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Molecular screening of ticks for the presence of *Rickettsia* species: A public health concern

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ABSTRACT

Objective: To profile ticks collected from domestic animals and vegetation for the presence of Rickettsial pathogens.
Methods: Ticks were collected from domesticated animals and from vegetation in some selected communities. A total of 900 adults and nymphs ticks were collected. They were identified by taxonomic morphological criteria and screened for the detection of *Rickettsia* genetic material using citrate synthase (*glt*A) gene specific primers to produced 610 bp amplicons which were sequenced and analyzed.
Results: The ticks collected were identified as belonging to three genera which were *Rhipicephalus*, *Amblyomma*, and *Hyalomma*, in decreasing order of their abundance. Rickettsial genetic material was detected in 15/900(1.67%) of the samples. Analyses of the generated sequences showed that five are phylogenetically related to members of the spotted fever group (SFG) rickettsia while the remaining ten sequences were Candidatus *Rickettsia* spp. whose pathogenic potentials are not known. The SFG rickettsia was of the genera *Rickettsia akari* and *Rickettsia raoultii* both being reported in South Africa for the first time and also in *Rhipicephalus* spp. as a vector.
Conclusions: This finding thus shows that ticks in South Africa harbor *Rickettsia akari* and *Rickettsia raoultii* and are capable of transmitting it to infested humans.

1. Introduction

Ticks generally are the second to mosquitoes as disease vectors, and they are involved in the transmission of a diverse array of diseases causing microbes such as bacteria, protozoa and viruses, all of which are pathogens of importance affecting both human and animal health globally[1,2]. Among the arthropod vectors, ticks are known to transmit the greatest variety of pathogens[3]. Different *Rickettsia* species that are pathogenic to humans have emerged as tick-borne diseases recently[1]. Members of the genus *Rickettsia* are pleomorphic, Gram-negative, intracellular parasites that consist of the spotted fever group (SFG) rickettsiae which are the etiologic agents of tick-borne rickettsioses of humans [4] as well as three other groups which are the typhus group (TG) rickettsiae, the transitional group (TGR) comprising *Rickettsia felia* and *Rickettsia akari* (*R. akari*) and the ancestral rickettsiae group (*Rickettsia bellii* and *Rickettsia canadensis*) which are not known to cause disease in human[5,6]. Numerous species of tick-borne *Rickettsia* that were hitherto thought as nonpathogenic are recently being implicated in human infections while many more species of unknown pathogenicity are frequently being described across the globe. As a result of geographical expansion of ticks into new ecological territories across the globe, many well described *Rickettsia* spp. and some partially described species of the bacterium which hitherto were considered to be confined to a particular tick or ecological location are now being reported from different parts of the globe and in different ticks[7].

Consequent upon this variation in the global distribution, availability and density in population of the tick vectors and reservoir hosts, the prevalence of tick-borne rickettsial pathogens varies from one ecological site to the other [8-11]. Across the globe, there are many studies that are currently being carried out with the sole aim of determining the significance for human health of tick-borne diseases. However, in South Africa, there are relative few systematic studies on ticks and tick-borne diseases. The Eastern Cape Province of South Africa is well reputed for animal rearing

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and these animals are kept in close proximity to peridomestic areas and tourist sites. Coupled with this, is the high prevalence of HIV/AIDS patients who are immune compromised and whose health could likely be more jeopardized and endangered with rickettsiosis arising from infected tick bites. This study was therefore conducted to document the current prevalence of rickettsial pathogens in ticks collected from animals that are in close proximity to human dwellings and by blanket dragging from open fields frequently visited by humans.

2. Materials and methods

Between the months of March 2016 to June 2016, ticks were collected from domesticated ruminants: cattle, sheep, and goats, and by blanket dragging in three localities in the Eastern Cape of South Africa. The study sites were chosen because of the abundance of these animals which are in close proximity to homes and tourist attraction sites. Ticks collection was done with the help of animal health assistants who are staff of the South African Department of Agriculture and Wildlife Services. Prior to sample collection, ethical clearance was obtained from the University of Fort Hare, ethics committee and permission was granted by farmers from whose farms ticks were collected. Strict adherence to rules as pertaining animal handling were observed. All ticks were collected into 50 mL Nalgene tubes containing 70% ethanol and transported to the laboratory of the Applied and Environmental Microbiology Research Group (AEMREG) for analyses.

2.1. Ticks identification and processing for bacterial DNA extraction

The collected ticks were identified based on their morphological criteria using the taxonomic criteria previously described by Hoogstral and Aeschlimann[12]. All ticks were stored at –70 ˚C until processed. Each individual or pool was sectioned with scalpel in a Petri dish containing Tris-EDTA buffer and then triturated prior to DNA extraction. The pooled ticks consisted of nymphs of about 10 in number while the engorged adults were processed individually taking into cognizance the animals, locations from which they were collected, and the tick type.

2.2. Bacterial DNA extraction

The ticks were washed with sterile water to remove the traces of the 70% ethanol into which they were collected. DNA was extracted from each individual engorged adult tick while the DNA from nymphs or non-engorged ticks was extracted from pools containing about 10 nymphs/adults per pool. Before DNA extraction, the ticks were chopped with a sterilized razorblade in a Petri dish containing Tris-EDTA. As for DNA extraction from non-engorged ticks, after maceration with sterile pistil, they were further subjected to detergent lysis overnight in the presence of proteinase K at 56 ˚C and then incubated for 20 min. Extraction of DNA from the macerated ticks was carried out by using the ZYMORESEARCH Quick-DNA Universal Kit (Madison, WI, USA) according to the manufacturer's instructions. The extracted DNA was stored at 4 ˚C until use as templates in PCR or at –20 ˚C until further use.

2.3. PCR detection of bacteria in ticks

Screening for the detection of *Rickettsia* spp. in the extracted DNA was performed by using the primers previously described in literature by Kollars *et al.*[13]. Briefly, the presence of *Rickettsia* DNA was assessed through PCR to generate a partial segment of 617 bp of the *Rickettsia* citrate synthase (*glt*A) gene using RK *glt*A F 5'-TTTGTAGCTCTTCTCATCCTATGGC-3' and RK *glt*A R 5'CCCAAGTTCCTTTAATACTTCTTTGC-3' primer pairs. PCR was performed in a 25 μL reaction mixture containing 12.5 μL of the master mix, 1 μL each of 10 pmol/L of the forward and reverse primers, 5.5 μL of RNase nuclease free water and 5 μL of DNA template. The cycling conditions were as follow; initial denaturation at 94 ˚C for 3 min, followed by denaturation at 93 ˚C for 30 sec, annealing at 50 ˚C for 30 sec with an elongation of 1 min at 72 ˚C and a final elongation at 72 ˚C for 5 min. The PCR products were separated by electrophoresis on 1.5% agarose gels in a 0.5X TBE buffer at 120 V for 45 min, stained with ethidium bromide, viewed under UV transilluminator and photographed.

2.4. DNA sequencing and sequence editing

PCR amplicon products were sequenced in a commercial sequencing facility and sequence editing was performed using Geneious R9.1.5 version (Biomatters Limited). The generated nucleotide sequences were subjected to homology search using the BLAST 2.0 program in the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/blast/ Blast.cgi), where our edited sequences were compared with genetic sequences of previously characterized *Rickettsia* spp. in the GenBank. *Rickettsia* spp. sequences that had high percentage similarity with our queried sequences were selected as reference sequences for phylogenetic analysis.

2.5. Phylogenetic analysis

The obtained DNA sequences were aligned with homologous sequences of other *Rickettsia* spp. obtained from the GenBank based on the percentage similarity of the homology search. Phylogenetic analysis was performed using Geneious Tree Builder as implemented in Geneious version 9.1.5 created by Biomatters. Available from http://www.geneious.com. Tree reliability was performed by bootstrap analysis with 1 000 replicates.

3. Results

We collected 790 adult *Ixodes* ticks and 110 nymphs in this study. The ticks were delineated by morphological characteristics into three genera, *Rhipicephalus*, *Amblyomma*, and *Hyalomma* comprising 6 species. *Rhipicephalus evasti evast* (*R. evasti evast*) 44.3% (350/790 adults) was the most prevalent species followed by *Rhipicephalus sanguineus* (*R. sanguineus*) 27.8% (180/790), *Rhipicephalus appendiculatus* (*R. appendiculatus*) 15.2% (120/790), *Amblyomma hebraeum* (*A. hebraeum*) 8.9% (70/790), and *Hyalomma marginatum rufipes* (*H. marginatum rufipes*) 7.6% (60/790) while all the 110 nymphs were identified as *Rhipicephalus* spp. There was similarity in the diversity of ticks collected at each site. The respective species and their number per each collection sites are shown in Table 1.

3.1. Prevalence of Rickettsia spp. detected in our study samples

Molecular screening of the ticks collected in this study for the presence of rickettsial pathogens revealed that out of the 900 adult and nymph ticks collected in this study from different animals and sites, 15 (1.7%) harboured the pathogen. Eight were from ticks collected from animals while seven were from questing ticks collected by blanket dragging. There was no evidence of genetic material of *Rickettsia* spp. from ticks collected from goats and horses and from questing ticks at Alice. Infection rates among cattle and sheep was 8/790 (1.01%) while that of questing ticks was 7/110 nymphs (6.4%). Homology search for similarity of the generated

sequences by BLAST in the NCBI database showed that all the *Rickettsia* sequences obtained in this study had high percentage similarity (ranging from 94%–100%) with other *Rickettsia* sequences in the GenBank. Phylogenetic analysis of sequences of our samples showed that they were *Rickettsia* as they clustered with references sequences obtained from the GenBank as can be seen in Figure 1. The reference name and accession number of reference sequences obtained from the GenBank that were used in the phylogenetic analysis are as follow: KF360027 Uncultured *Rickettsia* sp., KU499847 Candidatus *Rickettsia senegalensis*, KC993860 *Rickettsia monacensis* strain CN45kr, AF394896 *Rickettsia tamurae* strain, AT-1, RRU59721 *Rickettsia rhipicephali* 3-7-6, RMU59719 *Rickettsia massiliae* Mtu, RMU74756 *Rickettsia montana*, JQ697956 *Rickettsia* sp. Kagoshima 6 isolate Tick-6-Kagoshima-Hhys, DQ365803 *Rickettsia raoultii* (*R. raoultii*) strain Marne, RJU59724 *Rickettsia japonica* YM, AF178034 *Rickettsia heilongjiangii*, RAU59733 *Rickettsia africae* (*R. africae*), ESF-5, RPU59732 *Rickettsia parkeri* Maculatum, RCU59730 *Rickettsia conorii* Seven, RHU59723 *Rickettsia helvetica* C9P9, RSU59734 *Rickettsia sibirica* 246, RSU59725 *Rickettsia slovaca* (*R. slovaca*) N.A. 13-B, F018074 *Rickettsia honei*, RCU59713 *Rickettsia canadensis* 2678, RIRCITS *Rickettsia prowazekii*, RTU59714 *Rickettsia typhi* Wilmington, RBU59716 *Rickettsia bellii* 369L42-1, RAU59717 *Rickettsia akari* (*R. akari*) MK, KU310589 *R. raoultii*, KU310590 *R. raoultii*, KU310587 *Rickettsia sibirica*, GQ255903 *Rickettsia* sp. SGL01, KX077193 *Rickettsia* sp. ARAGAOI, KU171018 *R. raoultii*, RAU59717 *R. akari* MK (Kaplan), AY578115 Candidatus *Rickettsia principis* (*R. principis*), AY578114 Candidatus *R. principis*, KT825962 Candidatus *Rickettsia tarasevichiae*, KT873466 *Rickettsia aeschlimannii* (*R. aeschlimannii*), KT873465 *R. aeschlimannii*, KT873464 *R. aeschlimannii*, LT558852 *Rickettsia* sp. G1329, JQ697955 *Rickettsia* sp. Mie334, GQ255903 *Rickettsia* sp. SGL01, JQ697957 *Rickettsia* spp. Mie201, DQ365805 Candidatus *Rickettsia rara*, JQ697957 *Rickettsia* sp. Mie201, AY578115 Candidatus *R. principis*, AY578114 Candidatus *R. principis* from *Haemaphysalis japonica douglasi* 054.

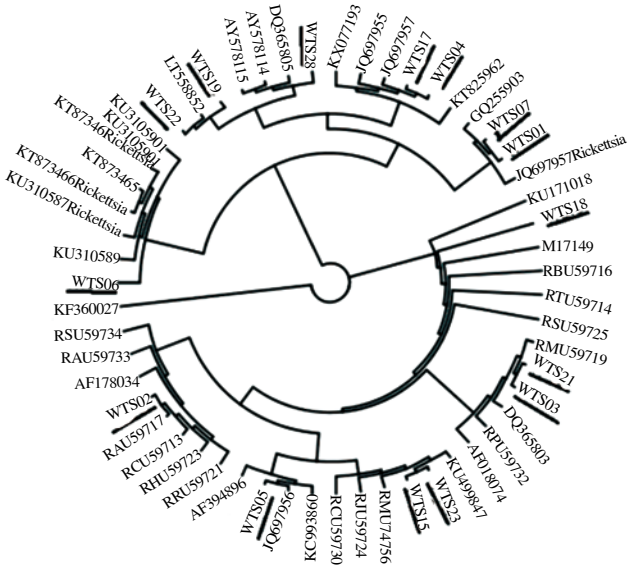

Figure 1. Phylogenetic relationship of various *Rickettsia* spp. based on the nucleotide sequences of the citrate synthase gene.

The underlined are the *Rickettsia* spp. sequences detected in this study. All the detected sequences in this study clustered phylogenetically with reference *Rickettsia* spp. *glt*A gene obtained from the GenBank. The tree was drawn with Geneious version 9.1.5 created by Biomatters. Available from http://www.geneious.com.

Sequence accession numbers: The nucleotide sequences analyzed here have been submitted to GenBank under the following accession numbers: KX891173–KX891187.

Table 1

Ticks diversity, developmental stages and prevalence of *Rickettsia* spp. in them.

Animal / Location	Tick species	Developmental stage	Number	Positive for *Rickettsia* spp.
Cattle	*R. evasti evast*	Adult	150	3 (WTS 01, WTS 23, WTS17)
	R. sanguineus	Adult	70	3(WTS03, WTS28, WTS06)
	R. appendiculatus	Adult	50	-
	Rhipicephalus spp.	nymph	0	-
	A. hebraeum	Adult	40	-
	H. marginatum rufipes	Adult	8	-
Sheep	*R. evasti evast*	Adult	140	-
	R. sanguineus	Adult	50	-
	R. appendiculatus	Adult	30	2 (WTS04, WTS18)
	Rhipicephalus spp.	nymph	0	-
	A. hebraeum	Adult	20	-
	H. marginatum rufipes	Adult	12	-
Goats	*R. evasti evast*	Adult	60	-
	R. sanguineus	Adult	60	-
	R. appendiculatus	Adult	40	-
	Rhipicephalus spp.	nymph	0	-
	A. hebraeum	Adult	30	-
	H. marginatum rufipes	Adult	30	-
Cofvimbaba	*R. evasti evast*	Adult	0	-
	R. sanguineus	Adult	0	-
	R. appendiculatus	Adult	0	-
	Rhipicephalus spp.	nymphx`	53	3 (WTS02, WTS07, WTS22)
	A. hebraeum	Adult	0	-
	H. marginatum rufipes	Adult	0	-
Whittlesea	*R. evasti evast*	Adult	0	-
	R. sanguineus	Adult	0	-
	R. appendiculatus	Adult	0	-
	Rhipicephalus spp.	nymph	32	4 (WTS05, WTS15,WTS21,WTS19)
	A. hebraeum	Adult	0	-
	H. marginatum rufipes	Adult	0	-
Alice	*R. evasti evast*	Adult	0	-
	R. sanguineus	Adult	0	-
	R. appendiculatus	Adult	0	-
	Rhipicephalus spp.	Nymph	25	-
	A. hebraeum	Adult	0	-
	H. marginatum rufipes	Adult	0	-

4. Discussion

The geographical distributions of rickettsioses which are emerging arthropod-borne zoonotic diseases are governed by the ecological distribution of their tick vectors. The two main groups in the genus *Rickettsia* are the SFG and the TG and this division is mainly based on their physical, chemical and immunological characteristics[7]. New species of *Rickettsia* are being continually discovered thus adding to the array of previously known members of the group that has zoonotic potentials.

We investigated the genetic prevalence of *Rickettsia* spp. in ticks collected from domesticated animals and by flagging in some locations within the Eastern Cape of South Africa. Genetic analysis of the obtained sequences revealed that they have very high sequence homology with the Rickettsial sequences in the GenBank. Out of the 15 sequences obtained in this study, two isolates (WTS03 and WTS21) clustered phylogenetically with *R. akari* (RMU59719) while WTS02 showed phylogenetic similarity by clustering with

another sequence of *R. akari* (RAU59717) obtained from the GenBank. *R. akari* is phylogenetically related to *Rickettsia felis* (*R. felis*) and *Rickettsia australis* (*R. australis*) as phylogenetic analysis based on the complete genomes of *Rickettsia* spp. by Parola *et al*.[7], showed them clustering in one branch. The first report on the pathogenicity of *R. australis* was from Queensland, Australia in 1946[7].

The identified ticks vectors of *R. australis* are *Ixodes tasmani*, *Ixodes holocyclus*, and *Ixodes cornuatus* and these ticks are distributed along the eastern states of Australia[14,15]. *R. akari*, a well characterized member of the spotted fever group *Rickettsia* is the ethiologic agent of rickettsialpox, a benign zoonotic illness which has similar clinical manifestation like that of murine typhus. *R. akari* has been reported to be very common in metropolitan cities of eastern United States, Argentina, Eastern European countries like Turkey, Croatia, Ukraine, Russia, Mexico, and Asia where they have been severally reported[16-27]. To the best of our knowledge, the presence of *R. akari* and its closest relatives *R. felis* and *R. australis* has not been reported anywhere in the African continent. *R. akari* is naturally transmitted by *Liponyssoides sanguineus*, a mite of mice and other rodents and it is frequently reported in the United States and many countries in Europe and Asia[20]. The involvement of other arthropod vectors in the transmission cycle of *Rickettsia* spp. other than their known host is possible and this does have tremendous potential for their transmission to humans. Rickettsial pathogens could adapt to a new host subject to the prevailing ecological environment and climatic conditions where it is found as it has previously been reported by Gracner *et al*.[28] and Houhamdi and Raoult[29] that *R. felis*, *R. rickettsii*, and *Rickettsia conorii* could easily adapt experimentally to new environment. The detection of *R. akari* in *Rhiphicephalus* spp. in our study is therefore corroborating the earlier report of its being recovered from *R. sanguineus* collected from an infected dog in Mexico[30]. The capacity to adapt to new hosts and ecological environment is, therefore a huge epidemiological potential of this emerging human pathogen.

R. slovaca and *R. raoultii* are two closely related *Rickettsia* spp. that are found in many European countries and the major vectors of these bacteria are *Dermacentor marginatus* and *Dermacentor* reticulatus ticks as a high percentage of these ticks have been reported to be infected with these bacteria[31-40]. In a study conducted in Spain, *R. slovaca* was reported to be 16% in the blood samples of sheep, over 21% in goats and cattle[6] while the bacterial DNA has also been detected in blood sample obtained from goat, thus suggesting the possibility of *R. slovaca* being haboured in domestic ruminants[41].

Human infections with *R. slovaca* have been reported in some European countries such as France, Slovakia, Italy, Germany, Hungary, Spain, and Poland and recently in China[42-45]. While in northern Africa, *R. slovaca* and *R. raoultii* have only been detected in Morocco and in Algeria in *Dermacentor marginatus* ticks[46,47] though, no cases of human infection has been detected in northern Algeria. The detection of *R. raoultii* has not been reported anywhere else in sub-Saharan Africa to the best of our knowledge. Two of our isolates WTS06 and WTS18 clustered closely with *R. raoultii* (KU310589 and KU171018) as shown in the phylogenetic tree in Figure1. Previous reports on the isolation or detection of the organism have been from *Dermacentor* ticks and from other geographical regions of the world except in sub-Saharan Africa, but here it was detected in *R. sanguineus* collected from cattle and *R. appendiculatus* from sheep thus supporting earlier reports that they are prevalent in sheep and cattle and proving that they have other tick hosts. The detection of this bacterium in *Rhipicephalus* spp. as well as in a region where it has not been reported previously, suggests a widening of its host and geographical range which does have epidemiological implications.

The possibility of being infected with Rickettsial pathogens upon tick bite in the study area is very high as genetic material of 7 incompletely described *Rickettsia* spp. of unknown pathogenic potential were detected from ticks collected by blanket dragging in this study. Out of these seven species detected, two of them clustered closely with Candidatus *Rickettsia senegalensis* (KU499847) that was previously isolated from cats in Senegal[48] though its pathogenicity in humans has never been reported. Equally detected in this study are various Candidatus *Rickettsia* spp. (WTS05) which phylogenetically clustered with other Candidatus *Rickettsia* spp. like *Rickettsia* sp. Mie201 (JQ697956), WTS19 and WTS22 with *Rickettsia* sp. G1329 (LT558852), WTS04 and WTS17 with *Rickettsia* sp. ARAGA01 (KX077193, JQ697955, and JQ697967), while WTS28 clustered closely with Candidatus *Rickettsia rara* (DQ365805) and sequences WTS01 and WTS07 were closely related phylogenetically with *Rickettsia* sp. SGL01 (GQ255903) which was also previously isolated in Senegal[49].

The geographical spread of rickettsial pathogens is directly related to the spread of its tick host. Ticks are capable of spreading into any territory that provides favorable conditions for their survival and provided that the temperature and relative humidity are optimal, ticks can reproduce and spread their bacterial pathogens. Several SFG have been reported in Africa which may be responsible for febrile fever of undetermined cause that in most cases could be attributed to malaria. Novel *Rickettsia* spp. have been discovered through large-scale profiling of ticks collected from animal and by blanket dragging in the field thus expanding the knowledge on this very important pathogen and making it an ideal paradigm for studying other emerging diseases[7]. Previous studies on tick collected from South Africa have shown that several SFG are circulating in the country. In a similar study recently conducted by Mtshali *et al*.[50] involving some of the study areas included in ours', they reported *R. africae* as the only SFG found in their study. Our findings are quite in contrast to theirs' as we did not detect *R. africae*; neither did they detect the *Rickettsia* spp. reported in this current study. The most likely reason for the dissimilarities in our findings could be attributed to the differences in protocols as different primer pairs we adopted in the studies. Other SFG rickettsial that have been detected in South Africa include *Rickettsia conorii conorii*, *R. aeschlimannii* and *R. sibrica monogolitimonae*[51,52] of which none was detected in our study.

In this study, the most abundant tick species was *R. evertsi evertsi* and it has also been previously reported by Sungirai *et al*.[53] and Mediannikov *et al*.[54] to be one of the most prevalent ticks that parasitize both domestic and wild animals. There are scanty reports on the ability of this species to transmit diseases in both humans and animal. However, it is a known vector agent of the protozoan *Babesia bigemina*, Crimean-Congo hemorrhagic fever virus (CCHFV), and *Ehrlichia ruminantium*[55-57]. Mediannikov *et al*.[54] has reported on the ability of *R. evertsi evertsi* to transmit rickettsial agents in ticks that were collected in Senegal. Our detection of *R. akari* genetic material in *R. eversti eversti* is in accordance with their findings thus confirming the role of *R. evertsi evertsi* in the epidemiology of SFG. In the study area, domestic animals live in close proximity with humans and the possibility of infection is increased if they and their children are bitten by infected ticks. Coupled with this, is the high rate of HIV/AIDS patients with compromised immune status thus making them more vulnerable to rickettsioses and exacerbating their precarious health condition. Also, many tourists visit the Eastern Cape of South Africa and reports have placed the incidence of rickettsial infections in travelers who visited sub-Saharan Africa

to be 5.6%[54,58-61]. The findings in this study confirm that ticks in the Eastern Cape, South Africa have the potential of transmitting an assortment of rickettsial pathogens to humans, particularly when the prevalent tick species (*R. eversti eversti* and *R. sanguineus*) also infest humans. As a result of high volume on international trades on livestock, there is always a continual introduction and geographical spread of tick hosts and the associated pathogens that they are capable of transmitting. Therefore, continual surveillance is highly recommended in order to have an up to date epidemiological data of these emerging tick-borne pathogens.

This study has shown for the first time that *R. raoultii*, *R. akari* and other Candidatus *Rickettsia* spp., belonging to the SFG *Rickettsia* are present in the Eastern Cape Province, South Africa and suggests a potential role for *R. eversti eversti* and *R. sanguineus* as vectors of *R. akari* and *R. raoultii* in these areas. Also, ticks collected in the study communities' habour rickettsial pathogens that could infect humans who might be infested by the ticks. It is highly recommended that sero-prevalence survey among the populace be undertaken so as to ascertain the risk associated with tick bite as this will enable the relevant governmental agencies formulate policies that will reduce tick population through regular dipping of domestic animals as well as educating the populace on the need for adequate self-protection in order to minimize exposure to ticks and their bites while in the field. The limitations of the study are that few study sites in one province were involved. Further studies involving wider communities' coverage need be undertaking to have a better picture of the prevalence of rickettsial pathogens in tick vectors in South Africa. In order to avoid being infected with *Rickettsia* pathogens, increased awareness of the populace coupled with improved regular surveillance, and the use of tick repellant like permethrin on clothes while in the wood remains the best option from being infested by ticks.

Conflict of interest statement

We declare that we have no conflict of interest.

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