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Molecular characterization of tick-borne pathogens of domestic dogs from communal areas in Botswana

by

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Summary

The occurrence of tick-borne pathogens in dogs on communal land in Botswana was studied using Reverse Line Blot (RLB). Eighty blood samples were collected from dogs that underwent a spay and neuter program in Maun, Botswana. These were sent to the Department of Veterinary Tropical Diseases, University of Pretoria, and were stored at the Molecular Laboratory. DNA was extracted from the samples followed by the Polymerase Chain Reaction (PCR) and the RLB. Six samples were found to be positive for *Ehrlichia canis*. Two of the samples were positive for a concurrent infection of *E. canis* and *Ehrlichia ruminantium*. There was one sample that was positive for the non-pathogenic *Ehrlichia* sp. Omatjenne. Although six samples were positive for the *Babesia/Theileria* catch-all probe, none hybridised with any of the species-specific probes.

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CHAPTER 1

1 Introduction

A number of tick-borne diseases affect domestic dogs in southern Africa. Treatment and surveillance of these diseases has been easy in towns and cities where private practitioners usually operate (Collett, 2000). Prevalence studies have so far not been conducted in rural areas where tick-borne diseases are known to affect many dogs because of the poor primary animal health care provided. Molecular characterisation of tick-borne diseases of dogs in southern Africa has mostly been done in South Africa (Matjila *et al.,* 2008a). Information on the occurrence of tick-borne pathogens in the communal areas of Botswana is currently lacking. There is a need to identify the species of tick-borne pathogens affecting dogs in these areas. The detection of these pathogens is important, as some of these pathogens cause morbidity and mortality in dogs. This research will not only benefit the academic arena but it may also have an impact on the economic and public-health sectors. This study will also enable the identification of the species that are endemic in Maun, Okavango delta, in Northern Botswana. Discovery of highly pathogenic species will be of importance in the selection of treatment regimens used by veterinarians in the specific area.

CHAPTER 2

2 Literature review

2.1 Babesiosis

Various tick-borne pathogens infect dogs. Diseases like canine babesiosis, hepatozoonosis, ehrlichiosis, rickettsiosis and borreliosis are a problem in the veterinary and public health fields. Different species of ticks play a role in the transmission of these diseases in dogs. *Rhipicephalus sanguineus*, commonly called the brown dog tick or the kennel tick, is a vector of *Babesia vogeli*, *Babesia gibsoni, Ehrlichia canis* and *Hepatozoon canis. Dermacentor reticulatus* transmits *B. canis* whilst *Haemaphysalis elliptica* transmits *Babesia rossi* (Schoeman, 2009; Neer and Harrus, 2006; Foldvari *et al.*, 2005; Baneth *et al.*, 1998).

The most important tick-borne pathogens of dogs in South Africa are *Babesia* and *Ehrlichia* (Collett, 2000; Rautenbach *et al.,* 1991; Van Heerden, 1982). Identification of the *Babesia* species has traditionally been based on host specificity and the morphology of the piroplasms. Canine piroplasms have been divided into two groups, the large (4–5 µm) *Babesia canis* and the small (1–2.5 µm) *Babesia gibsoni* (Foldvari *et al., 2*005). *Babesia canis* was formerly divided into three different subspecies comprising *Babesia canis canis, Babesia canis vogeli* and *Babesia canis rossi*. *Babesia canis canis* used to be endemic in southern Europe and it is now also found in Asia (Uilenberg *et al*., 1989). *Babesia canis rossi* is endemic in Sub-Saharan Africa and *B. canis vogeli* is endemic in tropical and subtropical areas world-wide. *Babesia canis vogeli* has been found in USA, Japan, France, Australia, South Africa and Brazil (Schoeman, 2009; Uilenberg *et al.*, 1989*)*. Studies using criteria like vector specificity, serology and cross immunity have led to suggestions that the three subspecies of *B. canis* can be elevated to the species rank (Duarte *et al*., 2008; Carret *et al*., 1999). Currently, the species names *B. rossi*, *B. vogeli* and *B. canis* are generally used in literature. In South Africa, canine babesiosis is mostly caused by *B. rossi* and to a lesser extent by *B. vogeli* (Matjila *et al*., 2004; Uilenberg *et al.,* 1989).

Babesia gibsoni, which is a small type of *Babesia*, has been reported in Asia, North America, North and East Africa and Europe. It is considered to be emerging in USA and has been detected in Italy, Hungary, South Africa and Australia (Muhlnickel *et al*., 2002; Caccio *et al.*, 2002; Matjila *et al*., 2007). It commonly appears as an

individual ring or as piriform bodies. It is difficult to distinguish *B.gibsoni* from other small parasites such as *Theileria equi* and *Babesia microti. Babesia gibsoni* causes a chronic disease that is characterised by progressive anaemia as the main sign (Caccio *et al.,* 2002).

The disease caused by *Babesia* species ranges from mild to severe. Clinical signs include pyrexia, anaemia and lethargy. Animals also present with lymphadenopathy, splenomegaly and thrombocytopenia. Cases of shock and also renal failure have been reported. The disease usually presents in two forms, the febrile and the haemolytic form. The haemolytic form involves bilirubinuria, jaundice and anaemia (Bourdoiseau, 2006; Jacobson, 2006). *Babesia rossi* has been found to be more virulent than the other *Babesia* parasites of dogs, causing cerebral form and other chronic forms (Rees & Schoeman, 2008). *Babesia vogeli* causes a clinically inapparent infection that is usually not virulent. Parasitaemia in *B. vogeli* seems to be low and therefore usually missed during blood smear examinations (Schoeman, 2009).

Parasitological diagnosis of cases infected with *Babesia* is achieved by demonstration of infected erythrocytes on Romanowsky-stained blood smears. False negatives diagnoses may be made where parasitaemia is low (Bourdoiseau, 2006). Serological diagnostic tests like the Indirect Fluorescent Antibody Test and ELISA can be used. Diagnoses should not be based on seropositivity, as clinically normal dogs from endemic areas can test positive serologically (Schoeman, 2009). ELISA with recombinant BgTRAP (*Babesia gibsoni* thrombospondin-related adhesive protein) has been used to detect antibodies to *B.gibsoni* in dogs. The results showed that ELISA with rBgTRAP can be used as a reliable test with or without combinations with PCR in diagnoses of *B gibsoni* (Goo *et al*., 2008). Future ELISA tests with recombinant genes can be reliable as research is ongoing. New technologies such as the Polymerase Chain Reaction (PCR) and Reverse Line Blot (RLB) are being used to diagnose false negatives, low parasitaemias or confirmation of already known cases (Duarte *et al.,* 2008; Schoeman, 2009).

2.2 Ehrlichiosis

Canine ehrlichiosis, also known as Canine Monocystic Ehrlichiosis (CME), is one of the tick-borne diseases that occur worldwide. It is caused by *Ehrlichia canis* which is transmitted by *Rhipicephalus sanguineus. Ehrlichia canis* is a Gram-negative coccoid bacterium that occur intracytoplasmically in clusters of organisms, usually described as morulae (Neer and Harrus, 2006). Three clinical stages have been differentiated: acute, subclinical and chronic. Clinical signs involve fever, generalised lymphadenopathy, splenomegaly and thrombocytopenia. Some clinical signs like depression, epistaxis, lethargy, stiffness, anorexia, oedematous limbs, dyspnoea and coughing are variable (Yabsley *et al.*, 2008).

Diagnosis can be confirmed by a haemogram in combination with many methods such as cytology of Giemsa-stained buffy coat, bone marrow and lymph node aspirate smears that reveal morulae in mononuclear leukocytes and in neutrophils (Mylonakis *et al*., 2008*)*. Immunofluorescent Antibody tests (IFA) can also be used to confirm diagnosis. New methods such as PCR have been developed to diagnose some of the species that affect dogs *(*Trapp *et al*., 2006).

Dogs are also affected by other *Ehrlichia* species. *Ehrlichia platys* infect platelets and causes infectious cyclic thrombocytopenia. *Ehrlichia chaffeensis* is known to affect man, although there is no known direct transmission from dogs to man (Anderson *et al.,* 1991). *Ehrlichia ruminantium,* the cause of heartwater in domestic and wild ruminants, or a closely related organism was detected in the blood of clinically ill dogs in Ethiopia, Mali, Uganda and Kenya (Neer and Harrus, 2006).

2.3 Other vector-borne pathogens

Hepatozoon canis and *Eperythrozoon* sp. are also vector-borne pathogens that cause disease in dogs in some countries. Canine hepatozoonosis is caused by a protozoan *Hepatozoon* of the phylum Apicomplexa in the family Hepatozoidae. Two species affect dogs, *Hepatozoon canis* and *H. americanum.* These species are distinct in their clinical, pathologic, genetic and antigenic aspects (Mundim *et al.,* 2008*).* The parasite is primarily found in white blood cells and parenchymal tissues. Manifestations vary from being subclinical to a severe and life-threatening illness associated with leukocytosis, fever, lethargy and anaemia (Baneth *et al.*, 1998).

Eperythrozoonosis is a febrile haemolytic disease caused by genus *Eperythrozoon* of the family Rickettsiaceae. It is host-specific and the species that affects dogs is *Eperythrozoon canis*. It is transmitted by ticks especially *Rhipicephalus sanguineus* (Rikihisa, 2008).

Theileria species that cause disease in dogs have been found. The only species that was associated with disease condition in dogs is the *Babesia microti*-like organism named *Theileria annae* (Zahler *et al*., 2000). Controversy surrounds this name as Goethert and Telford (2003) questioned the use of *Theileria* as a genus name. Other *Theileria* sp that have been reported from dogs are *Theileria annulata* and *Theileria equi*, with *T. equi* being isolated from three asymptomatic dogs and one symptomatic dog (Criado *et al*., 2006; Criado-Fornelia *et al*., 2003a, 2003b). Another *Theileria* species named as *Theileria* sp. (dog) was isolated from three dogs in South Africa. The three dogs had common clinical signs like anaemia, splenomegaly and a possible immune-mediated syndrome (Matjila *et al*., 2008b). The signs were similar to those seen in dogs infected with *T. annae* (Camacho Garcia, 2006).

CHAPTER 3

3 Materials and Methods

3.1 Collection of samples

Eighty canine blood samples originating from Maun, Okavango delta, in Northern Botswana, were collected in EDTA tubes during a spay campaign in 2006. The spay programme was run by the Maun Animal Welfare Society in Botswana. The primary objective of the exercise was to provide canine neutering and veterinary care to impoverished communities in the Maun and broader Okavango communal areas. The dogs were nominally owned but they led an essentially feral existence since almost all of them originated from low-income households.

The samples were then stored at -20 ºC at the molecular laboratory of the Department of Veterinary Tropical Diseases, University of Pretoria.

3.2 Experimental Design

Eighty blood samples were screened using molecular techniques for the presence of tick-borne pathogens. Known positive DNA controls were used to confirm positive blood samples results.

3.3 Experimental and Observations/analytical procedures

The Qiamp blood and tissue extraction kit (Qiagen, Hilden, Germany) was used for extracting DNA from 200 μl of whole blood. Extracted DNA was stored at -20 C. PCR was then conducted with a set of primers that amplify 460–540 bp fragment of the 18S SSU rRNA spanning the V4 region. The forward primer RLB-F2 (5′ -GAC ACA GGG AGG TAG TGA CAA G-3′) and the reverse primer RLB-R2 (biotin-5′-CTA AGA ATT TCA CCT CTG ACA GT-3′) were then used for amplification and hybridization of regions conserved for *Babesia* and *Theileria*. The *Ehrlichia* PCR amplified the V1 hypervariable region of the 16S SSU rRNA (Gubbels *et al*., 1999; Bekker *et al*., 2002). The primers were obtained from Isogen BV (Maarsen, The Netherlands).

The PCR reaction mixture (25 µl) contained 5 µl of DNA template, 12,5 µl PCR Supermix-UDG (Invitrogen, The Scientific Group, South Africa), 0.25 µl of each primer and 7µl of distilled water. These reactions were performed in an automated I-Cycler (Biorad, California, USA) with an initial step of 3 min at 37°C, 10 min at 94°C, 10 cycles of 94°C(20s)–67°C(30s)–72°C(30 s), with lowering of the annealing step after every second cycle with 2°C (touchdown) then followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s and extension at 72°C for 30 s.

Amplified PCR products were used to perform the Reverse Line Blot (RLB) as described by Gubbels *et al*. (1999) and Matjila *et al.* (2005) with the following modifications: denatured PCR products were diluted in 2 x SSPE -0.1% sodium docecyl sulphate (SDS), loaded onto the membrane and incubated at 42°C for one hour. Thereafter, the membrane was washed twice at 50°C instead of 42°C. The membrane was internally prepared in the laboratory.

Samples were screened against a number of pathogens as indicated by the probe list in Table 1 (Gubbels *et al*., 1999; Matjila *et al*., 2008a; Ros-Garcia *et al*., 2011; Pfitzer *et al*., 2011). Samples that were positive for pathogens not listed in our probes list were identified by the *Theileria/Babesia* (T/B) catch-all probe, which indicated that the sample was positive for an unknown *Theileria* and/or *Babesia* infection. The same applied to samples that reacted on the *Ehrlichia/Anaplasma* (E/A) catch-all probe indicating that the sample was positive for an unknown *Ehrlichia* and/or *Anaplasma* infection.

CHAPTER 4

4 Results

The 80 samples were separated into two groups. Batch 1 consisted of Samples 1–40 and Batch 2 consisted of Samples 41–80. Three of the 40 samples in Batch 1 were positive for *Ehrlichia canis.* One further sample hybridised on the *Ehrlichia/Anaplasma* catch-all probe without reacting on the species-specific probe. Three samples weakly hybridised on the *Theileria/Babesia* catch-all probe but did not react on specific species (Fig. 1, Table 2).

Batch 2 results are shown on Figure 2 and Table 3. Three samples were positive for *E. canis,* with two of these having a concurrent infection of *E. ruminantium.* One sample was positive for *Ehrlichia* sp. Omatjenne. Two other samples reacted on the *Ehrlichia/Anaplasma* catch-all probe without any species-specific reaction. One sample hybridised on *Ehrlichia/Anaplasma* catch-all, *Theileria/Babesia* catch-all and *Babesia* catch-all 1, but failed to react on the species-specific probes. Two samples reacted on the *Babesia* catch-all 1 and 2, but failed to react on the species-specific probes.

In total, six samples (7.5 %) were positive for *E. canis* and two of these were also positive for *E. ruminantium.* Four more samples failed to hybridise on the species specific probe but reacted on *Ehrlichia/Anaplasma* catch-all probe with one sample also reacting on the *Theileria/Babesia* catch-all probe. One sample (1.25%) was positive for *Ehrlichia* sp. Omatjenne. A total of four samples (5 %) reacted on the *Theileria/Babesia* catch-all probe. Two samples reacted on the *Babesia* catch-all 1 and 2, but failed to hybridise on the species specific probe.

CHAPTER 5

5 Discussion

Babesia rossi is a common tick-borne protozoan parasite in sub-Saharan Africa (Lewis *et al*., 1996) with *B. vogeli* occurring in South Africa (Matjila *et al*., 2004). Out of the whole batch that we received, only four samples tested weakly positive for *Babesia/Theileria* catch-all probe with an additional two testing positive on *Babesia* catch-all 1 and 2 probes but all the six samples were negative to our known species-specific probes. This could be due to lower concentrations of the parasite DNA in the blood. Lack of positive samples for *B. rossi* can also be attributed to an assumption that the vector, *H .elliptica*, may be found in small numbers in the area around Maun in Botswana as distribution is said to be limited to mainly northern Zimbabwe and Zambia when comparing with areas geographically close to the study area (Apanaskevich *et al*., 2007). *Babesia vogeli* may be present but in isolated cases as recent research has shown that it occurs in South Africa (Matjila *et al.,* 2004). *Rhipicephalus sanguineus,* the vector of *B. vogeli*, is present in the area, as indicated by the presence of *Ehrlichia*-positive cases.

The most significant finding was the presence of *E. canis.* A total of six samples reacted positively for *E. canis*, with two of the six also reacting positively for *E. ruminantium.* A further four samples hybridised on the *Ehrlichia/Anaplasma* catch-all probe*.* The clinicians who collected these samples on behalf of the Department had noticed that there were increased haemorrhagic complications in dogs that underwent neutering. No specific numbers of dogs or samples that had bleeding tendencies during ovario-hysterectomy were recorded during the procedures. *Ehrlichia canis* is the major pathogen causing Canine Monocytic Ehrlichiosis (CME). Ehrlichiosis in dogs is usually associated with haemorrhagic complications due to thrombocytopenia (Yabsley *et al.,* 2008). Haematologic abnormalities, like thrombocytopenia, are found in all phases of the disease, even before the other typical signs of ehrlichiosis appear (Neer and Harrus, 2006).

Two of the samples were positive to both *E. canis* and *E. ruminantium. Ehrlichia ruminantium* DNA or a closely related organism was detected in clinically ill and healthy dogs in Uganda, Kenya, Ethiopia and Mali. Its infectivity or virulence was not tested and it is therefore still unknown (Neer and Harrus, 2006). Another organism closely related to *E. ruminantium* was also recognized in imported dogs at a

quarantine station in South Africa (Allsopp and Allsopp, 2001). The presence of *E. ruminantium* in dogs from Botswana is then not unexpected. *Ehrlichia ruminantium* is the causative agent of heartwater in livestock and wildlife. It is transmitted by *Amblyomma* ticks (Mahan *et al.*, 2004). Maun is known for its wildlife richness with the whole of Botswana being a cattle-ranching country and suffering from a lot of tick-borne diseases (Fanikiso and Ndzinge, 1992). It is possible that the dogs got infected by *Amblyomma* ticks feeding on them. Adult *Amblyomma hebraeum* ticks have been found on dogs, especially in rural areas, in Mozambique and South Africa. Immature stages of *A. hebraeum* are even more common in dogs (De Matos *et al*., 2008). *Amblyomma hebraeum* is also known to occur in the eastern part of Botswana (Walker & Olwage, 1987).The presence of *Amblyomma* ticks in Maun is therefore not unexpected considering the distribution of the tick in other parts of Botswana.

Ehrlichia sp. Omatjenne is an *Ehrlichia*-like agent that was isolated from a *Hyalomma truncatum* tick from Omatjenne area in the Otjiwarongo District of Namibia. After several passages of this parasite through *Amblyomma* ticks, it was seen to be pathogenic to sheep, which developed clinical signs similar to heartwater (Du Plessis, 1990). This pathogen has also been isolated from nyala (*Tragelaphus angasii*) in areas where *H. truncatum* ticks are distributed (Du Plessis, 1990; Pfitzer, *et al.*, 2011). A 16s sequence identical to *Ehrlichia* sp Omatjenne was detected in goats in Mozambique (Bekker, *et al*., 2002). This may imply that *Ehrlichia* sp Omatjenne may be present in southern Africa wherever the vector *H. truncatum* occurs. The incidental finding of this pathogen in dogs is therefore not unexpected.

It would have been preferable to follow up on the four samples that tested positive on the *Theileria/Babesia* catch-all probe, the two samples that reacted on the *Babesia* catch-all 1 and 2 probe and the four *Ehrlichia/Anaplasma* catch-all positive reactions. However, due to time limitations and the scope of the study, these samples could not be processed further. Future work will involve the sequencing of these samples in order to evaluate their significance.

CHAPTER 6

6 Conclusion

The use of RLB assay detected 6 positive samples of *E. canis* and no species-specific *Babesia* parasites. The diagnosis of *Ehrlichia* means that ehrlichiosis could be an underlying problem in Botswana. *Ehrlichia canis* can be fatal in dogs and to survive, dogs may need aggressive treatment (Neer and Harrus, 2006). Dogs that are not showing signs of CME can be poor surgical candidates as complications tend to occur because of poor blood-clotting properties. This may prove a challenge to veterinarians who do not have any diagnostic equipment in remote and isolated areas. The occurrence of *E. ruminantium* is also an interesting scenario that may need comparison with the prevalence of heartwater in livestock and wildlife in the Maun area.

CHAPTER 7

7 Figures and Tables

Table 1: List of organisms and the corresponding probe sequences used to detect pathogen DNA.

ORGANISM	PROBE SEQUENCE FROM 5' TO 3'
Ehrlichia/Anaplasma catch-all	GGG GGA AAG ATT TAT CGC TA
Anaplasma centrale	TCG AAC GGA CCA TAC GC
Anaplasma marginale	GAC CGT ATA CGC AGC TTG
Anaplasma phagocytophilum	TTG CTA TAA AGA ATA ATT AGT GG
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG
Anaplasma ovis	ACC GTA CGC GCA GCT TG
Ehrlichia chaffeensis	ACC TTT TGG TTA TAA ATA ATT GTT
Ehrlichia sp. Omatjenne	CGG ATT TTT ATC ATA GCT TGC
Ehrlichia canis	TCT GGC TAT AGG AAA TTG TTA
Theileria/Babesia catch-all	TTA TGG TTA ATA GGA RCR GTT G
Theileria catch-all	ATT AGA GTG CTC AAA GCA GGC
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 divergens	ACT RAT GTC GAG ATT GCA C
Babesia microti	GRC TTG GCA TCW TCT GGA
Babesia bigemina	CGT TTT TTC CCT TTT GTT GG
Babesia bovis	CAG GTT TCG CCT GTA TAA TTG AG
Babesia rossi	CGG TTT GTT GCC TTT GTG
Babesia canis	TGC GTT GAC GGT TTG AC
Babesia vogeli	AGC GTG TTC GAG TTT GCC
Babesia major	TCC GAC TTT GGT TGG TGT
Babesia bicornis	TTG GTA AAT CGC CTT GGT C
Babesia caballi	GTT GCG TTK TTC TTG CTT TT
Babesia gibsoni	CAT CCC TCT GGT TAA TTT G
Theileria sp. kudu	CTG CAT TGT TTC TTT CCT TTG
Theileria sable	GCT GCA TTG CCT TTT CTC C
Theileria bicornis	GCG TTG TGG CTT TTT CTC C
Theileria annulata	CCT CTG GGG TCT GTG CA
Theileria buffeli	GGC TTA TTT CGG WTT GAT TTT
Theileria sp. buffalo	CAG ACG GAG TTT ACT TTG T
Theileria mutans	CTT GCG TCT CCG AAT GTT
Theileria parva	GGA CGG AGT TCG CTT TG
Theileria taurotragi	TCT TGG CAC GTG GCT TTT
Theileria velifera	CCT ATT CTC CTT TAC GAG T
Theileria equi	TTC GTT GAC TGC GYT TGG
Theileria lestoquardi	CTT GTG TCC CTC CGG G
Theileria ovis	TTG CTT TTG CTC CTT TAC GAG
Theileria annae	CCG AAC GTA ATT TTA TTG ATT TG
Babesia leo	ATC TTG TTG CCT TGC AGC T
Babesia occultans	GTG TGC CTC TTT TGG CCC ATC
Theileria separata	GGT CGT GGT TTT CCT CGT

(Symbols indicate degenerate positions: R = A/G, W = A/T, K = G/T)

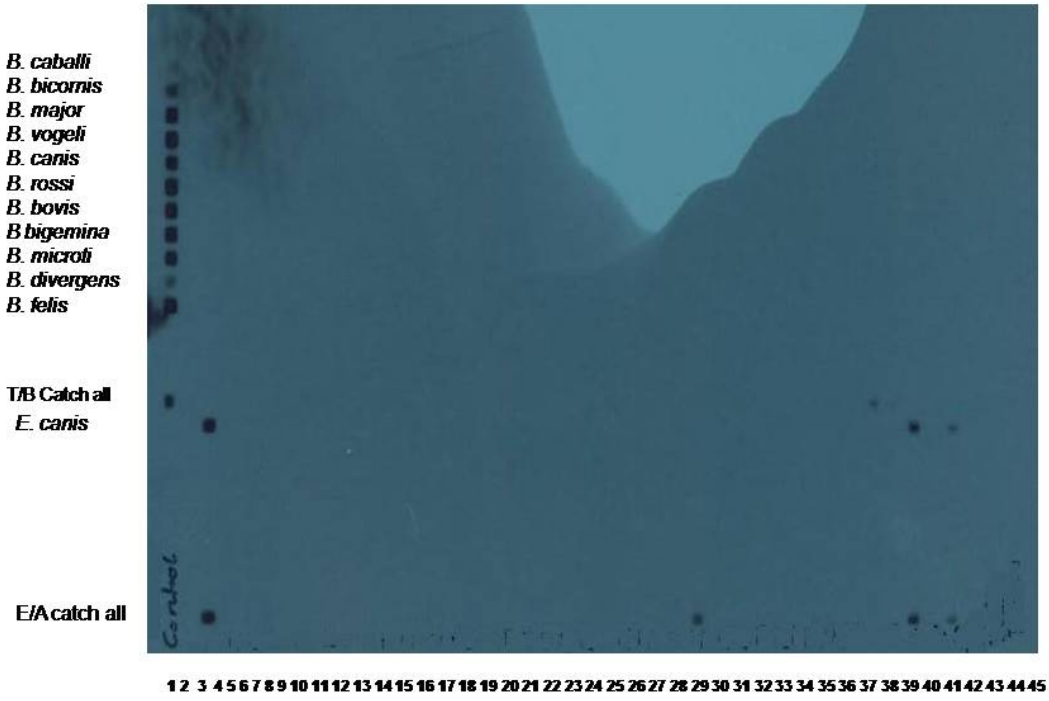

Figure 1: RLB results of the samples in Batch 1 indicating 12 species-specific oligonucleotides in the horizontal lanes and PCR products on the vertical lanes. From left to right are shown: *Babesia* plasmid control, negative control, 40 canine blood samples, negative control and 2 buffer lanes. A total of 38 species-specific oligonucleotides in the horizontal lanes were used but only 12 that reacted are shown in the figure.

Table 2: RLB report from Batch 1 samples as shown on Figure 1 indicating 4 *Ehrlichia/Anaplasma* catch-all positive samples of which 3 were species-specific positive for *E. canis* and 3 *Theileria/Babesia* catch-all positive samples that failed to hybridise on any species-specific probe. Samples 4 to 25 not shown on the table were negative.

Tested for		**Name and date of membrane**	**Date membrane was stripped**	***Date of procedure done***
Theileria/ Babesia X	*Ehrlichia/ Anaplasma* X	**Troskie 1/09**	**04/04/2009**	**21/04/2009**

MEMBRANE SAMPLE LIST

Lane	**Tube no.**	**Lab number**	**Results**	
1	1	Positive control *Babesia* plasmid	T/B catch-all, *B. felis, B. divergens, B. microti, B. bigemina, B. bovis, B. rossi, B. canis, B. vogeli, B. major, B. bicornis, B. caballi*	
2	2	Negative control	Negative	
3	3	1	E/A catch-all, *Ehrlichia canis*	
4	4	2	Negative	
5	5	3	Negative	
28	28	26	Negative	
29	29	27	E/A catch-all	
30	30	28	Negative	
31	31	29	Negative	
32	32	30	Negative	
33	33	31	Negative	
34	34	32	Negative	
35	35	33	T/B catch-all	
36	36	34	Negative	
37	37	35	T/B catch-all	
38	38	36	T/B catch-all	
39	39	37	E/A catch-all, *E. canis*	
40	40	38	Negative	
41	41	39	E/A catch-all, *E. canis*	
42	42	40	Negative	
43	43	Negative control	Negative	
44	44	Buffer	Negative	
45	45	Buffer	Negative	
PCR Positive Control Date and origin		*Babesia* Plasmid: Tropical Dept 04/04/2009		

Figure 2: RLB results of the samples in Batch 2 indicating 5 species-specific oligonucleotides in the horizontal lanes and PCR products in the vertical lanes. From left to right are shown: 40 canine blood samples (41 to 80), 3 *Babesia* positive controls, 1 negative control and 1 buffer lane. A total of 38 species-specific oligonucleotides in the horizontal lanes were used but only 5 that reacted are shown in the figure.

Table 3: RLB report from Batch 2 samples as shown on Figure 2 indicating 7 *Ehrlichia/Anaplasma* catch-all positive samples of which 2 were species-specific positive for *E. canis* and *E. ruminantium*, 1 was positive for *E. canis* and another one for *Ehrlichia* sp. Omatjenne. Three samples were positive for *Babesia* catch-all including one sample that was also positive for *Ehrlichia/Anaplasma* catch-all. Samples 41-53 and 60-67 not shown on the table were all negative.

Tested for		**Name and date of membrane**	**Date membrane was stripped**	***Date of procedure done***
Theilerial Babesia X	*Ehrlichial Anaplasma X*	**Troskie 1/09**	**22/04/2009**	**24/04/2009**

MEMBRANE SAMPLE LIST				
Lane	**Tube no.**	**Lab number**	**Results**	
14	14	54	Negative	
15	15	55	E/A catch-all T/B catch-all *Babesia* catch-all 1	
16	16	56	Negative	
17	17	57	E/A catch-all; *E. canis*	
18	18	58	E/A catch-all	
19	19	59	Negative	
28	28	68	E/A catch-all	
29	29	69	Negative	
30	30	70	Negative	
31	31	71	Negative	
32	32	72	Negative	
33	33	73	E/A catch-all, *E. ruminantium, E. canis*	
34	34	74	E/A catch-all, *E. ruminantium,E. canis*	
35	35	75	E/A catch-all, *E.* sp Omatjenne	
36	36	76	Negative	
37	37	77	*Babesia* catch-all 1 and 2	
38	38	78	*Babesia* Catch-all 1 and 2	
39	39	79	Negative	
40	40	80	Negative	
41	41	Positive control	Control *Babesia* catch-all 1 and 2, *Babesia gibsoni*	
42	42	Positive control	Control *Babesia* catch-all 1 and 2, *Babesia rossi*	
43	43	Positive control	Control *Babesia* catch-all 1 and 2, *Babesia rossi*	
44	44	Negative control		
45	45	Buffer		
PCR Positive Control Date and origin		*B. gibsoni*, *B. rossi* Tropical Dept, 04/04/2009		

CHAPTER 8

8 References

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