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Short communication

Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa

Paul Tshepo Matjila [a,*], Andrew L. Leisewitz [a], Frans Jongejan [a,b], Barend L. Penzhorn [a]

[a] *Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria, Private Bag x04, 0110 Onderstepoort, South Africa*

[b] *Utrecht Centre for Tick-borne Diseases (UCTD), Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL, Utrecht, The Netherlands*

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Abstract

A total of 1138 blood specimens were collected over a 6-year period (2000–2006) from domestic dogs in South Africa. Specimens from domestic dogs were obtained from the Onderstepoort Veterinary Academic Hospital (OVAH) in Pretoria, the Society for the Prevention of Cruelty to Animals (Johannesburg, Durban, East London and Bloemfontein) and private practices from four provinces (Gauteng, Mpumalanga, KwaZulu-Natal and Western Cape). All specimens were screened for *Babesia*, *Theileria*, *Hepatozoon* and *Ehrlichia/Anaplasma* species using PCR and Reverse Line Blot (RLB) assays. On RLB, 560/1137 domestic dog-specimens were positive for one or more parasites. Of the positive domestic dog-specimens, 420 (75%) were infected with *Babesia rossi*; 82 (15%) dogs were infected with *Theileria* sp. (dog); 18 (3%) dogs were infected with *Babesia vogeli*; 14 (3%) specimens were infected with *Ehrlichia canis*. Mixed infections were also found: *B. rossi* and *E. canis* were detected in 12 (2%) specimens; *B. vogeli* and *E. canis* occurred in 7 (1%) specimens; *Theileria* sp. (dog) and *E. canis* in 3 (0.5%) specimens; *B. rossi* and *B. vogeli* in one specimen. *B. rossi, B. vogeli* and *E. canis* occurred simultaneously in one dog. There was also one incidental finding of a dog positive for *Trypanosoma congolense*. The results indicate that a wide range of tick-borne pathogens are circulating in the canine populations in South Africa.

Keywords: Dogs; *Babesia*; *Ehrlichia*; RLB; South Africa

1. Introduction

Ticks transmit a wide variety of pathogens, including protozoa, bacteria and viruses. The most important tick-transmitted infections that cause severe clinical illness in South African dogs are babesiosis (Collett, 2000) and ehrlichiosis (Rautenbach et al., 1991; Van Heerden, 1982). *Babesia* parasites of dogs can be grouped into two groups based on morphology (Kuttler, 1988): the large babesias, known as the *Babesia canis* group (Uilenberg et al., 1989) and the small babesias, which include the *Babesia gibsoni* group, *Babesia conradae* and *Theileria annae* (Kjemtrup et al., 2000; Kjemtrup et al., 2006; Zahler et al., 2000). The large babesias of dogs have a wide distribution which includes South Africa (Uilenberg et al., 1989), while the small babesias of dogs occur in South-East Asia, North-East Africa, Spain and the USA (Kjemtrup et al., 2000, 2006; Zahler

* Corresponding author. Tel.: +27 125298424; fax: +27 125298312.
E-mail address: tshepo.matjila@up.ac.za (P.T. Matjila).

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et al., 1998). Canine babesiosis is an economically important disease of dogs in South Africa (Collett, 2000), hence the main focus of our study was to screen blood specimens for *Babesia* sp. infections.

Ehrlichia and/or *Anaplasma* species infecting humans and dogs have also been documented in South Africa (Pretorius and Kelly, 1998; Pretorius et al., 1999) although the results of these reports were based only on serological diagnostic assays. It is currently unknown whether the South African domestic dog population carries tick-borne infectious agents of human importance, which can be detected using molecular techniques. To date, we are aware of one report where a new *Anaplasma* species closely related to *Anaplasma phagocytophilum* was detected from canine blood (Inokuma et al., 2005). Therefore, the second focus of this report was to search for zoonotic *Ehrlichia* and *Anaplasma* species and thirdly, to evaluate the degree of co-infection with multiple tick-borne pathogens. Additionally, we aimed to identify tick-borne pathogens that were incidental or previously unknown pathogens of dogs.

2. Materials and methods

2.1. Collection of specimens

A total of 1138 domestic-dog blood specimens were collected from four inland provinces (Gauteng, North-West, Mpumalanga and Free State) and three coastal provinces (Kwa-Zulu-Natal, Eastern Cape and Western Cape) out of the nine provinces of South Africa (Table 1). Except for specimens from Mpumalanga, which were collected from known *Babesia*-positive animals, all specimens were collected randomly from domestic dogs. Blood specimens were obtained from dogs at the Onderstepoort Veterinary Academic Hospital (OVAH), the Society of the Prevention of Cruelty to Animals (SPCA) and private veterinary clinics. The OVAH is situated in the northern part of Gauteng, close to the North-West Province border. Therefore, blood specimens collected from OVAH could also represent specimens from North-West Province.

2.2. DNA extraction

DNA was extracted from 200 μl of each blood specimen. The QIAmp blood and tissue extraction kit (Qiagen, Hilden, Germany) was used for DNA extractions, following the manufacturer's protocols.

2.3. PCR

The *Babesia/Theileria/Hepatozoon* PCR was performed with primers RLB-F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′) and RLB-R2 (biotin-5′-CTA AGA ATT TCA CCT CTG ACA GT-3′) amplifying a fragment of 460–540 bp from the 18S rRNA gene spanning the V4 region (Gubbels et al., 1999; Matjila et al., 2004). The *Ehrlichia/Anaplasma* PCR was performed with the forward primer Ehr-F (5′-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3′) and Ehr-R (5′-biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3′) amplifying a fragment of 460–520 bp from the V1 hypervariable region of the 16S SSU rRNA gene (Bekker et al., 2002; Nijhof et al., 2005). The conditions for the PCR included an initial step of 3 min at 42 °C, 10 min at 94 °C, 10 cycles of 94 °C (20 s)–67 °C (30 s)–72° C (30 s), with lowering of annealing step after every second cycle with 2 °C (touchdown PCR). The reaction was then followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at

Table 1
Provinces and their localities in South Africa of areas where blood specimens of domestic dogs were collected

Localities	Gauteng and North West	Mpumalanga	Free State	KwaZulu-Natal	Eastern Cape	Western Cape	Total number of samples
OVAH	527						527
Johannesburg SPCA	90						90
White River private clinic		38					38
Bloemfontein SPCA			129				129
Durban SPCA				56			56
Durban private practice				4			4
Pietermaritzburg private practice				129			129
Hlhluwe private practice				1			1
East London SPCA					54		56
Cape Town private practice						47	47
Total numbers of samples	617	38	129	253	54	47	1138

Table 2
List of organisms and their corresponding probe sequences used to detect pathogen DNA

Anaplasma centrale	TCG AAC GGA CCA TAC GC
Anaplasma marginale	GAC CGT ATA CGC AGC TTG
Anaplasma ovis	ACC GTA CGC GCA GCT TG
Anaplasma phagocytophilum 1	TTG CTA TAA AGA ATA ATT AGT GG
Anaplasma phagocytophilum 3	TTG CTA TGA AGA ATA ATT AGT GG
Anaplasma phagocytophilum 5	TTG CTA TAA AGA ATA GTT AGT GG
Anaplasma phagocytophilum 7	TTG CTA TAG AGA ATA GTT AGT GG
Ehrlichia/Anaplasma catch-all	GGG GGA AAG ATT TAT CGC TA
Ehrlichia canis/Ehrlichia ovina	TCT GGC TAT AGG AAA TTG TTA
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG
Ehrlichia sp. (*Omatjenne*)	CGG ATT TTT ATC ATA GCT TGC
Hepatozoon catch-all	GCT TTG TAA TTG GAA TGA TAG A
Theileria/Babesia catch-all	TAA TGG TTA ATA GGA RCR GTT G
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG
Theileria annulata	CCT CTG GGG TCT GTG CA
Theileria bicornis	GCG TTG TGG CTT TTT TCT G
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT
Theileria catch-all	ATT AGA GTG CTC AAA GCA GGC
Theileria equi	TTC GTT GAC TGC GYT TGG
Theileria parva	GGA CGG AGT TCG CTT TG
Theileria sp. (*buffalo*)	CAG ACG GAG TTT ACT TTG T
Theileria sp. (*duiker*)	CAT TTT GGT TAT TGC ATT GTG G
Theileria sp. (*kudu*)	CTG CAT TGT TTC TTT CCT TTG
Theileria sp. (*sable*)	GCT GCA TTG CCT TTT CTC C
Theileria taurotragi	TCT TGG CAC GTG GCT TTT
Theileria velifera	CCT ATT CTC CTT TAC GAG T
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG
Babesia caballi	GTG TTT ATC GCA GAC TTT TGT
Babesia canis	TGC GTT GAC CGT TTG AC
Babesia canis 2	TGG TTG GTT ATT TCG TTT TCG
Babesia catch-all 1	ATT AGA GTG TTT CAA GCA GAC
Babesia catch-all 2	ACT AGA GTG TTT CAA ACA GGC
Babesia felis	TTA TGC GTT TTC CGA CTG GC
Babesia gibsoni Japan	TAC TTG CCT TGT CTG GTT T
Babesia gibsoni USA	CAT CCC TCT GGT TAA TTT G
Babesia microti	GRC TTG GCA TCW TCT GGA
Babesia ovis	TGC GCG CGG CCT TTG CGT T
Babesia rossi	CGG TTT GTT GCC TTT GTG
Babesia vogeli	AGC GTG TTC GAG TTT GCC

57 °C for 30 s and extension at 72 °C for 30 s. The PCR and restriction fragment length polymorphism assay (RFLP) for the species identification of the *Trypanosoma*-infected specimen was done as described by Delespaux et al. (2003). The amplification for this nested PCR was first done on the 18S gene using the forward primer 18STnF2 (CAACGATG-ACACCCATGAATTGGGGA) and 18STnR3 (TGCGCGACCAA-TAATTG-CAATAC) (Geysen et al., 2003). The second amplification was done using the forward primer 18STnF2 (CAACGATG-ACACCCATGAATTG-GGGA) of the first amplification with the reverse primer 18STnR2 (GTGTCTTGTTCTCACTGA-CATTGTAGTG). Nested products were then analysed using the RLFP for species identification as described by Delespaux et al. (2003).

2.4. Reverse line blot hybridisation

RLB was subsequently conducted on amplified products (*Babesia*, *Theileria*, *Hepatozoon*, *Anaplasma* and *Ehrlichia*) as previously described (Matjila et al., 2004). The list of probes and their sequences used for detecting pathogen DNA are listed in Table 2.

Table 3
Pathogen species detected from domestic dogs using the RLB

Pathogen	OVAH[a] (*n* = 527)	Southern Gauteng (*n* = 90)	Mpumalanga (*n* = 38)	Free State (*n* = 129)	KwaZulu-Natal (*n* = 253)	Eastern Cape (*n* = 54)	Western Cape (*n* = 47)
B. rossi	345	2	36	7	16	12	2
B. vogeli	5	–	–	13	–	–	–
E. canis	12	–	–	1	1	–	–
Theileria sp. dog	3	–	–	–	79	–	–
B. gibsoni	–	1	–	–	–	–	–
B. rossi and *E. canis*	8	1	1	–	1	–	1
B. vogeli and *E. canis*	7	–	–	–	–	–	–
Theileria sp. dog and *E. canis*	–	–	–	–	3	–	–
B. rossi and *B. vogeli*	1	–	–	–	–	–	–
B. rossi, *B. vogeli* and *E. canis*	1	–	–	–	–	–	–
T. congolense	–	–	–	–	1	–	–
Total number of positives	382 (72%)	4 (4%)	37 (97%)	21 (16%)	101 (40%)	12 (22%)	3 (6%)

[a] Onderstepoort Veterinary Academic Hospital (Northern Gauteng/North-West Province).

3. Results

The majority of specimens collected from OVAH (382/527; 72%) were infected with at least one pathogen (Table 3). Most specimens (65%) from this area were infected with *Babesia rossi*. A newly identified species (*Theileria* sp. dog) was detected in three specimens (Matjila et al., 2008). Mixed infections were also detected: eight specimens were co-infected with *B. rossi* and *Ehrlichia canis*; seven specimens were co-infected with *Babesia vogeli* and *E. canis*; one specimen was co-infected with *B. rossi* and *B. vogeli* and one specimen had a triple infection of *B. rossi*, *B. vogeli* and *E. canis*.

Almost all of the specimens collected from Mpumalanga (97%) were positive for *B. rossi*. Forty percent of specimens collected from KwaZulu-Natal were positive for at least one parasite. Sixteen specimens from this area were infected with *B. rossi*, 1 specimen with *E. canis* and 79 specimens with *Theileria* sp. dog. Mixed infections were detected in four specimens, one of which was co-infected with *B. rossi* and *E. canis* and three specimens were co-infected *Theileria* sp. dog and *E. canis*. One specimen collected from the northern part of KwaZulu-Natal (Hluhluwe) was confirmed positive for *Trypanosoma congolense*. Twenty-two percent of specimens collected from the Eastern Cape were positive, with only 12 infected *B. rossi* specimens. Sixteen percent of specimens collected from the Free State were positive: 7 specimens were *B. rossi* positive, 13 were *B. vogeli* positive and only 1 specimen was *E. canis* positive. Mixed infections were not detected in specimens collected from this area. Only 6% of specimens collected from Western Cape were positive: two specimens were positive for *B. rossi* and one specimen was co-infected with *B. rossi* and *E. canis*. Finally, only 4% of specimens collected from southern Gauteng were positive: two specimens were *B. rossi* positive and one specimen was co-infected with *B. rossi* and *E. canis*. We were also able to detect *B. gibsoni* in a specimen collected at a private practice in southern Gauteng from a pit-bull pup recently imported into South Africa (Matjila et al., 2007). No *Hepatozoon* infections were detected in domestic dogs.

4. Discussion

Specimens obtained from the OVAH were infected with a wide variety of pathogens. Our findings seem to correlate with a study on ixodid ticks collected from *B. canis*-infected dogs presented at OVAH (Horak, 1995). A majority of *Babesia*-infected dogs (41%) were only infested with *Haemaphysalis elliptica* (previously regarded as synonymous with *Haemaphysalis leachi*) (Apanaskevich et al., 2007), the only known vector of *B. rossi* (Lewis et al., 1996). An additional 35% of dogs were infested with *H. elliptica* as well as other ticks species. Based on the current understanding, the high incidence of *B. rossi* infections in our sampled dogs correlates with the high incidence of the tick-vector collected from dogs with *Babesia* infections presented at OVAH. In the study by Horak (1995), *Rhipicephalus sanguineus* ticks were collected from 15% of the dogs as pure infestations and a further 22% of dogs had mixed infestations including *R. sanguineus*. Since *R. sanguineus* is the known vector of *E. canis* (Groves et al., 1975) and *B. vogeli* (Uilenberg et al., 1989), it came as no surprise that *E. canis* and *B. vogeli* were

detected, albeit in less than 3% of our sampled dogs. *R. sanguineus* and *H. elliptica* have overlapping distributions and have been collected from the same host (Horak, 1995). Since these ticks transmit *B. vogeli*/*E. canis* and *B. rossi*, respectively, it follows that we were able to detect mixed infections of *B. rossi* and *E canis* in eight dog specimens and *B. vogeli* and *E. canis* in seven dog specimens. It remains unclear, however, why there were so few dogs co-infected with both *B. vogeli* and *B. rossi*. We detected only one specimen co-infected with *B. rossi* and *B. vogeli* and we also detected only one specimen co-infected with *B. rossi*, *B. vogeli* and *E. canis*. Of interest was the detection of a *Theileria* species from three specimens collected at OVAH, as we are not aware of any *Theileria* species ever having been isolated from dogs in South Africa (Matjila et al., 2008).

B. rossi was the common species detected from all sampled areas. The specimens collected from Mpumalanga were 97% *B. rossi* positive, as sampling in this area was biased to only *Babesia*-positive animals based on blood smear examination. We also detected co-infections of *B. rossi* and *E. canis* in all our sampled areas except in the Free State and Eastern Cape. This could indicate that *H. elliptica* and *R. sanguineus* have overlapping distribution and also feed on the same hosts in those areas where *B. rossi* and *E. canis* occurred as co-infections. Currently, *B. vogeli* infections have only been detected in specimens collected from Free State and OVAH (Matjila et al., 2004), which implies that *B. vogeli* infections are not as widely spread as *B. rossi* infections in South Africa. The same *Theileria* species isolated from 3 specimens collected at OVAH was detected in 79 specimens collected from Pietermaritzburg, KwaZulu-Natal. This species was also isolated in three specimens co-infected with *E. canis*. The vector of this *Theileria* species is still unknown. Additionally, an incidental *T. congolense* infection was detected from a specimen collected at Hluhluwe, northern KwaZulu-Natal. A recent report indicated that *Trypanosoma* infections isolated from cattle and buffalos are prevalent in the Hluhluwe-iMfolozi area (Van Den Bossche et al., 2006). To our knowledge this is the first report of a confirmed *T. congolense* infection from a dog originating from Hluhluwe.

Currently, the two tick-transmitted *Ehrlichia*/*Anaplasma* species known to cause human disease are *Ehrlichia chaffeensis* (Dumler et al., 1995; Paddock et al., 1997) and *Anaplasma phagocytophilum* (Wormser et al., 2006). Our RLB assay had probes that could detect and differentiate between the two zoonotic species (Table 2), but our results were negative. The reported detection of *E. chaffeensis* in dogs and in a human being from Bloemfontein (Pretorius et al., 1999) was based on serological assay and not on the detection of parasite DNA. Serological cross-reactivity between *Ehrlichia* infections is known to occur (Parola et al., 2001) which suggests that molecular assays should be used to support serological evidence. On the other hand, a species closely related to *A. phagocytophilum* has been identified from three dog specimens in South Africa (Inokuma et al., 2005). Perhaps if more dogs with confirmed ehrlichiosis could be sampled, the current picture may change.

5. Conclusion

Our current results indicate that the abundance of tick-vectors on domestic dog hosts encourages the cyclical transmission of tick-borne pathogens in the country. Molecular diagnostic techniques allows for previously unknown species to be identified. There is no doubt that if the current momentum of research is maintained, various other important pathogens will be discovered which will in return influence our understanding of the epidemiology, management and treatment of tick-borne pathogens of domestic dogs. These findings also underpin the need for effective control measures to prevent transmission of tick-borne pathogens to domestic dogs in South Africa.

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