with relapsing fever group with exception of the human louse-borne *Borrelia recurrentis* [18]. *Borrelia* species exist in enzootic cycles mostly involving ticks and several animals and bird hosts.

Most hard-bodied ticks belonging to genus *Ixodes* have been described to be major vectors transmitting pathogenic *Borrelia* spp. with *I. scapularis* transmitting *B. burgdorferi* in the mid-Atlantic, East, and upper Midwest of United State while *I. pacificus* transmitting *B. bissettii* and *B. burgdorferi* in the Western parts of USA, whereas in European, *I. ricinus* is known as a major vector for *B. afzelii*, *B. burgdorferi*, and *B. garinii* along with other non-pathogenic and potentially pathogenic *Borrelia* spp., and *I. persulcatus*, whose geographical distribution has been described to some extent overlaps with *I. ricinus*, which is a known vector of *B. garinii* and *B. afzelii* in eastern regions of Asia and Europe [19,20].

Owing to expansion in geographical boundaries by ticks into new ecological terrains worldwide, severally described *Rickettsia* spp. and *Borrelia* spp. that were previously considered to be endemic to a particular geographical location are now being described from different parts of the world and in different ticks [21]. The understanding of bacteria transmitted by ticks (potential reservoirs and vectors of microorganisms) in a given geographical location is a valuable marker for assessing the risk of infection in both humans and animals. This study therefore was aimed at investigating the prevalence of TBPs of *Rickettsia* and *Borrelia*

spp. in ticks parasitizing domesticated animals in the Amatole and O.R Tambo District Municipalities of Eastern Cape, South Africa.

Materials And Methods

Sampling Location

FIGURE 1: The map showing the geographical location of the sampling sites with their coordinates; Debe (Db) = 32°52'11.852"S, 27°1'14.171"E; Gxulu (Gx) = 32°40'26.702"S, 27°6'19.591"E; KwaMemela (Km) = 32°47'38.497"S, 26°44'10.889"E; Dwesa (Dw) = 32°13'50.916"S, 28°51'16.135"E; Umtata (Um) = 31°39'26.69"S, 28°48'0.194"E; Jambini (Jb) = 31°23'36.856"S, 29°29'46.921"E. Map created using **ArcMap 10.5.1**.

Ethical clearance

Ethical clearance for the study was obtained from the University of Fort Hare Research and Ethics Committee and permission to collect samples was sought from farmers and appropriate authority prior to sample collection.

Sample collection

With the assistance of the animal health technicians and animal farm workers, one thousand two hundred (1,200) feeding ticks were mechanically removed from farm animals (cattle, goats and sheep) into sterile 50 mL Nalgene tubes containing 70 % ethanol. The six different sampling sites selected for this study are known geographical locations for animal husbandry in Amatole and O.R Tambo District Municipalities of the Eastern Cape, South Africa. There was adherence to the University of Fort Hare Animal Ethics Committee regulations on animal handling, throughout the sampling period. The collected ticks were transported to Applied and Environmental Microbiology Research Group (AEMREG) laboratory, in the Department of Biochemistry and Microbiology at University of Fort Hare for analyses. Collected ticks from different animals and locations were properly labelled in different tubes for easy identification and to avoid possible mix up.

Tick identification and DNA extraction

Upon arrival at the laboratory, identification of tick species was carried out based on morphologic criteria such as scutum formation, capitulum formation and limbs formation [22,23,24]. Upon identification, the arthropods were washed in sterile distilled water for about 3 to 4 times for total removal of ethanol into which they were collected, chopped with a sterile blade in petri dish containing phosphate buffer saline (PBS), then transferred into a 2 mL centrifuge tube and vortexed. Following this process, DNA extraction was carried out using the commercially available kit, Promega ReliaPrep® gDNA Tissue Miniprep System (Madison, USA), and the manufacture's protocol was strictly adhered to. Engorged ticks were processed individually while non-engorged ticks of the same species from same animal were processed by pooling

using method previously described by James et al. [25]. Care was taken so that only ticks types collected from the same animal were processed as pools.

Molecular Identification of tick species

For the molecular identification of tick species previously identified morphologically, a fragment of 338 bp of mitochondrial 12S ribosomal RNA (rRNA) gene was amplified using a set of oligonucleotide 85F 12S [F:5'-TTAAGCTTTTCAGAGGAATTTGCTC-3'] and 2225 12S [R:5' TTTAAGCTGCACCTTGAC TTAA-3']. Polymerase chain reaction was performed in a 25µL reaction mixture comprising of 14 µL of master mix, 1 µL each of 10 pmol/L of the forward and reverse primers, 4 µL of RNase nuclease free water and 5 µL of DNA template. The cycling conditions used for the amplification was as followed; initial denaturation at 94 °C for 3 min, followed by denaturation at 93 °C for 30 sec, annealing at 51 °C for 30 sec, elongation at 72 °C for 60 sec with a final elongation at 72 °C for 5 min.

Molecular Detection of Zoonotic Tick-Borne Bacterial Pathogens

Rickettsia species

For the detection of *Rickettsiae* from the extracted DNA through polymerase chain reaction (PCR), a fragment of 631 bp of *Rickettsia* citrate encoding synthase (*glt*A) gene was amplified, using genus-specific primers [F:5'-TTTGTAGCTCTTCTCATCCTATGGC-3'] and [R:5'CCC AAGTTCCTTTAATA CTTCTTTGC-3'] as previously described by Pesquera et al. [26]. The reaction mixture containing 25 µL volume consisted of 14 µL of master mix, 1 µL each of 10 pmol/L of the forward and reverse primers, 4 µL of RNase nuclease free water and 5 µL of DNA template. DNA amplification was carried using Biorad T100® thermal cycler system, with the following cycling conditions; initial denaturation at 94 °C for 3 min, followed by denaturation at 93 °C for 30 sec, annealing at 49 °C for 30 sec, elongation at 72 °C for 60 sec with a final elongation at 72 °C for 5 min. Subsequently, all the positive samples were further subjected to screening for outer membrane protein A (*omp*A) and outer membrane protein B (*omp*B) which are structural protein that are imperative factors for rickettsial virulence and immunogen during infection [13], by PCR using oligonucleotides [F:5'-ATGGCGAATATTTCTCCAAAA-3'] and [R:5'- GTTCCGTTAATGGCAGCATCT-3'] to generate 631 bp of *omp*A gene, while [F: 5'-GTAACCGGAAGTAATCGTTTCGTAA-3'] and [R:5'-GCTTTATAACCAGCTAAACCACC-3'] primers was used to generate 511 bp of *omp*B gene. The PCR cycling conditions were as follow; initial denaturation at 94 °C for 3 min, followed by denaturation at 93 °C for 30 sec, annealing at 48 °C and 54 °C for 30 sec for *omp*A and *omp*B gene respectively, elongation at 72 °C for 60 sec with a final elongation at 72 °C for 5 min in a 25 µL reaction mixture comprising 14 µL of master mix, 1 µL each of the forward and reverse primers, 4 µL of RNase nuclease free water and 5 µL of DNA template, as previously described by Noh et al.[27] with modification of the annealing temperature.

Borrelia species

A two set of primers were used to amplify a partial region of *fla*B gene for *Borrelia* species; outward primer pairs of [F 5'-CCGTGCTAATTGTAGGGCTAA TAC-3'] and [R 5'-GAAGGTGCTGTAGCAGGTGCTGGCTGT-

3'] while the inward primers of *fla*B [F 5'- AARGAATTGGCAGTTCAATC-3'] and [R 5'-GCATTTTCAATTTTAGCAAGTG

ATG-3'] to eventually generate 380 bp in a 25 µL reaction volume containing 14 µL of enzyme master mix, 1 µL each of the forward and reverse primers, 4 µL of RNase nuclease free water and 5 µL of DNA template, under a thermo-cycling conditions of 3min at 94 °C for initial denaturation, followed by denaturation at 93 °C for 30 sec, annealing at 55 °C and 52 °C for 30 sec, for the outward and inward *fla*B gene respectively, elongation at 72 °C for 60 sec (40 cycles of amplification) with a final elongation at 72 °C for 5 min. All the PCR products were analyzed in 1% agarose gel electrophoresis in 0.5% TBE buffer, followed by staining with ethidium bromide. The gel was visualized under UV transilluminator (Samson et al., 2004). A negative control was included in the PCR so as to detect false positive or any possibility of cross contamination. Bi-directional sequencing was carried on all the positive ompB amplicons using ABI3500xl automated DNA sequencer with a 50cm Capillary array and POP7 (all supplied by Applied Biosystems).

Sequence Editing and Blast Search

Nucleotide sequences for both forward and reversed strands were assembled together and edited to generate consensus sequences for each positive PCR product, using the Geneious programme version 10.1.2 [28].

The consensus sequences data generated after editing were subjected to BLAST program in GenBank for homology search with other curated sequences (http://blast.ncbi.nlm.nih.gov). The search parameters were set on highly similar sequences, hence, *Borrelia* spp, and *Rickettsia* spp. were chosen separately as the organism option. Sequences with a percentage similarity above 97% were downloaded for phylogenetic analysis.

Results

A total of 1,200 ticks were mechanically removed from domesticated ruminants (718, 130 and 352 from cattle, sheep and goats respectively) from selected communal farms from Amatole and O.R Tambo District Municipalities. Nineteen species of ticks belonging to thre genera; *Rhipicephalus, Amblyomma* and *Haemaphysalis* were identified in this study (Table 1), with *Amblyomma hebraeum* having the highest occurrence of 335 (27.9%), followed by *Rh. appendiculatus*; 274 (22.8 %), *Rhipicephalus decoloratus*; 224 (18.7 %) and *Rhipicephalus eversti eversti*; 200 (16.7 %). For *Rickettsia* species, 320 (27%) genetic materials (DNA) were confirmed positive for *Rickettsia citrate encoding synthase (gltA) gene*, out of which 74 (23%) were further confirmed positive for both *omp*A and *omp*B gene while none was detected for *Borrelia*. A homology search for the generated sequences from the PCR data revealed a high percentage of identity between 96% - 100% with other homologous *omp*B of other *Rickettsia* sequences in GenBank (Table 2).

Table 1: Diversity of Tick Species Collected From the Animals in the Study Areas

Tick species	Number of tick species per animal			Total number of ticks (%)
	Cattle	Goat	Sheep	
A. hebraeum	235	80	20	335 (27.9)
Rh. decoloratus	129	70	25	224(18.7)
Rh.sanguineus	0	15	5	20 (1.7)
Rh. eversti eversti	140	40	20	200 (16.7)
Rh. microplus	70	40	20	130 (10.8)
Rh.appendiculatus	139	95	40	274 (22.8)
Rh. zambeziensis	5	0	0	5 (0.4)
H.spinulosa	0	12	0	12 (1.0)
Total	718	352	130	1,200

**Rh=Rhipicephalus, A=Amblyomma, H=Haemaphysalis*

FIGURE 2: The prevalence of tick species collected in the study. The figure shows the overall prevalence of tick species collected in all the sampling sites.

Molecular detection of zoonotic tickborne bacterial pathogens

For *Rickettsia* species, 320 (27%) genetic materials (DNA) were confirmed positive for *Rickettsia* citrate encoding synthase *(gltA)* gene, out of which 74 (23%) were further confirmed positive for both *omp*A and *omp*B gene, while no positive sample was confirmed for *Borrelia*. A homology search for the generated sequences from this study revealed a high percentage of identity between 96% - 100% with other homologous *omp*B of other *Rickettsia* sequences in GenBank (Table 2).

TABLE 2: Identities of rickettsial (*omp*B) sequences obtained from tick samples

Sample	Blast Homology (%)	Reference species	GenBank Accession Number
D219, D189	100.0	*R.africae*	KY124259,KX227790
B10, B26, D187	97.1-99.1	*Rickettsia* sp.	KX227788, KT032137
B12, B17, B20, B24, B218	96.8-99.8	*R.parkeri, R.sibrica*	KY113111, CP003341, KY124259, KY113111, HM050273
B13, B14, B15, B16, B22, D183, D186, D197, D200	96.7	*R.conorii*	AF123726
B24, D213, D214, D215	99.1	*R.peacockii, R.slovaca*	KJ675445,JX683122
B240,D211	96.0	*Rickettsia* sp.	KT032141, KT032136,KX227791
D191, D216, D127	97.6	*R.slovaca*	KJ663756,HQ232242,CP002428,AF123723
D219	98.1	*R.honei, R,rickettsi, R,rhipicephalis*	AF123724, CP006010,AF123719
D221	97.4	*Candidatus R.barbariae, R, slovaca, R.sibrica*	KY233284, KX227791,AF123722
D22	97.9-99.3	*R.peacockii, R.raoulti, R.philipii, R.rhiphicephali*	CP001227, HQ232277,CP003308, CP013133

***Only sequences that show 96.0%-100.0% similarities with other *omp*B reference strains are reported on the above table.**

The derived *Rickettsia* (*omp*B) sequences were further subjected to phylogenetic analyses with the following *Rickettsia omp*B reference strains from GenBank: KX227791- *R. africae* (Kenya), KU721071- *R. africae* (Austria), AF123721- *R. conorii* (France), AF149110- *R. conorii* (Australia), KF660534- *R. africae* (Kenya), KY124259-*R. parkeri* (USA), AF123726 (India), KY113111 (USA), KX891187 (South Africa), KY924884 (Ethopia), KX227788- *R. conorii* (Kenya), KU721071- *R. africae* (Tanzania), KT032137- *Rickettsia* sp. (Djibouti), KF660535- *R. africae* (Kenya), KT032137- *Rickettsia* sp. (USA), AF123722- *R. sibirica* (France), LT558854- *Candidatus Rickettsia wissemanii* (French Guiana), EF219461- *Rickettsia* sp. (Taiwan), CP001612- *R. africae* (France), X16353- *Rickettsia rickettsii* (USA), HQ232253- *Rickettsia raoultii* (Germany), AF123706-*R. africae* (France), CP003311- *Rickettsia rickettsii* (USA), EF219464- *Rickettsia* sp. (Taiwan), JQ792107- *Rickettsia raoultii* (China), KT835128- *Rickettsia* sp. (South Africa), CP003306- *Rickettsia rickettsii* (Colombia), KJ619633- *R. africae* (Gabon), KT835081- *Rickettsia* sp. (South Africa), LT558854- *Candidatus Rickettsia wissemanii* (Guiana), KY124259- *R.parkeri* (Western USA), AF123726-*R.conorii* (France), JX683122- *R. slovaca* (Romania), KJ675443-*R. peacockii* (Italy), EF219464-*R.Rickettsia* (Taiwan). The reference sequences were previously aligned with the derived sequences, using ClustalW in MEGA 7.0. version software (Kumar and Tamura, 2016), before generating the phylogenetic tree as shown in Figure 4.

FIGURE 3: Nucleotide sequence alignment of the sequences in the polymorphic region of the *omp*B gene of *Rickettsia* spp. with *Rickettsia rickettsii* serving as the reference sequence along with generated sequences in this study. Nucleotides that are identical for all species are highlighted in colour while those that vary between species and can be used for differentiation are not highlighted.

FIGURE 4: Evolutionary relationships of different *Rickettsia* spp. based on the nucleotide sequence of *omp*B gene. The evolutionary history was inferred using the Neighbor-Joining method. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test 1000 replicates is shown next to the branches. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA7 [29](Kumar et al., 2016).

Phylogenetic Analysis of rickettsial pathogens using *omp*B gene

Phylogenetic analysis showed that the obtained sequences clustered into three clades with other reference sequences from different geographical regions of the world. 26 sequences clustered in one clade with reference sequences from USA, France, Italy, Austria, Kenya and Gabon, with accession number LT558854-*Candidatus Rickettsia wissemanii*, KY124259-*R.parkeri,* AF123722-*R.sibirica,* EF21946*1-Rickettsia* sp., KJ675443-*R.peacockii*, AF123721-*R.conorii*, KJ619633-*Rickettsia* sp. A598, AF123722-*R.sibirica*, KT032137-uncultured *Rickettsia* sp., KX227791-*R. africae*, KX227788-*R. africae,* and AF123706-*R. africae*. 44 sequences were found to cluster in another clade with each other, while two other sequences clustered in

another clade closely with *R. rikettsii*- X16353, *R. conorii*- AF149110, and *R.africae*- KF660535 reference sequences from Australia, South Africa, Romania, and Germany with high genetic similarities (Fig 4).

FIGURE 5: Evolutionary relationships of tick species based on Nucleotide sequences of mitochondrial 12S ribosomal RNA gene. The evolutionary history was inferred using the UPGMA method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA7 [29]

Phylogenetic analysis of tick species

Phylogenetic analysis of generated tick sequences showed that the four genera; *Rhipicephalus, Amblyomma, Haemaphysalis* and *Boophilus* that were initially identified through morphologic criteria, clustered with different corresponding species of the reference sequences (Fig 5). Sequence T21 was shown to clustered in one clade with reference sequences KC503255-*Rh.australis*, KC503261-*Rh.microplus*, KC503259- *Rh.microplus,* and AB075954- *Haemaphysalis* sp. Also, the following obtained sequences; T29, T48, T29 were found to cluster closely with reference strain AF031859-*Rh.appendiculatus*, DQ801282-*Rh.appendiculatus*, KX276945-*Rh.appendiculatus*, DQ849237-*Rh.zambeziensis*, DQ849224-*Rh.zambeziensis*, MF3611814-*Rhipicephalis* sp., MF479197-*Rh.appendiculatus*. Likewise, sequence T01, T4, T13, T20, T25, and T32 clustered together and were found to cluster together in another clade with reference sequence KF583637-*H.longicornis*, AF31853-*H.longicornis*, HQ434625-*H.longicornis*, AF150049-*A.hebraeum*, MG076932-*A.maculatum*, AY342288-*A.triste*, AY342261-*Amblyomma* sp., KT386309-*Amblyomma* sp. AF150049-*A.hebraeum*. In addition, obtained sequence T40 revealed high closeness to reference sequence MF479198-*Rh. evertsi*, AF150052- *Rh. evertsi,* MF348105- *Rh. evertsi* while sequence T10 was found to be closely related to KY676830-*Rh.australis*, EU9217770-*Rh.microplus*, AF150045-*B.annulatus*, and AF031847-*B.microplus*.

The nucleotides sequences generated from this study were submitted to GenBank under the following accession numbers; MK347112 - MK347185, for *Rickettsia,* while eight representative sequences were deposited for *Amblyomma hebraeum, Rhipicephalus microplus, Boophilus annulata, Haemaphysalis longicornis* under accession number MK347205 - MK347212.

Discussion

Majority of people living in South Africa rural settlements are in close proximity with tick-infested ruminants, hence they are at high risk of being infected with arthropod-borne zoonotic pathogens. In addition, several cases of arthropod-borne diseases are commonly reported, especially in international travellers returning from South Africa, hence it is expedient to be aware of new vectors, host and pathogens [30].

Although, the detection of arthropod-borne pathogens in ticks that infest animals cannot automatically demonstrate the role of the host as reservoir or the tick as vector, however, both can hypothetically act as amplifiers, and are important for the epidemiology of tick-related infections [31].

In this study, a total of 1200 ticks comprising both adult and nymph that were mechanically removed from farm animals belonged to three genera; *Rhipicephalus, Amblyomma,* and *Haemaphysalis* were identified, with *Rhipicephalus* having the highest occurrence of 853 (71.1 %), followed by *Amblyomma*; 335 (27.9 %), and *Haemaphysalis*; 12 (1%) (Fig 2). The three genera had previously been reported from South Africa [32,33], thus our results is a confirmation that these genera are the predominant ticks species in the study areas. The emergence and geographical distribution of tick-borne zoonotic pathogens are controlled by the ecological distribution of their arthropod vectors.

Of recent, newly discovered species of arthropod-borne diseases are continually being added to the variety of groups of tick families/genera, owing to their chemical, physical and immunological features [34] which has made the species in each genus becoming endless, especially the species with zoonotic potentials. Globally, various species of *Amblyomma* have been reported to be vectors of both animal and human rickettsial pathogens, which have resulted in increased risk of spotted fever group (SFG) rickettsiosis of late [49]. As a rare acute and multi-systemic febrile disease, spotted fever has been described to have mortality rate of over 50% in the absence of proper prophylaxis (Prado et al., 2018). *A. hebraeum* with a significant aggression for biting humans has been documented as a well-known vector of *Rickettsia africae*, a zoonotic tick-borne bacterial pathogen that is the etiologic agent of African tick-bite fever in sub-Sahara Africa with a morbidity rate of > 50% [47]. Thus, *R. africae* infection has been described, after malaria, to be liable for most febrile illnesses diagnosed in tourists returning from southern Africa [21,34].

In addition, various species of *Rhipicephalus* and *Haemaphysalis* have also been documented in several geographical regions of the world to be vectors for various zoonotic tick-borne pathogens. *R. massiliae*, was first isolated from *Rhipicephalus sanguineus* (brown dog tick) in Europe, and it has been implicated in human cases of spotted fever in South America, Argentina and Europe [35,35].

The abundance of *Rhipicephalus* species in many West African countries has been described as currently have a magnitude higher than *Amblyomma* species (Biguezoton et al., 2016),which was formerly known as the main vector of heartwater *Ehrlichia ruminantium in the region. Over 70% of total number of Rhipicephalus genus has been described to be endemic to Afrotropical region. It has also been implicated in human cases of monocytic ehrlichiosis and Mediterranean spotted fever [37,38].*

Several mammals have been reported to be infested by *Haemaphysalis* ticks in countries like Italy, Turkey, Greece, Spain, Cyprus and Croatia [39,40,41,42], resulting in various bacterial, protozoan and viral infections. *Haemaphysalis* ticks have also been implicated as vector of human bacterial infections in Australia and China [43,44]. Sarih et al.[45], also reported *Haemaphysalis* ticks as being responsible for transmission of spotted fever group rickettsiosis in Morocco.

Furthermore, about nine pathogenic *Rickettsia* species (*R. africae, R. parkeri, R. mongolotimonae, R. conorii, R. honei, R. rickettsii, R. raoultii, R. australis, and R. rhipicephalis*) belonging to the spotted fever group *Rickettsia*e were detected in different tick samples collected in this study. *R. africae* had been previously reported from ticks removed from different animals and humans in South Africa, thus its detection was expected [34,46,47] from its well-known vector; *A. hebraeum*. Subsequently, about 24 sequences, from the 74

obtained sequences for *omp*B gene of *Rickettsia* sp., showed between 98.9% - 99.3% homology with *Rickettsia parkeri* (KY124259, CP003341, AF123717, and KY1131111).

Rickettsia africae, a causative agent of African tick-bite fever (ATBF), belonging to Spotted Fever Group (SFG) *Rickettsia*, has been described as an emerging infectious pathogen in the African continent, affecting both humans and animals with devastating effects on livestock production and human health. Several seroepidemiological studies across the continent have described residence in livestock production areas as the major risk factor for seropositivity in rickettsiosis antigen [48,49].

The risk has been attributed to the abundance of *Amblyomma* sp. in most African countries [50], as species of *Amblyomma* have severally been implicated as vectors of *R. africae* infection, hence the increase in percentage of infected ticks could increase the probability of humans being bitten by them thereby leading to increased rate of human rickettsisosis.

ATBF has also been recovered from American travellers returning from Southern Africa [51], with a history of tick bite during their adventure, as well as positive human serum samples in western Africa [52]. The detection of *R. africae* in the present study is supported by the findings of Mediannikov et al. [52] who reported high detection rate of *R.africae* from species of *Amblyomma* ticks collected from domesticated animals.

In addition, the first detection of *R. africae,* the most widespread spotted fever agent in sub-Saharan Africa, has recently been described in Corsica, France [53], through PCR, from ticks that were manually removed from cattle. Kernif et al. [54] also reported the detection of *R. africae* from *Amblyomma* specie in Algeria, using *glt*A and *omp*A gene amplification by PCR. In the same way, Keller et al.[49] equally reported a high incidence of *R. africae* from *Amblyomma* ticks, which have directly led to the human rickettsiosis among pregnant women, although in low incidence rate.

Similarly, the detection of *R. africae* by PCR on a skin biopsy of a returning 40-year-old Italian physician from Zimbabwe, who presented with fever, a tache noire on the left leg, and a neurological syndrome characterized by severe pain of the left leg was reported by Zammarchi et al. [55]. The global incidence rate of human rickettsisosis caused by *R. africae* has been reported to be above 5% among travellers in whom acute febrile infection developed after their returning from sub-Saharan Africa [52].

The presence of *R. africae* from *A. hebraeum* and species of *Haemaphysalis* and *Rhipicephalus* has long been established in South Africa [31,46], hence South Africa has been described as an endemic region for ATBF.

Rickettsia parkeri, the causative agent of spotted fever rickettsiosis in human, was first discovered to parasitize *A. maculatum* ticks in the United State in 2004 [56] with infection in humans having similar clinical symptoms with *R. rickettsii*. Infection of humans living in the Gulf Coast tick endemic region of USA is very high as clinical specimen of twelve patients living in the endemic region that were submitted for laboratory evaluation confirmed six samples positive for *R. parkeri,* the etiologic agent of spotted fever rickettsiosis [57].

R. parkeri has also been described as a causative agent of human rickettsiosis in other countries like Argentina [58] and Brazil [59] with *Amblyommatriste* ticks haven been implicated as vectors for this infection [60]. Similarly, Faccini-Martínez et al. [61] reported a case of *R. parkeri* infection from a Spanish traveller returning from Uruguay, who was confirmed bitten previously by *Amblyomma triste* tick, hence, it has been described to be the second most important cause of tick-borne rickettsiosis in the United States, Argentina and Brazil, after *R. rickettsia* [60,62].

Until now, *R. parkeri* has not been reported to infect humans in African continent; hence this is the first report of its existence in the continent, although from tick sample. However with the zoonotic potential of *R. parkeri* which has been described from other continents, it is expedient for the public to be aware of its existence and the appropriate authority to expedite action in preventing its outbreak.

Another spotted fever group pathogenic *Rickettsia* detected in this study was *R. mongolotimonae* which was first recovered from *Hyalomma asiaticum* tick from France in 1991[63] and in 1996, its pathogenicity in humans was first described in a female patient with an atypical tick-transmitted disease, followed by another human case from a 49 year old HIV patient in 1998 [64]. A similar human case was described in Greece from another immune-compromised patient, who reported no contact with animal except working in the field where he was collecting olives. During his hospitalization, an engorged female *Hyalomma anatolicum* tick was recovered from his scrotum by the physician [65].

In addition, human cases of *R. mongolotimonae* have recently been reported in Sri Lanka from a 30-year-old female who returned from travelling to a jungle and was examined as an outpatient for fever [66] and also in Cameroon, from a 54-year-old woman who presented a clinical symptoms of fever, headache, chills, myalgia and arthralgia [67]. Generally, *R. mongolitimonae* infection has been described to cause a mild, less fatal disease, nevertheless some complications have been reported, such as disseminated intravascular coagulation, shock, neurological disorders, atrial fibrillation, retinal vasculitis and acute renal failure [68].

Pretorius and Birtles [69] also reported the first human case of *R. mongolotimonae,* in South Africa, from a 34 year old patient who developed a severe headache and high fever, after he discovered a lesion on his right foot. This rickettsiosis was linked to bite from *H. truncatum with high* endemicity in the region where the patient had been working and is known to parasitize humans [70].

Other *Rickettsia* species associated with human diseases that have been described in South Africa include *R. conorii* and *R. sibirica* which are etiologic agents of African tick bite fever [69]. Despite the emergence and re-emergence of various species of *Rickettsia* with potential zoonosis, rickettsiosis is still considered as a neglected disease [21].

Furthermore, *R. conorii, R. honei, R. rickettsii, R. raoultii* were other related SFG pathogenic *Rickettsia* detected in this study. These SFG rickettsial pathogens have been described in several geographical regions of the world such as; USA [10, 71] ,Europe [63,72] Australia [73] as well as Asia [74,75] to infest both humans and animals, hence their prevalence is on the increase, globally.

In addition, *R. australis,* and *R.rhipicephali* are other closely related *Rickettsia* spp. found in the United States, Brazil, Italy, Uruguay, China and Australia [76,77,78,79,80].These obligate intracellular Gram negative bacteria have been associated with human infections, which still remain underdiagnosed and underreported especially in poorly scientific resource countries [81], hence the pathogenicity, prevalence, and diversity of different arthropod-borne agents is still not well understood [82] pointing out the need for a systemic microbial surveillance in tick vectors.

A study conducted in Kenya by Kimita et al. [83] reported that a partial fragment of *omp*B gene was found to be most identical to *Rickettsia rhipicephalus* with 99.0% homology as against a required homology of 99.2% to qualify it as *R. rhipicephalus,* thus suggesting the probability of *R. rhipicephalus circulating in African continent.*

Based on phylogenetic data, two rickettsial isolates from this study were found to be closely clustered with R. conorii (AF149110 and AF123726) from Australia and India respectively (Fig 4), this is supported by the findings of Essbauer et al. [21] who reported, for the first time, 16% prevalence of pathogenic R. conorri from rodent with zoonotic potential in South Africa, It had also been reported in Algeria [84].

Rhipicephalus spp. has been described as the main arthropod vectors for this bacterium in different geographical regions, which could probably be distributed by migratory birds and wild animals. Similarly, human infections with R. conorri have been described in some European countries such as France, Spain, Portugal, and Greece [3,63,85,86,87]. Fournier et al. [88](1998), also reported the detection *of R. conorri from French athletes, who returned from South Africa and presented with headache, fever, regional lymphadenopathies, and multiple inoculation eschars.*

The detection of R. conorri in different Rhipicephalus spp. in the study areas implies a wide-ranging of its host and ecological variation which does have epidemiological consequences. Also, the populace living in proximity with domesticated animals, in the study area is at high risk of rickettsial infections, if bitten by infected ticks, as the presence of genetic materials of the organisms detected in this study indicates probable zoonotic potential, hence systematic study is further required to establish the detection of these pathogens from human samples.

Several studies on ticks have been previously conducted in South Africa, which have shown that varieties of SFG are in circulation in the country [89,90]. Similarly, a recent study conducted by Essbauer et al. [21] showed that different species of pathogenic Rickettsia are in circulation in the country.

The most predominant tick species in this study was Amblyomma hebraeum and it had been formerly reported to be among the prevalent arthropod-vectors parasitizing different animals in South Africa, especially in the Eastern Cape [32,90, 91] followed by *Rhipicephalus* species [33, 92]*. The detection of R. africae genetic material in A. hebraeum and Riphicephalis species in this study is corroborated by the findings of Yawa et al. [32], thus confirming the role of A .hebraeum and Riphicephalis spp. in the epidemiology of spotted fever group Rickettsia.*

Findings from the study showed that the domesticated animals lived in close proximity with humans in the study area, apart from being released to graze freely in the vegetation, thus increasing the possibility of zoonosis from infected ticks to humans. Equally, with the increased prevalence of immuno-compromised individual in this region, rickettsioses could probably act as an opportunist infections taking advantage of their medical condition and aggravate their delicate health conditions. South Africa has been considered as destination for many tourists of which many have been diagnosed of rickettsiosis upon returning to their countries; hence the incidence of rickettsioses among travellers from sub-Saharan Africa is put at 21% [93]. Also, Dzelalija et al. [96] through GeoSentinel Surveillance Network reported SFG rickettsiosis among Croatian travellers to be 82.5%, thus confirming the potentiality of zoonosis of ticks, particularly when the prevalent tick species (Amblyomma hebraeum and Riphicephalus species) infest humans.

Conclusion

This study revealed the diversity of Rickettsia spp. and presence of R. parkeri, R. australis, and R.mongolotimonae; all belonging to the SFG Rickettsia for the first time in the Eastern Cape Province, South Africa, thus suggesting potential role for A. hebraeum and Riphicephalis species as vectors in the area, although, Borrelia species was not detected from the DNAs of the ticks. Occasioned by the high prevalence of rickettsial pathogens reported in this study, a systematic sero-prevalence study is proposed among the populations living in close proximity with domesticated animals as well as those with frequent exposure to vegetation, so as to establish the risk associated with tick bites in order to leverage appropriate legislative actions to be taken, hence reducing the tick-borne disease burden. Owing to increase in demand for livestock in international trades, systematic surveillance is highly recommended, for the update of epidemiological data of these emerging and re-emerging arthropod-borne pathogens.

Declarations

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Conflict of Interest

None declared by the authors.

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Figures

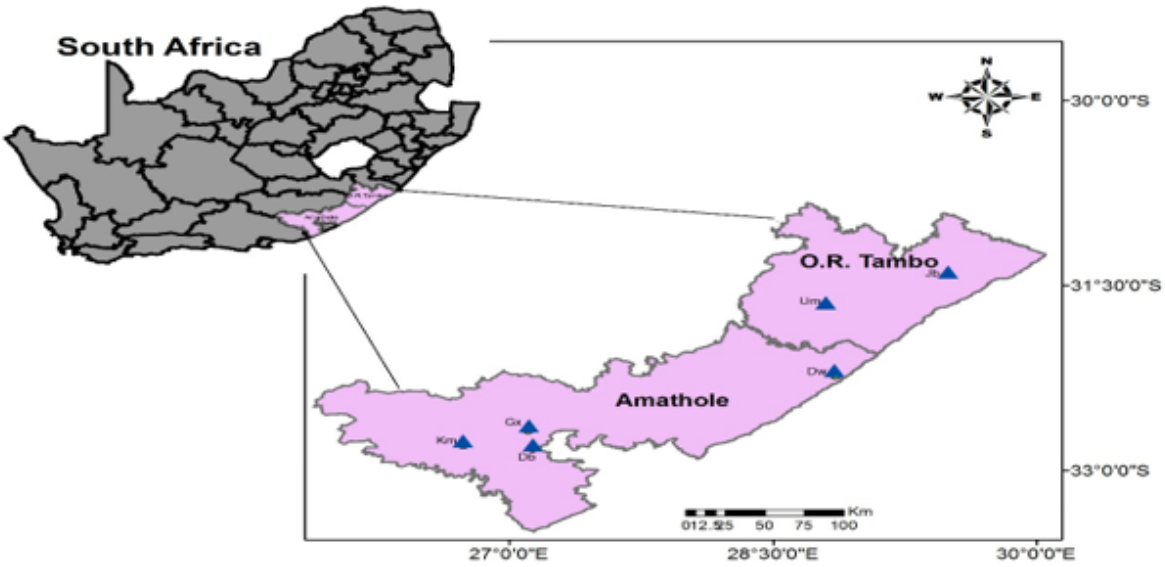

Figure 1

The map showing the geographical location of the sampling sites with their coordinates; Debe (Db) = 32°52'11.852"S, 27°1'14.171"E; Gxulu (Gx) = 32°40'26.702"S, 27°6'19.591"E; KwaMemela (Km) = 32°47'38.497"S, 26°44'10.889"E; Dwesa (Dw) = 32°13'50.916"S, 28°51'16.135"E; Umtata (Um) = 31°39'26.69"S, 28°48'0.194"E; Jambini (Jb) = 31°23'36.856"S, 29°29'46.921"E. Map created using ArcMap 10.5.1.

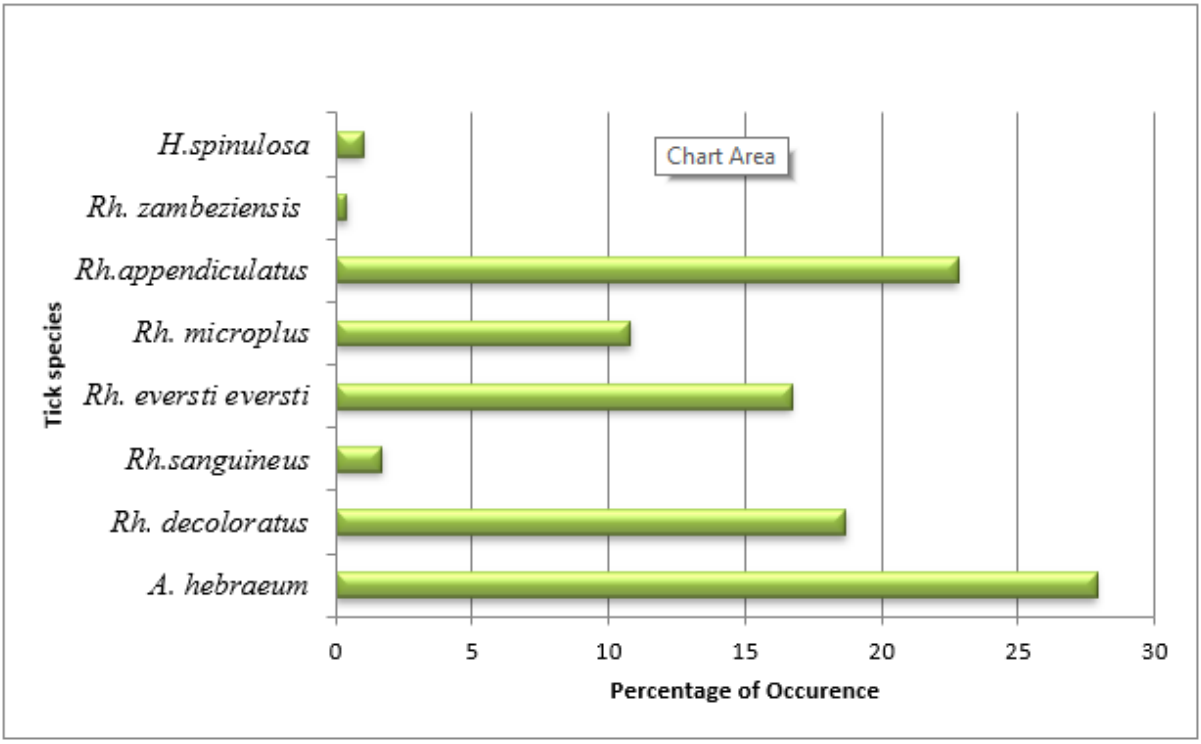

Figure 2

The prevalence of tick species collected in the study. The figure shows the overall prevalence of tick species collected in all the sampling sites.

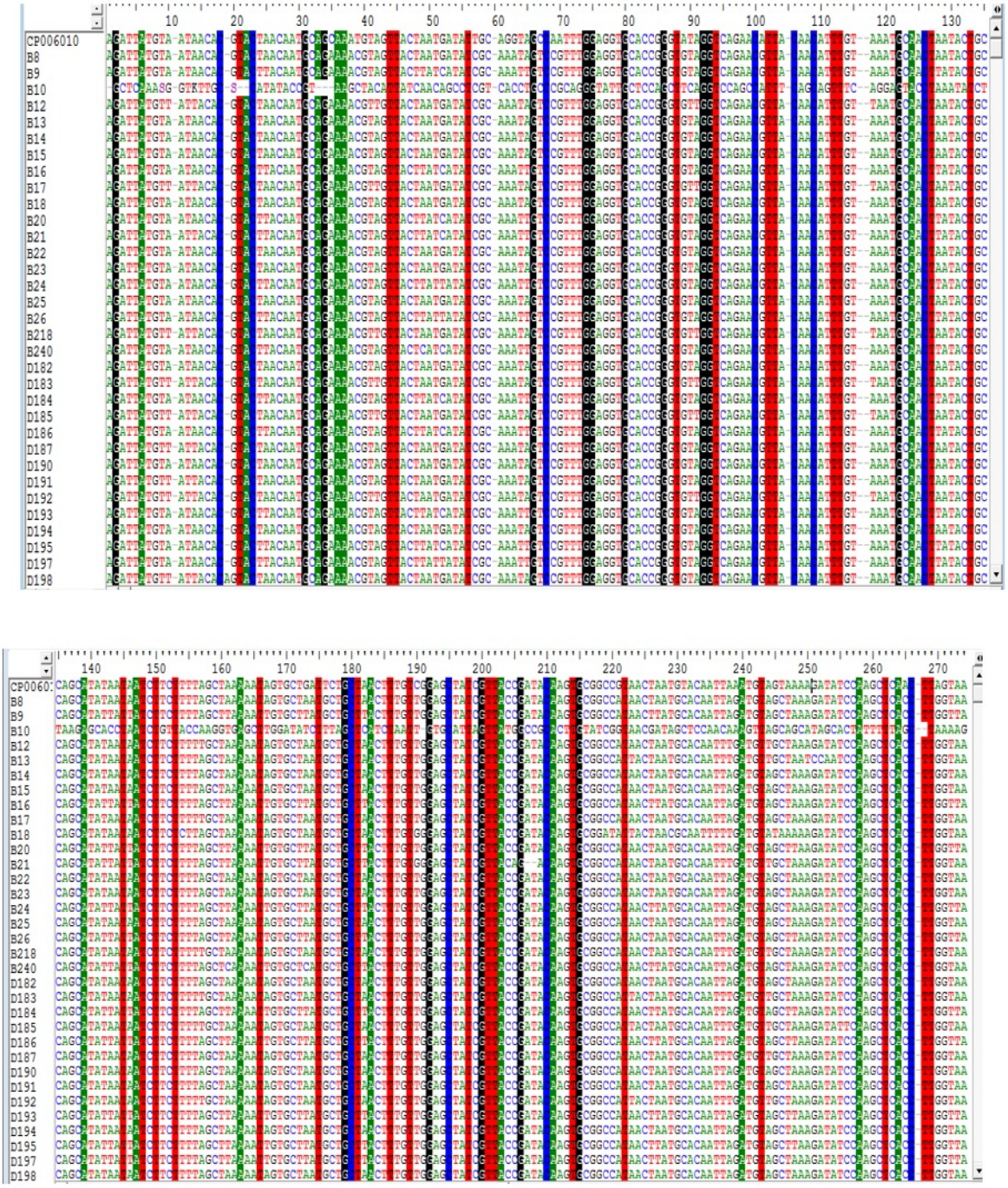

Figure 3

Nucleotide sequence alignment of the sequences in the polymorphic region of the ompB gene of Rickettsia spp. with Rickettsia rickettsii serving as the reference sequence along with generated sequences in this study. Nucleotides that are identical for all species are highlighted in colour while those that vary between species and can be used for differentiation are not highlighted.

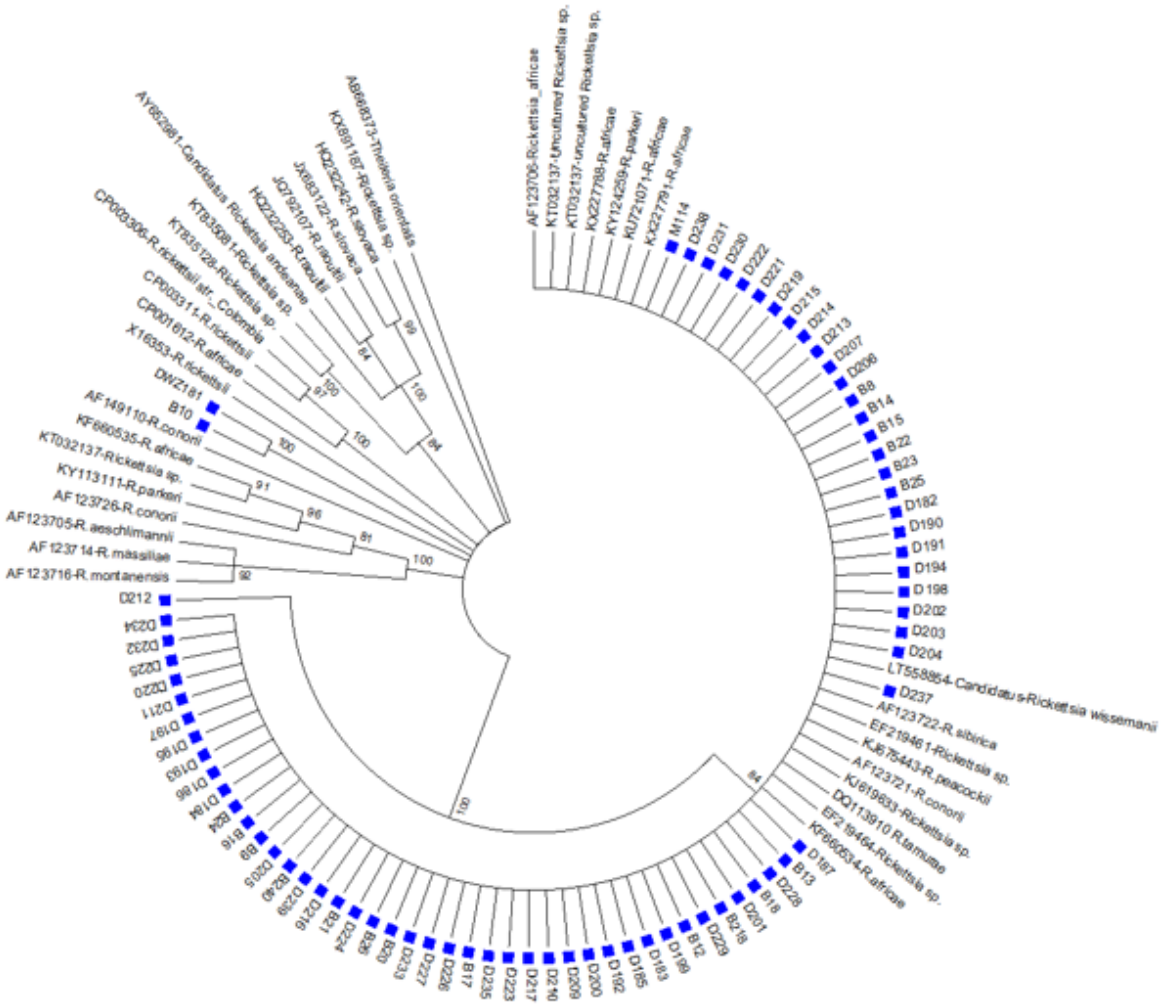

Figure 4

Evolutionary relationships of different Rickettsia spp. based on the nucleotide sequence of ompB gene. The evolutionary history was inferred using the Neighbor-Joining method. The confidence probability (multiplied by 100) that the interior branch length is greater than 0, as estimated using the bootstrap test 1000 replicates is shown next to the branches. The evolutionary distances were computed using the p-distance method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA7 29(Kumar et al., 2016).

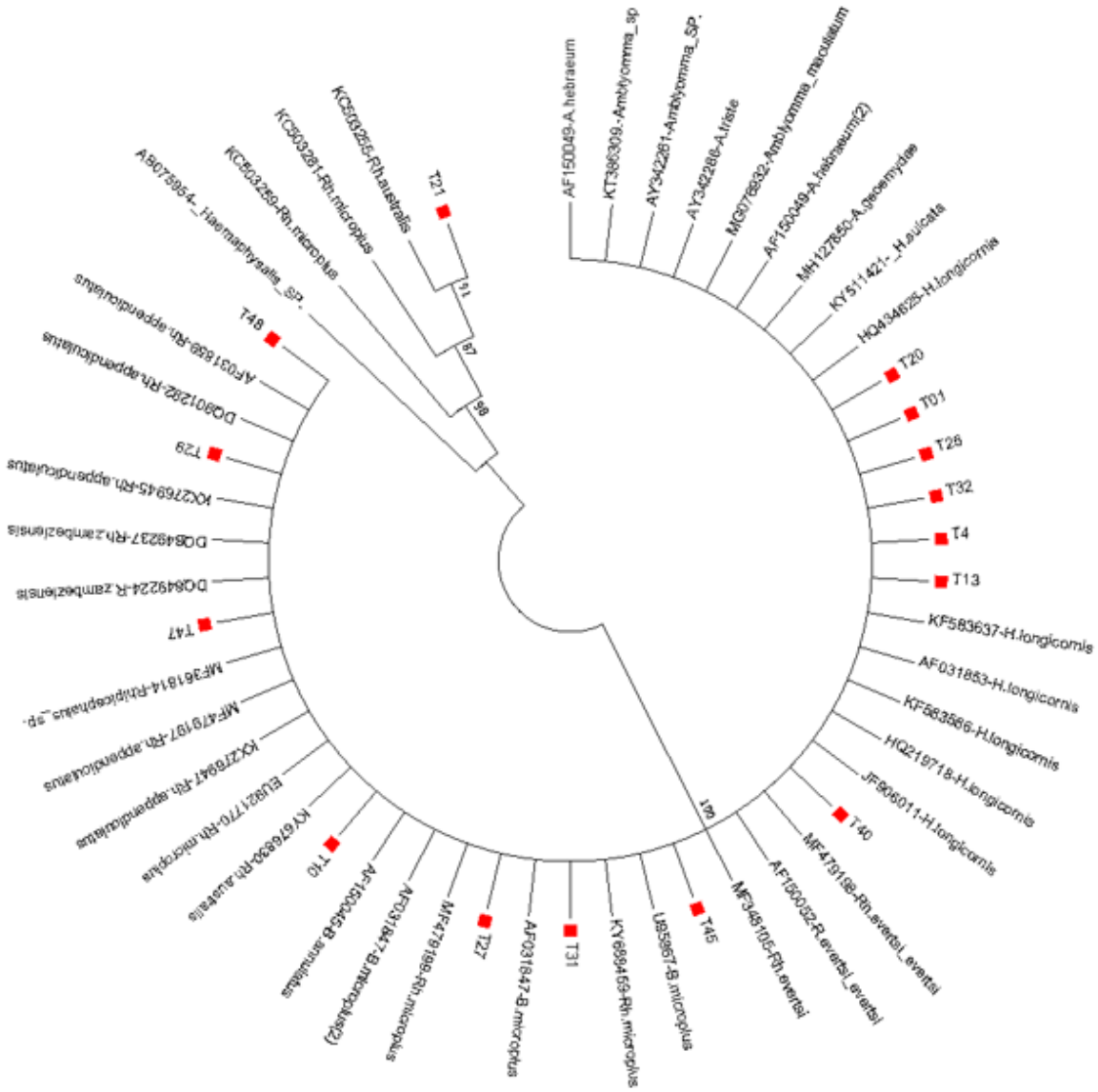

Figure 5

Evolutionary relationships of tick species based on Nucleotide sequences of mitochondrial 12S ribosomal RNA gene. The evolutionary history was inferred using the UPGMA method. The bootstrap consensus tree inferred from 1000 replicates was taken to represent the evolutionary history of the taxa analyzed. Branches corresponding to partitions reproduced in less than 70% bootstrap replicates are collapsed. The evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA7 29