Chapter 3: Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa.

3.1. Abstract

A total of 1138 blood samples were collected over a of six-year period (2000-2006) from domestic dogs in South Africa. Samples 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 4 provinces (Gauteng, Mpumalanga, KwaZulu-Natal and Western Cape). All samples were screened for *Babesia*, *Theileria*, *Hepatozoon* and *Ehrlichia / Anaplasma* species using PCR and Reverse Line Blot (RLB) assays. On RLB, 560/1137 samples were positive for one or more parasites. Of the positive samples, 420 (75%) were infected with *Babesia rossi*; 82 (15%) dogs were infected with *Theileria* sp. (dog); 18 (3%) dogs were infected with *Babesia vogeli* and 14 (3%) samples were infected with *Ehrlichia canis*. Mixed infections were also found: *B. rossi* and *E. canis* were detected in 12 (2%) samples; *B. vogeli* and *E. canis* occurred in 7 (1%) samples; *Theileria* sp. (dog) and *E. canis* in 3 (0.5%) samples; *B. rossi* and *B. vogeli* in one sample. *Babesia rossi, B. vogeli* and *E. canis* occurred simultaneously in one dog. There was also one 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.

3.2. 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, Boomker and Villiers, 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 *B. canis* (*senso lato*) group (Uilenberg, Franssen, Perie and Spanjer, 1989) and the small babesias, which include the *B. gibsoni* (*senso lato*) group, *B. conradae* and *Theileria annae* (Kjemtrup, Kocan, Whitworth, Meinkoth, Birkenheuer, Cummings, Boudreaux, Stockham, Irizarry-Rovira and Conrad, 2000; Kjemtrup, Wainwright, Miller, Penzhorn and Carreno 2006; Zahler, Rinder, Schein and Gothe, 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; Kjemtrup et al., 2006; Zahler, Schein, Rinder and Gothe, 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 samples 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, Venter, Ryst and Kelly, 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, Oyamada, Kelly, Jacobson, Fournier, Itamoto, Okuda, and Brouqui, 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.

3.3. Materials and Methods

3.3.1. Collection of samples

A total of 1138 (Table 3.1) domestic-dog blood samples were collected from four inland provinces (Gauteng, North West, Mpumalanga and Free State) and three coastal provinces (KwaZulu-Natal, Eastern Cape and Western Cape) (Fig 3.1) out of the nine provinces of South Africa. Except for samples from Mpumalanga, which were collected from known *Babesia*-positive animals, all samples were collected from domestic dogs that were available during our sampling days. Blood samples 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 samples collected from OVAH (n=527) could also represent samples from North West Province. All samples came from dogs presented with clinical disease requiring veterinary care. An additional 90 blood samples were collected in southern Gauteng. Samples from apparently healthy dogs were obtained from the Johannesburg SPCA,

while samples from sick dogs were obtained from private practices. Blood samples (n=38) collected from Mpumalanga were obtained from sick dogs at a private practice in White River. Blood samples (n=129) collected from the Free State were obtained from the Bloemfontein SPCA from apparently healthy dogs. A total 253 blood samples were obtained from KwaZulu-Natal's two major cities: 56 were from the Durban SPCA from apparently healthy dogs, 4 from sick dogs at a private practice in Durban and 192 were collected from private practices in the Pietermaritzburg area from healthy dogs involved in a study of tick-repellent impregnated dog collars. An additional sample confirmed positive on blood smear for *Trypanosoma* was obtained from a private clinic in Hluhluwe, northern KwaZulu-Natal. Blood samples (n=54) collected from Eastern Cape were obtained from the East London SPCA from apparently healthy dogs and blood samples (n=47) obtained from the Western Cape were collected from sick dogs at private practices in Cape Town. Blood samples were collected from the cephalic vein into EDTA tubes.

3.3.2. DNA extraction

Once collected, the blood samples were sent to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. DNA was extracted from 200 µl of each blood sample. The QIAmp blood and tissue extraction kit (Qiagen, Hilden, Germany) was used for DNA extractions, following the manufacturer's protocols.

3.3.3. 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 460-540bp from the 18S rRNA gene spanning the V4 region (Gubbels, de Vos, Van der Weide, Viseras, Schouls, de Vries and Jongejan, 1999; Matjila, Penzhorn, Bekker, Nijhof and Jongejan, 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 to 520 bp from the V1 hypervariable region of the 16S SSU rRNA gene (Bekker, de Vos, Taoufik, Sparagano and Jongejan, 2002; Nijhof, Pillay, Steyl, Prozesky, Stoltsz, Lawrence, Penzhorn and Jongejan, 2005). The conditions for the PCR included an initial step of 3 min at 42º C, 10 min at 94ºC, 10 cycles of 94ºC (20s)- 67 ºC (30s)- 72º C (30s), with lowering of annealing step after every second cycle with 2º C (touchdown PCR). The reaction was then followed by 40 cycles of denaturation at 94º C for 30s, annealing at 57º C for 30s and extension at 72º C for 30s. The PCR and restriction fragment length polymorphism assay (RFLP) for the species identification of the *Trypanosoma* infected sample was done as described by Delespaux, Ayral, Geysen and Geerts (2003). The amplification for this nested PCR was first done on the 18S gene using the forward primer 18STnF2 (CAA CGA TG-ACA CCC ATG AAT TGG GGA) and 18STnR3 (TGC GCG ACC AAT AAT TG-CAA TAC) (Geysen, Delespaux, and Geerts, 2003). The second amplification was done using the forward primer 18STnF2 (CAA CGA TG-ACA CCC ATG AAT TGG GGA) of the first amplification with the reverse primer 18STnR2 (GTG TCT TGT TCT

CAC TGA CAT TGT AGT G). Nested products were then analysed using the RLFP for species identification as described by Delespaux et al. (2003). Known positive samples were used as controls.

3.3.4. 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 3.2.

3.4. Results

The majority of samples collected from OVAH (382/527; 72%) were infected with at least one pathogen (Table 3.3). Most samples (65%) from this area were infected with *B. rossi*. A newly identified species (*Theileria* sp. dog) was detected in 3 samples (Matjila, Leisewitz, Oosthuizen, Jongejan and Penzhorn, 2008). Mixed infections were also detected: 8 samples were co-infected with *B. rossi* and *E. canis*; 7 samples were co-infected with *B. vogeli* and *E. canis*; one sample was co-infected with *B. rossi* and *B. vogeli* and one sample had a triple infection of *B. rossi*, *B. vogeli* and *E. canis*.

Almost all of the samples collected from Mpumalanga (37/38; 97%) were positive for *B. rossi*. Forty percent (101/253) of samples collected from KwaZulu-Natal were positive for at least one parasite. Sixteen samples from this area were infected with *B. rossi*, 1 sample with *E. canis* and 79 samples with *Theileria* sp. dog. Mixed infections were

detected in four samples, one of which was co-infected with *B. rossi* and *E. canis* and 3 samples were co-infected *Theileria* sp. dog and *E. canis*. One sample collected from the northern part of KwaZulu-Natal (Hluhluwe) was confirmed positive for *Trypanosoma congolense*. Twenty-two percent (12/54) of samples collected from the Eastern Cape were positive, with only 12 infected *B. rossi* samples. Sixteen percent (21/129) of samples collected from the Free State were positive: 7 samples were *B. rossi* positive, 13 were *B. vogeli* positive and only 1 sample was *E. canis* positive. Mixed infections were not detected in samples collected from this area. Only 6% (3/47) of samples collected from Western Cape were positive: 2 samples were positive for *B. rossi* and 1 sample was co-infected with *B. rossi* and *E. canis*. Finally, only 4% (4/90) of samples collected from southern Gauteng were positive: 2 samples were *B. rossi* positive and 1 sample was co-infected with *B. rossi* and *E. canis*. We were also able to detect *B. gibsoni* in a sample collected at a private practice in southern Gauteng from a pit-bull pup recently imported into South Africa (Matjila, Penzhorn, Leisewitz, Bhoora and Barker, 2007). No *Hepatozoon* infections were detected in domestic dogs.

3.5. Discussion

Samples 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* (*senso lato*) infected dogs presented at OVAH (Horak, 1995). A majority of *Babesia*-infected dogs (41%) were only infested with *H. elliptica* (previously regarded as synonymous with *H. leachi*) (Apanaskevich, Horak and Camica, 2007), the only known vector of *B. rossi* (Lewis, Penzhorn, Lopez-Rebollar and De Waal, 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, Dennis, Amyx and Huxsoll, 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. *Rhipicephalus 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 8 dog samples and *B. vogeli* and *E. canis* in 7 dog samples. It remains unclear, however, why there were so few dogs co-infected with both *B. vogeli* and *B. rossi*. We detected only one sample co-infected with *B. rossi* and *B. vogeli* and we also detected only one sample co-infected with *B. rossi*, *B. vogeli* and *E. canis*. Of interest was the detection of a *Theileria* species from 3 samples 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).

Babesia rossi was the most common species detected from all sampled areas. *Haemaphysalis elliptica* is present in the eastern half and southern regions of South Africa, even in lowveld and other arid areas where the average rainfall might seem too low for the tick's survival (Norval, 1984). Since the areas sampled correlated with the

distribution of *H. elliptica*, it would be logical to associate parasite occurrence with the distribution of the tick vector. The samples 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. Even though all the Mpumalanga samples were from dogs diagnosed with babesiosis, we could not amplify DNA from one sample. 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 samples collected from Free State and OVAH (Matjila et al., 2004), even though *R. sanguineus* has a wider distribution in South Africa (Horak, 1995). This implies that *B. vogeli* infections are not as widely spread as *B. rossi* infections in South Africa. The same *Theileria* species isolated from 3 samples collected at OVAH was detected in 79 samples collected from Pietermaritzburg, KwaZulu-Natal. This species was also isolated in three samples co-infected with *E. canis*. The vector of this *Theileria* species is still unknown. Additionally, *Trypanosoma congolense* infection was detected from a sample 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, Esterhuizen, Nkuna, Matjila, Penzhorn, Geerts and Marcotty, 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 *E. chaffeensis* (Dumler, Chen, Asanovich, Trigiani, Popov and Walker, 1995; Paddock, Sumner, Shore, Bartley, Elie, McQuade, Martin, Goldsmith and Childs, 1997) and *Anaplasma phagocytophilum* (Wormser, Dattwyler, Shapiro, Halperin, Steere, Klempner, Krause, Bakken, Strle, Stanek, Bockenstedt, Fish, Dumler and Nadelman, 2006). Our RLB assay had probes that could detect and differentiate between the two zoonotic species (Table 3.2), 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, Inokuma, Camicas, Brouqui and Raoult, 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 3 dog samples in South Africa (Inokuma et al., 2005). A more directed study considering *Ehrlichia*-infected dogs only may elucidate the existence of potentially zoonotic *Ehrlichia* species.

3.6. 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 allow 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 turn influence our understanding of the epidemiology, control 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.

3.7. Figures and Tables

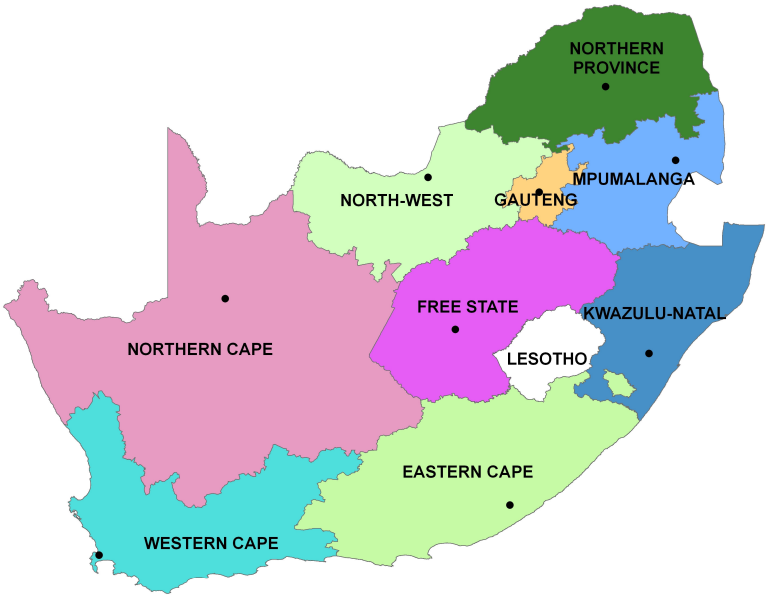

Figure 3.1: Map of South Africa, indicating provinces where blood samples were collected.

Table 3.2: The number (n=1138) of samples collected from dogs, by province and locality, in South Africa.

Localities	Gauteng and North West	Mpumalanga	Free State	KwaZulu-Natal	Eastern Cape	Western Cape
OVAH	527					
Johannesburg SPCA	90					
White River private clinic		38				
Bloemfontein SPCA			129			
Durban SPCA				56		
Durban private practice				4		
Pietermaritzburg private practice				192		
Hluhluwe private practice				1		
East London SPCA					54	
Cape Town private practice						47
Total numbers of samples	**617**	**38**	**129**	**253**	**54**	**47**

Table 3.2: 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

Table 3.3: Pathogen species detected from domestic dogs using the RLB.

Pathogen	OVAH* (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 & *E. canis*	8	1	1	-	1	-	1
B. vogeli & *E. canis*	7	-	-	-	-	-	-
Theileria sp. dog & *E. canis*	-	-	-	-	3	-	-
B. rossi & *B. vogeli*	1	-	-	-	-	-	-
B. rossi & *B. vogeli* & *E. canis*	1	-	-	-	-	-	-
T. congolense	-	-	-	-	1	-	-
Total number of positives	**382 (72%)**	**4 (4%)**	**37 (97%)**	**21 (16%)**	**101 (40%)**	**12 (22%)**	**3 (6%)**

* Onderstepoort Veterinary Academic Hospital (Northern Gauteng / North West Province)

3. 8. References

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