DETECTION OF *BABESIA ROSSI* GENOTYPES USING REAL-TIME PCR

by

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Declaration

I (.....) declare that this dissertation which I hereby submit to the University of Pretoria for the degree of Master of Science (Veterinary Science) is my own work and no part of it has been previously submitted by me for the degree at this or any other university.

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Proverbs 3:5-6
[5]Trust in the LORD with all your heart and lean not on your own understanding;
[6]in all your ways acknowledge Him, and He will make your path straight

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Abbreviations

A	Adenine
B	*Babesia*
BLAST	Basic Local Alignment Search Tool
bp	Base pair
*BrEMA*1	*Babesia rossi* Erythrocyte Membrane Antigen
C	Cytosine
D	Death
DNA	Deoxyribonulceic Acid
ECL	Enhanced Chemiluminescence Substrate
EDTA	Ethylenediamine tetra-acetic acid
e.g	Exepli gratia (for example)
ELISA	Enzyme-Linked Immunosorbent Assay
Fig	Figure
G	Guanine
IFAT	Indirect Fluorescent Antibody Test
ISA	In-Saline Agglutination
M	Molarity
ml	Millilitre
$NaHCO_3$	Sodium bicarbonate
nm	Nanometre
OVAH	Onderstepoort Veterinary Academic Hospital
POD	Peroxidase-labeled
R	*Rhipicephalus*

RLB	Reverse Line Blot
rRNA	Ribosomal ribonucleic acid
s	Second(s)
S	Survived
SDS	Sodium Dodecyl Sulfate
sp	Species
SPA	Soluble parasite antigens
SSPE	Sodium Chloride/Sodium Phosphate/EDTA
TM	Melting Temperature
T	Thymine
UDG	Uracil DNA Glycosylase
%	Percent
°C	Degrees Celsius
µl	Microliter

Dissertation summary

Detection of *Babesia rossi* genotypes using real-time PCR

by

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Babesia rossi is the most virulent *Babesia* species in dogs occurring in South Africa and is associated with severe clinical manifestation and mortalities. *Babesia rossi* is highly pathogenic and reacting dogs requires treatment to prevent mortalities. Mild, uncomplicated forms of the disease are effectively treated with antibabesial drugs. Complicated forms of the disease are difficult to treat with high mortality rate.This results in the disease being of economic importance in South Africa. There is a lack of information regarding the relationship between parasite genotype and disease phenotype. The broad objective of this study is to detect *B. rossi* genotypes using real-time PCR. This test is a method that can be used to monitor amplicon formation throughout the PCR reaction and to estimate the initial concentration of target DNA in samples. Polymerase chain reaction, sequencing and phylogenetic analysis were

used to determine the genotype detection of *Babesia rossi* isolated from infected dogs in South Africa. The correlation between the parasite genotype and disease phenotype was also investigated. Correlation between *B. rossi* Erythrocyte Membrane Antigen (*BrEMA*1) genotypes and age of dogs was studied in 44 cases. A total of 101 blood samples were tested using the reverse line blot (RLB) assay. Ninety six percent hybridized to the *Babesia rossi* species-specific probe.

Our findings demonstrate that the most encountered (*BrEMA*1) genotype is genotype29, followed by genotype28 and genotype19, with genotype29 associated with most of the severe clinical signs diagnosed compared to genotype19. The number of cases caused by genotype19 was low, constituting 22% of the cases. This is comparable with the 2009 report where genotype19 appeared highly prevalent and virulent, whereas the prevalence of genotype28/29 appeared moderate.

In this dissertation, we present the first report on the detection of an amplification product of *BrEMA*1 genotypes using real-time PCR. Samples which were below the detectable limit of conventional PCR and could not be sequenced probably due to low parasitaemia were also used and real-time PCR provided the ability to detect *B. rossi* positive animals. This was able to detect 10 *BrEMA*1 genotypes. However, it was not reliable enough in differentiating between various *BrEMA*1 genotypes.

When evaluating the relationship between *BrEMA*1 genotypes, clinical manifestation and age of the dogs, collapse was found to be a poor prognostic sign in dogs with

babesiosis. Genotype29 was associated with most of the collapsed cases and with high number of the dogs that died. Although *B. rossi* can infect dogs of all ages, young dogs showed to be more susceptible to canine babesiosis than older dogs. This is in agreement with the survey carried out from the Onderstepoort Veterinary Academic Hospital (OVAH) in 1994.

Since *B. rossi* is the most pathogenic species of the large babesias of dogs, the ability to manage the disease is dependent on rapid detection of the organism. Real-time PCR test is indeed a quicker method to confirm diagnoses of *B. rossi* infected dogs. It can detect *B. rossi* infection at a low DNA concentration (0.185 ng/µl) which provides a major advantage in detecting *B. rossi* infection in field blood samples.

CHAPTER 1

1. INTRODUCTION

Canine babesiosis caused by tick-borne protozoa, represents an important veterinary medical problem worldwide (Lobetti, 1998). Two species were traditionally identified as the aetiological agents of the disease in dogs: The *Babesia canis* group (i.e.*Babesia canis canis*, *Babesia canis rossi* and *Babesia canis vogeli*) and the *Babesia gibsoni* group, which correspond to large (3-5 µm) and small (0.5-2.5 µm) intraerythrocytic parasitic forms, respectively (Boozer and Macintire, 2003). Members of the *Babesia canis* group are now generally accorded species status.

Until recently, the only known *Babesia* species in dogs in South Africa was the virulent parasite, *B. rossi* (Matjila et al., 2004; Uilenberg et al., 1989). A recent molecular survey showed that the less virulent *B. vogeli* also occurs in South Africa, and is very common in some areas that exclude South Africa (Matjila et al., 2004). *Babesia canis* (senso stricto) is known to occur in Europe (Lobetti, 1998; Schetters et al., 1997; Taboada and Merchant 1991).

Demonstration of parasites from an infected animal by blood smear examination is an absolute and reliable method of diagnosis of clinical canine babesiosis (Casapulla et al., 1998; Ewing, 1963; Schuster, 2002; Suarez et al., 2001) but cannot distinguish amongst species (Ano et al., 2001). Nonetheless, molecular methods, such as polymerase chain reaction (PCR), present a higher sensitivity and specificity than the

peripheral blood smear evaluation in detecting babesial infection in peripheral blood (Macintire et al., 2002) and allow for specific identification.

A real-time PCR would, however, further improve the detection of these parasites. Therefore, the objectives of this study are to confirm and validate the previous results (Köster et al., 2009) by screening canine blood samples using the reverse line blot (RLB) hybridization assay for the presence of *Babesia* species, to determine the heterogeneity of *B. rossi* genotypes from infected dogs in South Africa using PCR, sequencing and phylogenetic analysis, to use a real-time PCR test for the detection of *B. rossi* genotypes and to evaluate the relationship between *BrEMA*1 genotypes, clinical manifestation and age of the dogs.

CHAPTER 2

2. JUSTIFICATION

2.1. Literature review

Babesia species are intraerythrocytic protozoan parasites affecting a wide range of vertebrate hosts. They are among the most common tick-borne pathogens of dogs in South Africa (Lobetti, 1998; Matjila et al., 2008). They are the second-most common blood-borne parasites of mammals after the trypanosomes (Telford et al., 1993).

More than 100 known *Babesia* species have been identified (Levine, 1971; Telford et al., 1993) which infect many types of mammalian hosts, mostly the order Rodentia, and also several avian species (Kakoma and Mehlhorn, 1994; Levine, 1971; Telford et al., 1993). Almost any mammal that serves as a host for a *Babesia*-infected tick is a potential reservoir (Telford et al., 1993). The first historical record of babesial infection may have been the biblical reference to a plague of murrain among cattle and other domestic animals (Exodus 9:3) (Krause, 2000). The *Babesia bovis* parasite was first described in 1888 in blood samples from cattle that suffered from hemolytic anaemia by the Romanian microbiologist Victor Babes (Babes, 1888).

Canine babesiosis is one of the most important tick-borne disease of dogs worldwide (Boozer and Macintire, 2003; Bourdoiseau, 2006; Jacobson, 2006). Canine babesiosis cases account for approximately 10% of the caseload in private clinics across South Africa, with the highest incidence in summer (Collett, 2000; Shakespeare, 1995). At the Onderstepoort Veterinary Academic Hospital (OVAH) on the outskirts of Pretoria, South Africa, around 12% of sick dogs presented are

diagnosed with babesiosis and around 31% of these are admitted for more intensive treatment (Shakespeare, 1995).

Nutall and Hadwen (1909) commented that the parasites used in experiments in Italy must have been much less virulent than South African isolate (Gonder, 1908; Nuttall and Hadwen, 1909). Furthermore, dogs that survived infection with a French isolate were fully susceptible to the South African one, prompting Laveran and Nattan-Larrier to conclude in 1913 that the African *Babesia* of dogs, if not a separate subspecies, was at least a variety distinct from the French one (Ciuca, 1913; Laveran and Nattan-Larrier, 1913). Uilenberg et al. (1989) proposed a trinomial system of nomenclature to differentiate the three subspecies of *B. canis* on a taxonomic level. Beyond the differently-induced pathogenicities in dogs among the three groups of B. *canis* (Uilenberg et al. 1989), a recent study demonstrated that *B. canis* and *B. rossi* exhibited differences in their *in vivo* and *in vitro* behaviour (Schetters et al. 1997).

Ehrlichia canis is also one of the most important tick-transmitted infectious diseases that cause severe clinical illness in South African dogs (Rautenbach et al., 1991; Van Heerden, 1982). In South Africa, dogs frequently become co-infected with both *B. canis* and *E. canis* (du Plessis, 1990).

2.1.1. Classification and life cycle of *Babesia* species

Babesia are classified as a member of the phylum Apicomplexa, suborder Piroplasmidea and family Babesiidae on the basis of their exclusive invasion of red blood cells, multiplication by budding rather than schizogony, and lack of haemozoin

(Hunfeld et al. 2008). Carret et al., (1999) confirmed that the three subspecies of *B. canis* could be taxonomically separated regardless of their morphological similarity. This suggested that the three subspecies of *B. canis* might be elevated to the rank of species. Further, evidence provided by several authors support the recognition of *B. rossi, B. vogeli* and *B. canis* as separate species (Schetters et al. 1997, Zahler et al, 1998). The life cycle of *Babesia* species are similar (Fig. 1). *Babesia* species are naturally transmitted by the bite of infected ticks (almost all ixodids rather than argasids) and the main life cycle difference amounts to the presence of transovarial transmission in some species (*Babesia* species *senso stricto*) and not in others (*B. microti*-like). Sporozoites represent the infectious stage of the parasite as they introduce these protozoa into the mammalian host and are injected into the mammalian host during the tick bite and directly infect erythrocytes (Fig. 1) (Chauvin et al., 2009; Hunfeld et al. 2008; Mehlhorn and Piekarski, 2002). This phenomenon separates *Babesia* species from *Theileria* species, where sporozoites do not readily infect erythocytes but initially enter a lymphocyte or macrophage in which development into schizonts takes place (Uilenberg, 2006).

In the host, *Babesia* sporozoites develop into piroplasms inside the infected erythrocyte resulting in the production of two merozoites by binary fission that leave the host cell to infect other erythrocytes until the host dies or the immunity of the host clears the parasites. The spleen with its lympho-reticular filter function removes infected cells from circulation because it is essential in resisting primary infections of *Babesia* species. This is probably through a combination of spleen microcirculation and stimulated phagocytic cell activity (de Vos et al., 1987; Gray and Weiss, 2008).

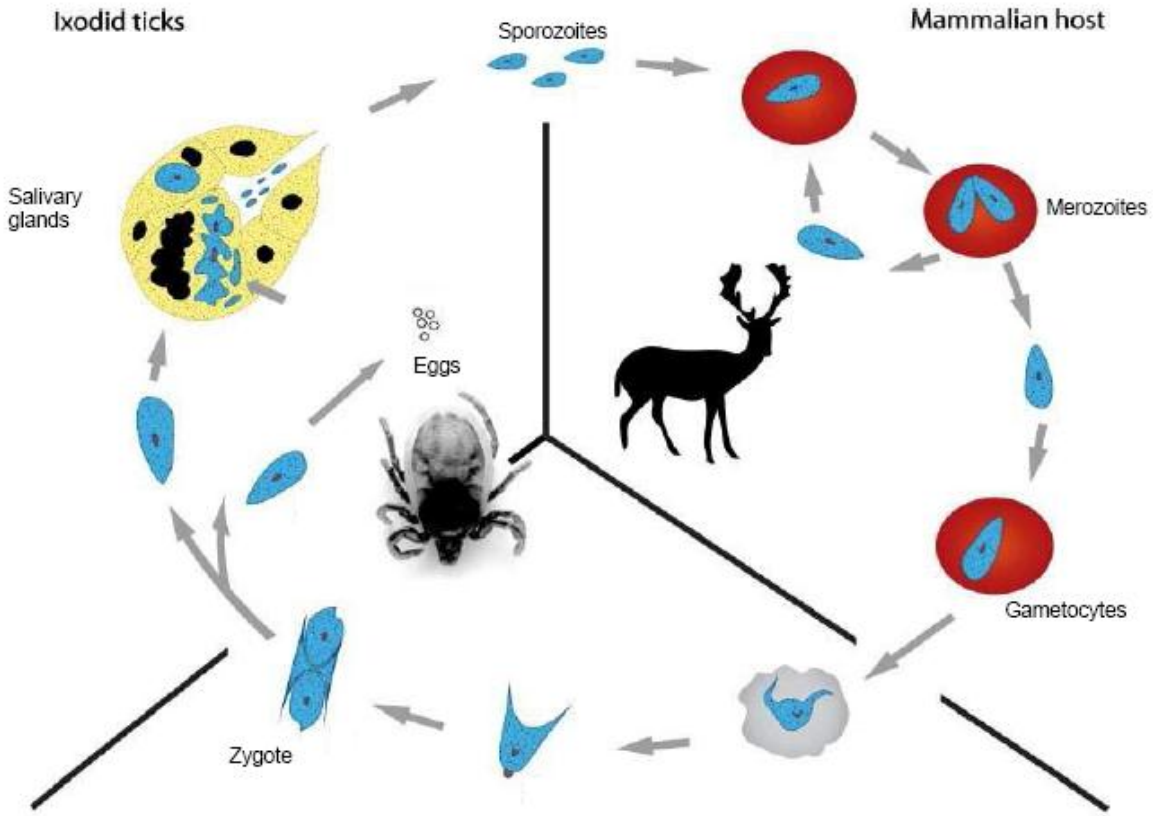

Figure. 1. General life cycle diagram of *Babesia* species (Hunfeld et al. 2008; Mehlhorn and Piekarski, 2002).

2.1.2. Morphology and Epidemiology

Individual *Babesia* species are typically characterized by the size and morphologic appearance of the intraerythrocytic forms, often referred to as piroplasms. The *Babesia canis* group are *B. canis* (senso stricto), *B. rossi* and *B. vogeli* that generally measure 3-5 µm in length, and usually occur as single pear-shaped piroplasms or in pairs of merozoites dividing by binary fission within the erythrocyte.

Historically, seven years after the Romanian scientist Victor Babes had described the first *Babesia* parasite as being the causative agent of heamoglobinuria in cattle, *Babesia canis* was described in dogs in Italy. Other *Babesia* species have been

described in a number of dogs with clinical signs and haematological parameters consistent with babesiosis (Birkenheuer et al., 2004, Lehtinen et al., 2008). *Babesia canis* (senso stricto) is transmitted by *Dermacentor reticulatus. Babesia canis* (senso stricto) is the main etiological agent in temperate regions of Europe (Cacciò et al., 2002). Although *D. reticulatus* is endemic to southern Europe (Estrada-Peña et al., 2004), reports have indicated that there are localized populations of the tick in several non-endemic north-western European countries including Switzerland, Germany, Belgium and the Netherlands (Duh et al., 2004; Losson et al., 1999; Nijhof et al., 2007; Zahler and Gothe, 1997). *Babesia canis* (senso stricto) has been reported from a variety of canids including coyotes (*Canis latrans*), wolves (*Canis lupis*), golden jackals (*Canis aureus*), and foxes (*Vulpes vulpes*) (Kakoma and Mehlhorn, 1994 and Kuttler and Ristic, 1988).

Babesia vogeli, the least virulent subspecies, is also present in Europe (Solano-Gallego et al., 2009) as well as in tropical or subtropical areas of Africa (Matjila et al., 2004). *Babesia vogeli* is transmitted by *Rhipicephalus sanguineus* (Cacciò et al., 2002).

Babesia rossi which was originally described from side-striped jackals (*Canis adustus*) in East Africa (Nuttall, 1910), is widespread in South Africa (Jacobson, 2006; Lobetti, 1998; Matjila et al., 2008; Reyers et al., 1998) and reacting dogs require treatment to prevent mortality (Jacobson, 2006). *Babesia rossi* trophozoites were seen in 2/29 (6.9%) of wild dog (*Lycaon pictus*), blood smear from the Kruger National Park, South Africa (Van Heerden et al., 1995). Other canids, eg. golden jackals, and wolves, are also susceptible to *B. rossi* (Breitschwerdt, 1990). *Babesia*

rossi is responsible for most canine babesiosis cases in South Africa and it is transmitted by *Haemaphysalis elliptica (*previously lumped with *Haemaphysallis leachi*; Apanaskevich et al., 2007). Recently, *B. rossi* has been reported in other regions of the African continent including Nigeria (Sasaki et al., 2007) and Sudan (Oyamada et al., 2005) where its vector ticks (*Haemaphysalis* species) are endemic. *Babesia rossi* can infect dogs of all ages, although in a survey from the Onderstepoort Veterinary Academic Hospital (OVAH), 77% of infected dogs were younger than 3 years of age (Van Zyl, 1994).

2.1.3. Clinical signs of canine babesiosis in South Africa

Canine babesiosis caused by *B. rossi* is clinically classified as either being uncomplicated or complicated. The disease is uncomplicated if the clinical changes could be attributed directly to a mild or moderate anaemia with no clinical evidence of organ dysfunction or failure. Complicated cases of the disease were those with problems that were not directly attributable to acute hemolysis (Jacobson, 2006). The clinical signs and pathology of the disease may include pyrexia, splenomegaly, anaemia, haemolysis and haemoglobinuria, icterus, circulatory collapse, multiple organ failure and neurological signs (Jacobson and Clark, 1994). The clinical signs of dogs infected with South African strains of *Babesia vogeli* have not been described, but this parasite usually causes mild or subclinical signs in adult dogs elsewhere in the world (Uilenberg et al., 1989). Some dogs are apparently more resistant to canine babesiosis without showing clinical signs. Nevertheless, they remain as asymptomatic carriers, presenting high antibodies titers and seem to be more resistant to further infections (Breitschwerdt et al., 1983; Masuda et al., 1983). This resistance is due to *Babesia* maintenance in the host. This carrier state is

known as premunition and it is believed to be the same as it would occur in *Babesia* infections of cattle and sheep (Penzhorn et al., 1995; Wlosniewski et al., 1997). The chronic asymptomatic carrier state in *Babesia* infection of domestic and wild animals has been recognized for many years (Lewis et al., 1995; Martinod et al., 1985). Chronically infected animals can act as reservoirs of the disease and they can keep elevated antibodies titers for long periods of time (de Vos et al., 1987).

2.1.4. Pathogenesis of canine babesiosis

The incubation period of canine babesiosis varies from 10 to 21 days for *B. canis* (senso stricto). The female ticks feed on their host for about one week only and have left the host by the time disease develops (Schoeman, 2009). The immunological response plays the most important role in pathogenesis of canine babesiosis. *Babesia* initiates a mechanism of antibody-mediated cytotoxin and destruction of circulating erythrocytes. Autoantibodies are directed against components of the membranes of infected and uninfected erythrocytes. This causes intravascular and extravascular haemolysis which leads to anaemia, haemoglobinaemia, hemoglobinuria and bilirubinuria (Irwin, 2005; Pedersen, 1999). Pyrexia which is attributed to the release of endogenous pyrogens from erythrolysis, parasite destruction, and activation of inflammatory mediators can also develop (Breitschwerdt, 1990; Maegraith et al., 1957). Antibody-coated erythrocytes are destroyed by macrophages in the spleen and liver (extravascular haemolysis). Intravascular haemolysis results from complement binding to an erythrocytic membrane by surface antigen-antibody reaction (Day, 1999). Haemolysis, severe systemic inflammation and associated pro-inflammatory cytokine production are

thought to contribute to disease pathogenesis in canine babesiosis (Reyers et al., 1998).

2.1.5. Control, prevention and treatment of canine babesiosis

The control of ticks is generally carried out by the use of acaricides (spot-on, pour-on or spray-on). Fiplonil and amitraz are among the active ingredients most used (Labruna and Pereira, 2001). Regular physical inspections should also be conducted to remove all attached ticks. Proper tick removal procedures (e.g., use of gloves) must be adopted (Gammons and Salam, 2002).

As variations in pathogenesis and clinical manifestations are known to exist between species and subspecies of the canine piroplasms, knowledge of their distribution is important for the therapy against canine babesiosis and also has potential implications for vaccine development (Schetters, 2005; Uilenberg et al., 1989). *In vitro* culture-derived soluble parasite antigens (SPA) have been used to protect dogs in Europe against *B. canis* (senso stricto) challenge in a commercially available vaccine since the 1980s, and it was reported in one study that the incidence of babesiosis decreased from 16% to near zero in populations of vaccinated dogs living in endemic regions over the three-year period of study (Moreau et at., 1988). In some experimental studies, SPA from different *Babesia* species have been shown to induce protective immunity when used as vaccine. When dogs were vaccinated with a vaccine comprising SPA from *B. rossi* combined with SPA from *B. canis*, protective immunity against experimental challenge infection was accomplished. Vaccination resulted in reduced clinical signs that resolved spontaneously, and reduction of parasites and SPA in the blood. Not a single infected erythrocyte could be found in

blood smears of *B rossi* challenged dogs that had been repeatedly boosted (three vaccinations in total) (Schetters et al., 2007).

Experimental studies suggested that a single dose of imidocarb dipropionate (6 mg/kg) protects dogs from *B. canis* challenge for up to 8 weeks (Uilenberg et al., 1981) and that doxycycline at 5 mg/kg/day ameliorates the severity of disease when challenged with virulent *B. canis* (Vercammen et al., 1996). The drugs of choice in South Africa against *B. rossi* are diminazene aceturate and imidicarb diproprionate. A single dose of imidocarb at 7.5 mg/kg or a single dose of diminazene at 3.5 mg/kg followed by a dose of 6 mg/kg imidocarb the following day have been shown to clear *B. rossi* infections (Penzhorn et al., 1995). Diminazene dose calculation should be meticulous due to its low therapeutic index, especially in puppies. The drug should not be repeated within an interval of shorter than a 3-week period (Miller et al., 2005).

2.1.6. Diagnosis of canine babesiosis

Historically, the microscopic identification of large piroplasms in canine erythrocytes was sufficient for the diagnosis of *Babesia* infection. Geographic location, differences in pathogenicity, antigenic variation, and differences in vector specificity have all been used to support the existence of three species of *Babesia canis: B. canis, B. rossi* and *B. vogeli* (Hauschild and Schein, 1996; Hauschild et al., 1995; Lewis et al., 1996; Uilenberg et al., 1989).

In South Africa, the diagnosis of canine babesiosis is made on examination of a capillary smear taken from the pinna (Keller et al., 2004; Malherbe and Parkin,

1951). Blood smears are typically used to quantify parasitaemia in *Babesia* research (Lewis et al., 1995; Schetters et al., 1994, 1996, 1997; Van Heerden et al., 1983).

Serology does not definitively discriminate species, as antibodies to *Babesia* species often cross-react (Birkenheuer et al., 2003). The Indirect fluorescent antibody test (IFAT) and the enzyme-linked immunosorbent assay (ELISA) are considered to be highly sensitive and moderately specific tools for detection of antibodies to *Babesia* parasites in dogs (Dell'Porto et al., 1993; Furuta et al., 2004; Yamane et al., 1993). Isolation of infected erythrocytes with a Percoll gradient can be used to enhance the recovery and identification of parasitized erythrocytes (Comazzi et al., 1999).

Recently, molecular techniques including PCR and sequence analysis have been used for the epidemiological study and phylogenetic analysis of tick-borne pathogens (Burkot et al., 2000; Cacciò et al., 2002; Duh et al., 2001; Holman et al., 2002; Inokuma et al., 2003; Kjemtrup et al., 2000; Matjila et al., 2009; Zahler et al., 1998). The advantages of molecular methods over other techniques are their sensitivity and specificity in detecting *Babesia* in peripheral blood and arthropod vectors. Furthermore, subsequent sequence analysis provides phylogenetic information on the pathogen. Because blood-sucking vectors contain infected host blood and the pathogen itself, analysis of these vectors is a reliable tool with which to demonstrate the existence of pathogens in a specific area (Roux and Raoult, 1999). Ticks have also being used for the epidemiological study of tick-borne pathogens (Sparagano et al., 1999).

2.1.7. *Babesia rossi* Erythrocyte Membrane Antigen (*BrEMA*1) gene

The South African canine babesiosis is a heterogeneous complex of disease presentations caused by *B. rossi* (Boozer and Macintire, 2003; Lewis et al., 1996; Lobetti, 1998). Preliminary evidence suggests that there might be a link between parasite genotype and disease phenotype (Matjila et al., 2009). A polymorphic phosphoprotein localized on the cytoplasmic surface of *B. rossi*-infected red blood cells was recently characterized and named *Babesia rossi* erythrocyte membrane antigen 1 (*BrEMA*1) (B. Carcy, unpublished data). Analysis of the *BrEMA*1 genes of various laboratory strains of *B*. *rossi* revealed that these code for polymorphic proteins that contain various numbers of repetitive hexapeptide motifs. Although the exact function of *BrEMA*1 is not known, it is suspected that this gene may be unique to the *B. rossi* parasites and it is absent in *B. vogeli* and *B. canis* (Matjila et al., 2009). Thus, since *B*. *rossi* is the most pathogenic species of the large babesias of dogs, it was hypothesized that the gene may have a role in virulence. This gene was used as a genetic marker to classify *B*. *rossi* isolates obtained from South Africa and to analyze the relationships between particular genotypes and the occurrence and severity of clinical manifestation of *B. rossi*-induced canine babesiosis. At present, at least 12 *B. rossi* genotypes (using *BrEMA*1) primers, sequencing and phylogenetic sequences have been identified, with genotype 19 reported to be the most virulent (Fig. 2). This genotype was associated with high numbers of dogs that were admitted at OVAH suffering from *B.rossi*-induced canine babesiosis and was the most common genotype occurring amongst solid-organ complication and fatal cases. Genotype19, 28, 29, and 11 are the most encountered amongst the 12 identified genotypes. These genotypes belong to three monophyletic groups (i.e. genotype19, genotype28/29 and genotype11); in agreement with their separation into three

distinct groups of genotypes on the basis of their frequency and virulence (Fig. 2). Our study aims to investigate further, the degree of variation within *B. rossi* genotypes.

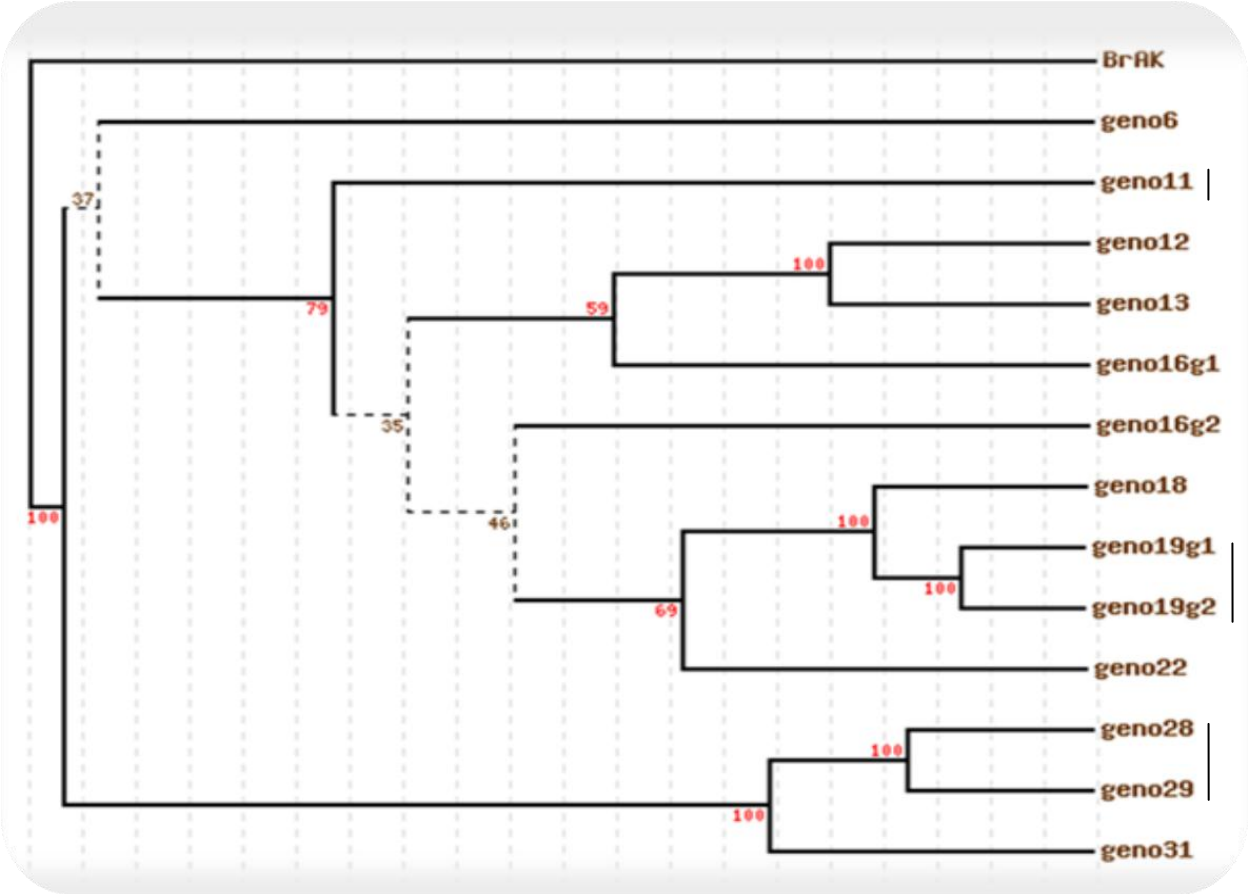

Figure. 2. Cluster algorithm tree showing the phylogenetic relationship between various genotypes based on the *BrEMA*1 gene sequences (Matjila et al., 2009). Numbers at the notes indicate bootstrap values.

2.2. Problem statement

Babesia rossi-induced canine babesiosis is associated with severe clinical manifestations and mortalities. There is a lack of information regarding the relationship between parasite genotype and disease phenotype.

2.3. Objectives of the study

The objectives of this study were:

(i) To confirm and validate the previous results (Köster et al., 2009) by screening canine blood samples using the reverse line blot (RLB) hybridization assay for the presence of *Babesia* species. This test can simultaneously screen for the presence of *Theileria, Anaplasma* and *Ehrlichia* species.

(ii) To determine the heterogeneity of *B. rossi* genotypes from infected dogs in South Africa using PCR, sequencing and phylogenetic analysis.

(iii) To use a real-time PCR for the detection of *B. rossi* genotypes and

(iv) To evaluate the relationship between *BrEMA*1 genotypes, clinical manifestation and age of the dogs.

CHAPTER 3

3. MATERIALS AND METHODS

3.1. Sample collection

In a previous study, 75 blood samples were collected from *Babesia*-positive dogs at Onderstepoort Veterinary Academic Hospital (OVAH), Faculty of Veterinary Science, University of Pretoria, South Africa. These samples were collected from the jugular vein of each dog into ethylenediamine tetra-acetic acid (EDTA) tubes. About 200 µl of blood was aliquoted into 1.5 ml Eppendorf tubes and stored at -20°C until DNA was extracted. These samples were confirmed *B. rossi*-positive in a previous study using blood smear examination, PCR and the reverse line blot hybridization assay (Köster et al., 2009). An additional 26 blood samples were recently collected from *Babesia*-positive dogs at Onderstepoort Veterinary Academic Hospital (OVAH), Faculty of Veterinary Science, University of Pretoria, South Africa. A total of 44 samples were used for correlation between *BrEMA*1 genotypes and age of dogs. These samples were used due to their known genotype status as determined by sequencing. These samples were grouped into two groups, i.e. 28 were from dogs aged 2 years and younger, and 15 were from dogs 3 to 9 years of age.

3.2. DNA extraction

DNA was extracted from 200 µl of EDTA-anticoagulated whole blood (stored at -20°C) using the QIAamp® DNA Mini Kit (Qiagen, Southern Cross Biotechnologies, South Africa), following the manufacturer's instructions. The concentration of the DNA was determined by measuring the absorbance of light (260 nm) in a spectrophotometer (Beckman Coulter™, DU® 530; Beckman Coulter, South Africa).

3.3. PCR

The PCR was conducted as described by Nijhof et al. (2003) and Nijhof et al. (2005). One set of primers was used to amplify a 460- to 520-base pair fragment of the 18S SSU rRNA spanning the V4 hypervariable region. The forward primer, RLB-F2 (5'-GAC ACA GGG TAG TGA CAA G -3'), and the reverse primer, RLB-R2 (biotin-5'-CTA AGA ATT TCA CCT CTG ACA GT -3'), were used for the amplification and hybridized with regions conserved for *Theileria* and *Babesia* (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 to 520 bp from the V1 hypervariable region of the 16S SSU rRNA gene (Bekker, et al., 2002; Nijhof et al., 2005). PCR reaction mixture consisted of 12.5 µl of Platinum® Quantitative PCR SuperMix-UDG (Invitrogen, The Scientific Group, South Africa), 20 pmol (0.25 µl) of each primer, 2.5 µl of DNA to a total volume of 25 µl. The Gene Amp®PCR System 9700 and 2700 (Applied Biosystems, South Africa) and the 2720 Thermal Cycler (Applied Biosystems, South Africa) were used for amplification. Amplification was done under the following conditions: Initial step of 3 min at 37°C, 10 min at 94°C, and 10 cycles of 94°C for 20 sec, 67°C for 30 sec, and 72°C for 30 sec, with lowering of the annealing step by 2°C after every second cycle (touchdown PCR), followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 57°C for 30 sec, and extension at 72°C for 30 sec. Two microlitres of DNA amplicon were loaded on 1.5% agarose gel electrophoresis containing 10 mg/ml ethidium bromide for verification.

3.4. Reverse line blot hybridization assay

Reverse Line Blot (RLB) hybridization was conducted on amplified products (*Babesia*, *Theileria*, *Ehrlichia* and *Anaplasma*) as previously described (Matjila et al., 2004). A known positive sample was used as a positive control to ensure that all genus and species-specific probes were correctly bound to the RLB membrane and that they were functional (Matjila et al., 2005).

3.4.1. Preparation of membrane

The genus-and species-specific oligonucleotides (Table 1) were diluted in 150 µl 0.5 M $NaHCO_3$ (pH 8.4). The membrane was then marked and incubated for 10 min in 16% EDTA at room temperature. It was then washed for 2 minutes with demineralized water. The species-specific oligonucleotides were then linked to the membrane by loading them onto the lanes of the miniblotter, followed by 1 minute incubation. The oligonucleotide probe solutions were aspirated and the membrane was inactivated by incubation in 100 ml of a 100 mM NaOH solution for 10 minutes at room temperature. The membrane was then washed with SSPE-0.1% sodium dodecyl sulfate (SDS) solution for 5 minutes at 60°C.

3.4.2. Reverse line blot hybridization

The membrane was incubated for 5 min in ~10 ml 2 X SSPE/0.1% SDS at room temperature. A volume of 25 µl of PCR product was diluted to an end volume of 150 µl 2 X SSPE/0.1% SDS. The products were denatured for 10 min at 100°C in a thermacycler, cooled immediately on ice and centrifuged. The membrane was placed in a miniblotter, with slots perpendicular to line pattern of applied probes. Slots were filled with diluted PCR product and the empty slots were filled with 2 X SSPE/0.1%

SDS, to avoid cross flow. The miniblotter was incubated for 60 min at 42°C for hybridization to take place. Samples were removed by aspiration, whereafter the membrane was removed from the blotter. The membrane was washed twice in preheated 2 X SSPE/0.5% SDS for 10 min at 50°C in a water bath under gentle shaking. It was then incubated with 10 ml 2 X SSPE/0.5% SDS + 2.5 µl streptavidin-POD (peroxidase-labeled) conjugate (Roche Diagnostics, South Africa) (1.25 U) for 30 min at 42°C. The membrane was then washed twice again in preheated 2 X SSPE/0.5% SDS for 10 min at 42°C in water bath under gentle shaking. The membrane was washed again twice with 2 X SSPE for 5 min at room temperature under gentle shaking. The membrane was then incubated for 1 min in 10 ml of ECL detection fluid (DNA Thunder™, Perkin Elmer, Separation Scientific, South Africa). The membrane was covered with overhead sheet and air bubbles were removed by rolling a tube over the sheet. It was placed between 2 (clean) overhead sheets and placed in the exposure cassette. The X-ray film (X-OMAT™ Blue XB-1, Kodak, Separation Scientific, South Africa) was exposed for 5-20 min and then developed for detection of hybridized PCR products, which were visualized by chemiluminescence. The film was placed in a grid and each sample lane correlated with the DNA probes.

After use, the PCR products were stripped from the membrane by two washes for 30 min each time at 80°C in 1% SDS solution. The membrane was rinsed in 20 Mm EDTA (pH 8.0) for 15 min and then stored in fresh 20 mM EDTA solution at 4°C for reuse (Gubbels et al., 1999).

The probes and their sequences used are listed in Table 1.

Table 1: The list of the probes and their sequences used for detecting pathogen DNA.

Probes	5'-3'
Ehrlichia/*Anaplasma* genus-specific	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
Anaplasma bovis	GTA GCT TGC TAT GRG AAC A
Ehrlichia ruminantium	AGT ATC TGT TAG TGG CAG
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* genus-specific	TAA TGG TTA ATA GGA RCR GTT G
Theileria genus-specific	ATT AGA GTG CTC AAA GCA GGC
Babesia genus-specific 1	ATT AGA GTG TTT CAA GCA GAC
Babesia genus-specific 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 CGT 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	GTG TTT ATC GCA GAC TTT TGT
Babesia gibsoni	CAT CCC TCT GGT TAA TTT G
Babesia leo	ATC TTG TTG CCT TGC AGC T
Babesia sp (sable)	GCT GCA TTG CCT TTT CTC C

Theileria sp. (kudu)	CTG CAT TGT TTC TTT CCT TTG
Theileria sp (sable)	GCT GCA TTG CCT TTT CTC C
Theileria bicornis	GCG TTG TGG CTT TTT TCT G
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 lestoguardi	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
Theileria separata	GGT CGT GGT TTT CCT CGT

Symbols used to indicate degenerate positions:

R = A/G, W=A/T, Y=C/T

3.5. *Babesia rossi* Erythrocyte Membrane Antigen PCR

Samples testing positive for *B. rossi* on RLB were re-amplified with primers Frep*BrEMA*1 (5'-CCA ACA TTG ATG ATG ACA A-3') and Rrep*BrEMA*1 (5'-CTG CAT GTC AGC TTA ATC A-3'). These primers were designed from the *B. rossi* Erythrocyte Membrane Antigen (*BrEMA*1) gene and amplifies an 18-nucleotide repetitive sequence whose number (from 16 to 31) and sequence have been shown to be variable between *BrEMA*1 genes. To check the specificity of *BrEMA*1 gene, two additional samples, positive for *B. vogeli* and *B. canis*, were included. PCR amplification was done with 2.5 µl of extracted DNA in a final volume of 25 µl of PCR reaction containing 10 µl PCR grade water, 10 µM of each primer, and 12.5 µl of

High Fidelity PCR Master. The cycling conditions consisted of an initial cycle 5 min at 94°C, 30 cycles of amplification (94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min) and 1 cycle of 7 min at 72°C.

3.6. Sequencing and phylogenetic analysis

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Germany) to remove excess PCR reagents that can influence the subsequent sequencing PCR reaction. PCR amplicons were directly sequenced using 3.2 pmol of the primer Frep*BrEMA1* and Rrep*BrEMA1* in the Equine Research Centre Genetic Lab of the Faculty of Veterinary Science and in Inqaba-Biotec, Pretoria, South Africa. ABI 3500XL genetic analyzer and BigDye Terminator v3.1 Cycle sequencing Kit were used (Applied Biosystems). Sequence data were assembled and edited using GAP 4 of the Staden package (Version 1.6.0 for Windows) and the sequences were aligned with sequences of related genera using CluctalX. Nucleotide sequences were translated to amino acids, aligned and phylogenetic trees were generated using the 'GeneBee' (www.//genebee.msu.su/genebee.html) programme. Phylogenetic analyses using cluster distance algorithm method were carried out (Phylip, Multiline) in to the program format from the aligned amino acid sequences. In the cluster algorithm, the notion of distance between groups of sequences was used for the setting of the branching order (www.//genebee.msu.su/genebee.html).

3.7. Real-Time PCR

Primers Frep*BrEMA1* and Rrep*BrEMA1* were used in a real-time PCR to specifically amplify the Br*EMA1* gene. The eBox LightCycler DNA Master SYBR Green 1 Kit was used (Roche Diagnostics). The real-time PCR reaction mixture consisted of 13.4 µl of water, PCR-grade, 3 mM $MgCl_2$, 1.0 µM of each primer, 2 µl LightCycler® DNA Master SYBR Green I and 1 µl of DNA to a total volume of 20 µl. The real-time PCR was performed in glass capillaries using a LightCycler 2.0 (Roche Diagnostics). The cycling conditions consisted of an initial cycle of 10 min at 95°C, 45 cycles of amplification (95°C for 10 sec, 55°C for 5 sec and 72°C for 20 sec), 1 cycle of melting curve (95°C for 0 sec, 65°C for 15 sec, 95°C for 0 sec) and 1 cycle of 30 sec at 40°C. Fluorescence values were measured at 530 nm.

3.7.1. Analytical specificity of real-time PCR in detecting *BrEMA*1 genotypes

The specificity of real-time PCR was tested using seven samples representing seven different *BrEMA*1 genotypes, genotype11, 18, 19g1, 19g2, 28g1, 28g2 and 29. In addition, DNA from *Babesia vogeli* and *Babesia canis* were tested.

3.7.2. Analytical sensitivity of the real-time PCR in detecting *BrEMA*1 genotypes

Babesia rossi DNA with a concentration of 185.4 ng/ul was subjected to 10-fold dilutions in water. All dilutions were prepared in triplicate for the real-time PCR. The identical samples were tested in two separate runs.

CHAPTER 4

4. RESULTS

4.1. DNA concentration

DNA concentration, determined by measuring the absorbance of light (260 nm) in a spectrophotometer (Beckman Coulter™, DU® 530; Beckman Coulter, South Africa), ranged from 3.57 ng/ul to 185.4 ng/ul.

4.2. RLB results

PCR/RLB assay results revealed that 98 (97%) of the 101 amplified parasite DNA hybridized to *Babesia rossi* species-specific probe and three (3%) to *Babesia vogeli* species-specific probe (Fig. 3 and Table 2.). Mixed infection was detected whereby one (1%) sample was co-infected with *B. vogeli* and *Ehrlichia canis*. The *Ehrlichia* positive sample was excluded in order to focus only on dogs that were *Babesia* positive. No signal was detected for the negative control sample.

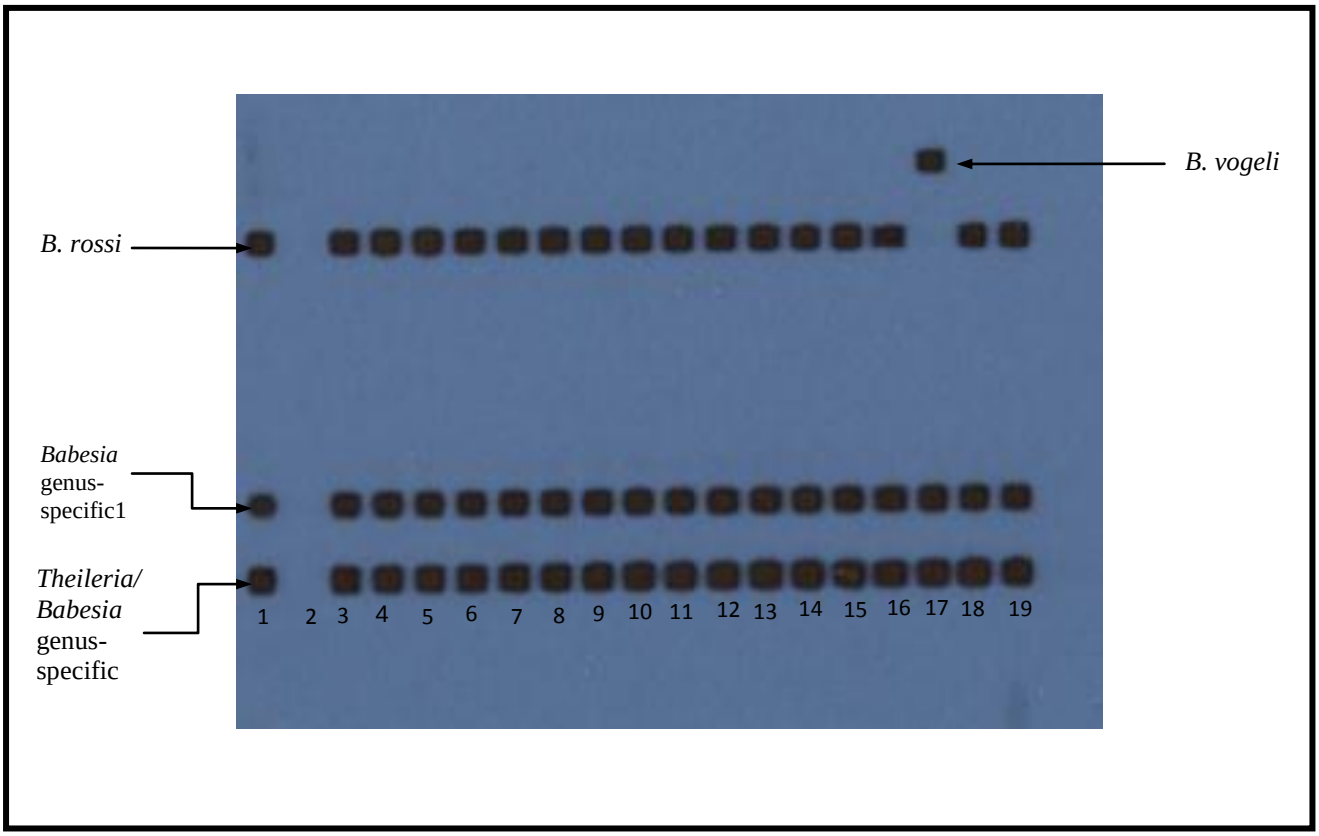

Figure. 3. Reverse line blot hybridization of *Babesia* PCR products. Species-specific oligonucleotides probe are applied in horizontal rows, and PCR products from samples and controls in vertical lanes. Lane 3 to 19: PCR products, lane 1: Positive control and lane 2: Negative control.

Table 2. RLB results of 101 samples

RLB number	Sample ID	Result
1.	LK1	*B. rossi*
2.	LK2	*B. rossi*
3.	LK3	*B. rossi*
4.	LK4	*B. rossi*
5.	LK5	*B. rossi*
6.	LK6	*B. rossi*
7.	LK7	*B. rossi*
8.	LK8	*B. rossi*
9.	LK9	*B. rossi*
10.	LK10	*B. rossi*

11.	LK11	*B. rossi*
12.	LK12	*B. rossi*
13.	LK13	*B. rossi*
14.	LK14	*B. rossi*
15.	LK15	*B. vogeli*
16.	LK16	*B. rossi*
17.	LK17	*B. rossi*
18.	LK18	*B. rossi*
19.	LK19	*B. rossi*
20.	LK20	*B. rossi*
21.	LK21	*B. rossi*
22.	LK22	*B. rossi*
23.	LK23	*B. rossi*
24.	LK24	*B. rossi*
25.	LK25	*B. rossi*
26.	LK26	*B. rossi*
27.	LK27	*B. rossi*
28.	LK28	*B. rossi*
29.	LK29	*B. rossi*
30.	LK30	*B. rossi*
31.	LK31	*B. rossi*
32.	LK32	*B. rossi*
33.	LK33	*B. rossi*
34.	LK34	*B. rossi*
35.	LK35	*B. rossi*
36.	LK36	*B. rossi*
37.	LK37	*B. rossi*
38.	LK38	*B. rossi*
39.	Lk39	*B. rossi*
40.	LK40	*B. rossi*
41.	LK41	*B. rossi*
42.	LK42	*B. rossi*
43.	LK43	*B. rossi*
44.	LK44	*B. rossi*
45.	LK45	*B. rossi*
46.	LK46	*B. rossi*
47.	LK47	*B. rossi*
48.	LK48	*B. rossi*

49.	LK49	*B. rossi*
50.	LK50	*B. rossi*
51.	LK51	*B. rossi*
52.	LK52	*B. rossi*
53.	LK53	*B. rossi*
54.	LK54	*B. rossi*
55.	LK55	*B. vogeli* and *E. canis*
56.	LK56	*B. rossi*
57.	LK57	*B. vogeli*
58.	LK58	*B. rossi*
59.	LK59	*B. rossi*
60.	LK60	*B. rossi*
61.	LK61	*B. rossi*
62.	LK62	*B. rossi*
63.	LK63	*B. rossi*
64.	LK64	*B. rossi*
65.	LK65	*B. rossi*
66.	LK66	*B. rossi*
67.	LK67	*B. rossi*
68.	LK68	*B. rossi*
69.	LK69	*B. rossi*
70.	LK70	*B. rossi*
71.	LK71	*B. rossi*
72.	LK72	*B. rossi*
73.	LK73	*B. rossi*
74.	LK74	*B. rossi*
75.	LK75	*B. rossi*
76.	CL1	*B. rossi*
77.	CL2	*B. rossi*
78.	CL3	*B. rossi*
79.	CL4	*B. rossi*
80.	CL5	*B. rossi*
81.	CL6	*B. rossi*
82.	CL7	*B. rossi*
83.	CL8	*B. rossi*
84.	CL9	*B. rossi*
85.	CL10	*B. rossi*
86.	CL11	*B. rossi*

87.	CL12	*B. rossi*
88.	CL13	*B. rossi*
89.	CL14	*B. rossi*
90.	CL15	*B. rossi*
91.	CL16	*B. rossi*
92.	CL17	*B. rossi*
93.	CL18	*B. rossi*
94.	CL19	*B. rossi*
95.	CL22	*B. rossi*
96.	CL23	*B. rossi*
97.	CL24	*B. rossi*
98.	CL25	*B. rossi*
99.	CH	*B. rossi*
100.	CL CON 20	*B. rossi*
101.	CL CON 21	*B. rossi*

4.3. *Babesia rossi* Erythrocyte Membrane Antigen PCR

The *BrEMA*1 gene of all samples that were positive for *Babesia rossi* on PCR/RLB could be amplified with primers FrepBr*EMA*1 (Fig. 4). The *BrEMA*1 gene of the *Babesia vogeli* and *Babesia canis* positive samples could not be amplified with the *BrEMA*1 primers using conventional PCR. In Fig. 4, lane 10 shows genotype12g2 with the smallest amplified product of *ca* 270 bp and genotype29 on lane 6 and 9 with the largest amplified product of *ca* 608 bp (Fig. 4).

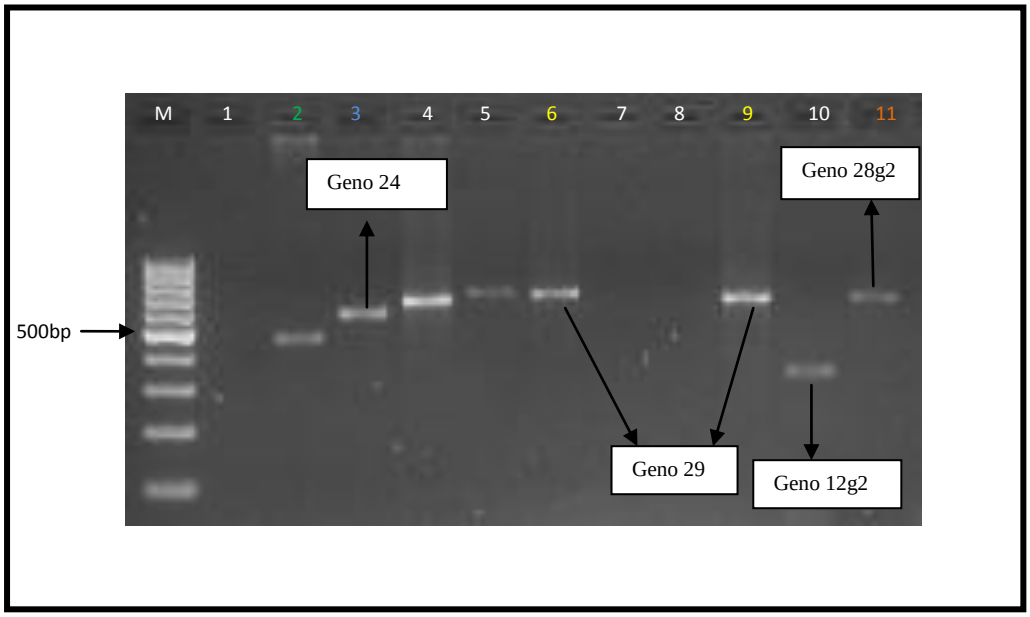

Figure.4. PCR amplification was performed with Frep*BrEMA1* and Rrep*BrEMA1* primers. Five µl of PCR products were loaded on a 1.5% agarose gel electrophoresis membrane. No product was obtained in lane 7 and 8 because amplification was done with *B. vogeli* and *B. canis* DNA respectively. Lanes 3, 10 and 11 represent new genotypes which are genotype24, genotype12g2, and genotype28g2 respectively with the most prevalent genotype29 in lane 6 and 9. Lane M is the O'GeneRuler 100 bp DNA ladder (Fementas) and lane 1 is the negative control.

4.4. Sequence and phylogenetic analysis

Parasite *BrEMA*1 consensus sequences obtained from 52 samples were compared with GenBank sequences using basic local alignment search tool (BLAST) (Altschul et al., 1990). These samples were used because they were the only samples out of 101 that passed quality control test for sequencing. Based on sequence analysis, 10 different genotypes were identified on the basis of *BrEMA*1 number of hexapeptide repeats, in agreement with previously published results and the existence of an additional 3 new genotypes: genotype24 (2%), identified on the basis of the size of number of repeat whereas genotype12g2 (2%) and 28g2 (2%) were identified on the

basis of their sequences within the initially described geno12 and geno28. We classified geno12 into two genetically distinct genotypes which are geno12g1, previously known as geno12 and geno12g2, a new genotype which is phylogenetically more related to geno11 and geno16g2, than to geno12g1 (Fig. 5). This relationship may be due to the fact that they are the genotypes containing the motif ASPGSV and two amino acid substitutions (IP rather than VL) downstream from the repetitive sequence of *BrEMA*1 (Matjila et al., 2009) (Table 3). Geno28 strains can now be separated into two groups, namely geno28g1, previously known as geno28 and geno28g2 which is a new genotype with a single amino acid mutation (Table 3 & 4). The most encountered genotype was genotype29 (33%), followed by 28 (27%, i.e., 19% genotype28g1 and 8% genotype28g2), 19 and 11 (Fig. 6).

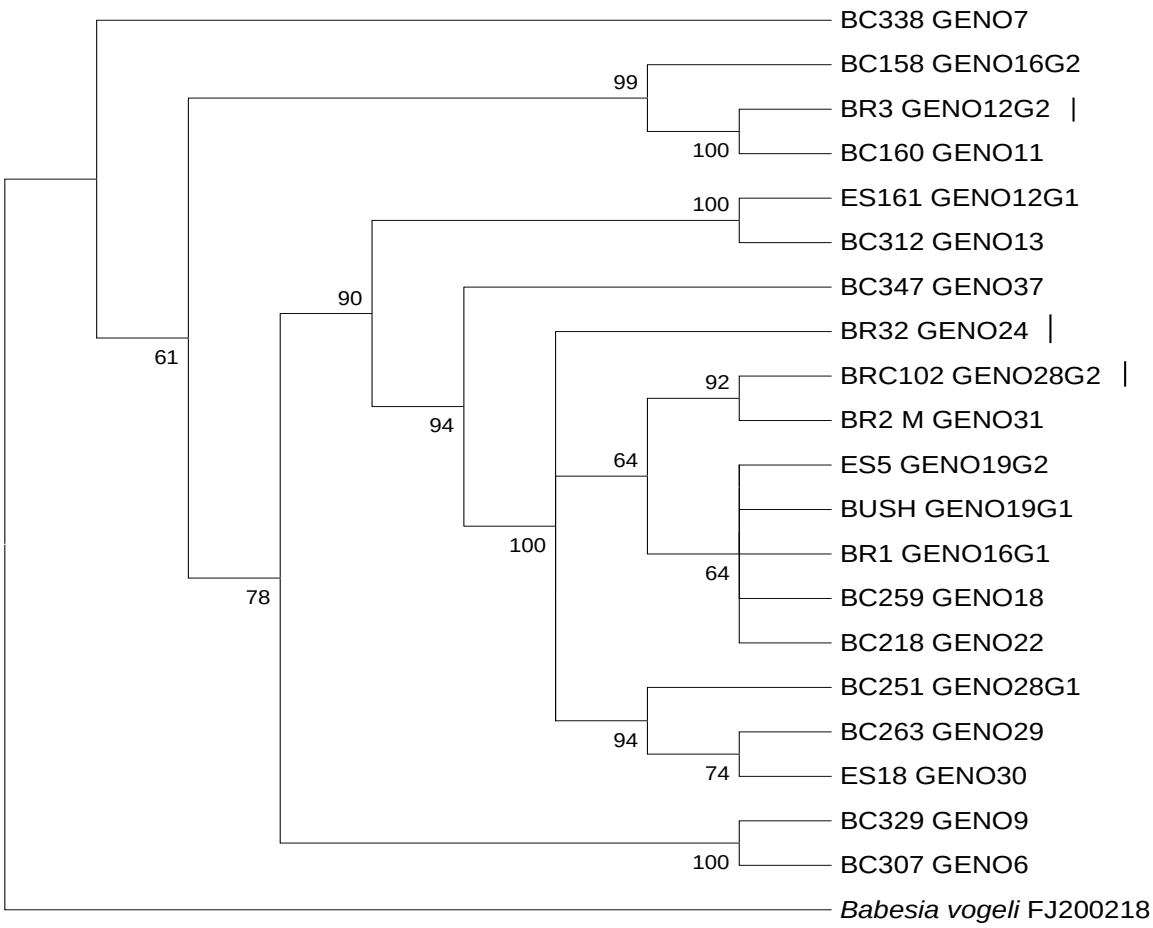

Figure. 5. Phylogenetic tree showing the phylogenetic relationship between *B. rossi BrEMA*1-derived genotypes including 3 new genotypes. Numbers below the branch indicate bootstrap support from 1000 replications.

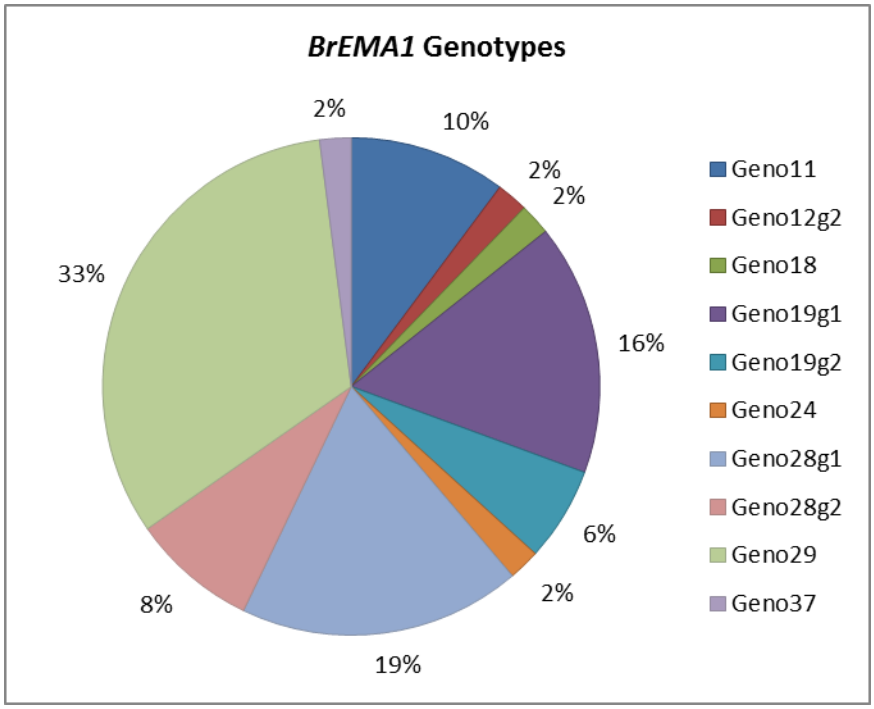

Figure. 6. Occurrence of 10 different *BrEMA*1 genotypes in 52 *Babesia rossi*-positive blood samples from OVAH.

Table 3. Consensus nucleotide and amino acid sequence of 10 identified *Babesia rossi* genotypes.

Genotype and sample name	**Consensus nucleotide sequence**	**Consensus amino acid sequence**
Geno12g2 Br3	caacattgatgatgacaaggccagtgtcaaatctg ctggttctgttagatctgcagactcggtagcatcc cctggttctgtcaagtctgcggcttctgttagatc tgcagactcggtagcatcccctggttctgtcaagt ctgcggcttctgttagatctgcagactcggtagca tcccctggttctgtcaagtctgcggcttctgttag atctgcagactcggtagaatccgttgcttctgtta gatctgcagatgcagacattccccatgacacccac cttgatgaagctgacatgcagc	NIDDDKASV KSAGSV RSADSV ASPGSV KSAASV RSADSV ASPGSV KSAASV RSADSV ASPGSV KSAASV RSADSV ESVASV RSADAD**IP***HDTHLDEADMQ
Geno24 Br32	caacattgatgatgacaaggccagtgtcaaatctg cagcctcgctcaaatccgctgattctgcaaaatcc gttgcttctgttagatctccagactcggtagaatc cgctggttctgtaaaatccgctgcttctgttagat ctgcagactcggtagaatccgctgcttccgcaaaa tccgctgcttctgttagatctgcagactcggtaga atccgctggttccgcaaaatccgttgcttctgtta gatctgcagactcggtagaatccgctggttctgca aaatccgttgcttctgttagatctgcagactcggt	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA

	agaatccgctggttctgcaaaatccgttgcttctg ttagatctgcagactcggtagaatccgctggttct gcaaaatccgttgcttctgttagatctgcagactc ggtagaatccgctggttctgcaaaatccgttgctt ctgttagatctgcagatgcagacgttcttcatgac acccatcttgatgaagctgacatgcag	KSVASV RSADADVLHDTHLDEADMQ
Geno28g2 BrCL02	caacattgatgatgacaaggccagtgtcaaatctg cagcctcgctcaaatccgctgattctgcaaaatcc gttgcttctgttagatctccagactcggtagaatc cgctggttctgcaaaatccgctggttctgtaaaat ccgctgcttctgttagatctgcagactcggtagaa tccgctggttccgcaaaatccgttgcttctgttag atctgcagactcggtagaatccgctggttctgcaa aatccgttgcttctgttagatctgcagactcggta gaatccgctggttctgcaaaatccgttgcttctgt tagatctgcagactcggtagaatccgctggttccg caaaatccgttgcttctgttagatctgcagactcg gtagaatccgctggttctgcaaaatccgttgcttc tgttagatctgcagactcggtagaatccgctggtt ctgcaaaatccgttgcttctgttagatctgcagac tcggtagaatccgctggttctgcaaaatccgttgc ttctgttagatctgcagatgcagacgttcttcatg acacccatcttgatgaagctgacatgcagc	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAASV RSADSV ESAGSA KS**V***ASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ
Geno11 Br16	aacattgatgatgacaaggccagtgtcaaatctgc tggttctgttagatctgcagactcggtagcatccc ctggttctgtcaagtctgcggcttctgttagatct gcagactcggtagcatcccctggttctgtcaagtc tgcggcttctgttagatctgcagactcggtagaat ccgttgcttctgttagatctgcagactcggtagaa tccgttgcttctgttagatctgcagatgcagacat tccccatgacacccaccttgatgaagctgacatgc agc	IDDDKASV KSAGSV RSADSV ASPGSV KSAASV RSADSV ASPGSV KSAASV RSADSV ESVASV RSADSV ESVASV RSADAD**IP**HDTHLDEADMQ
Geno18 BrC118	tgatgacaaggccagtgtcaaatctgcagcctcgc tcaaatccgctgattctgcaaaatccgttgcttct gttagatctccagactcggtagaatccgctggttc tgcaaaatccgctgcttctgttagatctgcagact cggtagaatccgctgcttccgcaaaatccgctgct tctgttagatctgcagactcggtagaatccgctgg ttctgcaaaatccgttgcttctgttagatctgcag actcggtagaatccgctggttctgcaaaatccgct gcttctgttagatctgcagactcggtagaatccgc tggttctgcaaaatccgttgcttctgttagatctg cagatgcagacgttcttcatgacacccatcttgat gaagcta	DDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSAASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEA
Geno19g1 Br18	caacattgatgatgacaaggccagtgtcaaatctg cagcctcgctcaaatccgctgattctgcaaaatcc gttgcttctgttagatctccagactcggtagaatc cgctggttctgcaaaatccgctggttctgtaaaat ccgctgcttctgttagatctgcagactcggtagaa tccgctgcttccgcaaaatccgctgcttctgttag atctgcagactcggtagaatccgctggttctgcaa	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSAASV RSADSV ESAGSA

	aatccgttgcttctgttagatctgcagactcggta gaatccgctggttctgcaaaatccgctgcttctgt tagatctgcagactcggtagaatccgctggttctg caaaatccgttgcttctgttagatctgcagatgca gacgttcttcatgacacccatcttgatgaagctga catgcagc	KSVASV RSADADVLHDTHLDEADMQ
Geno28g1 Br25	caacattgatgatgacaaggccagtgtcaaatctg cagcctcgctcaaatccgctgattctgcaaaatcc gttgcttctgttagatctccagactcggtagaatc cgctggttctgcaaaatccgctggttctgtaaaat ccgctgcttctgttagatctgcagactcggtagaa tccgctgcttccgcaaaatccgctgcttctgttag atctgcagactcggtagaatccgctggttctgcaa aatccgttgcttctgttagatctgcagactcggta gaatccgctggttctgcaaaatccgttgcttctgt tagatctgcagactcggtagaatccgctggttccg caaaatccgttgcttctgttagatctgcagactcg gtagaatccgctggttctgcaaaatccgttgcttc tgttagatctgcagactcggtagaatccgctggtt ctgcaaaatccgttgcttctgttagatctgcagac tcggtagaatccgctggttctgcaaaatccgttgc ttctgttagatctgcagatgcagacgttcttcatg acacccatcttgatgaagctgacatgcagc	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ
Geno29 Br42	caacattgatgatgacaaggccagtgtcaaat ctgcagcctcgctcaaatccgctgattctgca aaatccgttgcttctgttagatctccagactc ggtagaatccgctggttctgcaaaatccgctg gttctgtaaaatccgctggttctgtaaaatcc gctgcttctgttagatctgcagactcggtaga atccgctgcttccgcaaaatccgctgcttctg ttagatctgcagactcggtagaatccgctggt tctgcaaaatccgttgcttctgttagatctgc agactcggtagaatccgctggttctgcaaaat ccgttgcttctgttagatctgcagactcggta gaatccgctggttccgcaaaatccgttgcttc tgttagatctgcagactcggtagaatccgctg gttctgcaaaatccgttgcttctgttagatct gcagactcggtagaatccgctggttctgcaaa atccgttgcttctgttagatctgcagactcgg tagaatccgctggttctgcaaaatccgttgct tctgttagatctgcagatgcagacgttcttca tgacacccatcttgatgaagctgacatgcagc	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ
Geno37 BrC114	atgatgacaaggccagtgtcaaatctgcagcctcg ctcaaatccgctgattctgcaaaatccgttgcttc tgttagatctgcagactcggtagaatccgctggtt ctgcaaaatccgttgcttctgttagatctgcagac tcggtagaatccgctgattctgcaaaatccgttgc ttctgttagatctccagactcggtagaatccgctg gttctgcaaaatccgctgcttctgtaaaatccgct gcttctgttagatctgcagactcggtagaatccgc	DDKASV KSAASL KSADSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESADSA KSVASV RSPDSV ESAGSA KSAASV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA

	tgcttccgcaaaatccgctgcttctgttagatctg cagactcggtagaatccgctggttctgcaaaatcc gttgcttctgttagatctgcagactcggtagaatc cgctggttctgcaaaatccgttgcttctgttagat ctgcagactcggtagaatccgctggttccgcaaaa tccgttgcttctgttagatctgcagactcggtaga atccgctggttctgcaaaatccgttgcttctgtta gatctgcagactcggtagaatccgctggttctgca aaatccgttgcttctgttagatctgcagactcggt agaatccgctggttctgcaaaatccgttgcttctg ttagatctgcagactcggtagaatccgctggttct gcaaaatccgttgcttctgttagatctgcagatgc agacgttcttcatgacacccatcttgatgaagct	KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEA
Geno19g2 Br22	gcaacattgatgatgacaaggccagtgtcaaatct gcagcctcgctcaaatccgctgattctgcaaaatc cgttgcttctgttagatctccagactcggtagaat ccgctggttctgcaaaatccgctggttctgtaaaa tccgctgcttctgttagatctgcagactcggtaga atccgctgcttccgcaaaatccgctgcttctgtta gatctgcagactcggtagaatccgctggttctgca aaatccgttgcttctgttagatctgcagactcggt agaatccgctggttctgcaaaatccgttgcttctg ttagatctgcagactcggtagaatccgctggttct gcaaaatccgttgcttctgttagatctgcagatgc agacgttcttcatgacacccatcttgatgaagctg acatgcagc	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KS**V***ASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ

*Indicates differences in sequences.

Table 4. CLUSTAL 2.0.5 multiple alignment of amino acid sequence of 10 identified *Babesia rossi* genotypes.

```
                        10        20        30        40        50        60        70
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
geno28g2-BrCL02 NIDDDKASVKSAASLKSADSAKSVASVR----------------------------------SPDSVE
geno37-BrCL14   ---.......................SADSVESAGSAKSVASVRSADSVESADSAKSVASVR......
geno18-BrCL18   ---.......................-----------------------------------.......
geno19g1-Br18   ..........................-----------------------------------.......
geno19g2-Br22   ..........................-----------------------------------.......
geno28g1-Br25   ..........................-----------------------------------.......
geno29-Br42     ..........................-----------------------------------.......
geno24-Br32     ..........................-----------------------------------.......
geno12g2-Br3    ...........G------------...-----------------------------------------
geno11-Br16     -...........G------------...-----------------------------------------

                         80        90       100       110       120       130       140
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
geno28g2-BrCL02 SAGSAKSAGSVKSAASVR-----------------SADSVESAGSAKSVASVRSADSVESAGSAKSVAS
geno37-BrCL14   ........A.........SADSVESAASAKSAASVR.................................
geno18-BrCL18   ......------......------------------........A....A...................
geno19g1-Br18   ..................------------------........A....A...................
geno19g2-Br22   ..................------------------........A....A...................
geno28g1-Br25   ..................------------------........A....A...................
geno29-Br42     ..............G..KS-----------AASVR........A....A...................
geno24-Br32     ....V.------......------------------........A....A...................
geno12g2-Br3    ----------------------------------.....A.P..V..A.........A.P..V..A..
geno11-Br16     ----------------------------------.....A.P..V..A.........A.P..V..A..

                        150       160       170       180       190       200       210
                ....|....|....|....|....|....|....|....|....|....|....|....|....|....|
geno28g2-BrCL02 VRSADSVESAGSAKSVASVRSADSVESAGSAKSVASVRSADSVESAGSAKSVASVRSADSVESAGSAKSV
geno37-BrCL14   ................................................................................
geno18-BrCL18   ...............A.............----------------------------------------
geno19g1-Br18   ...............A.............----------------------------------------
geno19g2-Br22   ..............................----------------------------------------
geno28g1-Br25   ................................................................................
geno29-Br42     ................................................................................
geno24-Br32     ...............................................------------------.....
geno12g2-Br3    .......A.P..V..A.........----------------------------------------------
geno11-Br16     .........------..........----------------------------------------------
                        220       230       240       250
                ....|....|....|....|....|....|....|....|
geno28g2-BrCL02 ASVRSADSVESAGSAKSVASVRSADADVLHDTHLDEADMQ
geno37-BrCL14   ....................................---
geno18-BrCL18   -------------.......................---
geno19g1-Br18   -------------..........................
geno19g2-Br22   -------------..........................
geno28g1-Br25   ........................................
geno29-Br42     ........................................
geno24-Br32     ........................................
geno12g2-Br3    ----------------...........IP..........
geno11-Br16     ----------------...........IP..........
```

4.5. Real-time PCR

Real-time PCR was successful in amplifying 10 *BrEMA*1 genotypes using SYBR Green. All *Babesia rossi* positive samples had a melting temperature of between 76.75°C and 87.83°C with a majority of the samples melting around 86°C (Table 5).

Although we were successful in detecting all 10 *BrEMA*1 genotypes using real-time PCR, the test failed to reliably differentiate amongst the various genotypes. However, this approach was indeed shorter and quicker than the previously described techniques.

Table 5. List of samples and melting temperatures detected from 10 different genotypes as determined by sequencing and undetermined genotypes identified on real-time PCR.

A. sequenced samples, determined genotypes on real-time PCR

Geno11		Geno12		Geno18		Geno19g1		Geno19g2	
Sample name	**TM**	**Sample name**	**TM**	**Sample name**	**TM**	**Sample name**	**TM**	**Sample name**	**TM**
Br9	87.41°C	Br3	87.83°C	BrCL18	86.39°C	Br2	86.95°C	Br10	86.65°C
Br16	87.02°C					Br14	87.28°C	Br22	86.92°C
Br62	87.01°C					Br18	87.08°C	Br23	86.91°C
BrCL01	86.99°C					Br21	86.97°C	ES10	85.69°C
BrCL03	85.75°C					Br28	82.29°C	ES46	87.28°C
ES15	87.96°C					Br34	86.99°C	ES53	87.29°C
ES19	87.24°C					Br47	86.94°C	BC265	87.35°C
						BrCL04	87.09°C		
						ES78	87.24°C		
						ES80	85.62°C		
						ES90	87.71°C		

Geno24		Geno28g1		Geno28g2		Geno29		Geno37	
Sample name	**TM**	**Sample name**	**TM**	**Sample name**	**TM**	**Sample name**	**TM**	**Sample name**	**TM**
Br32	86.89°C	Br11	86.11°C	Br12	85.74°C	Br1	85.80°C	BrCL14	86.39°C
		Br25	86.45°C	Br31	86.59°C	Br5	85.72°C		
		Br33	86.48°C	BrCL02	85.74°C	Br7	85.52°C		

		Br36	86.81°C	BrCL06	85.79°C	Br17	85.88°C		
		Br40	82.79°C			Br19	85.67°C		
		Br68	86.46°C			Br30	85.90°C		
		BrCL07	86.66°C			Br41	85.77°C		
		BrCL10	86.83°C			Br42	85.85°C		
		BrCL16	86.61°C			Br43	85.13°C		
						Br45	85.82°C		
						Br64	76.75°C		
						Br66	85.42°C		
						Br75	85.94°C		
						BrCL05	76.75°C		
						BrCL09	85.42°C		

B. Samples not sequenced, undetermined Genotypes.

Sample name	**Tm**
Br4	85.47°C
Br5	86.10°C
Br8	78.68°C
Br20	86.72°C
Br24	87.09°C
Br26	87.52°C
Br27	86.15°C
Br29	87.00°C
Br30	86.38°C
Br35	86.44°C
Br37	86.23°C
Br38	86.42°C

Br44	86.31°C
Br46	86.71°C
Br48	86.33°C
Br49	85.88°C
Br50	85.92°C
Br51	86.33°C
Br52	86.46°C
Br53	86.47°C
Br54	85.49°C
Br58	86.59°C
Br59	86.14°C
Br60	86.74°C
Br61	86.91°C

4.5.1. Analytical specificity of Real-time PCR in detecting *BrEMA*1 genotypes

Although all six samples (BC265, ES19, ES53, ES78, ES90 and ES15) showed melting peaks, the test was not specific enough in detecting *BrEMA*1 genotypes. These samples were chosen due to their results illustrating clearly different genotypes melting at the same temperature. Melting curve analysis in Fig. 7a shows genotype11 and genotype19g1 melting at the same temperature and Fig. 7b shows various melting peaks of three different samples for genotype11.

No amplification was observed for DNA from the known positive *B. vogeli* and *B. canis* samples (Fig. 8). Five microlitres of a real-time PCR product were loaded on a 1.5% agarose gel electrophoresis to confirm the real-time PCR results. No product was obtained for *B. vogeli* and/or *B. canis* DNA represented in lane 4 and 5 respectively (Fig. 9). Lane 1 is a negative control (Fig. 9).

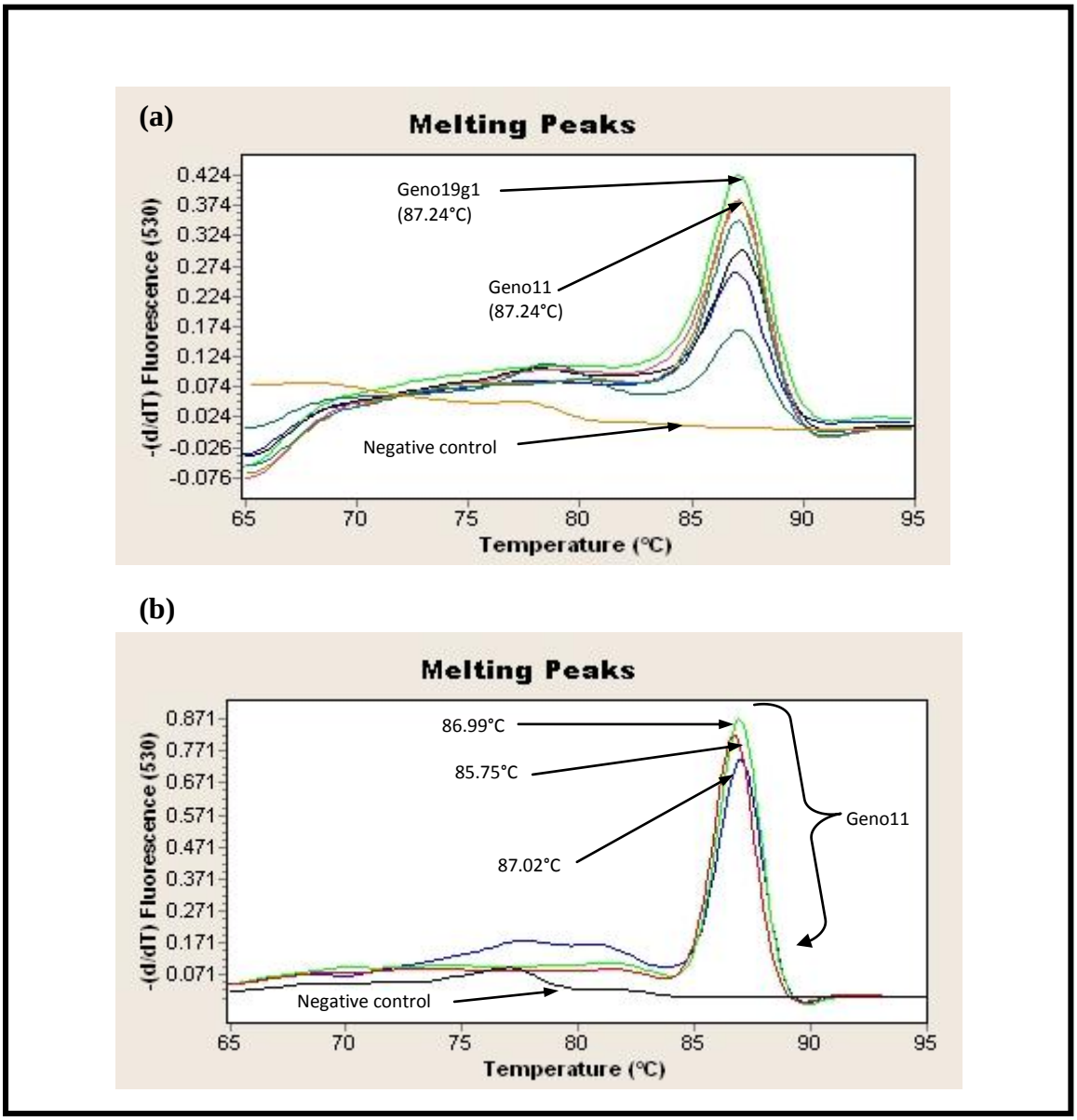

Figure. 7. Detection of *Babesia rossi* genotypes using real-time PCR with forward and reverse *BrEMA*1 primers. (a) Melting curve shows genotype11 and genotype19g1 melting at the same temperature (87.24°C) with no amplification in the negative control (b) Melting curve analysis shows various melting peak (86.99°C, 86.75°C and 87.02°C) of 3 different samples for genotype11.

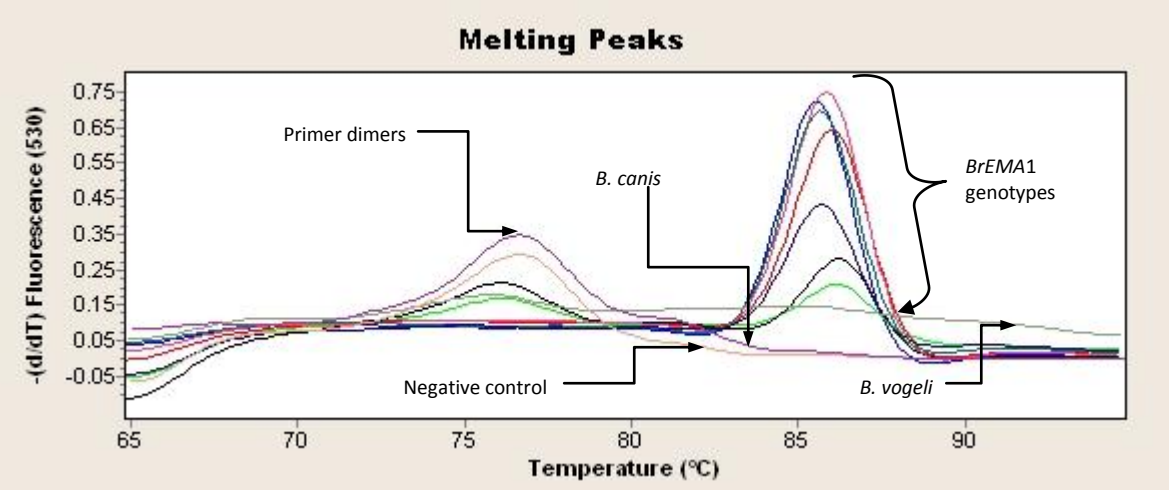

Figure. 8. Specificity of the *BrEMA*1 gene on real-time PCR using SYBR Green. No amplification was detected in *B. vogeli* and *B. canis* samples as well as in the negative control.

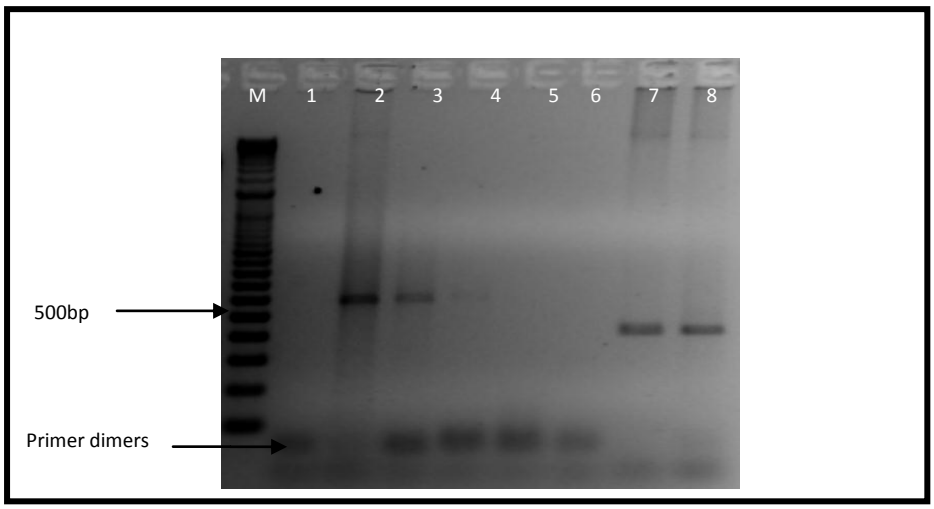

Figure. 9. Five microlitres of eight real-time PCR amplicons were loaded on a 1.5% agarose gel electrophoresis. Lane 2, 3, 4, 7 and 8 represent Br7, Br5, Br1, Br2 and Br10 respectively. Lane 5 and 6 contains amplicon with *B.vogeli* and *B. canis* respectively. Lane 1 was loaded with a negative control and Lane M is the O'GeneRuler 100bp + 500bp DNA ladder (Fementas).

4.5.2. Analytical sensitivity of the real-time PCR in detecting *BrEMA*1 genotypes

To determine the lower limit of detection of the real-time PCR for *Babesia rossi*, DNA concentration was determined. The test was determined using three replicates of serial 10-fold dilutions. Results demonstrated that amplifications were obtained up to the 10^{-3} dilution. The sensitivity of the test was 100% at 185.4 (10^{0}), 18.54 (10^{-1}) and 1.854 (10^{-2}) ng/µl DNA. At 0.185 (10^{-3}), the sensitivity of the test decreased by 33.3% (Fig 10, 11 & 12). These results were reproduced in two separate reactions.

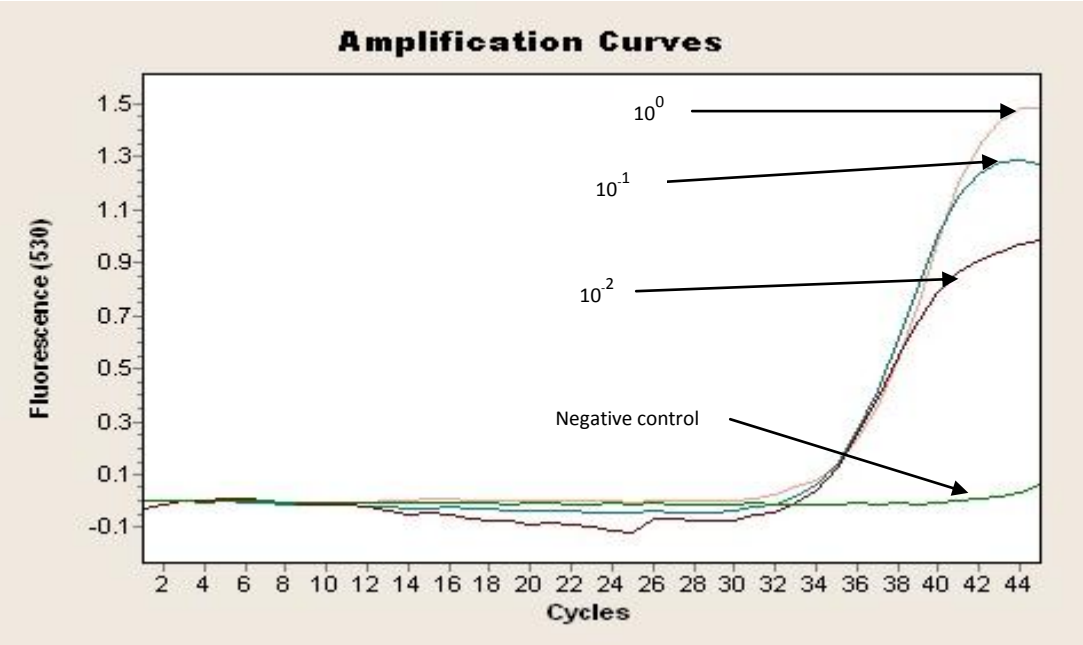

Figure 10. Sensitivity of *B. rossi* real-time PCR. The DNA with the concentration of 185.4 ng/ul was serially diluted with water and amplification was detected up to 1.854 ng/ul dilution.

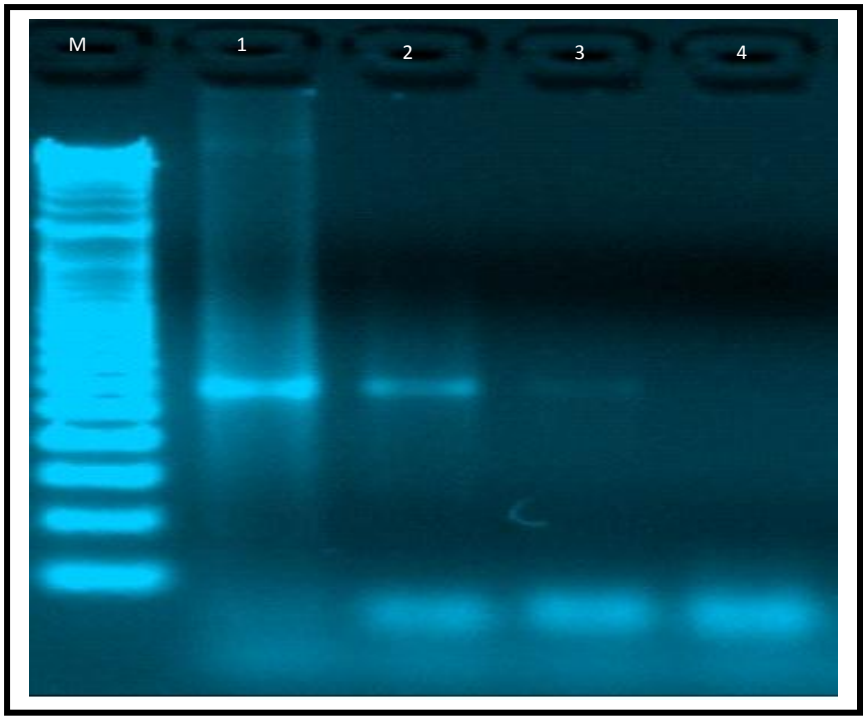

Figure 11. Five µl of PCR products were loaded on a 1.5% agarose gel electrophoresis. Lane1, 2, 3 represent DNA with the concentration of 185.4 ng/µl, 18.54 ng/µl and 1.854 ng/µl respectively. Lane 4 was loaded with a negative control and Lane M is the O'GeneRuler 100bp + 500bp DNA ladder (Fementas).

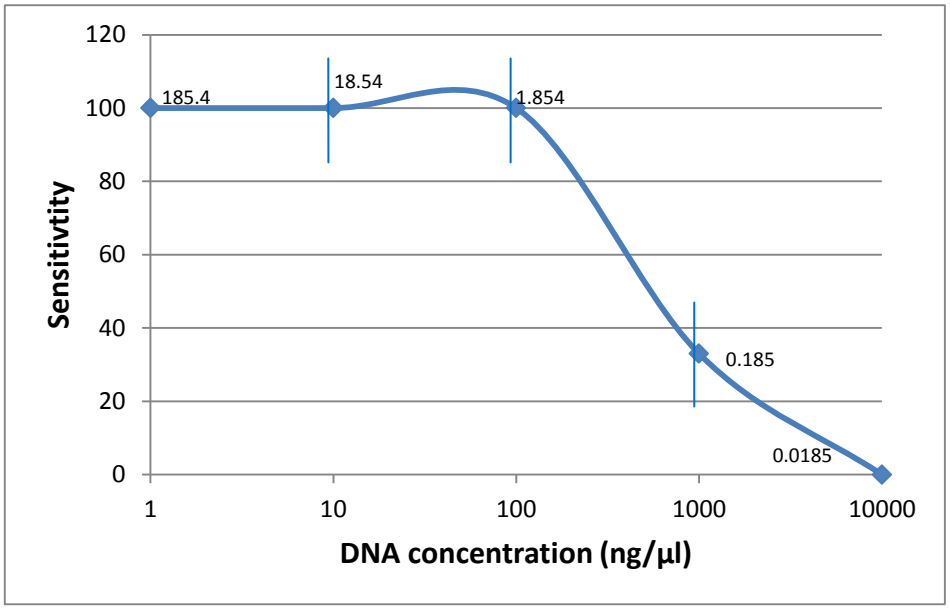

Figure 12. *B. rossi* sensitivity by DNA concentration using a 10-fold dilution series from 10^0 to 10^{-4}.

4.6. Relationship between *BrEMA*1 genotypes with clinical outcomes and dog breed

This was done from 44 samples that had a clinical history. Out of the 8 dogs that died, genotype29 was associated with five (62.5%) of the dogs that did not survive, genotype18g1 with 2 (25%) cases and genotype 19g2 with only 1 case (12.5%) case. Genotype19g2 and genotype 29 were associated with poor prognosis (Table 6). The most commonly encountered breeds were Boerboel at 15% followed by Jack Russel terrier, Chow Chow and German Shepherd dog at 9% each. Of the 8 dogs that died, 3 (37.5%) were Boerboel of which 2 cases were associated with genotype28g1 and 2 (25%) were Chow Chow associated with genotype 29 along with the remaining 3 (37.5%) cases (Table 6). There was no association of a specific breed with a specific genotype. No statistical analysis was attempted as our sample size was not sufficient to generate statistical results.

Table 6. *BrEMA*1 genotypes associated with clinical signs, age, outcomes and breed of dogs

GENOTYPES	SAMPLE NAME	CLINICAL SIGNS	AGE	CLINICAL OUTCOMES	BREED
Genotype11	Br9	Hypoglycaemia	6 yrs	S	Dashund
	Br16	ISA	1 yr	S	Jack Russel Terrier
	Br62	Hypoglycaemia and ISA	2 yrs	S	Saint Bernard
	BrCL01	Anaemia	2 yrs	S	_
	BrCL03	Anaemia	1 yr	S	_
Genotype12	Br3	Anorexia	3 months	S	Boerboel
Genotype18	BrCL18	Anaemia	9 yrs	S	_
Genotype19g1	Br2	Anorexia	1 yr	S	Terrier Cross
	Br14	Collapse and Icterus	8 months	S	Shar-pei
	Br18	Hypoglycaemia	2 yrs	S	Miniature

					Doberman
	Br21	ARDS and icterus	3 yrs	S	German Shepherd
	Br28	weight loss	1 yr	S	American Pitbull
	Br34	Hypoglycaemia and oliguria	2 yrs	S	Yorkshire Terrier
	Br47	ISA	4 yrs	S	Crossbreed
Genotype19g2	Br10(*)	Collapse, icterus and ISA	4 yrs	D	Boerboel
	Br22	Icterus and ISA	3 yrs	S	Siberian Husky
	Br23	Anorexia	2 yrs	S	Miniature Doberman
Genotype24	Br32	Icterus	3 yrs	S	Labrador Crossbreed
Genotype28g1	Br11	Gastro-intestinal	1 yr	S	German Shepherd
	Br33	Hypoglycaemia and pigmenturia	3 months	D	Boerboel
	Br40	ISA and congested mucous membrane	7 yrs	S	German Shepherd
	Br68	Icterus	3 yrs	D	Boerboel
	BrCL07	Anaemia	9 yrs	S	_
	BrCL10	Anaemia	9 yrs	S	_
	BrCL16	Anaemia	6 yrs	S	_
Genotype28g2	Br12	Collapse and icterus	1 yr	S	Fox Terrier
	Br25	Anorexia	3 months		Labrador Crossbreed
	Br36	Weakness	6 months	S	Pekingese
	BrCL02	Anaemia	1 yr	S	_
	BrCL06	Anaemia	1 yr	S	_
Genotype29	Br1	Collapse, ARDS		S	Pug
	Br5	Collapse and Hypoglycaemia	4 months	S	Jack Russel Terrier
	Br7	Hypoglycaemia and icterus	2 months	S	Boerboel
	Br17	Anorexia	7 months	S	Chow Chow
	Br19	Dyspnoea, ARDS and icterus	9 yrs	D	Pug
	Br30	Hypoglycaemia	2 months	S	Crossbreed

	Br41	Anorexia	3 months	S	Rottweiller
	Br42	Collapse and cerebral babesiosis	2 months	D	Chow Chow
	Br43	Collapse and ARDS	3 yrs	D	Boxer
	Br45	Collapse, cerebral babesiosis	6 months	D	Chow Chow
	Br66	Collapse and seizures	10 months	S	Husky
	Br75(*)	Collapse and Hypoglycaemia	2 yrs	D	Jack Russel Terrier
	BrCL05	Anaemia	1 yr	S	_
	BrCL09	Anaemia	3.5 yrs	S	_

ARDS: Acute respiratory distress syndrome

ISA: In-saline positive agglutination

S: Survived

D: Died/ anesthetized due to poor prognosis

(*): Samples associated with poor prognosis

(_): No data available

4.7. Correlation between *BrEMA*1 genotypes with clinical manifestation and age

The most encountered clinical sign was collapse followed by hypoglycaemia, icterus, in-saline agglutination (ISA) and acute respiratory distress syndrome (ARDS) (Fig. 13). The results presented here show that out of eight *B. rossi* infected dogs that died, five (62.5%) dogs were diagnosed with collapse followed by three (37.5%) dogs diagnosed with icterus. Although *B. rossi*-infected dogs attributed to genotype29 were diagnosed with 70% of collapse cases, there was no correlation between specific *BrEMA*1 genotypes with clinical manifestation. The majority of *B. rossi* infected dogs (65.1%) were younger or equal to 2 years of age. A further 34.9% of dogs were between the age of 3 and 9 years. Genotype29 is associated with 10 (35.7%) out of 28 young *B. rossi* infected dogs followed by genotype19 (g1 & g2) at

21.4% (Table 6). No statistical analysis was attempted as our sample size was not sufficient to generate statistically significant results.

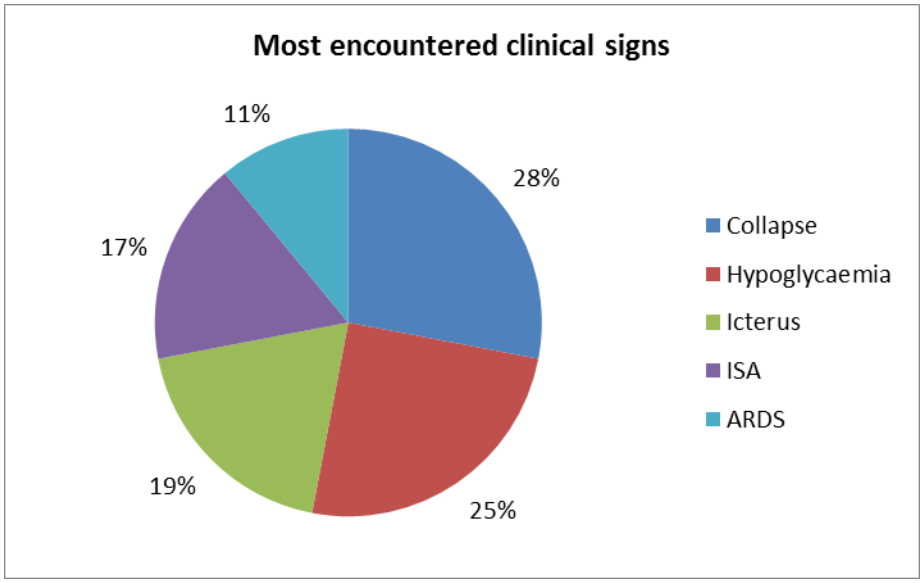

Figure. 13. Occurrence of clinical signs most encountered in 44 patients.

CHAPTER 5

5.1. DISCUSSION

Babesia rossi, the main causative agent of canine babesiosis in South Africa (Collett, 2000), was originally described from a side-striped jackal (*Canis adustos*) in East Africa (Nuttall, 1910). *Babesia rossi* infection causes a severe to fatal disease in domestic dogs (Jacobson, 2006) with a mortality rate of *ca* 12% (Schoeman, 2009). This results in the disease being of economic importance in South Africa. Data from a previous survey has indicated that owners could expect to pay approximately R 90.00 for the treatment of an uncomplicated case, R 230.00 for a complicated case and R 350.00 for one that is severely shocked (Collett, 2000). The costs involved in the treatment and management of canine babesiosis makes this an important disease. Currently, a number of techniques are used for genotyping which include PCR, sequencing, sequence analysis and phylogenetic analysis. These techniques are long and time consuming. In this study we detected *B. rossi* genotypes using a real-time PCR.

The majority of samples (98%) were infected with *B. rossi*. These results were expected because the sampling was biased to only *Babesia*-positive animals admitted to the OVAH. The animals that are presented at OVAH are at most those that are severely infected by *B. rossi* and show clinical signs. These animals require treatment to prevent fatalities (Jacobson, 1996). Very few animals infected with *B. vogeli* are presented at the hospital due to the fact that this parasite is rare and less virulent and shows mild or subclinical signs leading to the owners being unaware of the infection (Uilenberg et al., 1989). Co-infection with *Ehrlichia canis* was only

observed in 1 sample. Matjila et al. (2008) reported the co-infection of *B. rossi* and *E. canis* in 12 dog samples (2%) and *B. vogeli* and *E. canis* in 7 (1%) dog samples. One sample was co-infected with *B. rossi* and *B. vogeli* andone sample co-infected with *B. rossi*, *B. vogeli* and *E. canis.* Co-infection between *B. rossi* and *E. canis* was also reported by du Plessis et al. (1990) in 37/47 infected dog samples and by Van Heerden et al. (1983) in 3/6 infected dog samples. Mixed infection of *B. vogeli* and *E. canis* was also reported by Ewing, (1965) in 21/23 infected dog samples and by Ewing and Buckner (1965) in 8/10 infected dog samples. In comparison, co-infections were less commonly observed in our samples.

The genotyping was performed on the basis of amino acid sequence rather than nucleotide sequence due to the highly conserved nature of *BrEMA*1 gene making it impossible to design hybridization probes for all different genotypes.

In a previous study, Matjila et al. (2009) reported the existence of 12 *B. rossi* genotypes, with genotype19 being the one most commonly associated with high numbers of dogs that were admitted at OVAH suffering from *B. rossi*-induced canine babesiosis. The authors also showed that genotype19 was associated with most of the severe clinical signs diagnosed among the solid-organ complications and also the most fatal cases. This is not in agreement with our findings, which demonstrated that the most encountered *BrEMA*1 genotype was genotype29 (33%), followed by genotype28 (27%) and genotype19 (22%). Genotype29 was associated with most of the severe clinical signs diagnosed compared to genotype19. This study complements and extends previous research carried out by Matjila et al., 2009 using PCR, RLB, sequencing and phylogenetic analysis approaches to detect the

heterogeneity of *B. rossi* genotypes. Since we analyzed fewer numbers of samples as compared to the study of Matjila et al., (2009), however, the discrepancies could be as a result of varying sample sizes. This suggests that there might be more genotypes than expected. Furthermore, it was reported by Matjila et al, 2009 that the most prevalent genotypes amongst patients included in this study were genotype19, 28, 29 and 11, which is in line with our findings.

This study confirmed the existence of 10 *BrEMA*1 genotypes and identified three new genotypes by DNA sequence analysis from samples obtained from OVAH. Three newly identified genotypes where detected only in one sample each. These newly identified genotypes were different from genotypes identified previously by Matjila et al. 2009. Genotype12g2 contains amino acid IP downstream from the repetitive sequence of *BrEMA*1 compared to amino acid VL observed in genotype12g1. Genotype24 was different from other genotypes based on the size of number of repeat and genotype28g2 was different from genotype28g1 by a single amino acid in the hexapeptide KS(A/V)ASV (Matjila et al., 2009) (Tables 3 and 4).

Indeed, real-time PCR is a powerful tool for epidemiological research than previously described methods and provides the ability to detect positive animals with low parasitemia (Calder et al., 1996; Friedhoff and Smith, 1981). This test was able to detect 10 *BrEMA*1 genotypes. However, it could not differentiate between various *BrEMA*1 genotypes. Because SYBR green fluoresces strongly when bound to double-stranded DNA, the possibility exists that it will bind to non-specific double-stranded DNA as well as the target. The analysis was done based on the melting peak profiles to determine the specificity of the amplification reaction. There was no

consistency in the results; the melting temperatures of the same samples were fluctuating between separate reactions. Due to the highly conserved nature of the *BrEMA*1 gene, there was not much of a difference between the different genotypes since different genotypes melted at the same temperature making it impossible to differentiate between them. Primer-dimers also contributed to the fluorescent signal. The melting curve discriminated between primer-dimers and a specific PCR product. The specific product melted at a higher temperature than the primer-dimers and the fluorescence was displayed as melting peaks representing the characteristic melting temperature (T_m) of DNA product Fig. 8. The T_m was determined by GC-content of the DNA fragment.

Samples positive with *B. canis* and *B. vogeli* were tested for the specificity of *BrEMA*1 gene on real-time PCR using SYBR Green. This gene showed 100% specificity on 98 blood samples. The results in Fig. 8 shows that *BrEMA*1 gene is absent from both *B. canis* and *B. vogeli*-positive samples, which failed to amplify with Frep*BrEMA*1 and Rrep*BrEMA*1 primers. In this instance, SYBR green was used only to confirm the presence of *B. rossi* and not for determining genotypes.

Since sensitivity determinations using serial dilutions of infected blood at known parasitaemia were not performed, DNA with a known concentration was serially diluted to determine the sensitivity of *B. rossi* real-time. Real-time PCR proved to be sensitive in detecting *B. rossi.* It is reliable in detecting *B. rossi* at a DNA concentration as low as 1.854 ng/µl. However, there were some uncertainties at the concentration of 0.1854 ng/µl. Real-time PCR was able to detect the samples that

were below the detectable level on conventional PCR. This test can thus detect sub-clinical infections that could go undiagnosed by conventional PCR.

Table 5B shows the samples which could not be sequenced probably due to low piroplasm parasitaemiaReal-time PCR was able to detect *BrEMA*1 genotypes from these samples, however. This test can be implemented for epidemiological research in detecting *BrEMA*1 genotypes, but its use for differentiating between these genotypes not advised. The advantages of using this approach is that it is short and quicker than the previously described methods and is capable of detecting samples that were below the detectable limit on conventional PCR due to low parasitaemia.

Correlation between clinical signs and *BrEMA*1 genotypes was studied. Previous studies have shown that complicated babesiosis is considered common by 26% of practitioners, with the most reported complications being cerebral, enterorrhagia, hemoconcentration, acute renal failure and pulmonary oedema (Collett, 2000). In this study, the most encountered clinical sign was collapsed followed by hypoglycaemia, icterus and in-saline agglutination. Our results showed that genotype29 was associated with seven (70%) out of 10 collapse cases and was associated with 62.5% of the dogs that died. This confirmed the findings of Abdullahi et al., 1990 and Maegraith et al., 1957 that collapse is a poor prognostic sign in dogs with babesiosis. The most virulent strain, genotype19 was, less represented compared to the study carried out by Matjila et al. 2009, which found that genotype19 was associated with most of the severe clinical signs diagnosed among solid-organ complicationand was also associated with 13 out of 20 fatal cases..

Van Zyl (1994) reported that although *B. rossi* can infect dogs of all ages, the results obtained in a survey from the OVAH revealed that 77% of infected dogs were younger than 3 years of age. Results of the presented study indicate that 65.1% of *B. rossi*-infected dogs were 2 years of age and younger compared to 34.9% observed in old dogs between the age of 3 and 9 year. Van Zyl (1995) reported that 49% of cases presented at OVAH were less than 1 -year old and 23% were older than 3 years. These reports can therefore conclude that young dogs are more susceptible to canine babesiosis than older dogs.

5.2. CONCLUSION

The ability to quickly diagnose *Babesia rossi* infection is important for the rapid administration of effective treatment. Our study is the first to detect *BrEMA*1 amplification product using real-time PCR. Real-time PCR is an excellent tool with good sensitivity and specificity and has demonstrated to be more sensitive than conventional PCR, with the detection limit of 1.854 ng/ul concentration of target DNA. Conventional PCR lacks the sensitivity to diagnose infection when there is low parasitemia. Although it shows different profiles by examining the size of the amplicons which could be visualized on gel, it was not precise enough to distinguish between closely related genotypes due to highly conserved nature of *BrEMA*1. Our results show that real-time PCR is a much more rapid and specific method to detect *B. rossi* genotypes. However, it failed to reliably distinguish between different genotypes. This method can take less than four hours to give results from the time suspected samples are received by the laboratory and it is easy to perform. No-post PCR processing is needed.

CHAPTER 6

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