
Chapter 7: Molecular characterization of *Babesia gibsoni* infection from a pit-bull terrier pup recently imported into South Africa.

7.1. Abstract

Canine babesiosis caused by *Babesia gibsoni* was diagnosed in a three-month-old pit-bull pup during a routine clinical examination. The dog was a three-month old pit-bull puppy imported from Philadelphia, USA. On physical examination, the dog had no typical signs of clinical babesiosis, except for a temperature of 39°C. PCR targeting the 18S rRNA gene was conducted followed by the Reverse line blot (RLB) assay. *Babesia gibsoni* infection was confirmed by way of smear examination, PCR, Reverse Line Blot (RLB) and sequence analysis. Sequence analysis revealed that the sequence had a 100% homology with the Asian genotype (accession no. [AF205636](#)). Treatment was initiated with diminazene aceturate (Berenil RTU®) followed by 2 doses of imidocarb dipropionate (Forray-65®) 3 days and 14 days later, respectively. *Babesia gibsoni* DNA was still detectable two weeks post-treatment on the PCR/RLB test. A ten-day course of combination drug therapy using atovaquone and azithromycin was initiated. Blood samples taken on Day 1 and Day 40 after completion of treatment were negative for *B. gibsoni* DNA on PCR/RLB test. Increased travel and movement of animals into South Africa has increased the possibility of new tick-borne pathogens being introduced in non-endemic areas. Without vigilant surveillance and stringent import control, the presence of potential tick vectors increases the risk of this pathogen becoming established in South Africa.

7.2. Introduction

Canine babesiosis is caused by large or small *Babesia* parasites. The large piroplasms form the *B. canis (sensu lato)* group comprising three species: *B. canis* endemic in Southern Europe, *B. rossi* endemic in Sub-Saharan Africa and *B. vogeli* endemic in tropical and subtropical areas (Uilenberg, Franssen, Perie and Spanjer, 1989; Oyamada, Davoust, Boni, Dereure, Bucheton, Hammad, Itamoto, Okuda and Inokuma, 2005).

The small piroplasms, previously regarded as *Babesia gibsoni (sensu lato)*, have been reported to be endemic in Asia, North America, North and Eastern Africa, Europe and Australia (Yamane, Thomford, Gardner, Dubey, Levy and Conrad, 1993; Casapulla, Baldi, Avallone, Sannino, Pazzanese and Mizzoni, 1998; Muhlnickel, Jefferies, Morgan-Ryan and Irwin, 2002). There are at least three genetically distinct entities in this group: the Asian, North American (California) and Spanish isolates, respectively (Kjemtrup, Kocan, Whitworth, Meinkoth, Birkenheuer, Cummings, Boudreaux, Stockham, Irizarry-Rovira and Conrad, 2000). In the United States the most reported *B. gibsoni* infections are those of the Asian genotype (Irizarry-Rovira, Stephens, Christian, Kjemtrup, DeNicola, Widmer and Conrad, 2001; Kocan, Kjemtrup, Meinkoth, Whitworth, Murphy, Decker and Lorenz, 2001; MacIntire, Boudreaux, West, Bourne, Wright and Conrad, 2002; Birkenheuer, Levy and Breitschwerdt, 2003). The Spanish isolate was provisionally named *Theileria annae* (Zahler, Rinder, Schein and Gothe, 2000; Goethert and Telford, III, 2003) and the Californian isolate has been named *Babesia conradae* (Kjemtrup, Wainwright, Miller, Penzhorn and Carreno, 2006).

Clinical disease associated with *B. gibsoni* (*sensu lato*) may range from being a mild to a severe infection (Birkenheuer, Levy, Savary, Gager and Breitschwerdt, 1999; Macintire et al., 2002). The disease may follow a hyper-acute, acute or chronic course. The acute course, is characterized by fever, lethargy, anaemia, thrombocytopenia, lymphadenopathy and splenomegaly (Irizarry-Rovira et al., 2001; Matsuu, Kawabe, Koshida, Ikadai, Okano and Higuchi, 2004). The hyper-acute state is characterized by shock and extensive tissue damage (Conrad, Thomford, Yamane, Whiting, Bosma, Uno, Holshuh and Shelly, 1991; Freeman, Kirby, Panciera, Henik, Rosin and Sullivan, 1994). The chronic form has been reported in Australia and the USA (Hughes and Oz, 1995; Kjemtrup et al., 2000), and although its difficult to diagnose chronic *Babesia canis* (*sensu lato*) infections, PCR can be a useful tool in confirming chronic infections (Tuttle, Birkenheuer, Juopperi, Levy and Breitschwerdt, 2003).

Definitive diagnosis of *Babesia* infections in dogs depends on the demonstration of infected erythrocytes on Romanowsky-stained blood smears (Uilenberg et al., 1989). Although smear examination is useful, chances of false negatives are high in cases where parasitaemias are low. Indirect immunofluorescent antibody test (IFAT) can also be used for such cases (Uilenberg, Verdiesen and Zwart, 1981; Vercammen, De Deken and Maes, 1995). Also new technologies that include Polymerase Chain Reaction (PCR) and Reverse Line Blot (RLB) can be used to confirm *Babesia* infections (Matjila, Penzhorn, Bekker, Nijhof and Jongejan, 2004; Matjila, Nijhof, Taoufik, Houwers, Teske, Penzhorn, Lange and Jongejan, 2005).

Dogs with clinical babesiosis normally improve within 24 hours of treatment with an anti-babesial drug (Jacobson, Reyers, Berry and Viljoen, 1996). The drugs of choice in South Africa against *B. rossi* are diminazene aceturate and / or imidocarb dipropionate. 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, Lewis, De Waal and Lopez Rebollar, 1995). Diminazene and / or imidocarb are ineffective in treating *B. gibsoni* (Asian type) infections in dogs. The only therapy currently reported to successfully treat *B. gibsoni* infections in dogs is a combination of azithromycin and atovaquone (Birkenheuer, Levy and Breitschwerdt, 2004).

7.2.1. Case history

A three-month-old pit-bull terrier pup from the USA, imported into South Africa, was presented for routine microchip implantation and deworming. The owner indicated that the dog had good appetite and habitus. On physical examination, the dog had temperature of 39.4°C. A capillary blood smear was made and stained with Diff-Quick (Scientific Products Co., McGaw Park, USA). Numerous small piroplasms were seen on the smear (Fig. 7.1) which also revealed thrombocytopenia and a marked reticulocytosis. The dog was treated by subcutaneous injection of diminazene aceturate (Berenil RTU®) at a dose of 3.5 mg/kg.

Two days later the dog was returned to the clinic. On examination the dog had a rectal temperature of 38.6°C. A capillary blood smear was made and examined, and small

piroplasms were still present, although the parasitaemia was reduced. Based on blood smear examination thrombocyte numbers had increased and marked reticulocytosis persisted. On the 6th day, the dog's temperature was 38°C. A single infected erythrocyte was observed during smear examination as well as normal thrombocyte numbers, reticulocytosis and a monocytosis had developed. The dog was injected subcutaneously with imidocarb dipropionate (Forray-65®) at 6 mg/kg and the owner was requested to return the dog for a follow-up injection after 14 days.

Fourteen days later the dog had a rectal temperature of 39°C. Large numbers of small piroplasms were observed during smear examination, as well as a decrease in the number of thrombocytes and a marked increase in reticulocytes. A jugular blood sample was taken at this presentation and this was also the only point at which haematology was assessed. The same sample was also assessed using PCR/RLB test. The dog was again treated with imidocarb (Forray-65®) at a dose of 6 mg/kg.

The dog was examined again 14 days later. Blood smear investigations showed that the piroplasm parasitaemia had persisted. Treatment was initiated with azithromycin (10 mg/kg) given orally once daily for ten days and atovaquone (13.3 mg/kg) twice daily for ten days. Molecular evaluation of blood parasite DNA was carried out only on Day 1 and Day 40 after completion of treatment; on both occasions blood smears were examined and jugular blood samples were collected.

7.3. Materials and Methods

7.3.1. Collection of samples

Three blood samples collected in EDTA tubes at various intervals were sent to the Department of Veterinary Tropical Diseases, Faculty of Veterinary Science, for PCR and RLB tests. A blood sample in EDTA was also sent to a private veterinary clinical pathology laboratory for a full haematological examination. The first sample for PCR/RLB test and the only sample subjected to a haematological examination was collected two weeks before treatment with azithromycin and atovaquone began. The two other samples were taken on Day 1 and Day 40 after completion of treatment with azithromycin and atovaquone.

7.3.2. DNA extraction

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.

7.3.3. PCR

PCR was performed with primers RLB-F2 (5' -GAC ACA GGG AGG TAG TGA CAA G-3') and RLB-R2 (biotin-5'-CTA AGA ATT TCA CCT CTG ACA GT-3') amplifying a fragment of 460-540bp from the 18S rRNA gene spanning the V4 region (Gubbels, de Vos, Van der Weide, Viseras, Schouls, de Vries and Jongejan, 1999; Matjila et al., 2004). The conditions for the PCR included an initial step of 3 min at 37° C, 10 min at 94°C, 10 cycles of 94°C (20s)- 67 °C (30s)- 72° C (30s), with lowering of annealing step after every second cycle by 2° C (touchdown PCR). The reaction was then followed by 40

cycles of denaturation at 94° C for 30s, annealing at 57° C for 30s and extension at 72° C for 30s.

7.3.4. Reverse Line Blot hybridisation

PCR-amplified products were tested with the RLB, as previously described (Matjila et al., 2004). An additional plasmid control was used as an internal positive control to check whether all *Babesia* species-specific probes were correctly attached to the RLB membrane and functioning properly (Matjila et al., 2005).

7.3.5. Sequencing

Extracted DNA was PCR-amplified with the primers RLB-F2 and RLB-R2 and partial segments (400-540bp) of the PCR product were sequenced (Inqaba-Biotec, Pretoria, South Africa).

7.3.6. Phylogenetic analysis

Phylogenetic analysis was done to confirm the relationship between our positive *B. gibsoni* sample and other *Babesia* species (Fig. 7.2). Sequence data were assembled to a total length of 413bp using GAP4 of the Staden package (Version 1.6.0 for Windows). The sequences were aligned with sequences of related genera using ClustalX (Version 1.81 for Windows). The two parameter model of Kimura and the Jukes and the Cantor correction model for multiple base changes were used to construct similarity matrices (Jukes and Cantor, 1969; Kimura, 1980). Neighbor-joining (Saitou and Nei, 1987) and the maximum parsimony methods were used for the construction of phylogenetic trees

using the Mega 3.0 software package (Kumar, Tamura and Nei, 2004). The above methods were used in combination with the bootstrap method (Felsenstein, 1985).

7.4. Results

The blood sample collected prior to treatment with a combination drug therapy of atovaquone and azithromycin was confirmed positive for *B. gibsoni* by smear examination (Fig. 7.1), PCR and RLB. Sequence analysis (413bp) revealed that the sequence had a 100% homology with at least two full sequences (\pm 1600bp) of two genotypes: *B. gibsoni* (Japan, Aomori, [AB118032](#)) and *Babesia* sp. (Oklahoma, [AF205636](#)). Phylogenetic analysis procedures showed that the *B. gibsoni* (pit-bull terrier) sequence was closely related to *B. gibsoni* (Japan, Aomori, [AB118032](#)) and *Babesia* sp. (Oklahoma, [AF205636](#)) (Fig. 7.2). These procedures also showed that there were no significant changes in topology of trees or in bootstrap values using either the neighbor-joining or the maximum parsimony methods. Haematology results showed that the dog had a low PCV, a reduced erythrocyte count and a thrombocytopenia (Table 7.1).

The blood samples collected on Day 1 and Day 40 after completion of the combination drug therapy were negative on PCR/RLB test. All samples were negative for *Ehrlichia* infection, using PCR/RLB tests at all time points.

7.5. Discussion

In this study molecular techniques proved to be invaluable in confirming *B. gibsoni* infection in the imported dog. Prior to the blood samples being sent for PCR/RLB test it

was not clear which piroplasm was parasitizing the erythrocytes. Although the dog had no obvious signs of clinical babesiosis, microscopic examination of the capillary blood smears revealed the presence of piroplasms in the erythrocytes. Prior to the atovaquone / azithromycin therapy, infected erythrocytes could still be observed on capillary blood smears on all five occasions that the dog was examined. This was true for up to one month after treatment with diminazene and imidocarb. A number of researchers have reported that treatment with diminazene and / or imidocarb is ineffective against *B. gibsoni* (Asian genotype) infection (Birkenheuer et al., 1999; Stegeman, Birkenheuer, Kruger and Breitschwerdt, 2003). After completion of the atovaquone / azithromycin therapy, no infected erythrocytes were observed on capillary blood smear examination. PCR/RLB results were also negative. This treatment regimen was therefore successful in either clearing the infection or reducing the parasite load to below the detection limit of our PCR/RLB assay. Some dogs that had been treated with this combination drug therapy were negative on PCR assay up to 120 days post treatment (Birkenheuer et al., 2004).

The dog was moderately anaemic and thrombocytopenic. Dogs sub-clinically infected with *B. gibsoni* might be anaemic as a result of the host response to the parasite, immune-mediated erythrocyte destruction, or a combination of the host immune-mediated intravascular and extravascular haemolysis (Conrad et al., 1991; Adachi, Tateishi, Horii, Nagatomo, Shimizu and Makimura, 1995). Thrombocytopenia with variable leukocyte change is also a common feature of dogs infected with *B. gibsoni* (Conrad et al., 1991; Wozniak, Barr, Thomford, Yamane, McDonough, Moore, Naydan, Robinson and

Conrad, 1997; Birkenheuer et al., 1999; Wilkerson, Shuman, Swist, Harkin, Meinkoth and Kocan, 2001; Fukumoto, Suzuki, Igarashi and Xuan, 2005) .

Dogs imported into South Africa are subject to pre-import blood tests, including *B. gibsoni*, or exporting countries certifying freedom of disease, as this is a controlled disease in South Africa. Countries declaring freedom from *B. gibsoni* and other diseases are not required to do pre-importation blood testing. Only dogs with negative test results or those coming from *B. gibsoni*-free countries may be imported. In addition, dogs imported from certain countries are subjected to post-arrival quarantine and repeat serological blood testing on arrival in South Africa. *B. gibsoni* does occur in the USA and thus dogs from that country are tested prior to import but are not subjected to post-arrival quarantine. As this pit-bull terrier had tested negative in the USA within 30 days prior to importation, it was allowed to enter the country. The owner alleged that the dog had been in South Africa two weeks before it was taken to the veterinarian. The chances of the infection having been acquired locally are negligible since *B. gibsoni* is not endemic in South Africa. The translocation of infected dogs into *B. gibsoni*-free areas has been implicated as an important element in the spread of this parasite (Conrad et al., 1991).

Bull terrier-type dog breeds have a higher incidence of sub-clinical *B. gibsoni* infection than other breeds (Birkenheuer et al., 1999; Kocan et al., 2001). American pit-bull terriers are reported to be the most commonly reported *B. gibsoni*-infected breed in the USA, the reasons for this however remain unclear (Birkenheuer et al., 1999; Kocan et al.,

2001; MacIntire et al., 2002). Although transmission experiments have not yet proved which tick species is the vector for *B. gibsoni*, *Haemaphysalis bispinosa*, *H. longicornis*, *H. leachi* and *R. sanguineus* have been implicated in the transmission of the parasite (Wozniak et al., 1997; Kjemtrup et al., 2000). Both *H. elliptica* and *R. sanguineus* are endemic to South Africa (Horak, 1995) and they could be suitable vectors should *B. gibsoni* become established in South Africa.

Increased travel and movement of animals into South Africa have increased the possibility of new tick-borne pathogens being introduced in non-endemic areas. The risk of *B. gibsoni* becoming established in South Africa is limited, due to the requirements of pre-import testing of all dogs from countries that are not free of *B. gibsoni*. It is also crucial that blood testing should always include serological and molecular testing. Without vigilant surveillance and stringent import control, the presence of potential tick vectors increases the risk of this pathogen becoming established in South Africa. Furthermore, it has been suggested in Japan that in the absence of the tick vector, there is a possibility that *B. gibsoni* might be transmitted through blood contamination during dog fights (Matsuu et al., 2004). The dog reported in this case report was lost to further clinical follow up, as the dog had allegedly died of unknown causes.

7.6. Conclusion

This dog should not have been treated, as *B. gibsoni* is a controlled disease in South Africa and treatment is not allowed. Private veterinarians should be aware of possible diagnosis of similar diseases, which do not usually occur in South Africa, especially in

imported dogs. The diagnosis of any controlled disease in South Africa must be reported to the Veterinary Administration (National Department of Agriculture), and these diseases may only be managed and treated with express permission and under direction of the Senior Manager Animal Health, Department of Agriculture.

7.7. Figures and Tables

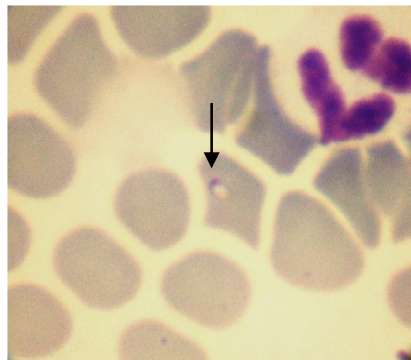
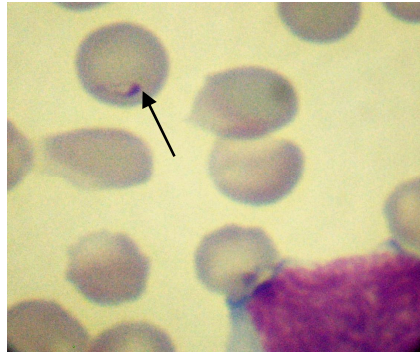


Figure 7.1: Giemsa-stained blood smears showing infected erythrocytes (magnification, x 1000).

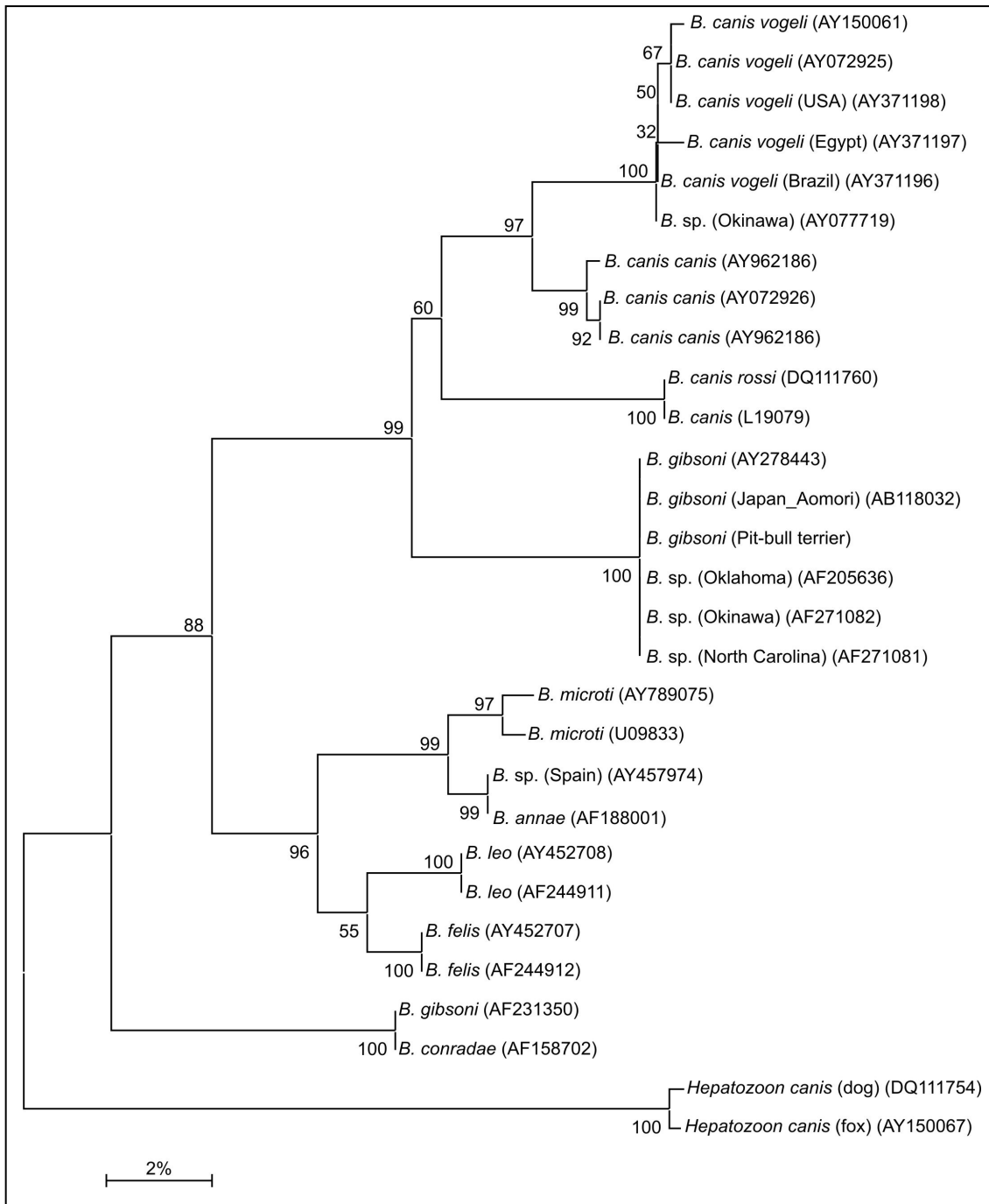


Figure 7.2: Neighbor-joining tree, based on the Kimura two-parameter distance calculation, showing the phylogenetic relationship of *B. gibsoni* (pit-bull terrier) to other *Babesia* sp. Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. The scale bar represents the % nucleotide difference. *Hepatozoon canis* was used as an outgroup. NB: *B. gibsoni* (AF231350): This sequence is another isolate of *B. conradae* (Kjemtrup et al., 2006).

Table 7.1: Haematological report indicating full blood counts 2 weeks prior to treatment of the dog with a combination of atovaquone and azithromycin.

		Normal range	
Red blood cell count	4.2 x 10 ¹² /l	5.5	8.5
Haemoglobin	9.55 g/l	12	18
Haematocrit (PCV)	29.00%	37	55
Mean cell volume (MCV)	69.00 fl	60	77
Mean cell haem cons (MCHC)	33.00 fl	32	36
White cell count	7.32 x 10 ⁹ /l	6	15
Platelet counts	83.00 x 10 ⁹ /l	200	500
Differential count			
Segmented neutrophils %	64	60.0	80
Band neutrophils %	2.00	0	3
Lymphocytes %	17.00	12	30
Monocytes %	15.00	3	10
Eosinophils %	1.00	2	10
Basophils%	1.00	0	3
Segmented neutrophils (abs)	4.68 x 10 ⁹ /l	3	1.5
Band neutrophils (abs)	0.15 x 10 ⁹ /l	0	.03
Lymphocytes (abs)	1.24 x 10 ⁹ /l	1	4.8
Monocytes (abs)	1.10 x 10 ⁹ /l	0.1	1.4
Eosinophils (abs)	0.07 x 10 ⁹ /l	0	1.2
Basophils (abs)	0.07 x 10 ⁹ /l	0	.02

7.8. References

- Adachi, K., Tateishi, M., Horii, Y., Nagatomo, H., Shimizu, T., Makimura, S., 1995. Immunologic characteristics of anti-erythrocyte membrane antibody produced in dogs during *Babesia gibsoni* infection. *Journal of Veterinary Medical Science* 57, 121-123.
- Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2004. Efficacy of combined atovaquone and azithromycin for therapy of chronic *Babesia gibsoni* (Asian genotype) infections in dogs. *Journal of Veterinary Internal Medicine* 18, 494-498.
- Birkenheuer, A.J., Levy, M.G., Breitschwerdt, E.B., 2003. Development and evaluation of a seminested PCR for detection and differentiation of *Babesia gibsoni* (Asian genotype) and *B. canis* DNA in canine blood samples. *Journal of Clinical Microbiology* 41, 4172-4177.
- Birkenheuer, A.J., Levy, M.G., Savary, K.C., Gager, R.B., Breitschwerdt, E.B., 1999. *Babesia gibsoni* infections in dogs from North Carolina. *Journal of the American Animal Hospital Association* 35, 125-128.
- Casapulla, R., Baldi, L., Avallone, V., Sannino, R., Pazzanese, L., Mizzoni, V., 1998. Canine piroplasmiasis due to *Babesia gibsoni*: clinical and morphological aspects. *Veterinary Record* 142, 168-169.
- Conrad, P., Thomford, J., Yamane, I., Whiting, J., Bosma, L., Uno, T., Holshuh, H.J., Shelly, S., 1991. Hemolytic anemia caused by *Babesia gibsoni* infection in dogs. *Journal of the American Animal Hospital Association* 199, 601-605.
- Felsenstein, J., 1985. Confidence limits on phylogenies: an approach using the bootstrap. *Evolution* 783-791.

- Freeman, M.J., Kirby, B.M., Panciera, D.L., Henik, R.A., Rosin, E., Sullivan, L.J., 1994. Hypotensive shock syndrome associated with acute *Babesia canis* infection in a dog. *Journal of the American Animal Hospital Association* 204, 94-96.
- Fukumoto, S., Suzuki, H., Igarashi, I., Xuan, X., 2005. Fatal experimental transplacental *Babesia gibsoni* infections in dogs. *International Journal of Parasitology* 35, 1031-1035.
- Goethert, H.K., Telford, S.R., III, 2003. What is *Babesia microti*? *Parasitology* 127, 301-309.
- 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.
- Horak, I.G., 1995. Ixodid ticks collected at the Faculty of Veterinary Science, Onderstepoort, from dogs diagnosed with *Babesia canis* infection. *Journal of the South African Veterinary Association* 66, 170-171.
- Hughes, W.T., Oz, H.S., 1995. Successful prevention and treatment of babesiosis with atovaquone. *Journal of Infectious Diseases* 172, 1042-1046.
- Irizarry-Rovira, A.R., Stephens, J., Christian, J., Kjemtrup, A., DeNicola, D.B., Widmer, W.R., Conrad, P.A., 2001. *Babesia gibsoni* infection in a dog from Indiana. *Veterinary Clinical Pathology* 30, 180-188.
- Jacobson, L.S., Reyers, F., Berry, W.L., Viljoen, E., 1996. Changes in haematocrit after treatment of uncomplicated canine babesiosis: a comparison between diminazene and trypan blue, and an evaluation of the influence of parasitaemia. *Journal of the South African Veterinary Association* 67, 77-82.

- Jukes, T.H., Cantor, C.R., 1969. Evolution of protein molecules. In: Munro, H.N. (Ed.), Mammalian Protein Metabolism. Academic Press, New York, pp. 21-132.
- Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16, 111-120.
- Kjemtrup, A.M., Kocan, A.A., Whitworth, L., Meinkoth, J., Birkenheuer, A.J., Cummings, J., Boudreaux, M.K., Stockham, S.L., Irizarry-Rovira, A., Conrad, P.A., 2000. There are at least three genetically distinct small piroplasms from dogs. *International Journal of Parasitology* 30, 1501-1505.
- Kjemtrup, A.M., Wainwright, K., Miller, M., Penzhorn, B.L., Carreno, R.A., 2006. *Babesia conradae*, sp. nov., a small canine *Babesia* identified in California. *Veterinary Parasitology* 138, 103-111.
- Kocan, A.A., Kjemtrup, A., Meinkoth, J., Whitworth, L.C., Murphy, G.L., Decker, L., Lorenz, M., 2001. A genotypically unique *Babesia gibsoni*-like parasite recovered from a dog in Oklahoma. *Journal of Parasitology* 87, 437-438.
- Kumar S, Tamura K, Nei M, 2004. MEGA3: Integrated software for Molecular Evolutionary Genetics Analysis and sequence alignment. *Brief Bioinformatics* 5, 150-163.
- MacIntire, D.K., Boudreaux, M.K., West, G.D., Bourne, C., Wright, J.C., Conrad, P.A., 2002. *Babesia gibsoni* infection among dogs in the south-eastern United States. *Journal of the American Veterinary Medical Association* 220, 325-329.

- 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.
- Matjila, T.P., Nijhof, A.M., Taoufik, A., Houwers, D., Teske, E., Penzhorn, B.L., Lange, T.D., Jongejan, F., 2005. Autochthonous canine babesiosis in The Netherlands. *Veterinary Parasitology* 131, 23-29.
- Matsuu, A., Kawabe, A., Koshida, Y., Ikadai, H., Okano, S., Higuchi, S., 2004. Incidence of canine *Babesia gibsoni* infection and sub-clinical infection among Tosa dogs in Aomori Prefecture, Japan. *Journal of Veterinary Medical Science* 66, 893-897.
- Muhlnickel, C.J., Jefferies, R., Morgan-Ryan, U.M., Irwin, P.J., 2002. *Babesia gibsoni* infection in three dogs in Victoria. *Australian Veterinary Journal* 80, 606-610.
- Oyamada, M., Davoust, B., Boni, M., Dereure, J., Bucheton, B., Hammad, A., Itamoto, K., Okuda, M., Inokuma, H., 2005. Detection of *Babesia canis rossi*, *B. canis vogeli*, and *Hepatozoon canis* in dogs in a village of eastern Sudan by using a screening PCR and sequencing methodologies. *Clinical Diagnostic Laboratory Immunology* 12, 1343-1346.
- Penzhorn, B.L., Lewis, B.D., De Waal, D.T., Lopez Rebolgar, 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.
- Saitou, N., Nei, M., 1987. The neighbor-joining method: a new method for reconstructing phylogenetic trees. *Molecular Biology and Evolution* 4, 406-425.

- Stegeman, J.R., Birkenheuer, A.J., Kruger, J.M., Breitschwerdt, E.B., 2003. Transfusion-associated *Babesia gibsoni* infection in a dog. *Journal of the American Veterinary Medical Association* 222, 959-963.
- Tuttle, A.D., Birkenheuer, A.J., Juopperi, T., Levy, M.G., Breitschwerdt, E.B., 2003. Concurrent bartonellosis and babesiosis in a dog with persistent thrombocytopenia. *Journal of the American Veterinary Medical Association* 223, 1306-1310.
- 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., Verdiesen, P.A., Zwart, D., 1981. Imidocarb: a chemoprophylactic experiment with *Babesia canis*. *Veterinary Quarterly* 3, 118-123.
- Vercammen, F., De Deken, R., Maes, L., 1995. Clinical and serological observations on experimental infections with *Babesia canis* and its diagnosis using the IFAT. *Parasite* 2, 407-410.
- Wilkerson, M.J., Shuman, W., Swist, S., Harkin, K., Meinkoth, J., Kocan, A.A., 2001. Platelet size, platelet surface-associated IgG, and reticulated platelets in dogs with immune-mediated thrombocytopenia. *Veterinary Clinical Pathology* 30, 141-149.
- Wozniak, E.J., Barr, B.C., Thomford, J.W., Yamane, I., McDonough, S.P., Moore, P.F., Naydan, D., Robinson, T.W., Conrad, P.A., 1997. Clinical, anatomic, and immunopathologic characterization of *Babesia gibsoni* infection in the domestic dog (*Canis familiaris*). *Journal of Parasitology* 83, 692-699.

Yamane, I., Thomford, J.W., Gardner, I.A., Dubey, J.P., Levy, M., Conrad, P.A., 1993.

Evaluation of the indirect fluorescent antibody test for diagnosis of *Babesia gibsoni* infections in dogs. American Journal of Veterinary Research. 54, 1579-1584.

Zahler, M., Rinder, H., Schein, E., Gothe, R., 2000. Detection of a new pathogenic

Babesia microti-like species in dogs. Veterinary Parasitology 89, 241-248.