Chapter 4: Preliminary evaluation of the *BrEMA*1 gene as a tool for correlating *Babesia rossi* genotypes and clinical manifestation of canine babesiosis.

4.1. Abstract

Babesia rossi, an intra-erythrocytic protozoan, causes a severe, often life-threatening, disease of domestic dogs. Dogs treated early for *B. rossi* infection usually recover from the disease, but dogs left untreated or treated at a later stage of infection seldom survive. Dogs infected with *B. rossi* have varied clinical manifestations that can be categorised as uncomplicated (with a good prognosis) or complicated (with a poor prognosis). One hundred and twenty one (121) blood samples were collected from dogs presented to the Onderstepoort Veterinary Academic Hospital (OVAH) and diagnosed with babesiosis on thin blood smear. An additional twenty (20) samples were obtained from private clinics around the Onderstepoort, Johannesburg, Durban, White River and Cape Town areas. The samples were screened by PCR targeting the *Babesia rossi* Erythrocyte Membrane Antigen (*BrEMA*1) gene and sequencing of the polymorphic region (i.e. a variable number of hexapeptide repeats). Analysis of PCR products revealed 11 different gene profiles, visualised by gel electrophoresis. Twelve distinct *BrEMA*1 genotypes were identified by sequencing, however, of which numbers of hexapeptide repeats varied from 6 to 31 (classified as genotype6 to genotype31). The genotypes were retrospectively compared to the clinical case data. The most frequently encountered *B. rossi* parasites were those attributed to genotype19 (36.2%), genotype28 and 29 (20.6% each) and genotype11 (12.7%). These genotypes were also the only ones associated with poorest prognosis. This

preliminary finding suggests clinically important differences between the various *B. rossi* genotypes identified.

4.2. Introduction

The large *Babesia* parasites, *Babesia rossi* and *Babesia vogeli,* are two of the most frequently encountered blood parasites of dogs in South Africa (Böhm, Leisewitz, Thompson and Schoeman, 2006; Matjila, Leisewitz, Jongejan and Penzhorn, 2008; Matjila, Penzhorn, Bekker, Nijhof and Jongejan, 2004). *Babesia rossi* is the most frequently encountered species detected in dogs presented with clinical babesiosis at the Outpatients Clinic of the Onderstepoort Veterinary Academic Hospital (OVAH). *Babesia vogeli* infections are reported to be rare and less virulent than *B. rossi* infections (Böhm et al., 2006; Uilenberg, Franssen, Perie and Spanjer, 1989). Canine babesiosis caused by *B. rossi* is clinically classified as either being uncomplicated or complicated. Clinical hallmarks of *Babesia* infections in dogs always include fever and splenomegaly. The disease is said to be uncomplicated if the clinical changes could be attributed directly to a mild or moderate anaemia with no clinical evidence of organ dysfunction or failure (Jacobson and Clark, 1994). Dogs with mild anaemia are usually treated with an antibabesial drug and discharged. Dogs with moderate anaemia may receive a blood transfusion and are then discharged. The survival rate in this manifestation of disease is almost 100% (Jacobson, 2006). Examples of complicated cases of the disease are those where clinical presentation is complicated by evidence of non-solid organ failure (non-solid organ complication [SOC]) characterized by severe anaemia and haemoconcentration or organ dysfunction or failure (solid-organ complications, [SOC]). Examples of complicated disease include acute renal failure (ARF), acute respiratory distress syndrome (ARDS), cerebral

involvement, coagulopathy, icterus and hepatopathy, immune-mediated haemolytic anaemia, peracute manifestations, haemoconcentration, pancreatitis, rhabdomyolysis and shock (Jacobson and Lobetti, 1996; Jacobson, 2006). These dogs all require admission to an intensive-care facility where advanced medical treatment is essential. The mortality rate in this group is around 15%, irrespective of the nature of the treatment administered (Shakespeare, 1995).

The mechanisms that result in *B. rossi* parasites being associated with such a range of diverse clinical signs and severe disease in the host are still unknown. As has been suggested for *B. canis* (*senso stricto*), there might be genotypic differences among *B. rossi* strains (Bourdoiseau, 2006) that could be associated with (and / or explain) variable virulence. A polymorphic phosphoprotein localised on the cytoplasmic surface of *B. rossi*-infected red blood cell has recently been characterised and named *Babesia rossi* Erythrocyte Membrane Antigen 1 (*BrEMA*1) (B. Carcy, personal communication). Analysis of the *BrEMA*1 genes of various laboratory strains of *B. rossi* revealed that these code for polymorphic proteins that contain variable numbers of repetitive hexapeptide motifs. We used this gene as a genetic marker to classify *B. rossi* isolates obtained from South Africa and analysed the relationship between particular genotypes and the occurrence and severity of clinical manifestations of *B. rossi*-induced canine babesiosis. The molecular and the biochemical characterization of the *BrEMA*1 will be reported elsewhere by B. Carcy, University of Montpellier.

4.3. Materials and Methods

4.3.1. Sample origin and grouping

Canine blood samples (n=121) were obtained from the Outpatients Clinic of the Onderstepoort Veterinary Academic Hospital (OVAH). These dogs were diagnosed with canine babesiosis based on clinical signs and microscopic confirmation of infected red blood cells on a blood smear. Blood samples were collected prior to treatment, into EDTA tubes from the cephalic vein. If the presence of *B. rossi* was confirmed in our laboratory, clinical data of a specific dog were retrieved from patient files. A further 20 blood samples were obtained from geographically dispersed private clinics (Onderstepoort area, Johannesburg, Durban, White River and Cape Town) from dogs diagnosed with canine babesiosis and sent to our laboratory for molecular characterisation of the infection. Dogs sampled from the non-OVAH sites were not included in the clinical part of the study. These samples were used only for establishing parasite genotypes. Dogs sampled at OVAH were grouped according to their clinical presentation and outcome as follows: Group H (=Home): Dogs that were treated with an antibabesial drug during consultation and discharged immediately; Group A (=Admitted): Dogs admitted for treatment and that survived until discharged; Group D (=Died): Dogs that died despite treatment or that were euthanised owing to poor prognosis (Böhm et al., 2006). Follow-up examinations were conducted on dogs in Group H that were sent home, to confirm that these dogs had indeed recovered completely. Canine babesiosis cases were diagnosed and treated in a similar manner by all clinicians at the OVAH. The diagnostic criteria used for the diagnoses of the various complications include: In-saline positive agglutination (ISA) for immune-mediated haemolytic anaemia; respiratory distress as evidenced by hyperpnoea or blood-gas evidence of lung

oedema for the diagnosis of ARDS; a rise in haematocrit above 50% or fall below 10% (identifying haemoconcentration and severe anaemia, respectively); the development of any central nervous system signs that could not be attributed to any other cause (especially hypoglycaemia) for the diagnosis of cerebral disease; clinical collapse for the diagnosis of shock; the rise of blood-creatinine levels above 150 µmol/L or anuria for the diagnosis of ARF and hypoglycaemia (glucose < 3.3 mmol/L). Based on the above criteria, dogs that were admitted with complications were then separated into two groups, i.e. those with solid-organ complication (SOC) and those without SOC. Finally, dogs from both groups were separated into dogs that survived until discharged and dogs that died despite treatment or that were euthanized owing to poor prognosis.

Two additional samples that were known positives of *B. canis* and *B. vogeli* were obtained from The Netherlands and Bloemfontein, respectively, for the purpose of evaluating whether *BrEMA*1 could be detected in these species.

4.3.2. DNA extraction and PCR

Once collected, the blood samples were sent to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, University of Pretoria. DNA was extracted from 200 µl of each blood sample using the QIAmp® blood and tissue extraction kit (Qiagen, Hilden, Germany), following the manufacturer's protocols. As a first step for the molecular diagnosis of *Babesia* species infecting each dog, a PCR was performed with Reverse Line Blot (RLB) primers F2 (5′-GAC ACA GGG AGG TAG TGA CAA G-3′) and RLB-R2 (biotin-5′-CTA AGA ATT TCA CCT CTG ACA GT-3′). The conditions for the PCR included an initial step of 3 min at

37º C, 10 min at 94ºC, 10 cycles of 94ºC (20s)- 67 ºC (30s)- 72º C (30s), with lowering of annealing step after every second cycle by 2º C (touchdown PCR). The reaction was then followed by 40 cycles of denaturation at 94º C for 30s, annealing at 57º C for 30s and extension at 72º C for 30s. The PCR-amplified products, a 460-540bp fragment from the 18S rRNA gene spanning the V4 region (Gubbels, de Vos, van der Weide, Viseras, Schouls, de Vries and Jongejan, 1999; Matjila et al., 2004) were tested with the RLB, as previously described (Matjila et al., 2004).

The *B. rossi* genetic diversity was then analysed on genomic DNA from samples that tested positive for *B. rossi* on the RLB. These samples 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 TTC ATC A-3'). These primers were designed from the *B. rossi BrEMA*1 gene (Accession number AJ416994). They amplified an 18-nucleotide repetitive sequence whose number (from 16 to 31, i.e., size fragment from 375 to 645bp) and sequence were initially shown to be variable between *BrEMA*1 genes from 3 laboratory strains (B. Carcy, personal communication). To test whether the *BrEMA*1 gene was unique to *B. rossi* isolates, 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 25 µl of PCR reaction containing 0.5 µl of each primer (10 µM), 4 µl of dNTP mix (1.25mM each), 2.5 µl of 10X PCR buffer and Taq DNA polymerase (1U). The conditions of the PCR included in the program were 1 cycle of 5 min at 94°C, 30 cycles of amplification (94°C 30s, 55°C 30s and 72°C 1 min) and 1 cycle of 7 min at 72°C.

4.3.3. Sequencing, phylogenetic and statistical analysis

PCR products (5µl) were loaded and visualised on a 1.5% agarose gel. Samples with distinct bands were purified with a QIAquick PCR purification kit (Qiagen, Germany). The sequencing reaction was prepared with 3.2 pmol of the primer Frep*BrEMA*1 and Rrep*BrEMA*1. Sequencing was performed in the Genetics Section of the Faculty of Veterinary Science. Sequence data for the full *BrEMA*1 sequences were assembled using the GAP 4 of the Staden package (Version 1.6.0 for Windows). Sequence alignments were manually edited using Bioedit (version 5.0.9). DNA sequences were translated into amino acid sequences and genotyping was done according to the number of hexapeptide repeats. Sequence alignment of the amino acid sequences and phylogenetic trees were generated using the website: www.genebee.msu.su/services/malign_reduce.html. The Cluster and Topological algorithm methods were used for the construction of phylogenetic trees (Phylip, multiline) into the phylogram format from the alignment sequences. The *B. rossi BrAK* (adenosine kinase) sequence (Carret, Walas Carcy, Grande, Precigout, Moubri, Schetters and Gorenflot, 1999) was used as an outgroup. Genotype frequencies were compared using a two-tailed binomial test. Proportions were compared using a two-tailed Fisher's exact test. Analyses were done using Stata 8.2 (StataCorp, College Station, TX, U.S.A.).

4.3.4. Nucleotide sequence accession numbers

The *BrEMA*1 gene nucleotide sequences (designated genotype6, 11, 12, 13, 16g1, 16g2, 18, 19g1, 19g2, 22, 28, 29 and 31) have been submitted to EMBL, GenBank and DDBJ Nucleotide Sequence databases and can be retrieved under the accession numbers FM164393 to FM164405 respectively.

4.4. Results

4.4.1. Genetic analysis B. rossi isolates

All the blood samples (n=141) were positive for *B. rossi* on the PCR/RLB assay. These samples were further analysed to determine the genetic heterogeneity of the *BrEMA*1 gene.

4.4.1.1. Diversity of BrEMA1 genotypes

PCR amplification of the polymorphic region of the *BrEMA*1 gene revealed a total of 11 distinct profiles by examining the size of the amplicons which could be visualised on gel electrophoresis. The profile of the amplicons seemed to correlate to a specific genotype. The smallest amplified product was 200bp (lane 8) and the largest *ca* 645bp (lane 15&17) (Fig. 4.1). Sequencing results of the amplicons showed 13 different consensus sequences that ranged in size between 6 and 31 hexapedtide repeats, which in fact reflected 12 distinct genotypes (Table 4.1). Samples with a repetitive region composed of 16 or 19 hexapeptide repeats, respectively, were subdivided into two groups (designated g1 or g2). Genotype16g1 and genotype16g2 corresponded with two distinct sequences, indicating that they are genetically distant. In contrast, genotype19g1 and genotype19g2 differed by a single conservative amino acid mutation, A or V, in the hexapeptide KS (A/V) ASV. Finally, sequence analysis of genotype11 and genotype16g2 indicated that they might be genetically related. This is based on the fact that they are the only genotypes containing the motif ASPGSV and 2 amino acid substitutions (IP rather than VL) downstream from the repetitive sequence of *BrEMA*1 (Table 4.1).

The two positive *B. vogeli* and *B. canis* samples could not be amplified with the *BrEMA*1 primers and were therefore negative for the *BrEMA*1 gene.

4.4.1.2. Phylogenetic relationship of BrEMA1 sequences

The low bootstrap values obtained at certain nodes, especially those <50, strongly suggest that the phylogenetic analysis of *BrEMA*1 genotypes presented in our study is incomplete (Fig. 4.2). Based on the 13 *BrEMA*1 genotype sequences available to date, it follows that the genotypes could belong to 6 distinct monophyletic groups: (i) group A which includes genotype6; (ii) group B which includes genotype11; (iii) group C which includes genotype12, genotype13 and genotype16g1; (iv) group D which includes geno16g2; (v) group E which includes genotype18, genotype19 (i.e., 19g1 and 19g2) and genotype22; and lastly (vi) group F which includes genotype28, genotype29 and genotype31 (Fig. 4.2).

4.4.1.3. Occurrence of BrEMA1 genotypes among all samples

So far, the total analysis of the 141 blood samples revealed 12 different *BrEMA*1 genotypes that clustered in 6 monophyletic groups (see above). The most abundant were genotype19 (36%, i.e., 22% genotype19g1 and 14% genotype19g2); genotypes 28 and 29 (both at *ca* 20%) and genotype11 (12.8%) (Fig. 4.3). The remaining genotypes represented 10% of the cases. Genotype16g2 represented 3.5% of cases, whereas each of the remaining genotypes represented less than 1.5% of the cases (data not shown).

4.4.2. Occurrence of BrEMA1 genotypes among OVAH samples

Results revealed that there were 4 frequently occurring genotypes: genotype19 represented *ca* 40% of cases (i.e., 25% genotype19g1 and 15% genotype19g2); genotype28 and 29 both represented *ca* 22% and 23%, respectively, and genotype11 represented *ca* 12% (Fig. 4. 4a). The other genotypes occurred less frequently, representing only 3.3% of the cases. Using the two-tailed binomial test, genotype19 was more frequent than genotype11 ($P<0.001$), genotype28 ($P=0.02$), genotype29 ($P=0.03$) and the remaining genotypes ($P<0.001$).

4.4.2.1. Analysis of BrEMA1 genotype and clinical parameters

The analysis of the association of *BrEMA*1 genotypes with clinical parameters was performed only on the dogs that were seen at the OVAH and followed up (n=121). Of these, 86 dogs (71%) were admitted with complications (non-SOC or SOC; Table 4.2). The remaining 35 dogs (29%) were treated and sent home immediately after consultation. There were no significant differences in the *BrEMA*1 frequencies of admitted dogs versus dogs that were sent home immediately after consultation ($P=0.48$, Fisher's exact test). The percentage of dogs diagnosed with SOC was 24% and the mortality rate was estimated at 16.5%.

4.4.2.2. Occurrence of BrEMA1 genotypes in complicated cases

The distribution of the various *B. rossi* genotypes in the complicated cases (n=86) was compared to that in the entire group (Fig. 4.4b; linear regression, $R^2=0.99$, $p<0.05$). There was no specific association of particular *BrEMA*1 genotype with complicated disease.

4.4.2.3. Occurrence of BrEMA1 genotypes in SOC cases

Regarding the distribution of the various *BrEMA*1 genotypes, significant differences (*P=0.01*) were found using Fisher's exact test, between dogs with non-SOC (n=57) and SOC (n=29) amongst admitted dogs. *Babesia rossi* genotypes 19, 28, 29 and 11 were all associated with SOC (29 dogs; Table 4.2). *Babesia rossi* genotype19 represented 62% of SOC cases (38% genotype19g1 and 24% genotype19g2). *Babesia rossi* genotypes 28 and 29 were associated with *ca* 30% of SOC cases (20% and 7% respectively) and genotype11 with 10% (Fig. 4.4c). In the SOC group, significantly more dogs were infected with genotype19 than with the other genotypes combined (*P=0.02*, Fisher's exact test).

4.4.2.4. Occurrence of BrEMA1 genotypes in fatal cases

The number of dogs infected with genotype19 that died of complications (n=13) was significantly higher than the number of fatal cases (n=7) of dogs infected with the remaining genotypes (Fisher's exact test; p=*0.01*) (Fig. 4.4d). The distribution of *B. rossi* genotypes in the fatal cases (n=20) approximated to the distribution of *B. rossi* genotypes in the SOC group (n=29) (Fig. 4.5). This suggested an association between SOC and fatal outcome. This correlation was statistically significant (linear regression, R^2=0.95; p<0.05).

4.4.2.5. Correlation between BrEMA1 genotypes and clinical signs

Frequency of clinical signs and associated genotypes were analysed among the 29 dogs diagnosed with SOC and among the 19 dogs that died from SOC-induced death (Table 4.3). Genotypes 19 and 28 were associated with most of the clinical signs. The two most frequently encountered clinical signs, ARDS and shock, were found

preferentially associated with genotype19 (Table 4.3). Genotype19 was associated with >70% (11/16 amongst SOC and 8/10 amongst fatal cases) of ARDS cases (against *ca* 10% for genotype28) and with >60% (6/9 amongst SOC and 5/8 amongst fatal cases) of shock cases (against *ca* 20% for genotype28).

4.5. Discussion

It should be noted that a relative large number of disease phenotypes and parasite genotypes were studied in a relative small population. This means that associations between disease phenotype and parasite genotype, although interesting, requires a much larger prospective study to elucidate them further.

This study identified the existence of 12 *B. rossi* genotypes, the most common being genotype19. This genotype was also associated with high numbers of dogs that were admitted at OVAH suffering form *B. rossi*-induced canine babesiosis. Furthermore, our data have shown that *B. rossi* genotype19 is associated with most of the severe clinical signs diagnosed among SOC and fatal cases. Although not unique to genotype19, ARDS was the most common clinical sign associated with genotype19. Since genotype19 appears to be more virulent, this might suggest a correlation between this genotype and severe clinical signs in admitted cases of canine babesiosis.

Statistical analysis appears to suggest that SOC can be a precursor to fatal outcome. In agreement to published data (Shakespeare, 1995), we estimated that once a dog is *B. rossi* infected, its chance to develop SOC would be *ca* 24% with a 16.5% chance of dying from these complications.

Phylogenetic analysis of the *BrEMA*1 sequences identified 6 monophyletic groups. The low bootstrap values (<50) obtained at certain nodes of the *BrEMA*1 phylogenetic tree imply that the diversity of *B. rossi* in the field is higher than described in this report. These data, however, indicate that genotypes19, 28, 29 and 11 are the most prevalent amongst the 12 identified genotypes. These 4 genotypes belong to 3 monophyletic groups (i.e. genotype19, genotype 28/29 and genotype11), in agreement with their separation into 3 distinct groups of genotypes on the basis of their prevalence and virulence. In comparison, genotype19 appears highly prevalent and virulent, genotypes28/29 appear moderate in prevalence and virulence, whereas the prevalence and virulence of genotype11 appears low.

Our analysis also indicates that the *BrEMA*1 gene might be a good genetic marker for investigating *B. rossi* virulent genotypes in endemic areas, especially since the less pathogenic species, *B. canis* and *B. vogeli,* were found not to have the *BrEMA*1 gene. This finding is likened to related studies through which specific genes have been found to be unique to virulent protozoal species. For example, the virulence of *Plasmodium falciparum* and *Babesia bovis* is influenced by their ability to adhere to capillary endothelium, a phenomenon known as sequestration (Allred and Al-Khedery, 2004; Miller, Baruch, Marsh and Doumbo, 2002; O'Connor, Long and Allred, 1999). Sequestration has been linked to proteins expressed on the surface of infected red blood cells of both *P. falciparum* and *B. bovis*, which seem to alter the adhesive properties of these cells (Cooke, Mohandas and Coppel, 2001; O'Connor et al., 1999). Preliminary evidence has suggested that the expression of parasite-derived antigens (with adhesive properties) on the extra-cellular surface of *B. rossi*-infected red blood cells may be responsible for their ability to sequester *in vivo* and to

aggregate *in vitro* (Schetters and Eling, 1999). This has not been proven, however. Furthermore, there is no evidence related to the involvement of the *BrEMA*1 in sequestration. It has been demonstrated, though, that both adhesive and mechanical properties of *P. falciparum* or *B. bovis*-infected red blood cells play a pivotal role in their ability to sequester and consequently in the virulence of these parasites (Cooke et al., 2001; Hutchings, Li, Fernandez, Fletcher, Jackson, Molloy, Jorgensen, Lim and Cooke, 2007). If virulence is indeed directly related to cytoadhesion, as suggested in the *B. bovis* model (Allred and Al-Khedery, 2004; Allred and Al-Khedery, 2006), then more work should be undertaken to correlate the relationship between the virulent *BrEMA*1 genotypes and cytoadhesion or to identify auxiliary genes that may play a role in this phenomenon.

4.6. Conclusion

The significant suggestion of this work is that different parasite genotypes may cause differing host responses to infection (i.e. there could be a relationship between parasite genotypes and disease pathogenesis). Changing disease outcome through treatment depends heavily on understanding of disease pathogenesis. In this sense, this preliminary data may eventually prove clinically significant.

4.7. Figures and Tables

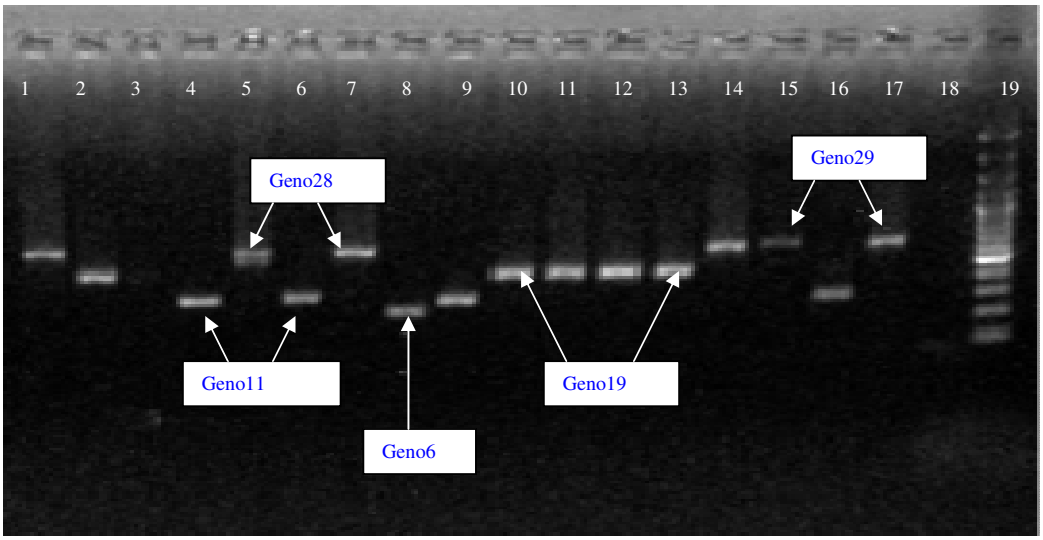


Figure 4.1**:** Field polymorphism of *B. rossi* strains in South Africa evidenced by the PCR amplification of *BrEMA*1 repeats region with primers Frep*BrEMA*1 / Rrep*BrEMA*1. PCR products were loaded on a 1.5% agarose gel. PCR profiles of the most encountered genotypes (11, 19, 28&29) and that of genotype6 are indicated on the picture as follows: lanes 15 and 17 represent genotype29; lanes 1, 5, 7 and 14 represents genotype28; lanes 2, 10, 11, 12 and 13 represents genotype19; lanes 4, 6, 9 and 16 represents genotype11; lane 8 represents genotype6 and lane 19 is the 100bp marker.

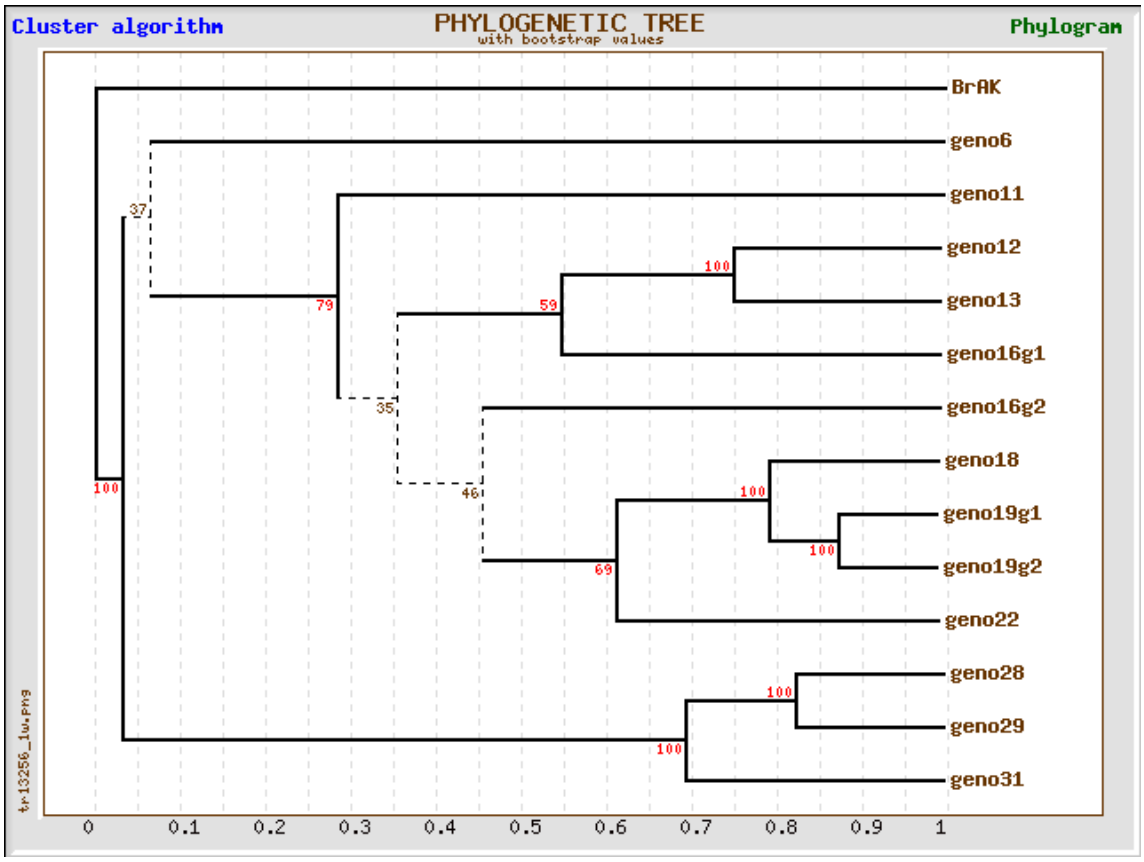


Figure 4.2: Cluster algorithm tree, showing the phylogenetic relationship between various genotypes based on the *BrEMA*1 gene sequences. *BrAK* (Adenosine kinase from *B. rossi;*AJ223322) was used as an outgroup.

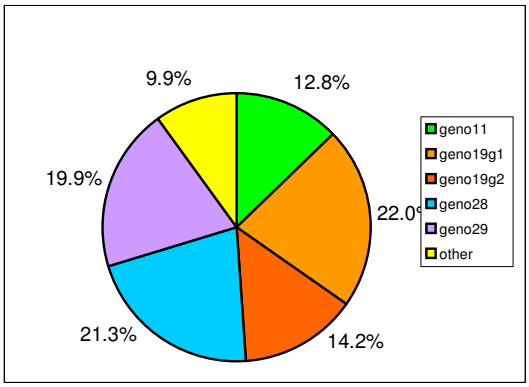
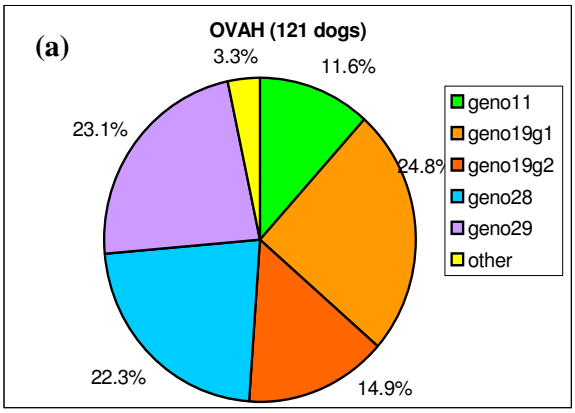
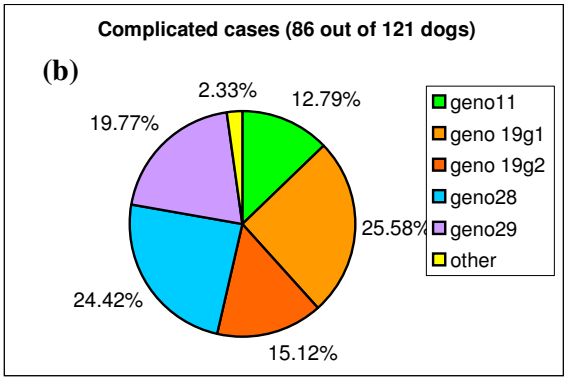
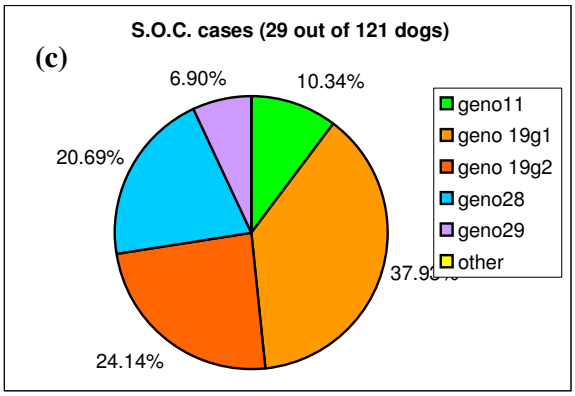


Figure 4.3: Occurrence of *B. rossi BrEMA*1 genotypes among all samples (141 dogs).







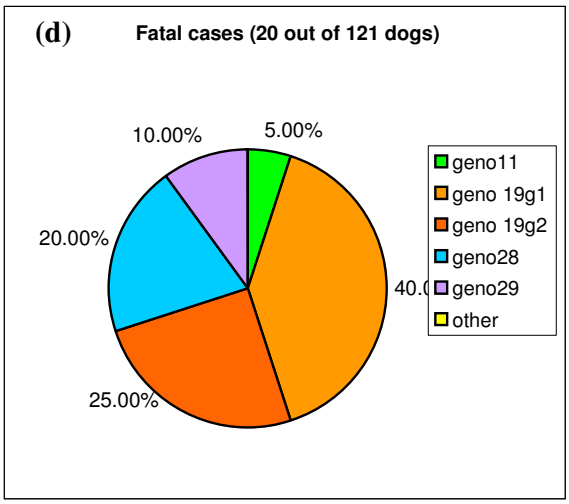


Figure 4.4: Occurrence of *B. rossi BrEMA*1 genotypes among (a) total (b) complicated (c) Solid organ complications (S.O.C). and (d) fatal cases.

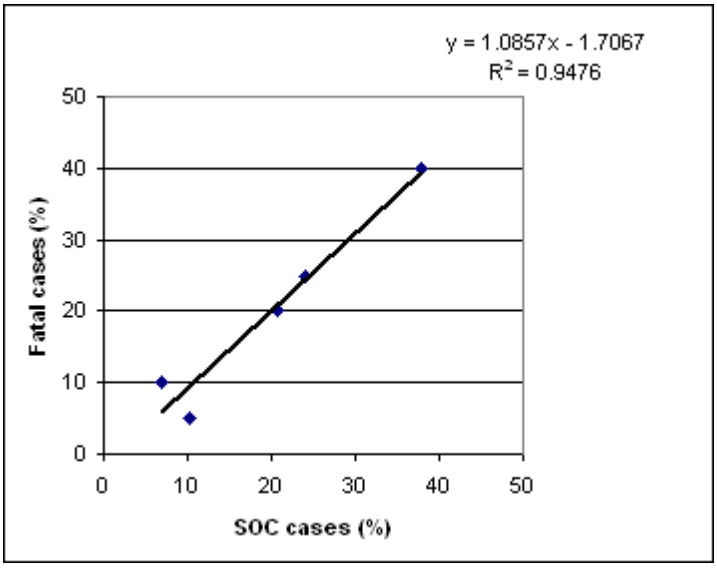


Figure 4.5: Relationship between occurrence of fatalities (n=20) and occurrence of SOC-cases (n=29).

Table 4.1: Consensus amino acid sequence of the 13 *BrEMA*1 genotypes**.** Sequencing of the repetitive region of the gene was performed on 141 dogs diagnosed with *B. rossi* infections.

Genotype		**sample origin**	**Consensus amino acid sequence**
Geno6		OVAH (1)	NIDDDKASV KSADSL KSAGSA KSVASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ 64
Geno11		OVAH (14) Ondersterpoort (4)	NIDDDKASV KSAGSV [RSADSV ASPGSV KSAASV]**2** RSADSV ESVASV RSADAD**IP**HDTHLDEADMQ 94
Geno12		OVAH (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSVASV [RSADSV ESAGSA KSVASV]**2** RSADADVLHDTHLDEADMQ 100
Geno13		Onderstepoort (1)	NIDDDKASV KSAASL KSADSA KSVASV RSADSV ESAGSA KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSAASV RSADADVLHDTHLDEADMQ 106
Geno16	Geno16g1 (**Repeated region from sequence AJ416994**)	Johannesburg (1) OVAH (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAASV KSAASV RSADSV ESAASA KSAASV [RSADSV ESAGSA KSVASV]**2** RSADADVLHDTHLDEADMQ 124
	Geno16g2	Durban (1) White River (4)	NIDDDKASV KSAGSV [KSAASV RSADSV ASPGSV]**4** KSAASV RSADSV ESVASV RSADAD**IP**HDTHLDEADMQ 124
Geno18		OVAH (1) Onderstepoort (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA [KSVASV RSADSV ESAASA]**3** KSVASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ 136
Geno19	Geno19g1 (**Repeated region from sequence AJ416997**)	OVAH (30) Onderstepoort (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KS**A***ASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ 142
	Geno19g2	OVAH (18) Onderstepoort (1) White River (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KS**V***ASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ 142
Geno22		Onderstepoort (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAASV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA KSAASV RSADSV ESAGSA KSVASV RSADSV ESAGSA KSAASV RSADSV ESAGSA KSVASV RSADADVLHDTHLDEADMQ 160
Geno28		OVAH (27) Onderstepoort (3)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA [KSVASV RSADSV ESAGSA]**5** KSVASV RSADADVLHDTHLDEADMQ 196

Geno29	OVAH (28)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV KSAGSV KSAASV RSADSV ESAASA KSAASV RSADSV ESAGSA [KSVASV RSADSV ESAGSA]**5** KSVASV RSADADVLHDTHLDEADMQ 202
Geno31 **(Repeated region from sequence AJ416996)**	Cape Town (1)	NIDDDKASV KSAASL KSADSA KSVASV RSPDSV ESAGSA KSAGSV [KSAASV RSADSV ESAGSA]**2** [KSVASV RSADSV ESAGSA]**6** KSVASV RSADADVLHDTHLDEADMQ 214

( ): number of samples sequenced
*Differences in a single conservative amino acid mutation

Table 4.2: Frequency of *B. rossi BrEMA*1 genotype identified from blood samples and clinical outcomes of dogs presented at OVAH.

	FOLLOW-UP(OVAH)						
	Complicated cases (admitted)				Home	Total	
	S.O.C.		Non S.O.C.				
	Died	Survived	Died	Survived			
geno11	1	2		8	3	14	14
geno19g1 (AJ416997)	8	3		11	8	30	48
geno19g2	5	2		6	5	18	
geno28	4	2		15	6	27	55
geno29	1	1	1	14	11	28	
geno6					1	1	4
geno12				1		1	
geno13						0	
geno18				1		1	
geno16g1 (AJ416994)					1	1	
geno16g2						0	
geno22						0	
geno31 (AJ416996)						0	
Total	*19*	*10*	*1*	*56*	*35*	*121*	

S.O.C.: Solid-Organ Complication

Table 4.3: Clinical signs from complicated cases with solid-organ complications and their associated *BrEMA*1 genotypes.

Sample No	Genotype	Outcome	Complication
ES15	11	S	ARDS
BC214(*)	11	S	ARDS
ES80	19g1	S	ARDS
ES90	19g1	S	ARDS
ES46	19g2	S	ARDS
BC232(*)	19g1	D	ARDS
BC289	19g1	D	ARDS
BC298(*)	19g1	D	ARDS
ES78	19g1	D	ARDS
BC238	19g1	D	ARDS
BC228 (*)	19g1	D	ARDS
BC265	19g2	D	ARDS
ES53	19g2	D	ARDS
ES1	28	S	ARDS
BC302 (*)(h)	28	D	ARDS
BC303(*)	29	D	ARDS
BC232(*)	19g1	D	Shock
BC298(*)	19g1	D	Shock
BC228(*)	19g1	D	Shock
ES10	19g2	D	Shock
ES76	19g2	D	Shock
BC138	19g2	S	Shock
BC302 (*)(h)	28	D	Shock
BC251 (*)(h)	28	D	Shock
BC303(*)	29	D	Shock
ES19	11	D	ARF
ES84 (*)	19g1	D	ARF
BC288 (h)	28	S	ARF
BC251 (*)(h)	28	D	ARF
BC303 (*)	29	D	ARF
BC268(*)	28	D	Icterus and hypoglycaemia

BC168(*)	28	D	Icterus and hypoglycaemia
BC228 (*)	19g1	D	Neurological signs
ES84 (*)	19g1	D	Pancreatitis
BC214(*)	11	S	Splenomegaly
BC211(*)	19g1	S	Icterus and ISA
BC217(*)	29	S	Icterus and ISA
ES63 (**)	29	D	Haemoconcentration

(*) Sample associated with two distinct clinical signs; (h) Sample associated with haemolysis; (S) Survived; (D) Died; (**) Sample associated with non-solid organ complications
ARDS: Acute respiratory distress syndrome
ARF: Acute renal failure

4.8. References

Allred, D.R., Al-Khedery B., 2004. Antigenic variation and cytoadhesion in *Babesia bovis* and *Plasmodium falciparum*: different logics achieve the same goal. Molecular and Biochemical Parasitology 134, 27-35.

Allred, D. R., Al-Khedery B., 2006. Antigenic variation as an exploitable weakness of babesial parasites. Veterinary Parasitology 138, 50-60.

Böhm, M., Leisewitz, A.L., Thompson, P.N., Schoeman, J. P., 2006. Capillary and venous *Babesia canis rossi* parasitaemias and their association with outcome of infection and circulatory compromise. Veterinary Parasitology 141, 18-29.

Bourdoiseau, G., 2006. Canine babesiosis in France. Veterinary Parasitology 138, 118-125.

Carret, C., Walas F., Carcy, B., Grande, N., Precigout, E., Moubri, K., Schetters T. P., Gorenflot, A., 1999. *Babesia canis canis*, *Babesia canis vogeli, Babesia canis rossi:* differentiation of the three subspecies by a restriction fragment length polymorphism analysis on amplified small subunit ribosomal RNA genes. The Journal of Eukaryotic Microbiology 46, 298-303.

Cooke, B.M., Mohandas, N., Coppel R.L., 2001. The malaria-infected red blood cell: structural and functional changes. Advances in Parasitology 50, 1-86.

Gubbels, J.M., de Vos, A.P., van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. Journal of Clinical Microbiology 37, 1782-1789.

Hutchings, C.L., Li, A., Fernandez, K.M., Fletcher, T., Jackson, L.A., Molloy, J. B., Jorgensen, W.K., Lim, C.T., Cooke, B.M., 2007. New insights into the altered

adhesive and mechanical properties of red blood cells parasitized by *Babesia bovis*. Molecular Microbiology 65, 1092-1105.

Jacobson, L.S., Clark, I.A., 1994. The pathophysiology of canine babesiosis: new approaches to an old puzzle. Journal of the South African Veterinary Association 65, 134-145.

Jacobson, L.S., Lobetti R.G., 1996. Rhabdomyolysis as a complication of canine babesiosis. The Journal of Small Animal Practice. 37, 286-291.

Jacobson, L.S., 2006. The South African form of severe and complicated canine babesiosis: Clinical advances 1994-2004. Veterinary Parasitology 138, 126-139.

Matjila, P.T., Leisewitz, A.L., Jongejan, F., Penzhorn, B.L., 2008. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. Veterinary Parasitology 155, 152-157.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P., Nijhof, A.M., Jongejan F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Veterinary Parasitology 122, 119-125.

Miller, L. H., Baruch, D. I., Marsh, K., Doumbo, O. K., 2002. The pathogenic basis of malaria. Nature 415, 673-679.

O'Connor, R.M., Long, J.A., Allred, D.R., 1999. Cytoadherence of *Babesia bovis*-infected erythrocytes to bovine brain capillary endothelial cells provides an *in vitro* model for sequestration. Infectious Immunology 67, 3921-3928.

Schetters, T. P., Eling W.M., 1999. Can *Babesia* infections be used as a model for cerebral malaria? Parasitology Today 15, 492-497.

Shakespeare, A.S., 1995. The incidence of canine babesiosis amongst sick dogs presented to the Onderstepoort Veterinary Academic Hospital. Journal of the South African Veterinary Association 66, 247-250.

Uilenberg, G., Franssen, F. F., Perie, N.M., Spanjer A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Veterinary Quarterly 11, 33-40.

Chapter 5: Autochthonous canine babesiosis in the Netherlands.

5.1. Abstract

Outbreaks of autochthonous babesiosis, caused by *Babesia canis*, occurred in the Netherlands in the spring and autumn of 2004 affecting 23 dogs. Blood samples were taken from dogs that had been brought to the clinics with signs and symptoms associated with clinical babesiosis. The samples originated from the surroundings of the two cities in the Netherlands, The Hague and Arnhem. These samples were sent to the Department of Companion Animal Medicine and to the Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University for diagnostic purposes. Samples were tested for *Babesia* and *Ehrlichia* parasites by blood or buffy coat smear investigation, Indirect flourescent antibody test (IFAT), Polymerase chain reaction (PCR) followed by Reverse Line Blot (RLB). Twenty-three cases of autochthonous babesiosis were confirmed by a combination of clinical history, positive blood smears, IFAT and PCR/RLB. Nineteen animals recovered after treatment, whereas 4 dogs died. Adult *Dermacentor reticulatus* ticks collected from these dogs indicate that canine babesiosis could become endemic in the Netherlands.

5.2. Introduction

During the spring of 2004, outbreaks of babesiosis occurred in dogs simultaneously in two different residential areas in the Netherlands. Previously, canine babesiosis had only been diagnosed from dogs returning from endemic areas outside the Netherlands. The only known autochthonous cases of canine babesiosis associated with *Dermacentor reticulatus* were recorded in 1985 from five dogs (Uilenberg, Top, Arends, Kool, van Dijk, van Schieveen and Zwart, 1985). In the current study, twenty dogs, which had not been outside the Netherlands, were affected; 13 in the municipality of Rijkerswoerd, situated just south of Arnhem (51º56' N; 005º53' E) (Fig. 5.1) in the east of the Netherlands, and another 7 dogs acquired the infection in a recreational area in The Hague (52º05' N; 004º17' E) (Fig. 5 1) further to the west of the country. There were four fatal cases; two in each locality, which had not been recognised as babesiosis and hence had been left untreated. Typical symptoms in each of these fatal cases (dog no. 5, 7, 9, and 17) were lethargy, anorexia, fever, haemoglobinuria/dark urine, and vomiting. Haematological abnormalities included anaemia and thrombocytopenia. Moreover, bilirubinuria, icterus, petechiae and splenomegaly were also noticed. *Babesia* infection was only confirmed after the dogs had died and no post-mortem examinations were carried out.

A subsequent notice to all veterinary clinicians in the Netherlands through the Royal Dutch Veterinary Association, concerning outbreaks of canine babesiosis of an autochthonous origin, prevented any further casualties. Another 16 dogs exhibited clinical symptoms associated with an acute haemolytic disease typical for babesiosis

(Boozer and Macintire, 2003). Symptoms included lethargy, anorexia, fever, anaemia and abdominal tenderness. Blood smear examination revealed the presence of a large *Babesia* species in erythrocytes. All animals were successfully treated with imidocarb dipropionate (Imizol®), Schering-Plough, Utrecht, the Netherlands. Two partly fed ticks and one almost engorged female of *D. reticulatus* were collected from three different dogs in both areas.

During the autumn of 2004, three additional clinical cases of babesiosis (dog no. 21, 22 and 23, see Table 5.1) were diagnosed in dogs that had also not been outside the Netherlands. These dogs were frequent visitors of the same recreational area in The Hague, where the previous outbreak had occurred. This time typical symptoms were recognised early and the dogs were successfully treated with imidocarb dipropionate.

5.3. Materials and Methods

5.3.1. Collection of samples

Twenty samples were collected in the spring and three in the autumn of 2004. Samples were collected from 10 dogs involved in the outbreaks in The Hague and from 13 dogs involved in the outbreak near Arnhem. Blood smears, serum samples as well as EDTA blood samples for PCR were collected from each dog. In addition, 13 dogs were sampled again between 4 and 10 weeks after treatment to obtain a second blood sample for PCR to determine the carrier status of the animals after treatment. Blood smears were stained with Giemsa and examined for *Babesia* parasites. The indirect fluorescent antibody test (IFAT) was carried out as described previously by Uilenberg, Franssen, Perie and Spanjer

(1989). PCR and reverse line blotting for simultaneously detection and differentiation of *Babesia* sp. was essentially performed as described previously (Gubbels, de Vos, Van der Weide, Viseras, Schouls, de Vries and Jongejan, 1999; Matjila, Penzhorn, Bekker, Nijhof and Jongejan, 2004) with some modifications.

5.3.2. DNA extraction

DNA was extracted from 200 µl of each blood sample, from three collected *D. reticulatus* ticks, as well as from the eggs produced by the engorged *D. reticulatus* female tick which had been kept at 27 ºC in an incubator at 80 % relative humidity for oviposition. The QIAmp blood and tissue extraction kit (Qiagen, Hilden, Germany) was used for DNA extractions, following the manufacturer's protocols. DNeasy tissue kit was used for the extraction of DNA from the ticks with the following modifications. Surface sterilised ticks were cut into small pieces and triturated in liquid nitrogen. The material was subsequently lysed at 55º C and further treated according to the manufacturer's protocols.

5.3.3. PCR

PCR was performed with primers RLB-F2 and RLB-R2 amplifying a fragment of 460-540bp from the 18S rRNA gene spanning the V4 region (Gubbels et al., 1999; Matjila et al., 2004). The conditions for the PCR included an initial step of 3 min at 37º C, 10 min at 94ºC, 10 cycles of 94ºC (20s)- 67 ºC (30s)- 72º C (30s), with lowering of annealing step after every second cycle with 2º C (touchdown PCR). The reaction was then followed by 40 cycles of denaturation at 94º C for 30s, annealing at 57º C for 30s and extension at 72º C for 30s.

5.3.4. Reverse line blot hybridisation

RLB was subsequently conducted on amplified products as previously described (Matjila et al., 2004). The method was improved by the addition of a novel plasmid control, which was used as an internal positive control to check whether all *Babesia* species-specific oligonucleotides were correctly attached to the RLB membrane and functioning properly. Three fragments (A, B & C), each containing four RLB-probe sequences flanked by a restriction enzyme recognition sequence for sticky end cloning and their reverse complement, were synthesized by Isogen-Lifescience (Maarssen, the Netherlands) (Table 5.2 and 5.3). The oligonucleotides were diluted in MilliQ to a working solution of 20 pmol/µl. Equal volumes of fragment A and its reverse complement were allowed to hybridise for 15 min at room temperature and subsequently ligated in cloning vector pUC19 digested with the accompanying restriction enzymes EcoRI and KpnI using standard techniques. The ligated plasmid was transferred to competent *E. coli* cells by electroporation. Transformants were checked for successful incorporation of the fragment by performing a PCR using biotin labelled M13 primers and subsequent testing of the PCR product on a RLB membrane. Positive clones were then digested with BamHI and SalI and ligated with hybridised fragment B. Using the same methods, positive clones for fragment A and B were digested with PstI and HindIII to ligate hybridised fragment C. All fragments were successfully incorporated (Fig. 5 2).

5.4. Results

Twenty-three cases of autochthonous babesiosis were confirmed by a combination of clinical history, positive blood smears, PCR/RLB hybridization and detection of *B. canis*-

specific antibodies. Eighteen out of 23 cases were confirmed to be caused by *B. canis* only by PCR/RLB (Fig. 5 2). Although DNA samples were not available for the remaining 5 animals, dogs numbered 1, 4, and 6 were shown to be infected indirectly by a high IFAT antibody titre against *B. canis* (Table 5.1). Dog no. 10 was confirmed positive by a combination of clinical history, positive response to anti-*Babesia* treatment and a *B. canis* positive PCR test conducted by another laboratory. Finally, dog no. 19 was the only dog that was found positive based on blood smears only in combination with the clinical history and positive response to treatment. Thirteen dogs were retested within 4 to 10 weeks after treatment. All were found negative on blood smear examination as well as in the PCR/RLB assay, and titres had either declined (dog 6, 8, 14, 16) or disappeared (dog 1). Three *D. reticulatus* ticks collected from the dogs were also positive for *B. canis* only (Fig. 5.2). The egg progeny derived from the engorged *D. reticulatus* female was also positive (Fig. 5.2).

DNA from the four fatal cases was PCR-amplified using primers RLB-F2 and RLB-R2 and subsequently sequenced (BaseClear, Leiden, The Netherlands). The resulting sequences were submitted to Genbank (AY703070, AY703071, AY703072 and AY703073) and were 100% homologous with *B. canis* 18S sequences deposited in Genbank.

5.5. Discussion

In the spring of 2004, thirteen dogs in Arnhem and 7 dogs in The Hague were involved in outbreaks of babesiosis, whereas in the autumn another three dogs acquired the infection

in The Hague. Pathogen DNA isolated from 18 cases (including four fatalities) was demonstrated to be *B. canis* only (Fig. 5.2). This is based on the observation that no hybridization with any other *Babesia* species was found in the RLB (Fig. 5.2). Although additional tick-borne parasites have been reported in dogs, for instance *B. gibsoni* (Casapulla, Baldi, Avallone, Sannino, Pazzanese and Mizzon, 1998), *Theileria annae* (Zahler Rinder, Schein and Gothe 2000a) and a large *Babesia* recently discovered (Birkenheuer, Neel, Ruslander, Levy and Breitschwerdt, 2004), the catch-all probe would hybridize with all *Babesia* or *Theileria* species or variants for which no species-specific probe is included in the RLB. The cloned plasmid control that was added to the assay, considerably enhanced the reproducibility of the assay and eliminated the need to include pathogen DNA from clinical cases as positive controls (Fig. 5.2). In addition to 18 confirmed *B. canis* cases using PCR/RLB, material from 5 other cases was insufficient to conduct a full analysis. However, babesiosis could be confirmed either by serological means or by clinical and parasitological evidence (Table 5.1).

However, of critical importance here was to obtain confirmation that these dogs had indeed not been outside the Netherlands. Each owner was contacted in order to confirm this; with the exception of four dogs (nos. 10, 11, 12, and 16), no dogs had been out of the country. Dog no. 10 had been in France once in 1995, dog no. 11 had been to Turkey in 2000, dog no. 12 visited France in August 2003 and dog 16 had been to Switzerland in 2001. None of these dogs had ever been treated or diagnosed with canine babesiosis prior to the current outbreak. It is unlikely that dog no's 10, 11, or 16 had contributed to the current outbreak either as asymptomatic carriers or as carriers for ticks, since at least

three years had elapsed since these visits took place. Finally, dog no. 12 was diagnosed with babesiosis in the outbreak in April 2004, ten months after the animal returned from France. The fact that it was negative on IFAT at the onset of the disease (Table 5.1) indicates that the infection had been acquired in the Netherlands.

After the first 4 fatal cases occurred in the spring of 2004, a subsequent warning to all clinicians in the Netherlands prevented any further casualties. It can also be derived from Table 5.1 that the duration between the onset of clinical signs and subsequent treatment became progressively shorter after this warning had been issued. All remaining dogs exhibiting clinical symptoms typical for babesiosis were successfully treated. It is important to note that 13 dogs that were retested several weeks after treatment were all negative in the RLB (Table 5.1). This would indicate that the infection had been cleared, although in theory organisms could still be present under the detection limit of the assay. According to the literature, a single dose (7.5 mg/kg) of imidocarb dipropionate (Penzhorn, Lewis, De Waal and Lopez Rebollar, 1995; Boozer and Macintire, 2003) or two doses (7mg/kg) with a 14-day interval (Brandao, Hagiwara and Myiashiro, 2003) will sterilize the infection. In addition to the life-saving ability of the drug, sterilising the infection in dogs in the Netherlands would render the dogs non-infective for ticks. Moreover, it would also ease matters with respect to blood-component therapy, which has become more accessible in veterinary practice over the recent years (Reine, 2004).

On the other hand, it has been shown that sterilization of infection by imidocarb creates higher susceptibility to re-infection and that untreated animals are more resistant to

homologous challenge (Penzhorn et al., 1995; Lewis, Penzhorn and Lopez Rebollar, 1995). Vaccination, which would sufficiently reduce clinical symptoms but not prevent infection, would be the preferred preventive measure in an endemic area (Schetters, Kleuskens, Scholtes, Pasman and Goovaerts, 1997). The future policy for the control of babesiosis in the Netherlands would depend upon the establishment of the vector tick.

In 1985, *Dermacentor reticulatus* ticks were reported in the Netherlands associated with the first five autochthonous cases of babesiosis (Uilenberg et al., 1985). In several non-endemic European countries including Switzerland, Germany and Belgium (Pfister, Schwalbach, Chuit, Liz and Aeschlimann, 1993; Zahler and Gothe, 1997; Losson, Mollet, Avez, Malaise and Mignon, 1999; Zahler, Steffen, Lutz, HShnel, Rinder and Gothe, 2000b; Duh, Tozon, Petrovec, Strasek and Avsic-Zupanc, 2004) outbreaks of *B. canis* have been reported associated with *D. reticulatus* ticks. It is interesting to note that localized populations of *D. reticulatus* do occur in southwest England, Belgium and Germany, in similar ecological conditions as are present in the Netherlands (Hillyard, 1996; Zahler et al., 2000b). However, there is a lack of detailed distribution maps for *Dermacentor* ticks in North-western Europe, as recently shown by Trotz-Williams and Trees (2003).

Dermacentor reticulatus is a three-host tick of which only the adult stage feeds on dogs; larvae and nymphs feed primarily on small mammals and also on birds (Estrada-Peňa, Bouattour, Camicas and Walker, 2004). It is likely that *Dermacentor* ticks are spread in Europe on dogs traveling to and from endemic areas. In addition, an estimated 1500 dogs

are imported every year into the Netherlands, the majority originating from southern Europe, where *D. reticulatus* occurs (Estrada-Peña et al., 2004). In theory, several wildlife species and migrating birds could also play a role. Without horizontal transfer of adult *Dermacentor* ticks between dogs, engorged female ticks would have to be introduced into each area in the year preceding the current outbreaks. After immature stages had successfully fed on small mammals, subsequent adult ticks emerged in the spring of 2004. Evidence to support this theory is the demonstrated transovarial passage of *B. canis* through the eggs of *D. reticulatus* (Fig. 5.2).

5.6. Conclusion

Although the second, smaller outbreak in the autumn of 2004 was predicted based on the seasonal dynamics of the tick in France (Martinod and Gilot, 1991) it remains to be shown whether further outbreaks should be anticipated. In order to clarify this situation, a survey was initiated to identify ticks from dogs presented at veterinary clinics combined with a survey for *B. canis* using PCR/RLB. Preliminary data from this survey showed that although *Ixodes ricinus* was the predominant tick found on dogs (510 specimens), *Ixodes hexagonus* (33 ticks), *Rhipicephalus sanguineus* (20 ticks) and one additional *D. reticulatus* were also found. These findings would suggest including other pathogens, such as *Ehrlichia canis* transmitted by *R. sanguineus* ticks, in future surveys. In fact, *E. canis* has already been found in dogs introduced into the Netherlands either alone or in combination with *B. canis* (Zandvliet, Teske and Piek, 2004). All clinical cases of babesiosis included in this case report were negative for *E. canis* (data not shown). The establishment of *D. reticulatus* in the Netherlands would also mean that *Rickettsia*

slovaca, causing TIBOLA in humans, would have to be taken into consideration (Raoult, Lakos, Fenollar, Beytout, Brouqui and Fournier, 2002).

In conclusion, future strategies to control ticks and tick-borne diseases in companion animals in the Netherlands need to be reassessed in case populations of *D. reticulatus* ticks become established in the Netherlands. To this date, a recent survey has shown that *Dermacentor* ticks have established themselves at several locations in the Netherlands and in large numbers (Nijhof, Bodaan, Postigo, Nieuwenhuijs, Opsteegh, Franssen, Jebbink and Jongejan, 2007).

5.7. Figures and Tables

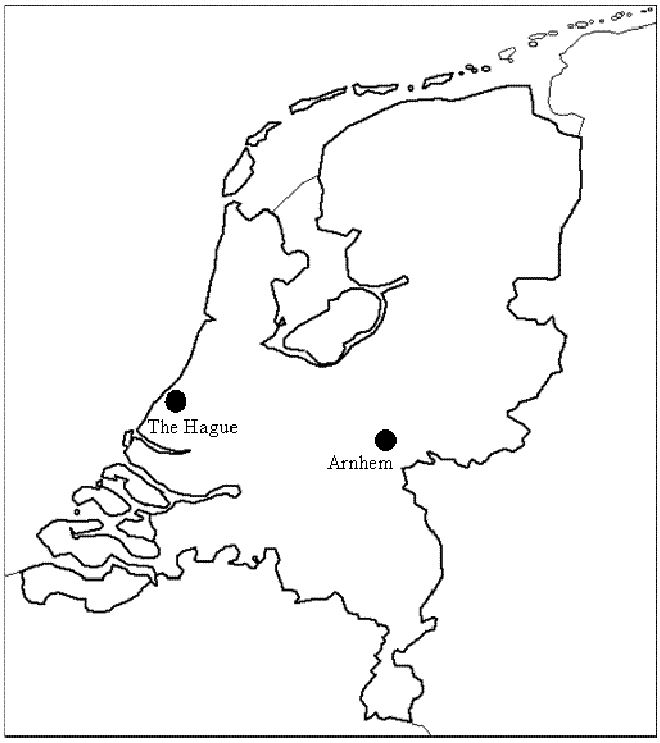


Figure 5.1: Map of the Netherlands indicating the two locations where cases of canine babesiosis were detected.

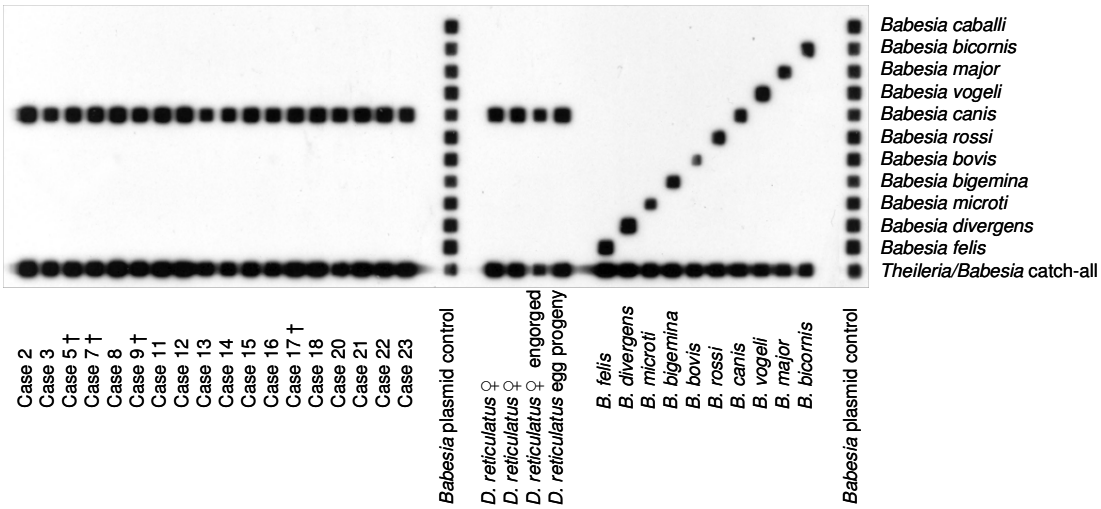


Figure 5.2: RLB results displaying 11 species-specific oligonucleotides of the 18S rRNA gene in the horizontal lanes and PCR products in the vertical lanes. From left to right are shown: 18 canine blood samples from clinical cases, *Babesia* plasmid positive control, 4 tick specimens, 11 positive *Babesia* sp. DNA controls and a second *Babesia* plasmid positive control.

Table 5.1: Twenty-three confirmed cases of autochthonous babesiosis caused by *Babesia canis* in the Netherlands in 2004.

Dog	Location	Onset of disease	1st Imizol treatment	Date blood collection	Blood Smear	PCR / RLB	IFAT
1	Arnhem	23 Feb	2 Apr	2 Apr	NI	NI	1:640
				10 Jun	-	-	-
2	Arnhem	4 Mar	1 Apr	30 Mar	-	+	NI
				29 Jun	-	-	-
3	Arnhem	9 Mar	16 Apr	2 Apr	NI	+	1:640
4	Arnhem	19 Mar	8 Apr	25 Mar	NI	NI	1:1280
				10 Jun	-	-	NI
5 [a]	The Hague	21 Mar	None	21 Mar	+	+	-
6	Arnhem	23 Mar	2 Apr	2 Apr	NI	NI	1:1280/2560
				10 Jun	-	-	1:80/160
7 [a]	Arnhem	24 Mar	None	29 Mar	NI	+	-
8	Arnhem	25 Mar	1 Apr	30 Mar	+	+	1:2560
				28 Jun	-	-	1:320
9 [a]	Arnhem	26 Mar	None	26 Mar	NI	+	NI
10	The Hague	30 Mar	7 Apr	2 Apr	NI	+	NI
				3 Jun	-	-	NI
11	The Hague	5 Apr	5 Apr	6 Apr	NI	+	1:80/160
				9 Jun	-	-	NI
12	The Hague	6 Apr	9 Apr	8 Apr	+	+	-
				9 Jun	-	-	NI
13	The Hague	7 Apr	13 Apr	13 Apr	+	+	1:1280
14	Arnhem	10 Apr	20 Apr	19 Apr	-	+	1:1280
				28 Jun	-	-	1:320
15	Arnhem	14 Apr	14 Apr	14 Apr	+	+	NI
				28 Jun	-	-	-
16	Arnhem	27 Apr	10 May	16 Apr	-	+	1:640
				29 Jun	-	-	1:160
17 [a]	The Hague	28 Apr	29 Apr	29 Apr	+	+	-
18	The Hague	29 Apr	29 Apr	29/Apr	+	+	-
19	Arnhem	4 May	7 May	5 May	+	NI	-
				10 Jun	-	-	NI
20	Arnhem	5 May	26 May	13 May	+	+	1:640
21	The Hague	13 Oct	16 Oct	16 Oct	+	+	NI
22	The Hague	23 Oct	8 Nov	8 Nov	+	+	NI
23	The Hague	2 Nov	4 Nov	4 Nov	+	+	NI

RLB: Reverse line blot
IFAT: Indirect fluorescent antibody test
NI: not investigated
[a] Indicates a fatal case

Table 5.2: Composition of the *Babesia* plasmid control with three fragments (A, B and C) each containing four RLB-probe sequences flanked by the restriction enzyme recognition sequence for sticky-end cloning.

Fragment	**Synthesized oligonucleotides**	**Restriction enzymes**
A	5'-AATTC(sequence probe 1 to 4)GGTAC-3' 5'-C(reverse complement probe 4 to 1)G-3'	EcoRI and KpnI
B	5'-GATCC(sequence probe 5 to 8)G-3' 5'-TCGAC(reverse complement probe 8 to 5)G-3'	BamHI and SalI
C	5'-G(sequence probe 9 to 12)A-3' 5'-AGCTT(reverse complement probe 12 to 9)CTGCA-3'	PstI and HindIII

Table 5.3: RLB-probes incorporated in the *Babesia* plasmid control.

Probe	Oligonucleotide probe specificity	Oligonucleotide sequence (5'→3')
1	*Babesia* catch-all	TAATGGTTAATAGGA(AG)C(AG)GTT
2	*Babesia bicornis*	TTGGTAAATCGCCTTGGT
3	*Babesia bigemina*	CGTTTTTTCCCTTTTGTTGG
4	*Babesia bovis*	CAGGTTTCGCCTGTATAATTGAG
5	*Babesia canis*	TGCGTTGACGGTTTGAC
6	*Babesia rossi*	CGGTTTGTTGCCTTTGTG
7	*Babesia vogeli*	AGCGTGTTCGAGTTTGCC
8	*Babesia felis*	TTATGCGTTTTCCGACTGGC
9	*Babesia major*	TCCGACTTTGGTTGGTGT
10	*Babesia caballi*	GCTTGATTTTCGCTTCGCTT
11	*Babesia divergens*	ACT(AG)ATGTCGAGATTGCAC
12	*Babesia microti*	GACTTGGCATCTTCTGGA

5.8. References

Birkenheuer, A.J., Neel, J., Ruslander, D., Levy, M.G., Breitschwerdt, E.B., 2004. Detection and molecular characterization of a novel large *Babesia* species in a dog. Veterinary Parasitology 124, 151-160.

Boozer, A.L., Macintire, D.K., 2003. Canine babesiosis. The Veterinary clinics of North America. Small Animal Practice 33, 885-904.

Brandao, L.P., Hagiwara, M.K., Myiashiro, S.I., 2003. Humoral immunity and reinfection resistance in dogs experimentally inoculated with *Babesia canis* and either treated or untreated with imidocarb dipropionate. Veterinary Parasitology 114, 253-265.

Casapulla, R., Baldi, L., Avallone, V., Sannino, R., Pazzanese, L., Mizzoni, V., 1998. Canine piroplasmosis due to *Babesia gibsoni*: clinical and morphological aspects. Veterinary Record 142, 168-169.

Duh, D., Tozon, N., Petrovec, M., Strasek, K., Avsic-Zupanc, T., 2004. Canine babesiosis in Slovenia: molecular evidence of *Babesia canis canis* and *Babesia canis vogeli*. Veterinary Research 35, 363-368.

Estrada-Peňa, A., Bouattour, A., Camicas, J.-L., Walker, A.R., 2004. Ticks of domestic animals in the Mediterranean region, a guide to the identification of species. University of Zaragoza, Spain. (Book,Monograph).

Gubbels, J.M., de Vos, A.P., Van der Weide, M., Viseras, J., Schouls, L.M., de Vries, E., Jongejan, F., 1999. Simultaneous detection of bovine *Theileria* and *Babesia* species by reverse line blot hybridization. Journal of Clinical Microbiology 37, 1782-1789.

Hillyard, P.D., 1996. Ticks of North-West Europe; keys and notes for identification of species. Synopses of the British Fauna, New Series, 52, 176-176.

Lewis, B.D., Penzhorn, B.L., Lopez Rebollar, L.M., 1995. Immune responses to South African *Babesia canis* and the development of a preliminary vaccine. Journal of the South African Veterinary Association 66, 61-65.

Losson, B., Mollet, J.J., Avez, F., Malaise, F., Mignon, B., 1999. Description of three cases of canine babesiosis in Belgium. Annales de Médecine Vétérinaire 143, 119-124.

Martinod, S., Gilot, B., 1991. Epidemiology of canine babesiosis in relation to the activity of *Dermacentor reticulatus* in southern Jura (France). Experimental and Applied Acarology 11, 215-222.

Matjila, P.T., Penzhorn, B.L., Bekker, C.P., Nijhof, A.M., Jongejan, F., 2004. Confirmation of occurrence of *Babesia canis vogeli* in domestic dogs in South Africa. Veterinary Parasitology 122, 119-125.

Nijhof, A.M., Bodaan, C., Postigo, M., Nieuwenhuijs, H., Opsteegh, M., Franssen, L., Jebbink, F., Jongejan, F., 2007. Ticks and associated pathogens collected from domestic animals in the Netherlands. Vector-Borne and Zoonotic Diseases 7, 585-596.

Penzhorn, B.L., Lewis, B.D., De Waal, D.T., Lopez Rebollar, L.M., 1995. Sterilisation of *Babesia canis* infections by imidocarb alone or in combination with diminazene. Journal of the South African Veterinary Association 66, 157-159.

Pfister, K., Schwalbach, B., Chuit, P.A., Liz, J., Aeschlimann, A., 1993. Präliminäre Untersuchungen zur endemischen Ausbreitung von *Babesia canis* und der Zecke *Dermacentor reticulatus* in der Schweiz. Mitteilungen der Österreichischen Gesellschaft für Tropenmedizin und Parasitologie 1-6.

Raoult, D., Lakos, A., Fenollar, F., Beytout, J., Brouqui, P., Fournier, P.E., 2002. Spotless rickettsiosis caused by *Rickettsia slovaca* and associated with *Dermacentor* ticks. Clinical Infectious Diseases 34, 1331-1336.

Reine, N.J., 2004. Infection and blood transfusion: a guide to donor screening. Clinical Techniques in Small Animal Practice 19, 68-74.

Schetters, T.P.M., Kleuskens, J.A.G.M., Scholtes, N.C., Pasman, J.W., Goovaerts, D., 1997. Vaccination of dogs against *Babesia canis* infection. Veterinary Parasitology 73, 35-41.

Trotz-Williams, L.A., Trees, A.J., 2003. Systematic review of the distribution of the major vector-borne parasitic infections in dogs and cats in Europe. Veterinary Record 152, 97-105.

Uilenberg, G., Franssen, F.F., Perie, N.M., Spanjer, A.A., 1989. Three groups of *Babesia canis* distinguished and a proposal for nomenclature. Veterinary Quarterly 11, 33-40.

Uilenberg, G., Top, P.D., Arends, P.J., Kool, P.J., van Dijk, J.E., van Schieveen, P.B., Zwart, D., 1985. Autochthonous babesiosis in dogs in the Netherlands? Tijdschrift voor Diergeneeskunde 110, 93-98.

Zahler, M., Gothe, R., 1997. The risk of *Babesia canis* infection, transmitted by *Dermacentor reticulatus* becoming endemic in Germany. Tierärztliche Praxis. Ausgabe K, Kleintiere/Heimtiere 25, 666-670.

Zahler, M., Rinder, H., Schein, E., Gothe, R., 2000a. Detection of a new pathogenic *Babesia microti*-like species in dogs. Veterinary Parasitology 89, 241-248.

Zahler, M., Steffen, T., Lutz, S., HShnel, W.C., Rinder, H., Gothe, R., 2000b. *Babesia canis* and *Dermacentor reticulatus* in Munich: a new endemic focus in Germany. Tierärztliche Praxis. Ausgabe K, Kleintiere/Heimtiere 28, 116-120.

Zandvliet, M.M., Teske, E., Piek, C.J., 2004. *Ehrlichia* and *Babesia* infections in dogs in The Netherlands. Tijdschrift voor Diergeneeskunde 129, 740-745.