

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* spp. and *Borrelia* spp. 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 *gltA*, *ompA* and *ompB* genes for *Rickettsia* and *flaB* gene for *Borrelia* spp. Thereafter, the positive ticks, *Borrelia* and *Rickettsia ompB* 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 *ompA* and *ompB* genes. Phylogenetic analysis of *ompB* 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 [8].

Rickettsia and *Borrelia* spp. are both transmitted by ticks and are among the numerous zoonotic pathogens responsible for febrile illness among humans [8,9]. Rickettsial diseases, caused by organisms of the genus *Rickettsia* are classified into three biogroups the first bio-group is known as spotted fever

group (SFG) which includes Rocky Mountain spotted fever (RMSF) caused by *R. rickettsia*, 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) rickettsiae 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, a translational group which includes *Rickettsia felis*, *Rickettsia australis*, and *Rickettsia akari* (Johnson et al., 2018). Rickettsial diseases have been reported to be very challenging to diagnose, owing to similar symptoms and epidemiology shared with several other febrile illnesses. Thus, suggesting that the overall reported cases of rickettsial diseases are probably inaccurate as they are often times underreported [12].

Borrelia, a genus of bacteria belonging to the spirochete phylum [13], 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* [14]. *Borrelia* species exist in enzootic cycles mostly involving ticks and several animals and bird hosts.

Most hard 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 Europe, *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 [15,16].

Owing to expansion in geographical boundaries by ticks into new ecological terrains worldwide, TBPs belonging to the *Rickettsia* and *Borrelia* genera 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 [17]. 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) adult ticks were manually removed from farm animals [cattle (n=718), goats (n=352) and sheep (n=130)] 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 (Fig 1). 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 [18,19,20]. 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. Each tick sample was processed using method previously described by [21].

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.

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***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 (*gltA*) gene was amplified, using genus-specific primers [F:5'-TTTGTAGCTCTTCTCATCCTATGGC-3'] and [R:5'CCC AAGTTCCTTTAATA CTTCTTTGC-3'] as previously described by [22]. 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. A positive control of *Rickettsia* species (KX891173) was added to the reaction. Subsequently, all the positive samples were further subjected to screening for outer membrane protein A (*ompA*) and outer membrane protein B (*ompB*) which are structural protein that are imperative factors for rickettsial virulence and immunogen during infection (Johnson, 2018), by PCR using oligonucleotides [F:5'-ATGGCGAATATTTCTCCAAA-3'] and [R:5'- GTTCCGTTAATGGCAGCATCT-3'] to generate 631 bp of *ompA* gene, while [F: 5'-GTAACCGGAAGTAATCGTTTCGTAA-3'] and [R:5'- GCTTTATAACCAGCTAAACCACC-3'] primers was used to generate 511 bp of *ompB* 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 *ompA* and *ompB* 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. (2017), with modification of the annealing temperature.

***Borrelia* species**

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

gene respectively, elongation at 72 °C for 60 sec (40 cycles of amplification) with a final elongation at 72 °C for 5 min. All amplified PCR products were visualised via transillumination on 1.5 % agarose gel stained with ethidium bromide. 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 [23].

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, *Rickettsia* spp. was 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 manually 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. Eight species of ticks belonging to three 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 positive for *Rickettsia citrate encoding synthase (gltA) gene*, out of which 74 (23%) were further confirmed positive for both *ompA* and *ompB* 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 *ompB* of other *Rickettsia* sequences in GenBank (Table 2).

TABLE 1 Diversity of Collected from the Animals in the Study Areas

Tick species	Number of tick species per animal			Total number of ticks (%)	Numbers of positive samples for <i>Rickettsia</i> spp. (%)
	Cattle	Goat	Sheep		
<i>A. hebraeum</i>	235	80	20	335 (27.9)	140 (42.0)
<i>Rh. decoloratus</i>	129	70	25	224 (18.7)	
<i>Rh. sanguineus</i>	0	15	5	20 (1.7)	
<i>Rh. eversti eversti</i>	140	40	20	200 (16.7)	78 (39.0)
<i>Rh. microplus</i>	70	40	20	130 (10.8)	
<i>Rh. appendiculatus</i>	139	95	40	274 (22.8)	102 (37.2)
<i>Rh. zambeziensis</i>	5	0	0	5 (0.4)	
<i>H. spinulosa</i>	0	12	0	12 (1.0)	
Total	718	352	130	1,200	

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.

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For *Rickettsia* species, 320 (27%) genetic materials (DNA) were positive for *Rickettsia* citrate encoding synthase (*gltA*) gene, out of which 74 (23%) were further confirmed positive for both *ompA* and *ompB* gene, while no positive sample was detected for *Borrelia*. A homology search for the generated sequences

from this study revealed a high percentage of identity between 96% - 100% with other homologous *ompB* of other *Rickettsia* sequences in GenBank (Table 2).

Table 2 Identities of rickettsial (*ompB*) sequences obtained from tick samples

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

The derived *Rickettsia* (*ompB*) sequences were further subjected to phylogenetic analyses with the following *Rickettsia ompB* 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 (Ethiopia), 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. slovacica* (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 [24], before generating the phylogenetic tree as shown in Figure 4.

Phylogenetic Analysis of Rickettsial Pathogens Using *ompB* Gene

Phylogenetic analysis showed that the obtained sequences clustered into three clades with other reference sequences from different geographical regions of the world. 28 sequences clustered in one clade with reference sequences from France, Austria, Kenya and French Guiana, with accession number AF123706-*R. africae*, KU721071- *R. africae*, KX227788-*R. africae*, KX227791-*R. africae*, KT032137- uncultured *Rickettsia* sp. and LT558854-*Candidatus Rickettsia* 44 sequences were found to cluster in another clade with each other, while two other sequences clustered in another clade closely with CP001612-*R. R.africae* and CP003311- *R. rickettsii*, reference sequences from France and USA with high genetic similarities (Fig 4). Sequences obtained in this study are labelled with circles

Figure 3: 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 analysed. The evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA7 [24]. Tick sequences obtained in this study are marked with square dots.

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 3). Sequence T01, T32 and T29 were shown to clustered with reference sequences AY342261-*Amblyomma*. sp. Sequence T13 was

shown to cluster closely with reference strain HQ434625-*Haemaphysalis longicornis*. Likewise, sequence T47 clustered with reference strain KX276947- *Rhipicephalus appendiculatus* and *Rhipicephalus* sp. Lastly, sequence T45, T40 and T48 were found to cluster with reference sequence AF031847- *Boophilus 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.

Figure 4: Evolutionary relationships of different *Rickettsia* spp. based on the nucleotide sequence of *ompB* gene. The evolutionary history was inferred using the UPGMA 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 Maximum Composite Likelihood method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA7 [24].

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. 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 [25,26].

Ticks collected in this study 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 [27,28,29], thus our result is a confirmation that these genera are the predominant tick species in the study areas.

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 [30,31,32]. 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 [33]. *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-Saharan Africa with a morbidity rate of > 50% [34,35]. Thus, *R. africae* infection has been described, after malaria, to be liable for most febrile illnesses diagnosed in tourists returning from southern Africa [17,36].

The abundance of *Rhipicephalus* species in many West African countries has been described to 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.

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 [27], followed by Rhipicephalus species [29]. The detection of R. africae genetic material in A. hebraeum and Rhipicephalis species in this study is corroborated by the findings of [27], thus confirming the role of A. hebraeum and Rhipicephalis spp. in the epidemiology of spotted fever group Rickettsia.

The 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, increase the possibility of zoonosis from infected ticks to humans. Equally, with the increase 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 approximately 21% [25].

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 *Rickettsiae* 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 [35,36,37], from its well-known vector; *A. hebraeum*. Subsequently, about 24 sequences, from the 74 obtained sequences for *ompB* 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 [38,39]. The risk has been attributed to the abundance of *Amblyomma* sp. in most African countries [40], as species of *Amblyomma* have been implicated as vectors of *R. africae* infection, hence the increase in percentage of infected ticks could increase the probability of humans being bitten thereby leading to increase rate of human rickettsiosis.

ATBF has also been recovered from American travellers returning from Southern Africa [41], with a history of tick bite during their visit, as well as from positive human serum samples in western Africa [42]. The detection of *R. africae* in the present study is supported by the findings of [42], who reported high detection rate of *R. africae* from species of *Amblyomma* ticks collected from domesticated animals.

Likewise, [43], reported the detection of *R. africae* from *A. variegatum* in six Caribbean Islands based on DNA sequences of *ompA* gene recovered from ticks.

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 [44], through PCR, from ticks that were manually removed from cattle [45], also reported the detection of *R. africae* from *Amblyomma* species in Algeria, using *gltA* and *ompA* gene amplification by PCR. In the same way, [39] equally reported a high incidence of *R. africae* from *Amblyomma* ticks, which has 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 and a neurological syndrome characterized by severe pain of the left leg was reported by [46]. The global incidence rate of human rickettsiosis caused by *R. africae* has been reported to be above 5% among travellers who developed acute febrile infection after their returning from sub-Saharan Africa [42]. The presence of *R. africae* from *A. hebraeum* and species of *Haemaphysalis* and *Rhipicephalus* has long been established in South Africa [37], 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 [47], with infection in humans having similar clinical symptoms with *R. rickettsii*. Infection of humans living in the Gulf Coast (a tick endemic region) 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 [48].

R. parkeri has also been described as a causative agent of human rickettsiosis in other countries like Argentina [49], and Brazil [50] with *Amblyommatriste* ticks haven been implicated as vectors for this infection [49]. *R. parkeri* has equally been described as an emerging zoonotic pathogen in Mexico [51]. Similarly, [52] reported a case of *R. parkeri* infection from a Spanish traveller returning from Uruguay, who was confirmed bitten previously by *Amblyomma triste* tick. Infection by this pathogen has also been reported in Canada, 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* [49,53].

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 that the public 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 [54] 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 [55]. 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 [56].

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 [57] and also in Cameroon, from a 54-year-old woman who presented a clinical symptoms of fever, headache, chills, myalgia and arthralgia [58]. Generally, *R. mongolotimonae* 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 [59].

The first human case of *R. mongolotimonae*, has been reported 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 a bite from *H. truncatum* with high endemicity in the region where the patient had been working and is known to parasitize humans [60]. 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 [61,62]. Despite the emergence and re-emergence of various species of *Rickettsia* with potential zoonosis, rickettsiosis is still considered as a neglected disease [17].

A study conducted in Kenya by [63], reported that a partial fragment of *ompB* gene was found to be the 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. *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. conorii* have been described in some European countries such as France, Spain, Portugal, and Greece [54,64]. [65] also reported the detection of *R. conorii* from French athletes, who returned from South Africa and presented with headache, fever, regional lymphadenopathies, and multiple inoculation eschars.

The detection of R. conorii in different Rhipicephalus spp. in the study areas implies a wide range 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 [28,66]. Similarly, a recent study conducted by [17], showed that different species of pathogenic Rickettsia are in circulation in the country.

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 *Rhipicephalis* 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.

List Of Abbreviations

TBDs Tick Borne Diseases

OMP Outer Membrane Protein

Declarations

Acknowledgment

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Conflict of Interest

None declared by the authors.

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Figures

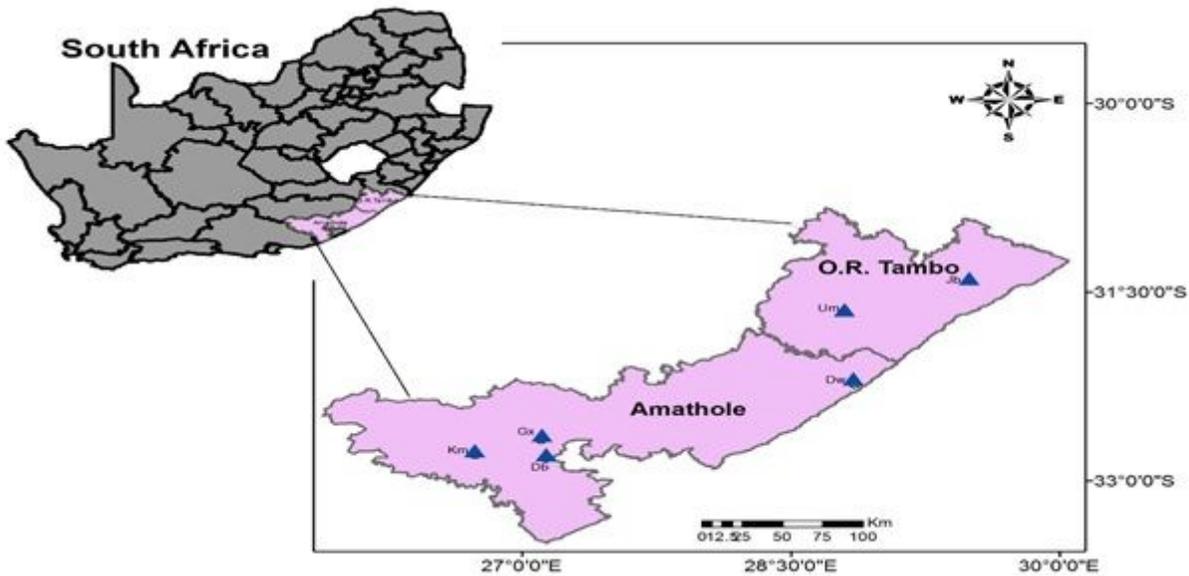


Figure 1

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.

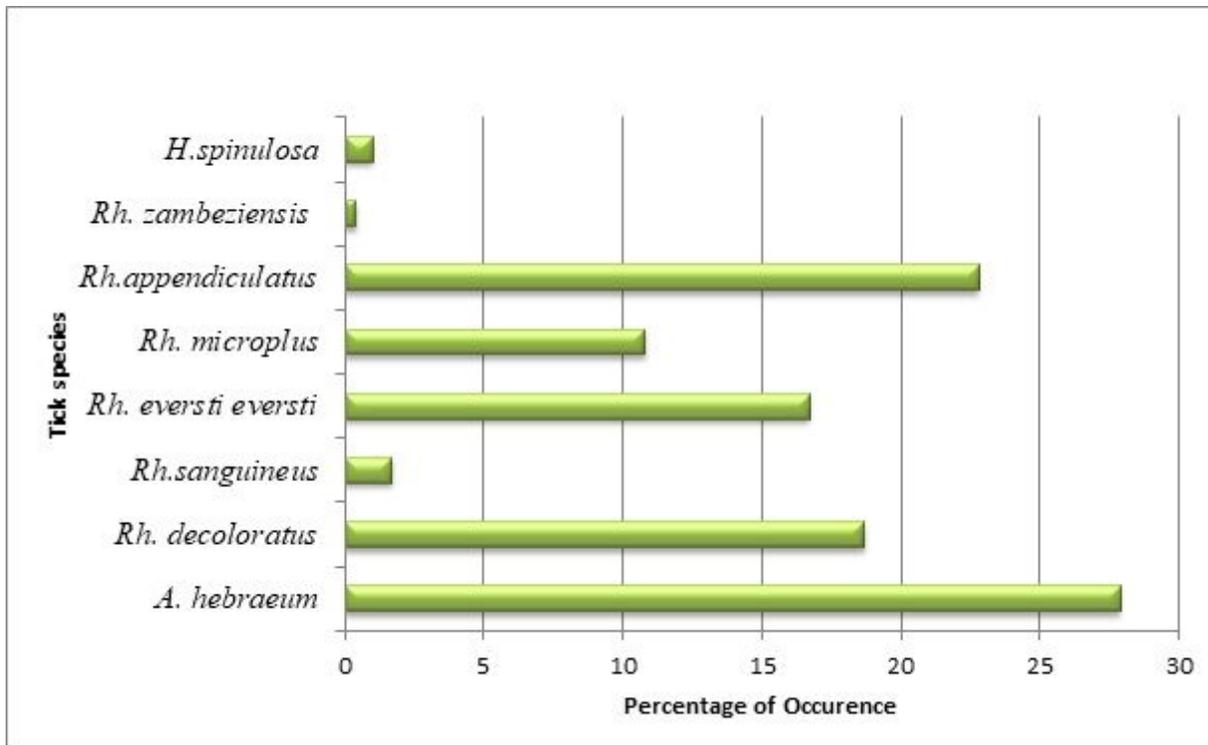


Figure 2

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

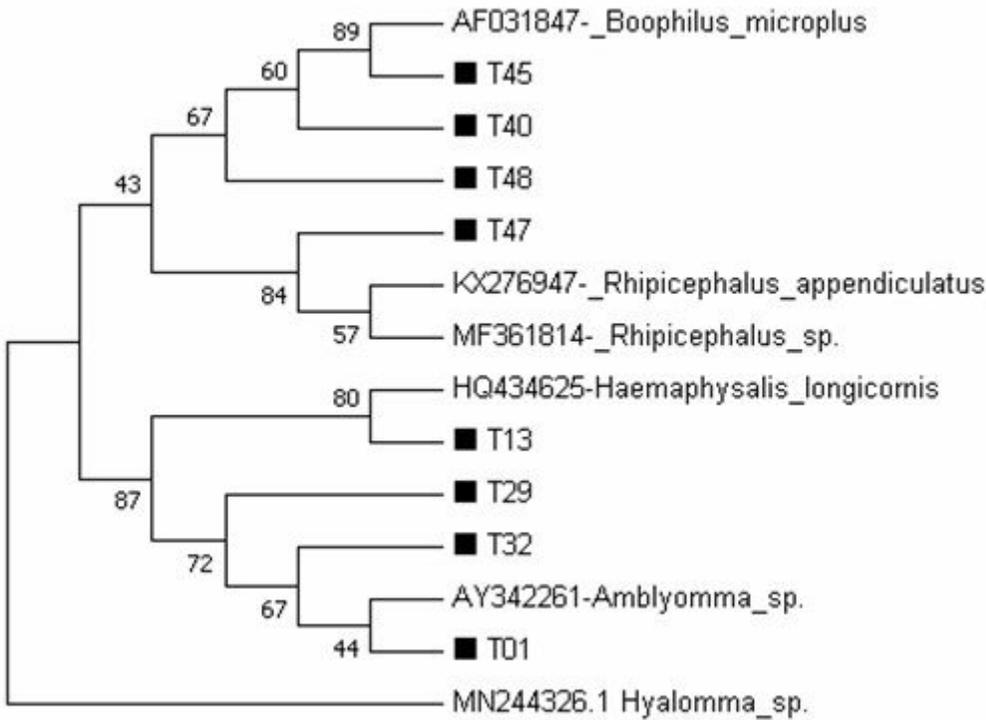


Figure 3

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 analysed. The evolutionary distances were computed using the Maximum Composite Likelihood method. Evolutionary analyses were conducted in MEGA7 [24]. Tick sequences obtained in this study are marked with square dots.

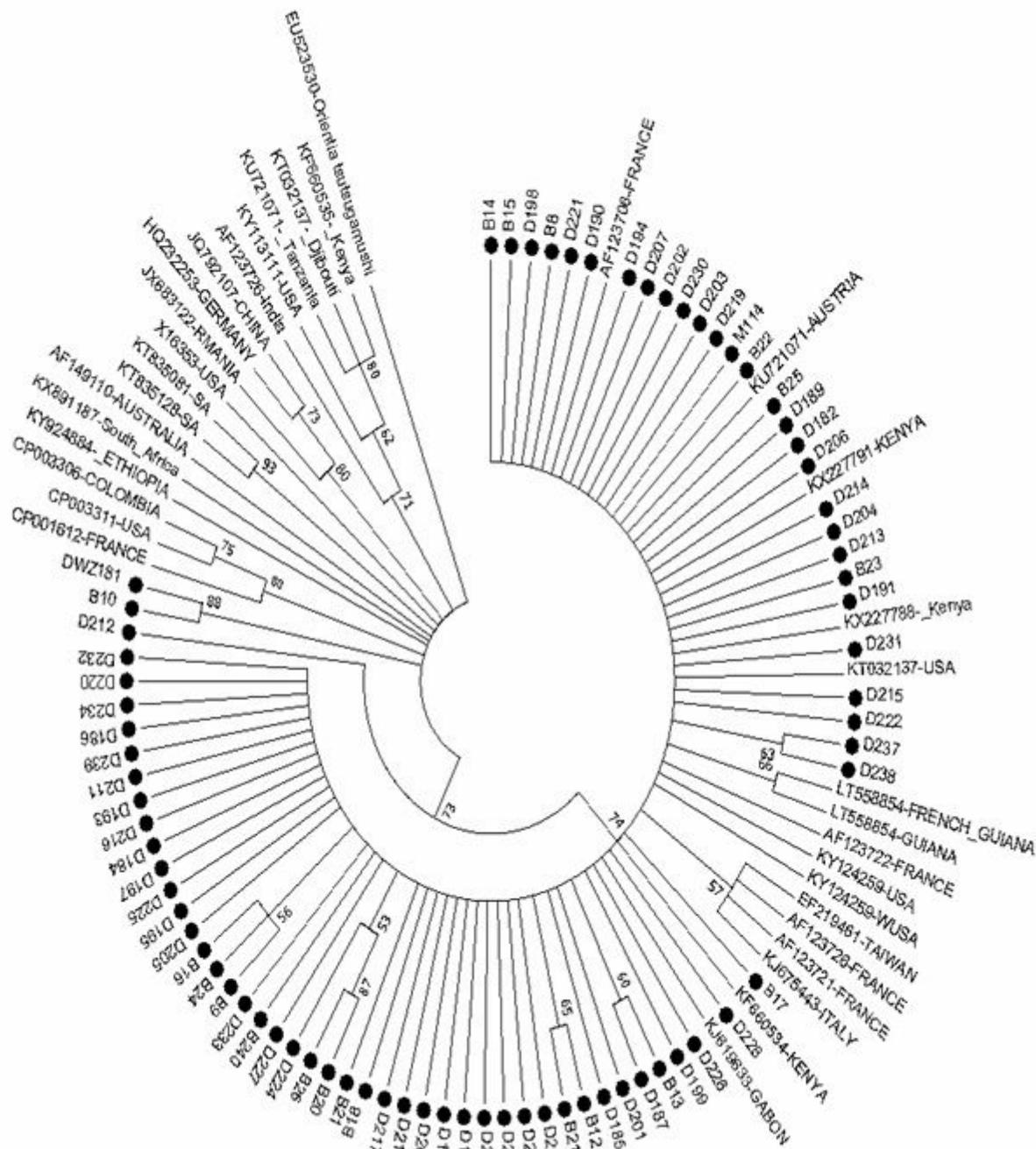


Figure 4

Evolutionary relationships of different *Rickettsia* spp. based on the nucleotide sequence of *ompB* gene. The evolutionary history was inferred using the UPGMA 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 Maximum Composite Likelihood method and are in the units of the number of base differences per site. Evolutionary analyses were conducted in MEGA7 [24].