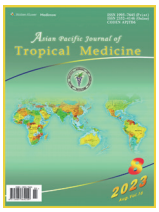

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Molecular evidence and phylogenetic delineation of spotted fever group *Rickettsia* species in *Amblyomma* ticks from cattle in Gauteng and Limpopo Provinces, South Africa

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ABSTRACT

Objective: To determine the prevalence of tick-borne pathogens with a particular focus on *Rickettsia* spp. in ticks collected from cattle in Gauteng and Limpopo Provinces, South Africa.

Methods: A total of 200 ticks were collected from cattle within the Madala livestock, Pretoria, Gauteng Province and in Mankweng Township, Polokwane, Limpopo Province in 2019. The ticks were morphologically identified and processed individually for a total genomic DNA extraction. Specific primers targetting *ompA*, *ompB*, and the *17KDa* genes were used for a molecular screening and delineation of *Rickettsia* from the extracted genetic materials using polymerase chain reaction (PCR) technique. PCR amplicons of positive samples were sequenced bidirectionally using the Sanger sequencing method. Sequences generated were processed and analysed using appropriate bioinformatics software.

Results: The ticks were morphologically identified as *Amblyomma* spp. PCR profiling of the genomic DNA samples revealed the presence of the *Rickettsia* pathogen in 42 (21%) of the ticks collected from both Provinces. Out of the genes profiled, 14 (7%) were positive for *17KDa*, 42 (21%) for *ompA* and 32 (16%) were positive for *ompB* genes respectively. The nucleotide blast of the sequenced genomes showed high similarity, as high as 100% with other reference *Rickettsia (R.) africae* in the GenBank. The phylogenetic analysis of the sequences further validated them as *R. africae* with their characteristic clustering pattern with related reference sequences.

Conclusions: There is an abundance of *R. africae* in *Amblyomma* ticks collected from cattle in the study areas. This has serious public health implications as individuals who accidentally get infested with the ticks could acquire *R. africae*. Hence, adequate precautions in terms of sensitization of farmers about the risk and mass mobilization drive to control the vectors in the areas are highly recommended to safeguard public health.

KEYWORDS: *Amblyomma ticks*; Tick-borne pathogen; *Rickettsia*; Cattle; South Africa

1. Introduction

Ticks are blood-sucking ectoparasites belonging to the phylum

Significance

This study aimed at profiling for spotted fever *Rickettsia* pathogens in ticks collected from cattle. The findings showed that *Rickettsia africae* is prevalent in the ticks and since the host cattle are kept near homes, human exposure is very high. This has a huge public health implication and efforts should be made towards control of ticks in order to reduce human exposure and infestation.

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Arthropoda and order Acari. They are recognized globally as one of the most significant disease vectors in the livestock industry[1]. Although the global public health impact of these arthropods on lives and productivity loss is mostly unmeasured, it has been asserted that ticks and their associated diseases cause harm to about 80% of the cattle population worldwide and are responsible for an estimated losses of about US$22-$30 billion annually[2]. Ticks are effective carriers of a wide range of microorganisms, many of which are the cause of newly emerging zoonotic illnesses[3].

These tick-borne diseases are widespread in rural areas of several sub-Saharan African nations, where there is continuous interaction between people and domestic animals that serve as hosts for the ticks[4]. Ticks are the second most well-known carriers of human and animal diseases after mosquitoe[5], with about 800 identified species worldwide[3].

One of the prominent microbes associated with ticks is the *Rickettsia* spp., belonging to the order Rickettsiales. The taxonomy of the Rickettsiales is intricate and is constantly being revised. The Rickettsiaceae family is sustained in nature through a cycle involving arthropod vectors and reservoirs in mammals[6]. Except for *Rickettsia (R.) prowazekii*, humans do not contribute to rickettsial circulation in the environment[7]. *Rickettsia* species are obligate Gram-negative bacterial pathogens that are only capable of growing inside eukaryotic cells. Since Rickettsiaceae is a relatively diverse group of organisms with many variations, they cannot be categorized as a single homogeneous group[6].

The term "rickettsioses" is frequently used to refer to the illnesses caused by *Rickettsia* spp[6], and the organisms within this genus can be divided into four subgroups namely: typhus group (TG), the transitional group, the ancestral group, and the spotted fever group (SFG)[4]. The typhus group consists of two species which are *R. prowazekii* vectored by louse found on human body, and *R. typhi* which is transmitted by the rat's flea[6]. The transitional group contains *R. australis*, *R. akari*, and *R. felis* which are transmitted by ticks, mites, and fleas, while the ancestral group is made up of *R. bellii* and *R. canadensis*, both of which are also tick-borne. The spotted fever group includes numerous species that are associated with human infections globally, including *R. rickettsii*, *R. conorii*, *R. parkeri*, *R. africae*, *R. japonica*, *R. philipii*, *R. heilongjiangensis*, *R. honei* and *R. amblyommatis*[4,7].

According to Katwara and Mukaratirwa[8], annually, more than 100 000 cases of zoonotic vector-borne illnesses are reported globally. The SFG rickettsiosis, which is caused by *R. africae*, *R. rickettsii* and others, is one of the most common emerging and re-emerging tick-borne illnesses. Large mammals play a crucial role as the tick's definitive hosts, while humans are usually unintentional and ineffective hosts[8].

Hard ticks are typically implicated in the transmission of tick-borne rickettsiosis. In the tropical areas of the world, the *Amblyomma*, *Hyalomma*, *Rhipicephalus*, *Ixodes*, *Dermacentor*, and *Haemaphysalis* are the ticks' genera responsible for the transmission of rickettsial illnesses[4,9].

Four of the already mentioned spotted fever group (SFG) *Rickettsia* are known to cause human illness in southern Africa: *R. africae* which is the etiological agent of African tick bite fever (ATBF), *R. conorii* responsible for the Mediterranean spotted fever, *R. aeschlimannii* causing innominate rickettsioses, and *R. sibirica* subsp. *mongolotimonae* which causes lymphangitis-associated rickettsioses[10]. According to the Department of Agriculture, Forestry, and Fisheries, South Africa is an agricultural exporting country that relies mostly on cattle production for subsistence. However, the nation also has game reserves, which provide favorable habitats for ticks and are frequently visited by both tourists and residents who contract diseases from tick bites[11].

ATBF is reportedly prevalent among overseas travelers, although it has received less attention from resource-constrained local livestock farmers in South Africa and other southern African nations[12]. There are few reports of this fever in local African populations; however, there is a high number of cattle herders and patients with acute febrile illness who have tested positive for *Rickettsia* infection in the past based on their serology, suggesting a high risk of infection in rural areas where the main vector, *Amblyomma (A.) hebraeum*, is frequently found[13,14].

Since infected females of some tick species give birth to at least one positive egg 100% of the time, SFG *Rickettsia* are comparatively uncommon in their excellent transovarial transmission. As a result, ticks themselves might be regarded as reservoirs for SFG *Rickettsia*, since they are less dependent on vertebrate hosts as disease reservoirs[10]. It has been demonstrated that *Rickettsia* can be transmitted transovarially and transstadially within the tick vector[15]. According to the studies, *R. africae* was kept alive by naturally infected *A. hebraeum* ticks for two generations by transovarial and transstadial transmission[15]. In a related investigation, three generations of *A. variegatum* ticks showed 100% transovarial transmission of *R. africae*. There is a paucity of data on the effectiveness of *R. africae* transovarial transmission in *A. hebraeum*, as well as infection rates at various tick developmental stages in the targeted areas in this study, which is near Kruger National Park where many international visitors contract ATBF after visiting the park[15].

Most reported cases have non-specific signs and symptoms that mirror influenza or malaria, leading to misdiagnosis, delayed identification, and treatment[12]. In many parts of the world, insufficient surveillance for ticks and tick-borne illnesses can lead

to underdiagnosis and gaps in the data on tick-borne infections. An essential first step in the successful research and management of diseases caused by ticks is to monitor ticks and the pathogens they harbor[10]. The objective of this study was to determine the prevalence of tick-borne pathogens with a particular focus on *Rickettsia* spp. in ticks collected from cattle in Gauteng and Limpopo Provinces, South Africa.

2. Materials and methods

2.1. Study area and tick collection

Ticks were collected from cattle within the Madala livestock, Pretoria in the Gauteng Province, and Mankweng Township, Polokwane, Limpopo Province between April and October 2019. With the permission of the livestock owners and the help of animal health technicians, a total of 200 ticks' samples were collected from cattle into 50 mL Nalgene tubes containing 70% ethanol. They were transported to the biology laboratory at Sefako Makgatho Health Sciences University and were stored at 4 ℃ until further processing.

Why Limpopo and Pretoria were selected for the study? The study sites were selected because of their unique locations. Mankweng (Latitude, 23° 53' 7.88"S ; Longitude, 29° 43' 36.04"E) was selected because of her closeness to Kruger National Park where many international visitors go for holiday and several wild animals that are host to ticks abound with close contacts with domestic animals. While Madala livestock (Latitude: -25° 37' 0.91" S Longitude: 27° 59' 40.96" E.) in GaRankuwa, Pretoria is located in close proximity to human dwelling and cattle are brought from different parts of Gauteng, Mpumalanga and the suburbs for sale. These sites, therefore, provide ideal locations for ticks-borne pathogens study.

2.2. Identification of ticks and total genomic DNA extraction

All collected ticks (adults and nymphs) were morphologically identified using appropriate taxonomic keys as described by Voltzit and Keirans, 2003[16]. Sequel to this, they were washed with sterile distilled water, chopped individually, and total genomic DNA was extracted from each processed sample using Promega ReliaPrep™ gDNA Tissue Miniprep System (Promega, Madison, USA) following the protocol provided by the manufacturer. Sex, feeding status and developmental stages were not taken into consideration in the DNA extraction process and the downstream analyses of the samples.

2.3. Molecular screening and identification of Rickettsia spp. from tick samples

To detect the presence of SFG rickettsial pathogens, the extracted genomic DNA samples were screened for *Rickettsia* spp. through polymerase chain reaction (PCR), using primer pairs targeting the *ompA*, *ompB*, and *17kDa* genes of the organism as shown in Table 1. The PCR reaction mixture had a final volume of 25 μL, containing 2 μL of each primer (forward and reverse), 12.5 μL of enzyme Master mix containing Taq polymerase, 5.5 μL of nuclease-free water, and 5 μL of DNA template. A negative control that contains all other reagents excluding the DNA template was added. The PCR process was performed in a Bio-Rad Thermal cycler with an initial heating block at 95 ℃ for 5 minutes followed by 30 seconds of denaturation at 94 ℃, annealing at different temperatures for each primer pair (Table 1) for 30 seconds followed by 35 cycles of elongation at 72 ℃ for 1 minute each, and final extension at 72 ℃ for 5 minutes. Following amplification, PCR products were electrophoresed on a 1.5% agarose gel in 0.5× TBE buffer stained with ethidium bromide. A Chemidoc imaging system was used to visualize and photograph the electrophoresed gels.

2.4. Sequencing of amplified genomes, processing, and phylogenetic analysis

The amplified PCR products were sequenced bidirectionally using both forward and reverse primers earlier used for the PCR amplification by employing Sanger sequencing method in a commercial sequencing facility. Sequenced DNA was edited and assembled to generate consensus sequences from both the forward and reverse strands by using Geneious v2023.0.4[18]. The identities of the generated sequences were analyzed using the Basic Local Alignment Search Tool (BLAST) as implemented in Geneious. Sequences obtained in this study and equivalent reference sequences representing the different species of *Rickettsia* from the GenBank

Table 1. Primers used for the screening and characterization of *Rickettsia* spp. from ticks.

Primer name	Gene	Primer sequence (5′⟶3′)	Specificity	Screen	TM	Reference
Rr.190 70P	*rompA*	ATGGCGAATATTTCTCCAAAA	Genus	Primary	52.5	[17]
Rr.190 602N	*rompA*	AGTGCAGCATTCGCTCCCCCT	Genus	Primary	64.9	[17]
BG1-21	*rompB*	GGCAATTAATATCGCTGACGG	Genus	Alternate	55.6	[17]
BG2-20	*rompB*	GCATCTGCACTAGCACTTTC	Genus	Alternate	55.2	[17]
Primer 1	*17kDa*	GCTCTTGCAACTTCTATGTT	Genus	Alternate	52.3	[17]
Primer 2	*17kDa*	CATTGTTCGTCAGGTTGGCG	Genus	Alternate	57.9	[17]

were prepared as data set for each of the sequenced genome. A ClustalW multiple sequence alignment was done using Bioedit software[19] and aligned data set was subjected to phylogenetic analysis using a distance-based Neighbour-Joining algorithm as implemented in MEGA 6 using 1 000 replicate bootstrapping and the evolutionary distances were computed using the Jukes-Cantor method.

3. Results

A total of 200 ticks of various stages of feeding-engorged and non-engorged, both male and female were collected from cattle's predilection sites (the udder, hind legs, tails, ear, scrotum, back and belly) and their morphological identifications showed them to be *Amblyomma* spp. All samples screened through PCR for *Rickettsia*

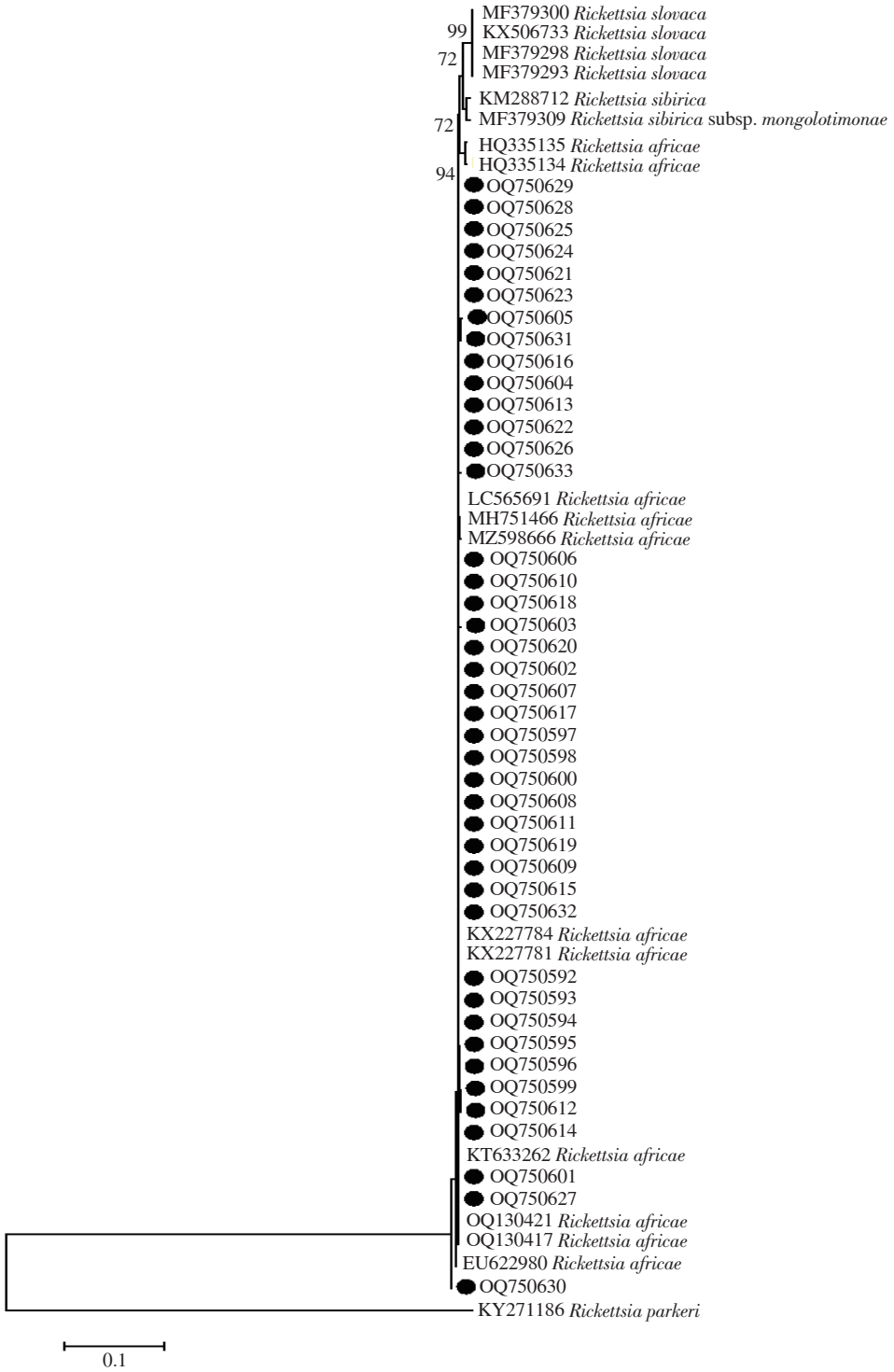

Figure 1. Phylogenetic analysis based on the 42 partial *ompA* gene sequences (in black dots) and 18 reference sequences representing different *Rickettsia* spp. from the NCBI GenBank. The GenBank accession numbers and species are indicated. Neighbour-Joining method was used and Bootstrap values ≥70% are shown.

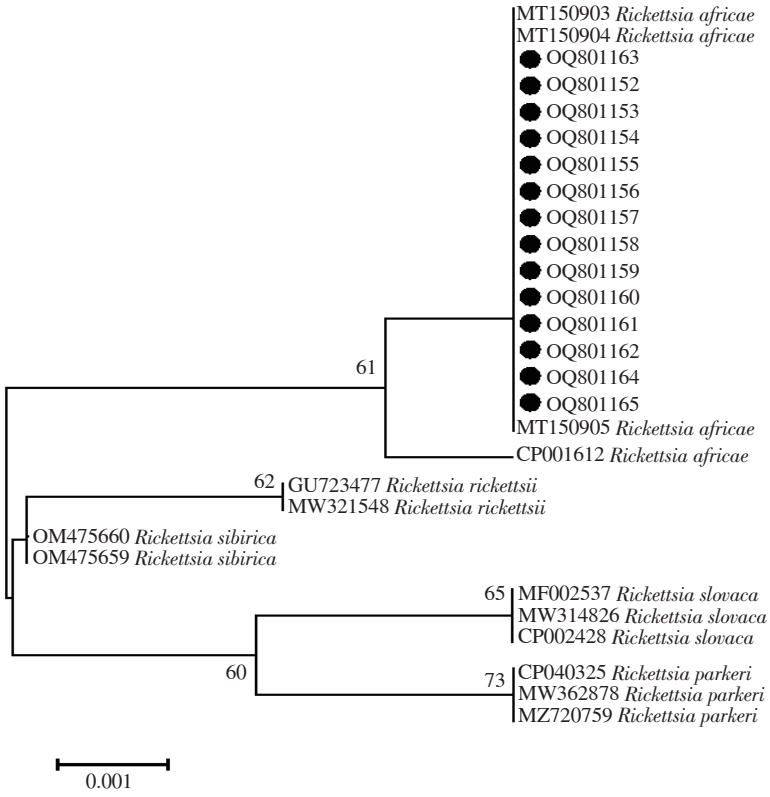

Figure 2. Phylogenetic tree of *17kDa* gene sequences (in black dots) in red generated from the study with the related reference sequences obtained from NCBI GenBank. The GenBank accession numbers and species are indicated. Neighbour-Joining method was used and Bootstrap values ≥60% are shown.

spp., targeting three different genes showed a prevalence of 21% with 9% prevalence in samples collected from Pretoria and 12% in Limpopo samples. Out of the genes profiled, 14/200 were positive for *17kDa*, 42/200 for *ompA*, and 32/200 were positive for *ompB* genes respectively. PCR profiling of the DNA samples revealed the presence of the *Rickettsia* pathogen while phylogenetic analyses of the generated sequences validated them as *R. africae* with >70% bootstrap values in all genes analyzed and their clustering patterns as shown in Figures 1-3.

4. Discussion

ATBF, a common febrile illness in humans, is caused by *R. africae*, a member of the spotted fever group (SFG) of Rickettsiaceae family. *A. variegatum* and *A. hebraeum* are the main vectors of this pathogen, with the latter being the most prevalent in South Africa, where it is widely dispersed[15]. The distribution of *R. africae* in South Africa is dependent on the geographical distribution of its vector, *A. hebraeum*[12].

This study presents evidence of the presence of *Rickettsia* species in *Amblyomma* ticks. In the current study, a total of 200 *Amblyomma* ticks were collected and analyzed for the presence of rickettsial pathogens. The extracted DNA was screened for *ompA*, *ompB*, and *17KDa* genes using a polymerase chain reaction. A total of 7% (14/200), 21% (42/200) and 16% (32/200) of the samples were positive for *17KDa*, *ompA* and *ompB* genes respectively. All study sequences from the three genes analyzed, formed monophyletic clusters with representative homologous sequences obtained from GenBank as presented in Figures 1-3[12,15,20]. This is in accordance with multiple studies that indicate rickettsial infections in *Amblyomma* ticks. Comparatively to our findings, a study conducted by Mazhetese *et al*.[15] on rickettsial infection rates in *A. hebreuam* ticks in the Mnisi area of South Africa found a 13.2% infection rate of *R. africae*. This is congruent with data from a related study that was published, in which PCR was used to detect *R. africae* in 15.7% (28/178) of *A. hebraeum* taken from goats in the Minisi community area[15,20]. A study by Mtshali *et al*.[11] also reported a 20% prevalence of *R. africae* in *A. hebraeum* collected from domestic animals in South Africa. Contrary to the findings of this study, a previous study by Pillay and Mukaratirwa[12] conducted in the Eastern Cape area of South Africa found a 59.5% *R. africae* prevalence in adult *A. hebreaum* ticks. Similarly, Maina *et al*.[21] also reported in a study conducted in Kenya where rickettsial infection rate was 92.6% in *A. varigatum*, indicating that *Amblyomma* is the primary vector of the pathogen in most countries in East and Southern Africa countries.

Ixodidae ticks pose a huge threat to the environment due to female

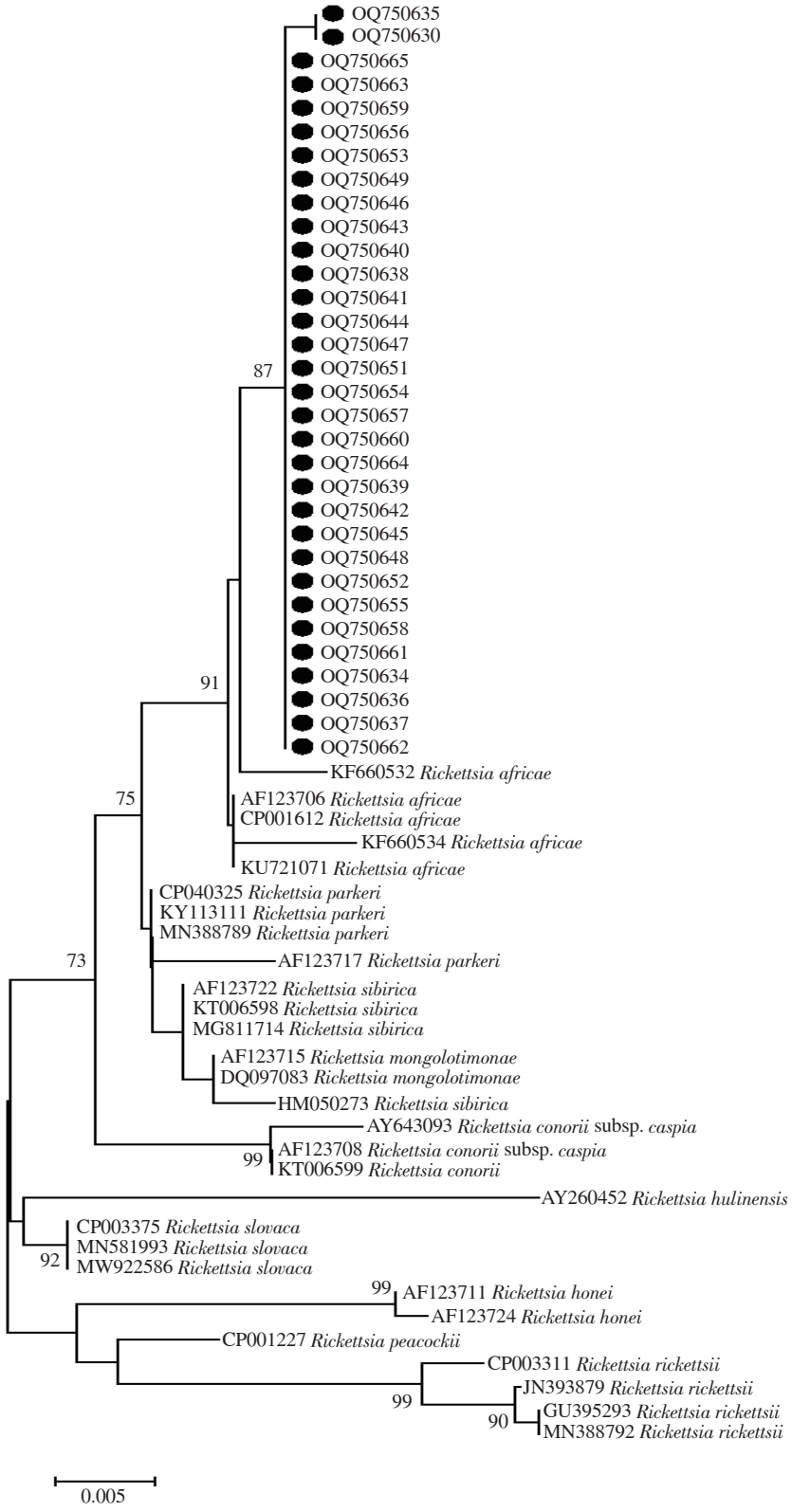

Figure 3. Phylogenetic tree showing the *ompB* gene sequences (in black dots) generated from this study with the related reference sequences obtained from NCBI GenBank. The GenBank accession numbers and species are indicated. Neighbour-Joining method was used and Bootstrap values ≥70% are shown.

hard ticks being able to lay more than 2 000 eggs in a single batch[16], while the *Amblyomma* ticks could lay as much as 20 000 eggs[2]. This is especially alarming due to ticks' ability to transovarially transmit pathogens.

In a study by Mazhetese *et al*[15], they observed an average transovarial transmission rate of 85% which according to the study, is comparable to transovarial transmission rates of *R. africae* observed in *A. hebraeum* and *A. variegatum*. In the absence of a host with the rickettsial disease, transovarial transmission of *R. africae* has been reported to continue for multiple generations. However, it

is still not known whether the disease can be transmitted this way indefinitely in the absence of an amplifying host. If this happens to be the case, the tick may serve as both a reservoir and a vector for the bacterial pathogen. In the same study, several *Rickettsia* spp. were also examined for transovarial transmission with naturally infected *R. sanguineus* ticks under laboratory conditions and was reported to have 100% transovarial transmission of *R. conorii conorii* across four generations[21]. Similarly, a study done by Snellgrove *et al*.[22] found that 100% of transovarial transmission and 99% of filial infections occurred in *R. sanguineus* throughout 12 generations in naturally infected *R. sanguineus* ticks. Also, the *Dermacentor nuttalli* ticks collected from Mongolia showed a 91% transovarial transmission rate for *R. raoultii*[23,24]. The etiologic agent of Rocky Mountain spotted fever, *R. rickettsii*, equally showed a 100% transovarial transmission rate over four generations in *A. aureolatum*[15]. The findings of our study keep up the trend of *Amblyomma* ticks being known to transmit rickettsial infections.

There is an abundance of *R. africae* in *Amblyomma* ticks collected from cattle in the study areas. The persistent and unceasing rise in the incidence of ticks and the pathogens they carry has been attributed to some factors. These include factors such as subpar veterinary and medical treatment, insufficient tick and tick-borne disease monitoring and surveillance systems, habitat destruction by humans and invasion by animals, tick resistance to acaricides, and climate change. These variables have also been attributed to most probable causes of newly emerging zoonotic illnesses[5]. This has serious public health implications as individuals who accidentally get infested with ticks could acquire *R. africae* with ultimate development and manifestations of symptoms of ATBF. Therefore, farmers who have regular contact with animals should be mindful of ticks' infestations and need to visit a clinician for monitoring when bitten by a tick.

Equally, health care workers should consider tick-borne diseases in their regular diagnoses of infections especially those presenting with flu-like symptoms. As ticks are vectors of many pathogens, concerted efforts should be made by the Department of Agriculture, Land Reform and Rural Development to carry out a regular control of ticks through animal dipping in acaricides. It is important to note that, though the use of acaricides when properly applied could be efficient in controlling ticks, however, it does have its disadvantages. Chemical residues of the acaricides can be found in the meat and dairy products of the animals undergoing dipping, and the chemicals could also pollute the environment. There have also been reports indicating acaricide resistance in mostly but not only *Rhipicephalus*[2]. A solution to this problem would be urgent development of more efficient but less environmentally harmful acaricides. There are studies suggesting the rearing of indigenous cattle breeds as they have shown some level of resistance to certain diseases; a good example of such is the N'Dama cattle's tolerance for trypanosomiasis. The Tanzania shorthorn zebu may be tolerant to ticks and East Coast Fever, according to preliminary research, whereas the Small East African zebu was reported to be resistant to *R. appendiculatus* ticks in Kenya. Unfortunately, the fundamental foundation for these conclusions has not been thoroughly explored in the scholarly literature[5].

In conclusion, SFG rickettsioses are tick-borne emerging human diseases caused by bacteria of the genus *Rickettsia*. They are obligate intracellular pathogens that are very common in *Amblyomma* ticks found in Africa. As a result of rapid increase in international travel and ecotourism, the disease is becoming very common among travelers who return from visits to sub Saharan African. In this study, we observed a 21% prevalence of SFG *Rickettsia* in *Amblyomma* ticks collected from cattle in Pretoria and Polokwane in South Africa. As the surge in international travel and ecotourism continues, it is therefore very advisable that travelers to South Africa should be very cautious of tick infestation and physicians should consider treating against SFG rickettsioses in patient with systemic febrile illnesses who traveled and are returning from visits to sub-Saharan Africa. We have established that *Amblyomma* spp. are the major vectors of SFG *Rickettsia* in the study areas in this study.

Conflict of interest statement

We hereby make a declaration that there is no conflict of interest as regards the publication of this research article.

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Availability of data

All data associated with this article are readily available in the manuscript while the generated sequences have been submitted to the GenBank repository.

Authors' contributions

BCI and LCO conceptualized the study; BCI, NM and LCO collected the samples; BCI, KM, KOA and NM performed the experiment and analyzed the results; BCI, KM and KOA wrote the manuscript; LCO provided the funding and proofread the manuscript.

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