Molecular detection of zoonotic tick-borne pathogens from ticks collected from ruminants in four South African provinces

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ABSTRACT. Ticks carry and transmit a remarkable array of pathogens including bacteria, protozoa and viruses, which may be of veterinary and/or of medical significance. With little to no information regarding the presence of tick-borne zoonotic pathogens or their known vectors in southern Africa, the aim of our study was to screen for *Anaplasma phagocytophilum*, *Borrelia burgdorferi*, *Coxiella burnetii*, *Rickettsia* species and *Ehrlichia ruminantium* in ticks collected and identified from ruminants in the Eastern Cape, Free State, KwaZulu-Natal and Mpumalanga Provinces of South Africa. The most abundant tick species identified in this study were *Rhipicephalus evertsi evertsi* (40%), *Rhipicephalus species* (35%), *Amblyomma hebraeum* (10%) and *Rhipicephalus decoloratus* (14%). A total of 1634 ticks were collected. DNA was extracted, and samples were subjected to PCR amplification and sequencing. The overall infection rates of ticks with the target pathogens in the four Provinces were as follows: *A. phagocytophilum*, 7%; *C. burnetii*, 7%; *E. ruminantium,* 28%; and *Rickettsia* spp., 27%. The presence of *B. burgdorferi* could not be confirmed. The findings of this study show that zoonotic pathogens are present in ticks in the studied South African provinces. This information will aid in the epidemiology of tick-borne zoonotic diseases in the country as well as in raising awareness about such diseases in the veterinary, medical and tourism sectors, as they may be the most affected.

KEY WORDS: *Anaplasma phagocytophilum, Coxiella burnetii, Ehrlichia ruminantium, Rickettsia* species, zoonoses

Ticks are excellent vectors for disease transmission; they are second in importance only to mosquitoes as vectors of human diseases, both infectious and toxic [6]. Apart from being agricultural pests, ticks can also carry pathogens that are transmissible to humans via bites or direct contact with infected animals, causing diseases known as zoonoses [15].

Anaplasma phagocytophilum and *Borrelia burgdorferi sensu lato*, the causative agents of human granulocytic anaplasmosis (HGA) and Lyme disease (LD), respectively, are common mainly in the U.S. and Europe [8, 22]. Reports of *A. phagocytophilum* are scarce in Africa, with one published report in Egypt [14]. In South Africa, there is one confirmed case report of the pathogen isolated from whole blood samples of dogs [19]. While information regarding the specific tick vector of *B. burgdorferi* in South Africa is currently unavailable, it has been suggested that the abundance

of tick species in the country would favor establishment of the disease [9]. Previous reports have speculated about the seroprevalence of the bacterium in patients, dogs and horses [10, 40]. Despite reports on these pathogens, their true prevalence has not been properly investigated.

Although *Coxiella burnetii* has been isolated from several arthropods (mainly ticks), the rate of arthropod-borne transmission of Q-fever in people is considered to be low [37]. Q-fever attracts relatively little attention because of the assumed low disease incidence in both humans and animals; however, one of the greater challenges is that it remains asymptomatic [1]. Cattle, sheep and goats are reported as traditional sources of human infection [7]. Although widespread in South Africa, it is far less a cause of disease in humans compared with *Rickettsia africae* [11].

It has been recently found that several rickettsial species are transmitted in southern Africa, the most common being *R. africae* [11]. The true reservoir is wide and includes mammals, birds and arthropods, mainly ticks. Cattle, sheep and goats are most commonly identified as sources of human infection, and the disease is prevalent in mostly rural areas worldwide [25], while up to 75% of infected *Amblyomma* ticks, serve as both reservoirs and vectors [34].

Ehrlichia ruminantium is the causative agent of heartwater disease in cattle, goats and some wild ruminants [4]. It is one

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of the most important tick-borne pathogens infecting wild and domestic ruminants throughout sub-Saharan Africa [3]. It is generally transmitted by ticks in the genus *Amblyomma*. In South Africa, the only known vector is *A. hebraeum* [4], although gene segments have been found, by PCR, in other ticks including *Rhipicephalus evertsi evertsi*, *Hyalomma truncatum* and *H. marginatum*; however, the organism has not been isolated [30].

South Africa is an agro-exporting nation and is mainly dependent on livestock productivity for subsistence according to the Department of Agriculture, Forestry and Fisheries. The country also boasts game reserves, which can be likened to safe havens for ticks, where more often than not, tourists and locals alike fall victim to tick bites and contraction of diseases [13, 35]. Data on the prevalence of these pathogens in ticks would therefore aid in understanding the epidemiology of the diseases they transmit as well as in raising awareness in the veterinary, medical and tourism sectors. In this study, we used PCR to screen for the presence of zoonotic pathogens (*A. phagocytophilum*, *B. burgdorferi*, *C. burnetii*, *E. ruminantium* and *Rickettsia* spp.) in ticks collected from various livestock in selected South African provinces.

MATERIALS AND METHODS

Study area and sample collection: Ticks were collected from livestock and vegetation from locations in four (4) provinces of South Africa (Fig. 1). They were collected from cattle, goats and sheep in the following specific areas; Hooningkloof (farm at a livestock-wildlife interface), Sekoto village, Seotlong Hotel & Agricultural School and Kestell in Free State (FS); uMsinga Mountain view dip tank in KwaZulu-Natal (KZN); Amathole District Municipality in Eastern Cape (EC); and Kameelpoort-KwaMhlanga area in Mpumalanga (MP). At Sekoto village in Free State, ticks were also collected from horses, and flagging was used to collect ticks at the Qwaqwa Campus of the University of the Free State, Free State, South Africa. All the tick collections were conducted by qualified animal health technicians from the government of South Africa's Department of Agriculture, Forestry and Fisheries (DAFF). The animals were handled according to the regulations of the Animal Ethics Committee of University of the Free State (SANS10386).

DNA extraction from ticks: The ticks were surface sterilized twice with 75% ethanol, washed once in phosphate buffered saline (PBS) solution, dissected and gutted (the engorged) or crushed whole (the males) in individual sterile Eppendorf tubes (Hamburg, Germany) and then preserved in PBS and stored at −34°C until further use. Ticks of the same species collected from the same animal were pooled to form one sample in preparation for DNA extraction. Some ticks laid eggs within the collection vials. The eggs were also washed in PBS, spun down at full speed ($16,000 \times g$) in a microcentrifuge, crushed and stored as described above. DNA was extracted from the processed samples using the salting out method as described by Miller *et al.* [26].

Polymerase chain reaction (PCR): To screen for the presence of *A. phagocytophilum*, *B. burgdorferi*, *C. burnetii*, *E.*
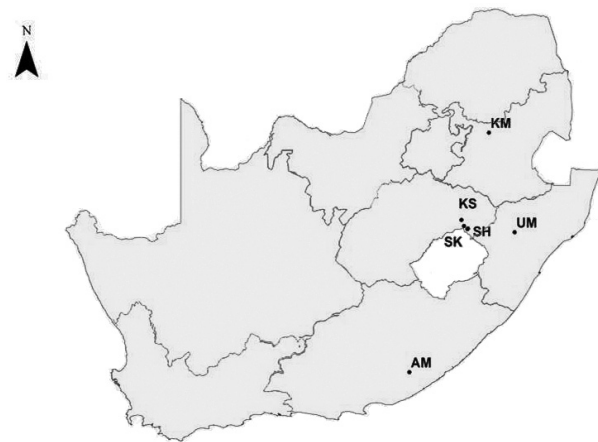

Fig. 1. Map indicating exact locations based on the GPS coordinates taken for the collection sites in the different provinces of South Africa. KM=Kameelpoort; KS=Kestell; SK=Sekoto village; SH= Seotlong Agriculture and Hotel School; UM=uMsinga Mountain View dip site; AM=Amathole District Municipality. Map created with ArcGIS (Esri, 2013).

ruminantium and *Rickettsia* spp., tick DNA was subjected to PCR amplification using published oligonucleotide sequences (shown in Table 1). The reactions were performed using AmpliTaq Gold® 360 Master Mix (Applied Biosystems Thermo Fisher Scientific, Waltham, MA, U.S.A.) as follows: initial denaturation at 95°C for 10 min; 35 cycles of denaturation at 95°C for 30 sec; annealing at varying temperatures (indicated in Table 1) for 30 sec, extension at 72°C for 60 sec; final extension at 72°C for 7 min and hold at 4°C (infinite). The reactions were incubated using Veriti®Thermal cycler (Applied Biosystems Thermo Fisher Scientific), and the PCR products were electrophoresed on 1.5% agarose gels, stained with GelRed and/or ethidium bromide and size fractionated using a 100 bp DNA ladder.

The positive PCR products were purified using USB ExoSAP-IT Enzymatic PCR Product Clean-Up (Affymetrix Japan K. K., Tokyo, Japan). The forward and reverse primer pairs in Table 1 were utilized in direct sequencing of the purified PCR products. Cycle sequencing reactions were performed using an ABI Prism BigDye Terminator Cycle Sequencing Kit (Applied Biosystems Thermo Fisher Scientific) on an ABI 3130 DNA Sequencer. The sequence data of the PCR products were analyzed using BLAST 2.0 (National Center for Biotechnology Information, Bethesda, MA, U.S.A.; http://www.ncbi.nlm.nih.gov/blast/) for homology searching. The CLCMain Workbench ver 7.5.1. (CLC bio, Aarhus, Denmark) package was used for sequence analysis and construction of a phylogram. The sequences used in the alignment were obtained from the NCBI GenBank Database (http://www.ncbi.nlm.nih.gov/genbank/).

RESULTS

A total of 1,634 ticks were collected from the designated study areas; a breakdown of species numbers is given in

Table 1. Oligonucleotide sequences used in the study for PCR reactions

Pathogen	Oligonucleotide sequences	Annealing Temperature	Product size (bp)	References
Anaplasma phagocytophilum	EHR521-5'TGT AGG CGG TTC GGT AAG TTA AAG'3 EHR747-5'GCA CTC ATC GTT TAC AGG GTG'3	60°C	250	[41]
Borrelia burgdorferi sensu lato	FL6-5'TTC AGG GTC TCA AGC TTG CAC T'3 FL7-5'GCA TTT TCA ATT TTA GCA AGT GAT G'3	55°C	276	[32]
Borrelia burgdorferi	B1-5'ATG CAC ACT TGG TGT TAA CTA'3 B2-5'GAC TTA TCA CCG GCA GTC TTA'3	63°C	126	[27]
Coxiella burnetii	CB-1: 59-5'ACT CAA CGC ACT GGA ACC GC'3 CB-2: 59-5'TAG CTG AAG CCA ATT CGC C'3	57–62°C	257	[31]
Ehrlichia ruminantium	pCS2 F3-5'CTT GAT GGA GGA TTA AAA GCA'3 pCS20B3-5'GTA ATG TTT CAT GTG AAT TGA TCC'3	57 °C	279	[28]
Rickettsia spp.	RpCS-877p-5'GGG GAC CTG CTC ACG GCG G'3 RpCS 1273r-5'CAT AAC CAG TGT AAA'3	55 °C	380	[20]
Rickettsia spp.	CS78-5'GCA AGT ATC GGT GAG GAT GTA'3 CS323-5'GCT TCC TTA AAA TTC AAT AAA TC'3	55°C	401	[29]

Table 2. Ticks and species collected in the four sampled provinces

Tick species	Number of tick species per province				Total number of ticks (%)
	KZN	FS	EC	MP	
Amblyomma hebraeum	81	0	87	3	171 (10)
Hyalomma marginatum rufipes	0	1	0	3	4 (0.2)
Rhipicephalus species	213	204	151	2	570 (35)
Rh. decolaratus	*	129	99	1	229 (14)
Rh. evertsi evertsi	*	620	23	7	650 (40)
Rh. appendiculatus	*	10	0	0	10 (0.6)
Total	294	964	360	16	1,634

*Grouped under *Rhipicephalus* species, KZN, KwaZulu-Natal; FS, Free State; EC, Eastern Cape and MP, Mpumalanga.

Table 2. A total of 590 DNA samples were processed for PCR screening. The overall infection rates of ticks with *A. phagocytophilum*, *C. burnetii*, *Rickettsia* spp. and *E. ruminantium* per sampled province are illustrated in Fig. 2.

The prevalence of *A. phagocytophilum* in ticks was 7% in the four provinces. This pathogen was detected in ticks infesting cattle, sheep and goats only and not from questing ticks. Of the positive tick samples collected from ruminants, the rates of detection of the bacterium were 50% for *Rhipicephalus* spp.; 23% for *Rh. e. evertsi*; 19.2% for *Rh. decoloratus;* and 7.7% for *A. hebraeum*. The crushed-egg DNA samples (n=10) and questing ticks (n=16) were all negative for *A. phagocytophilum*. The sequences were 98% identical to published sequences of *A. phagocytophilum* [GenBank, DQ648489.1]. Although the primers were synthesized to amplify a variable region of the 16S rRNA gene sequence specific for *E. equi, E. phagocytophila* and the HGA agent, which have since been renamed *A. phagocytophilum* [17], some of the generated sequences were 99% identical to *A. marginale, Anaplasma* sp. HLJ-14, *A. ovis* and *A. central*e [GenBank, LC007100.01, KM20273.1, KJ410246.1 and KC189839.1, respectively], as shown in Fig. 3. Our sequences (7, 13, 14, 21_EHR) show regions of conservation
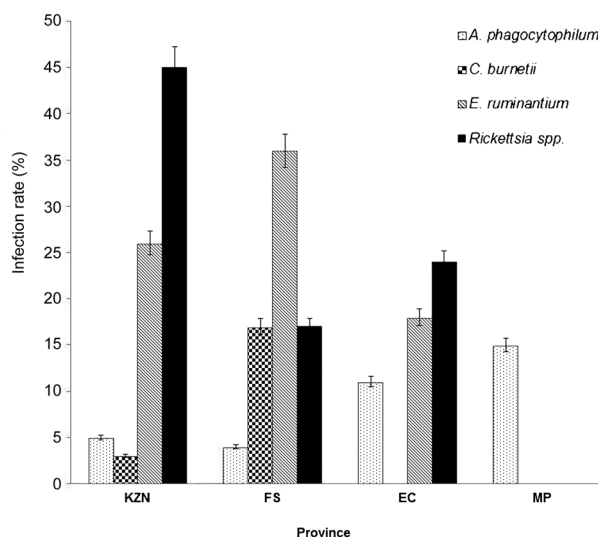

Fig. 2. Overall infection rates of ticks with *A. phagocytophilum*, *Coxiella burnetii*, *Ehrlichia ruminantium* and *Rickettsia* species per province. KZN=KwaZulu-Natal, FS=Free State, EC=Eastern Cape, MP=Mpumalanga.
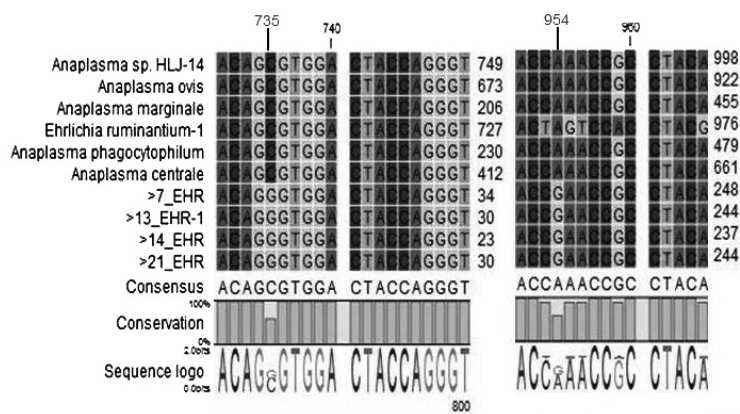
Fig. 3.   The *A. phagocytophilum* alignment with reference species *A. marginale*
(GenBank, LC007100.01), *Anaplasma* sp. HLJ-1 (GenBank, KM20273.1), *A. ovis* (GenBank, KJ410246.1) and *A. centrale* (GenBank, KC189839.1). The
sequences generated from the 16S rRNA region are similar throughout except
for the bases in lines 735 and 954 which were conserved between the amplified
sequences in the current study.

for bases in lines 735 and 954 that are different from the similar sequences used in the alignment.

The overall tick infection rate with *C. burnetii* was very low (7%) throughout the sampled areas and absent in the EC Province samples and in questing ticks. The highest infections recorded were in ticks infesting sheep (32%) followed by goat ticks (6%), and the lowest infections reported were in cattle ticks (3%). The *C. burnetii* PCR-positive samples were sequenced, and they revealed a 99% maximum identity to *Coxiella burnetii*_CbUKQ154 (GenBank, CP00102), *Coxiella burnetii* R.S.A. 331 (GenBank, EU448153.1) and Namibia genome (GenBank, CP007555.1).

The prevalence of infection of ticks with *Ehrlichia* spp. ranged between 0 and 64% within the provinces. The highest infections recorded were in ticks infesting goats (68%) followed by sheep ticks (33%), and the lowest infections recorded were in cattle ticks (18%). Questing ticks were all negative for *E. ruminantium*. The sequences generated were 100% identical to the pCS20 ribonuclease region of the *E. ruminantium* Welgevonden strains [GenBank, AY236058.1 and CR767821.1], the type species obtained from an *A. hebraeum* tick collected in the former north eastern Transvaal in South Africa. *Rh. evertsi evertsi* had the highest infection rate of 55.4%, followed by *Rhipicephalus* spp. (34.6%), *A. hebraeum* (6.2%) and lastly *Rh. decoloratus* (3.8%).

The prevalence of infection of ticks with *Rickettsia* spp. ranged between 17 and 45% within the provinces. In questing ticks, the rate of infection with *Rickettsia* spp. was 16%. One of the horse-tick DNA samples screened positive for *Rickettsia* species DNA. Infection rates were highest amongst sheep ticks (32%). Sequences generated had a 99% maximum identity to *Rickettsia africae* (GenBank, JN043505.1), *R. raoultii* isolate (GenBank, KM288492) and *R. sibirica* (GenBank, JX945526.1). The sequence alignment and phylogenetic analysis shows that the *Rickettsia* spp., detected in this study, are closely related to *R. africae*

(Fig. 4). Of the positive samples, 36%, 35% and 20% were from *Rhipicephalus* spp., *Rh. e. evertsi* and *A. hebraeum* ticks, respectively, and the rest were from *Rh. decoloratus*, *Rh. appendiculatus* tick species and eggs of *Rhipicephalus* spp. (9%).

We could not positively confirm the presence of *B. burgdorferi* with the two sets of primers used in this study. The bands viewed on agarose gel were shorter than the expected amplicon size of 276 bp, and the sequences generated ranged between 80 and 120 bases long and could not be used for homology searching in the databases. These observations were made in samples from some of the cattle and sheep ticks. The rates of infection of ticks collected from various sources with the target pathogens are summarized in Fig. 5.

DISCUSSION

The first report of the presence of *A. phagocytophilum* in South Africa was in whole blood specimens from three dogs in Bloemfontein [19, 24]. While most of the published literature show that infections with this pathogen are common in the U.S. and Europe [8], the data from Africa are sparse. An Egyptian study reported *A. phagocytophilum* infection rates for ticks collected from dogs and sheep (13.7%) and from goats (5.3%) [14], comparable to those of the current study i.e. 6, 17 and 1.25% in ticks from cattle, goat and sheep, respectively. In the absence of *I. persulcatus*, *I. ricinus*, *I. scapularis* and *I. pacificus* (recognized vectors), *Rhipicephalus* spp., *Rh. e. evertsi*, *Rh. decoloratus* and *A. hebraeum* should be considered possible vectors/reservoirs of the pathogen amongst livestock populations in the country. The true prevalence of *A. phagocytophilum*, however, remains unknown in South Africa and requires further investigations of all nine provinces and assessment of potential risk factors for infection in humans.

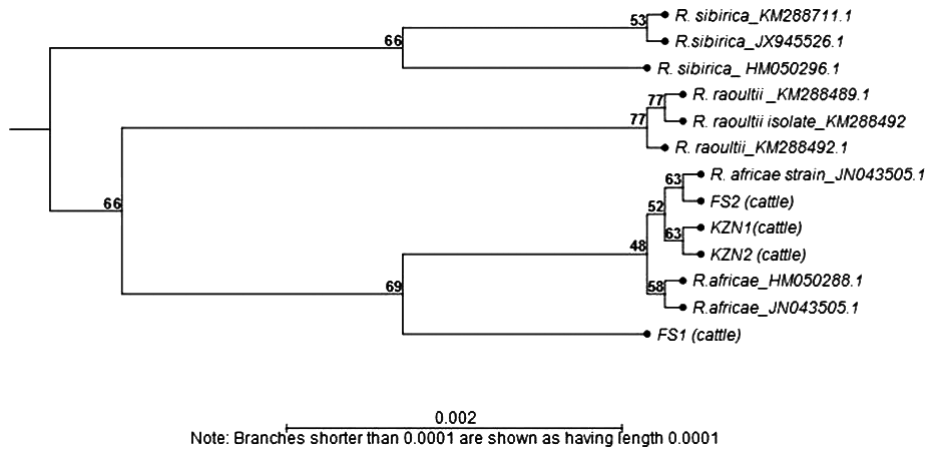*Coxiella burnetii* infections in South Africa have been

Fig. 4.   The evolutionary history of *Rickettsia* species from the generated sequences was inferred using the UPGMA algorithm. The optimal tree had a total branch length=0.002. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1,000 replicates) is shown next to the branches. The evolutionary distances were computed using the maximum composite likelihood method and recorded as the number of base substitutions per site using CLC Workbench Main ver 7.5.1. (CLC bio, Aarhus, Denmark).
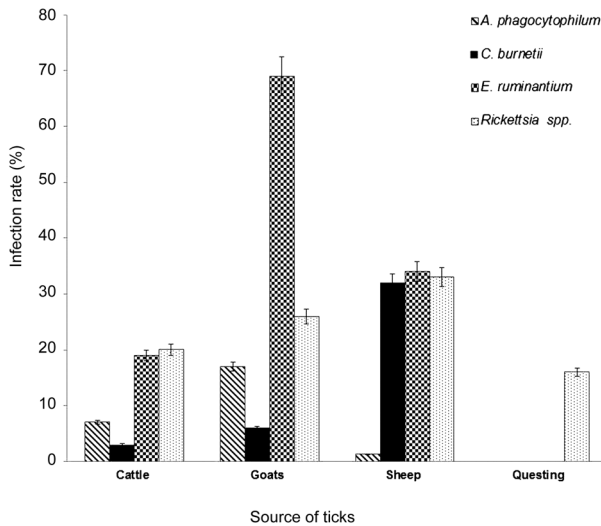

Fig. 5.   Overall Infection rates of ticks with *A. phagocytophilum*, *Coxiella burnetii*, *Ehrlichia ruminantium* and *Rickettsia* species per sampled group. The groups include cattle, goats, sheep and vegetation from which questing ticks were collected.

demonstrated only serologically in cattle, goats and sheep [21, 39], making the current study the first of its kind. The seropravelence of *C. burnetii* amongst cattle in South Africa is reported to be between 8 and 93% [11, 16, 39]. No previous published reports of tick rates were found. Here, we report a very low overall infection rate of tick infections (7%), supporting claims made in the early 50s that Q-fever in South Africa was apparently kept at levels below a certain threshold amongst livestock populations, especially in cattle,

and because of this low incidence, Q-fever was considered to have reached a state of endemic stability [16, 39]. We also suspect this, as the rate of infection among ticks was as low as 3% among this group. In contrast, the 32% infection rate observed in sheep ticks could prove significant, as sheep in certain areas, together with goats are considered an important source of human infections due to their extensive raising and close contact with humans. They have a predisposition to abortion similar to goats when infected, and they shed *C. burnetii* persistently in vaginal secretions, urine and feces, thus continually contaminating the environment [33].

Rickettsia africae was present in ticks infesting all groups of sampled animals and in questing ticks; it was detected in most tick species collected with varying rates of infection. It was also detected from *A. hebraeum* ticks as expected. Although *R. africae* has been detected in *Amblyomma* ticks and in patients from more than 14 African countries [5, 12], evidence is accumulating that tick-borne rickettsioses are underreported and underappreciated causes of illness in sub-Saharan Africa and that most reported cases are the result of an outbreak [35, 38]. Up to 11% of infections have been reported amongst international travelers returning from South Africa [5]. Although this study demonstrated *R. africae* infection in ticks by PCR and DNA sequence analysis, the best way to confirm infection amongst populations is by detecting the pathogen in blood or tissue samples of suspected patients. This remains to be achieved in South Africa, especially amongst endemic populations. It could be speculated from this study, however, that sheep, cattle, horses, goats and some ticks act as reservoirs of this infection in the country and that people may get infected by being unwittingly bitten by ticks questing on vegetation, specifically *A. hebraeum*.

Although there has been speculation regarding *B. burgdorferi* infections in South Africa [10, 40], the causative

agent of the disease in dogs and horses could not be confirmed as *B. burgdorferi*, because sera were not screened against other *Borrelia* that occur in Africa, such as *B. duttoni* and *B. theileri*. The organism amplified in the current study could have easily been any of the pathogens mentioned or something totally different; therefore, further investigation is needed to prove or disprove speculations based on this wealth of anecdotal information.

In conclusion, ticks, as ectoparasites of both humans and animals, play a major role in transmission of various pathogens, including hemoparasites, bacteria and viruses [2, 18, 23], some of which are agents of zoonosis. Lice have also been suspected as playing a role in transmission zoonotic pathogens, such as *Rickettsia* [36]. With the exception of *B. burgdorferi,* most of the targeted pathogens were detected amongst the tick samples collected from ruminants in four South African provinces. The pathogens should be considered as part of routine screening for patients presenting with fevers of unknown origin who have recently been exposed to ticks or livestock. Furthermore, studies concerning all the pathogens detected in this study as well as their vectors should be conducted to characterize and determine their prevalences in the country and factors influencing their epidemiology. Data obtained from this study further highlight the importance of formulating and managing successfully an effective tick control strategy for livestock.

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