Molecular detection of zoonotic rickettsiae and *Anaplasma* spp. in domestic dogs and their ectoparasites in Bushbuckridge, South Africa

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Running Title:

Rickettsial pathogens from dogs, South Africa

Abstract

Members of the order Rickettsiales are small, obligate intracellular bacteria that are vector-borne and can cause mild to fatal diseases in humans worldwide. There is little information on the zoonotic rickettsial pathogens that may be harboured by dogs from rural localities in South Africa. To characterize rickettsial pathogens infecting dogs we screened 141 blood samples, 103 ticks and 43 fleas collected from domestic dogs in Bushbuckridge Municipality, Mpumalanga Province of South Africa between October 2011 and May 2012 using the reverse line blot (RLB) and *Rickettsia* genus and species-specific qPCR assays. Results from RLB showed that 49% of blood samples and 30% of tick pools were positive for the genus-specific probes for *Ehrlichia/Anaplasma*; 16% of the blood samples were positive for *Ehrlichia canis*. Haemoparasite DNA could not be detected in 36% of blood samples and 30% of tick pools screened. Seven (70%) of tick pools and both flea pools were positive for *Rickettsia* spp; three (30%) of tick pools were positive for *R. africae* and both flea pools (100%) were positive for *R. felis*. Sequencing confirmed infection with *R. africae* and *Candidatus* Rickettsia asemboensis; a *Rickettsia felis*-like organism from one of the *R. felis*- positive flea pools. *Anaplasma* sp. South Africa dog strain (closely related to *Anaplasma phagocytophilum*), *A. phagocytophilum*, and an *Orientia tsutsugamushi*-like sequence were identified from blood samples. The detection of emerging zoonotic agents from domestic dogs and their ectoparasites in a rural community in South Africa highlights the potential risk of human infection that may occur with these pathogens.

Keywords:

Introduction

Rickettsioses are a group of infectious diseases caused by bacteria of the order Rickettsiales (Raoult and Roux 1997, Parola, et al. 2005). They are transmitted by arthropod vectors which include ticks, fleas, mites and lice (Kelly, et al. 2002). Rickettsial organisms include members of the spotted fever

group of rickettsiae (SFGR), typhus group of rickettsiae (TGR) and scrub typhus group of orientiae (STGO). *Orientia tsutsugamushi* (formerly known as *Rickettsia tsutsugamushi*) the cause of scrub typhus has previously been considered only endemic to Asia, northern Australia and the Pacific Islands, though serological evidence suggests it or a related Orientia sp. exists in Africa (Parola and Raoult 2006, Thiga et al. 2015). Spotted fever rickettsiosis has been recognized in South Africa since the beginning of the 20th century (McNaught 1911) with *Rickettsia conorii* the agent of Mediterranean spotted fever having long been associated with human disease in the country (Pretorius and Birtles 2002). *Rickettsia aeschlimannii* which causes a Mediterranean spotted fever-like illness has also been identified in South Africa (Beati, et al. 1997). African tick-bite fever (ATBF) caused by *R. africae* is prevalent in South Africa and is the leading cause of fever among travellers to South Africa (Jensenius, et al. 2003). Game hunting and travelling to Southern Africa from November through April increases the risk for ATBF among travellers and contact with tick-infested cattle and game in areas endemic for certain spotted fever group rickettsiae may also increase the risk of disease (Jensenius, et al. 2004). *Rickettsia mongolotimonae* which causes a lymphangitis-associated rickettsiosis has also been reported in South Africa (Pretorius and Birtles 2004). *Rickettsia felis* the cause of flea-borne spotted fever (FBSF) has not yet been reported in South Africa but has been recognized as an emerging pathogen especially in sub-Saharan Africa (Parola 2011).

Other rickettsial agents include *Anaplasma phagocytophilum* from the family Anaplasmataceae which causes canine and human anaplasmosis (Parola and Raoult 2006), and *Ehrlichia canis* which causes canine monocytic ehrlichiosis (CME) and is also responsible for some cases of human ehrlichiosis (Nicholson, et al. 2010). A new strain of *Anaplasma* sp. closely related to *Anaplasma phagocytophilum* has also been detected in canine blood samples in South Africa (Inokuma, et al. 2005). To identify and characterise rickettsial pathogens infecting dogs we used molecular techniques to screen blood samples and ectoparasites collected from domestic dogs in the Mnisi community area, in Mpumalanga Province, South Africa.

Materials and Methods

Study site

The Mnisi community, is situated in the north-eastern corner of the Bushbuckridge Municipal Area, Mpumalanga Province, South Africa; and located at the livestock/wildlife/human interface of the western boundary of the Kruger National Park (KNP). The study area falls within the savannah region and is adjacent to the Andover and Manyeleti game reserves (Figure 1). The geographic coordinates are S -24° 39´, E 31° 20´. A Health and Demographic Surveillance System in Dogs (HDSS-Dogs) was established in the community in 2011. This study sampled dogs from 400 dog-owning compounds that are enrolled in the HDSS project. Further description of the dog population and the HDSS-Dogs can be found in Conan et al. (2015).
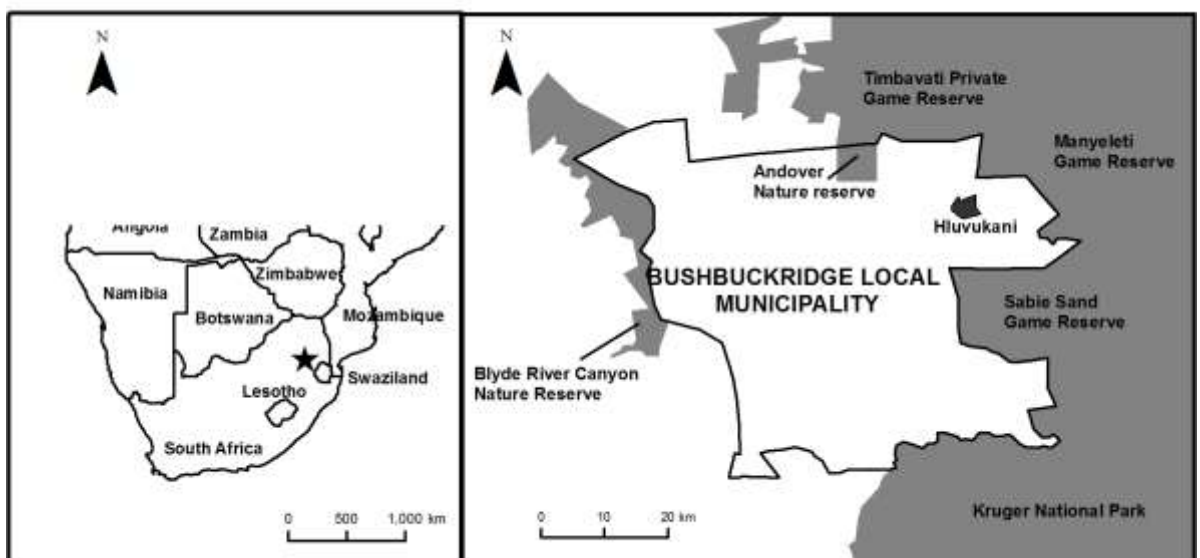

FIG. 1.   Map of study site showing area where dog-owning households are located.

Collection of blood samples

Blood samples were collected from owned, free roaming, apparently healthy dogs present at households visited by the HDSS-Dogs field team, during routine quarterly visits. Convenience sampling of dog-owning households was done from October 2011 to May 2012. During the first two sample collection periods (October and December, 2011), blood was collected directly in capillary

tubes (n=85) and stored on FTA filter paper (Whatman®, USA). During the third period (April/May 2012), blood was collected in EDTA vacutainer tubes (Lasec, South Africa), and thereafter transferred to FTA cards (n=56). Blood samples stored on FTA cards were then sent to the Department of Veterinary Tropical Diseases, University of Pretoria for analysis.

Collection of ectoparasites

Dogs were inspected for the presence of ectoparasites by brushing the hair with a plastic comb or brush and a white paper was used for collection of fleas. Live adult ticks were removed from the animals manually using forceps. A total of 103 ticks and 43 fleas were collected. All ectoparasites were preserved in 70% ethanol and identified to species level under a stereomicroscope, according to standard morphological identification guides (Segerman 1995, Walker, et al. 2003). Ticks and fleas were pooled into 10 pools of ticks and 2 pools of fleas according to their species. Large numbers of ticks from the same species were placed in separate pools, to reduce possible dilution if only a small number of ticks in a pool are positive. The maximum number of ticks in a pool was 14. The tick pools were collected from 64 dogs. Dogs that had more than 1 tick collected from them were 16 in number.

DNA extraction and RLB hybridization assay

DNA was extracted from 141 blood samples spotted on filter cards using the QIAamp DNA mini kit® (QIAGEN) according to the manufacturer's instructions to a final elution volume of 100 µl. The ticks and flea samples were homogenized using a Tissue Lyser® (QIAGEN) and DNA extraction was performed using the QIAamp DNA mini kit® (QIAGEN) according to the manufacturer's instructions. PCR was conducted with a set of primers (Ehr-F and Ehr-R) that amplify the V1 hypervariable region of the 16S rRNA gene of *Ehrlichia* and *Anaplasma* species (Bekker, et al. 2002). The PCR was performed as previously described (Gubbels, et al. 1999) on the Gene Amp® PCR system 9700 (Applied Biosystems). PCR amplicons were then screened using the reverse line blot hybridization assay (RLB) as described by Gubbels, et al. (1999). The *Ehrlichia* and *Anaplasma*, genus-specific and species-specific oligonucleotide probes used for the assay are shown in Table 1.

TABLE 1. Probe sequences for specific detection of parasite species

Genus/Species Target	Probe Sequence 5[1]-3[1]
Ehrlichia/Anaplasma catch all	GGG GGA AAG ATT TAT CGC TA
A. centrale	TCG AAC GGA CCA TAC GC
A. marginale	GAC CGT ATA CGC AGC TTG
A. phagocytophilum	GRA TAR TTA GTG GCA GAC GGG T
E. ruminantium	AGT ATC TGT TAG TGG CAG
A. bovis	CTT GCT ATG AGA AYA ATT AGT GGC
E. chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT
Anaplasma sp. omatjenne	CGG ATT TTT ATC ATA GCT TGC
E. canis	TCT GGC TAT AGG AAA TTG TTA
Neoehrlichia catch-all	GGAATAGCTGTTAGAAATGACAGG
N. mikurensis	CGAACGAATTGTARYTRTAGTTTACT

Detection of rickettsiae

A *Rickettsia* genus-specific assay amplifying a 115 bp fragment of the 17 kDa surface protein gene of *Rickettsia* was used to screen blood and ectoparasites pools. Two species-specific quantitative real-time PCR (qPCR) assays targeting the *ompB* gene of *R. africae* and *R. felis* were used to screen ectoparasite DNA samples as previously described (Jiang, et al. 2012, Maina, et al. 2014, Henry, et al. 2007). Species-specific assays were not performed on blood samples because they were all negative on the *Rickettsia* genus assay. Specific plasmid DNA were used as positive controls for each assay and PCR grade water replaced the DNA template in negative control reactions.

Sequencing and phylogenetic analysis

Five randomly selected samples (97, 98, 107, 115 and T3) that tested positive with genus-specific probes for *Anaplasma*, *Ehrlichia*, and *Rickettsia* spp., by RLB or qPCR assays were further analyzed by DNA sequencing (INQABA Biotechnologies, South Africa) to determine specific spp. For sequencing reaction the same primers used for PCR amplification were used, except that they had no biotin incorporated. Furthermore, three other samples (106, 116 and 125) were randomly selected to identify specific *Anaplasma* and *Ehrlichia* infections. These samples which had strong signals on the RLB assay for *Anaplasma* and *Ehrlichia* were selected for next-generation sequencing (NGS)

using DNA barcoding. The tick pool T8 which was positive for *Rickettsia* spp. but negative on the species assay was also selected, as well as F1 which was selected as a representative pool from the flea samples, were analysed using the similar approach. For NGS using DNA bar coding, the 16S rRNA gene was amplified from genomic DNA of selected samples using universal primers 341F and 785R, modified with Illumina specific adapters (Klindworth, et al. 2012). Sequencing was performed on the Illumina's MiSeq platform using a MiSeq v3 kit.

The 16S rRNA sequences obtained from PCR amplicons were prepared for assembly using the PreGap4 program of the Staden package (version 2.0 for Windows) and BLAST searches were performed using MegaBlast from the Basic Local Alignment Search Tool (BLAST) (http://www.ncbi.nlm.nih.gov/BLAST). Phylogenetic relationships were inferred using the neighbour-joining method with MEGA version 6 (Tamura, et al. 2013). High throughput NGS data from the Illumina pipeline was analyzed using CLC Genomics Workbench 5.1 and assembled using the *de novo* assembly algorithm of the CLC workbench, to create simple sequence contigs which were then updated based on mapped reads. BLAST searches were performed to detect the identity of sequence contigs. Sequence alignments and phylogenetic analyses were done as described above.

GenBank accession numbers

The sequences of the molecular isolates (97, 98, 107, 115, T3, 125, F1, 106,116, and T8) obtained from blood samples, *Rhipicephalus sanguineus*, *Ctenocephalides felis strongylus* and *Haemaphysalis elliptica* pools have been submitted to GenBank with accession numbers KP823591-KP823600 for 16S rRNA gene.

Results

Detection of hemoparasites in blood

The results of the RLB hybridization analysis for DNA prepared from 141 blood samples spotted on FTA filter cards showed the presence of *Ehrlichia* and *Anaplasma*, species detected either

as single or mixed infections. *Ehrlichia/Anaplasma* species was detected in 70 (50%) samples,

Ehrlichia canis was detected in 23 (16%) samples and 51 (36%) samples were negative.

Detection of hemoparasites from ticks and fleas

The tick species collected included *Haemaphysalis elliptica* (n=30, pools T1-T3),

Amblyomma hebraeum (n=27, pools T4 & T5), *Rhipicephalus sanguineus* (n=27, pools T7 & T8),

Rhipicephalus simus (n=18, pools T9 & T10), and one unspeciated *Ixodes* (T6). The fleas included

Ctenocephalides felis strongylus (n=23, pool F1) and *Echidnophaga gallinacea* (n=20, pool F2). The

results of the RLB hybridization analysis of DNA from pooled tick and flea samples also showed the

presence of *Ehrlichia* and *Anaplasma* species. Table 2 shows results of RLB analysis of ectoparasites.

TABLE 2. RLB hybridization assay results from ticks and fleas

Sample number	Ectoparasite	Species name	Haemoparasite detected by RLB analysis
T1	Tick	*Haemaphysalis elliptica*	Negative
T2	Tick	*H. elliptica*	Negative
T3	Tick	*H. elliptica*	*Ehrlichia/Anaplasma* genus-specific probe
T4	Tick	*Amblyomma hebreum*	Negative
T5	Tick	*A. hebreum*	*Ehrlichia/Anaplasma, E. ruminantium*
T6	Tick	*Ixodes*	Negative
T7	Tick	*Rhipicephalus sanguineus*	Negative
T8	Tick	*R. sanguineus*	*Ehrlichia/Anaplasma, Neoehrlichia* genus-specific probe
T9	Tick	*Rhipicephalus simus*	Negative
T10	Tick	*R. simus*	Negative
F1	Flea	*Ctenocephalides felis strongylus*	Negative
F2	Flea	*Echidnophaga gallinacea*	Negative

Detection of *Rickettsia* by qPCR

DNA samples from blood and ectoparasites were subjected to a *Rickettsia* genus-specific qPCR assay

for detection of rickettsial infections prior to testing with species-specific qPCR assays which identify

R. africae and *R. felis* DNA. *Rickettsia* DNA was not detected from any of the DNA samples prepared

from blood when using the *Rickettsia* genus-specific assay. By contrast, 7/10 (70%) tick pools and 2/2

(100%) flea pools tested positive for *Rickettsia* DNA using the same assay (Table 3). Analysis with

species-specific qPCR assays revealed that 3/10 (30%) tick pools were positive for *R. africae* and 2/2

(100%) flea pools positive for *R. felis*. Rickettsial DNA was not detected in three tick pools (T1, T2

&T7). The minimum infection rate (MIR) which assumes that a positive pool contains a single infected pool (Cowling, et al. 1999) was calculated for the positive *H. elliptica* pool as 3.3%, MIR for T4 an *A. hebraeum* pool was 7.7%, MIR for T5 the second *A. hebraeum* pool was 7.1%, MIR for *R. sanguineus* pool T8 was 3.6% and MIR for *R. simus* pools (T9 and T10) was 11.1%.

TABLE 3. Summary of qPCR *Rickettsia* assays

| Sample ID	Sample type	Species	*Rickettsia* genus assay	*R. africae* assay	*R. felis* assay
1-141	Blood	Canine	-	Not tested	Not tested
T1	Tick pool (n=10)	*Haemaphysalis elliptica*	-	-	-
T2	Tick pool (n= 10)	*Haemaphysalis elliptica*	-	-	-
T3	Tick pool (n=10)	*Haemaphysalis elliptica*	+	+	-
T4	Tick pool (n=13)	*Amblyomma hebraeum*	+	+	-
T5	Tick pool (n=14)	*Amblyomma hebraeum*	+	+	-
T6	Sample (n=1)	*Ixodes* spp.	+	-	-
T7	Tick pool (n=13)	*Rhipicephalus sanguineus*	-	-	-
T8	Tick pool (n=14)	*Rhipicephalus sanguineus*	+	-	-
T9	Tick pool (n=9)	*Rhipicephalus simus*	+	-	-
T10	Tick pool(n=9)	*Rhipicephalus simus*	+	-	-
F1	Flea pool (n=23)	*Ctenocephalides felis strongylus*	+	-	+
F2	Flea pool (n=20)	*Echidnophaga gallinacea*	+	-	+

+: qPCR positive

-: qPCR negative
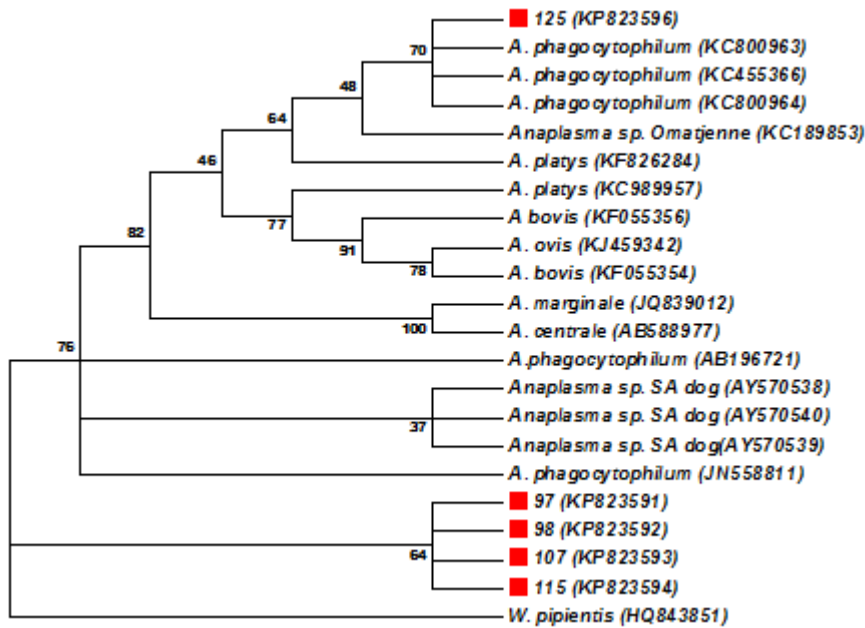
Sequence and phylogenetic analysis

Analysis of the 16S rRNA gene revealed that a sequence obtained from DNA of *H. elliptica* pool (T3) was 99% (461/466) similar to *R. africae* and was placed in the same clade as *R. africae* 95.2 (accession number: JF949783) (Figure 2). The sequence from *R. sanguineus* pool (T8) was found to have 96% (291/303) sequence identity to *R. peacockii* (NR118837) and was 95.7% (290/303) similar to other rickettsiae. This sequence (T8) was placed between the typhus group of rickettsiae and the scrub typhus group of orientiae. Analysis showed that the sequence of one of the positive flea pools (F1) (264bp) and blood sample 106 (265 bp) were identical and portrayed 100% sequence identity with *Ca.* R. asemboensis (JN315973 and JN315967). The sequence of blood sample 116 identified *O. tsutsugamushi* with 96.1% (247/257) homology and was 95.7% (246/257) similar to the closest/other

rickettsiae. This sequence was placed in the same clade as *O. tsutsugamushi* Kawasaki strain

(accession number: D38625) (Figure 2).
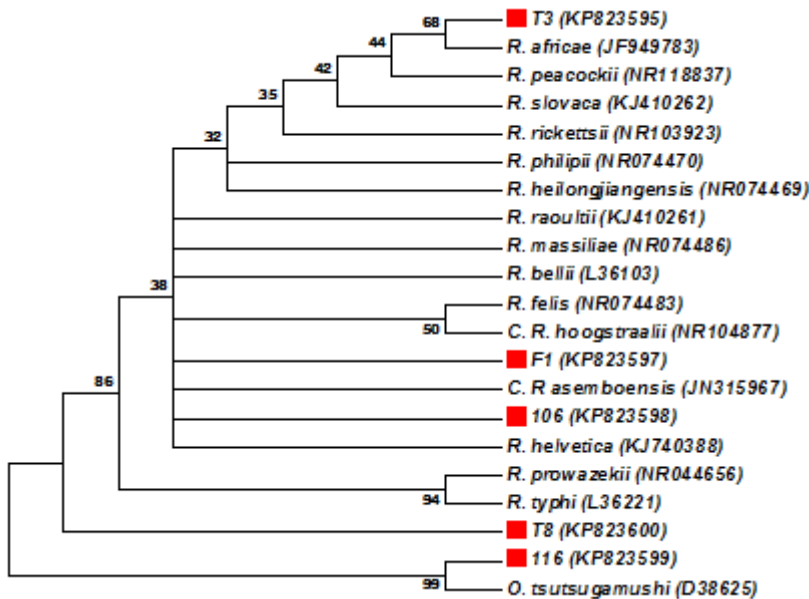
FIG. 2. **Neighbor-joining phylogenetic tree of the 16S rRNA gene sequences of *Rickettsia* species generated from this study together with homologous sequences from GenBank. Bootstrap analyses were performed with 1000 replications (MEGA software version 6). *Gray square* indicates sequences obtained from the study.**

Phylogenetic analyses of the *Anaplasma* species 16S rRNA sequences from blood samples

97, 98, 107 and 117 showed that these sequences share 99% similarity with *Anaplasma* sp. South

Africa dog 1076, 1108 and 1245 (accession numbers: AY570539, AY570538 and AY570540) and

clustered together with the latter (Figure 3). The sequence of blood sample 125 had a 99% identity to

sequences of *A. phagocytophilum* strains ApGDr1, ApGDr2 and GDR4 (accession numbers:

KC800963, KC800964 and KC455366) and was placed in the same clade with *A. phagocytophilum*

species (Figure 3).

FIG. 3. **Neighbor-joining phylogenetic tree of the 16S rRNA gene sequences of *Anaplasma* species generated from this study together with homologous sequences from GenBank. Bootstrap analyses were performed with 1000 replications (MEGA software version 6).** *Gray square* **indicates sequences obtained in the study.**

Discussion

We detected *R. africae* in *A. hebraeum* and *H. elliptica* ticks tested with the *R. africae*-specific qPCR assay; this is consistent with previous molecular studies in Africa where the organism was detected in Chad, Burundi, Ethiopia, Senegal and in southern Africa (Jensenius, et al. 2003). *Rickettsia africae* is the cause of African tick-bite fever (ATBF), an emerging infectious disease transmitted by ticks (Jensenius, et al. 2003). Many cases of human infections with *R. africae* have been acquired from South Africa which has many wildlife tourist centers usually situated in areas in which tick vectors are endemic (Chmielewski, et al. 2013). In South Africa, *R. africae* is transmitted mainly by *A. hebraeum* (Jensenius, et al. 2003). Other tick species have been found to harbour *R. africae* (Mediannikov, et al. 2012, Parola, et al. 2001) however this is the first time that the organism has been detected in *H. elliptica* thereby extending the known host range of the organism. The detection of *R. africae* from *A. hebraeum* a tick known for readily biting humans and *H. elliptica* one

of the most common ticks infesting domestic dogs in South Africa underpins the potential risk of human infection with this pathogen in the study area.

This is the first report of members of the *R. felis* clade being detected in South Africa. *Rickettsia felis* is an emerging zoonotic agent that has been found in several countries globally causing flea-borne spotted fever (FBSF) in humans (Parola 2011). The cat flea *C. felis* is the only biological vector and reservoir of *R. felis* so far identified (Reif and Macaluso 2009). In Africa *R. felis* has been detected in humans and arthropods in Gabon, Tunisia, Egypt, Congo, Algeria (Parola 2011) and Kenya where substantial work on the organism has been carried out (Jiang, et al. 2013). Sequence analysis of one of the flea pools (F1) positive for *R. felis* on the species-specific qPCR assay however revealed a 100% homology to *Ca.* R. asemboensis, a newly described rickettsiae and a *R. felis*-like organism (RFLO) previously identified from fleas in Kenya (Jiang, et al. 2013). In that study *Ca.* R. asemboensis was detected in 60% of flea pools tested. Detection of *Ca.* R. asemboensis in pool F1 in our study shows that the *R. felis* species-specific qPCR assay (Henry, et al. 2007) is not specific to *R. felis* but is able to detect other RFLOs. We found the same organism in a dog blood sample (sample106) - the first time that this putative new species has been detected in a canine host. Recently, this agent has been detected in the blood of cynomolgus monkeys without signs of infection (Tay et al. 2015). Sample T6 (*Ixodes* sp.) and tick pools T9 and T10 (*Rhipicephalus simus*) were positive with the *Rickettsia* genus-specific assay but negative on the two species-specific assays used, implying that they could be positive for species of *Rickettsia* other than *R. africae* or *R. felis*. Sequencing would have to be performed in follow up studies to determine the specific species. *Ixodes ricinus* is a known vector and reservoir host of *R. helvetica* (Parola, et al. 2005) while *Rhipicephalus simus* is more known as a vector of *Anaplasma marginale* and *A. centrale* (Potgieter, 1983).

Sequence analysis of the 16S ribosomal RNA gene using high throughput sequencing technology detected rickettsiae sequences in two dog blood samples. The samples were positive for the *Ehrlichia*/*Anaplasma* genus-specific probe only on the RLB hybridisation assay but were negative on the *Rickettsia* genus-specific qPCR assay based on the 17-kD antigen gene (Jiang, et al. 2012). This means that the qPCR assays used in this study may not be as sensitive in detecting *Rickettsia* spp.

as the 16S rRNA gene high throughput sequencing technology using the Illumina platform. The *Rickettsia* qPCR assays (Jiang, et al. 2012, Maina, et al. 2014, Henry, et al. 2007) are able to detect 3-10 copies of the target DNA fragment per reaction whereas NGS, using the MiSeq Illumina platform, is more sensitive because of its high sequencing depth of about 1.6 gigabases per run/60 mega bases per hour so the detection threshold is far above the qPCR assays, especially in cases of multiple pathogen infections. The inability to amplify rickettsial DNA from blood samples collected from dogs using the *Rickettsia* genus-specific qPCR and species-specific qPCR assays could also be attributed to several factors. It is possible that rickettsial DNA was present in the samples tested but at a copy number that was below the detection limits of the assays used (Hawley, et al. 2007). Rickettsial DNA may be cleared from the blood of the dogs by a fast and effective immune response as has been hypothesized by (Bayliss, et al. 2009). It is also possible that the dogs were infected with rickettsial pathogens but the organisms were enclosed in other tissues like the vascular endothelium, dermis or spleen (Hawley, et al. 2007).

An agent closely related to *O. tsutsugamushi* strain Kawasaki was detected in a dog blood sample. *Orientia tsutsugamushi* causes scrub typhus a febrile disease transmitted by larval stage mites *Leptotrombidium akamushi* and *L. deliense* commonly called "chiggers" (Parola and Raoult 2006). The disease occurs in the Asia-Pacific region of the world which includes the north of Australia, India, Korea, Japan, Papua New Guinea and Pakistan. However two recent reports indicate that scrub typhus occurs outside this region, one case occurred in a patient visiting UAE and Chile (Izzard, et al. 2010, Balcells, et al. 2011). Moreover, there have been reports suggesting that scrub typhus occurs in Africa (Ghorbani, et al. 1997, Osuga, et al. 1991) with the most recent report from Kenya (Thiga, et al. 2015). Infected mites serve as the vector and reservoir of this organism, though the vector/host for the *Orientia* spp. detected in scrub typhus patients from UAE (*Orientia chuto)* and Chile (*Orientia* sp.), is unknown (Izzard, et al. 2010, Balcells, et al. 2011). Clinical signs of the infection in humans include fever, headaches, maculopapular rash, eschar, lymphadenopathy and neurological signs, cough and interstitial pneumonia in some cases (Parola and Raoult 2006). There is a recent case report of *O. tsutsugamushi* infection in a lethargic dog in an area of Japan showing that dogs can be naturally

infected and may play a role as a host of the organism (Namikawa, et al. 2014). To our knowledge this is the first report of an *O. tsutsugamushi*-like organism in South Africa.

A *Rickettsia peacockii*-like agent was detected in the 16S rRNA gene sequence obtained from the *R. sanguineus* pool with a 96% sequence identity to *R. peacockii* and 95.7% similarity to other rickettsiae. *Rickettsia peacockii* first detected in *Dermacentor andersoni* ticks in Montana, USA is generally considered to be a non-pathogenic spotted fever group rickettsiae and is mainly transmitted transovarially from one tick generation to the next (Simser, et al. 2001). An endosymbiont of *D. andersoni* ticks, its presence in the tick is related to the reduced prevalence of *Rickettsia rickettsii* the cause of Rocky Mountain spotted fever in dogs and humans in the Americas (Felsheim, et al. 2009). Phylogenetic analysis of 16S rRNA gene sequence obtained from the *R. sanguineus* tick pool against other homologous rickettsial sequences published from the GenBank showed that the sequence was placed between *O. tsutsugamushi* and a SFGR but in a separate clade, suggesting that it may potentially be a new species of *Rickettsia*. Further characterization will however be needed to determine if this is indeed a new species of *Rickettsia* and to determine whether this organism may be pathogenic to dogs and/or humans.

The results of the RLB hybridization assay showed that 49% of the blood samples and 30% of tick pools were positive for the genus-specific probes of *Ehrlichia*/*Anaplasma* species. Sequence analysis of the 16S ribosomal RNA gene of four blood samples positive for the *Ehrlichia*/*Anaplasma* genus-specific probes revealed a 99% sequence homology to an *Anaplasma* sp. (South African dog strain) closely related to *A. phagocytophilum* (Inokuma, et al. 2005). In another blood sample positive for *Ehrlichia*/*Anaplasma* genus-specific probes, next-generation sequence analysis of the 16S rRNA gene revealed a 99% sequence identity to *A. phagocytophilum* strains ApGDr1 (KC800963), ApGDr2 (KC800964) and GDR4 (KC455366). *Anaplasma phagocytophilum* was first detected in dogs in the United States in the 1980s (Madewell and Gribble 1982) with several genetic variants subsequently described based on molecular typing. *Ixodes* spp. ticks are the vectors implicated for transmitting *A. phagocytophilum* (Woldehiwet 2010). However, in this study only one out of 103 ticks collected from domestic dogs was identified as an unspeciated *Ixodes*. This suggests that other genera of ticks may be

responsible for the transmission of *Anaplasma* spp. to dogs in Bushbuckridge. *Anaplasma*

phagocytophilum is a known zoonotic pathogen causing human anaplasmosis (formerly known as

human granulocytic ehrlichiosis) with disease in humans characterized by fever, myalgia, headache,

an increase in liver function enzymes, thrombocytopenia and disorientation *(*Bakken and Dumler

2008). *Anaplasma* spp. are transmitted to dogs and humans by tick vectors (Nicholson, et al. 2010).

The diseases caused by *Anaplasma* spp. can be prevented by effective tick control coupled with

awareness of tick and tick bite prevention (Nicholson, et al. 2010). Doxycycline is the drug of choice

for treatment of *A. phagocytophilum* infections (Nicholson, et al. 2010). In the present study, almost

half of the samples were positive for *Anaplasma* spp. as confirmed by sequencing. The finding of *A.*

phagocytophilum suggests a potential risk of human infection with this rickettsial pathogen in the

study area.

Conclusions

The detection of rickettsial agents *Ca.* R. asemboensis and an *O. tsutsugamushi*-like agent in

canine blood suggests that dogs may play a role in the life cycle of rickettsiae. Our study provides

preliminary information about the occurrence of zoonotic rickettsiae in domestic dogs and their

ectoparasites in a South African rural community and highlights the potential risk of human infection

with these pathogens. Further work is needed to characterize *Anaplasma* sp. South Africa dog strain

and *Ca.* R. asemboensis to determine their pathogenic potential.

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