Molecular characterisation of *Babesia gibsoni* infection from a Pit-bull terrier pup recently imported into South Africa

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ABSTRACT

Canine babesiosis caused by *Babesia gibsoni* was diagnosed in a 3-month-old Pit-bull pup during a routine clinical examination. Diagnosis was confirmed by way of smear examination, PCR, Reverse Line Blot (RLB) and sequence analysis which showed 100% homology with *B. gibsoni* (Japan AB118032) and *Babesia* sp. (Oklahoma) (AF205636). Haematology showed moderate anaemia and severe thrombocytopenia. Treatment was initiated with diminazene aceturate (Berenil RTU®) followed by 2 doses of imidocarb diproprionate (Forray-65®) 3 days and 14 days later, respectively. *Babesia gibsoni* DNA was still detectable 2 weeks post-treatment on the PCR/RLB test. A 10-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. The implications of a possible introduction of *B. gibsoni* into South Africa are discussed.

Key words: atovaquone, azithromycin, *Babesia gibsoni,* controlled disease, Pit-bull terrier pup.

INTRODUCTION

Canine babesiosis is caused by large or small *Babesia* parasites. The large piroplasms form the *B. canis* group comprising 3 subspecies: *B. canis canis* endemic in southern Europe, *B. canis rossi* endemic in Sub-Saharan Africa and *B. canis vogeli* endemic in tropical and subtropical areas[26,31].

The small piroplasms, previously regarded as *Babesia gibsoni,* have been reported to be endemic to Asia, North America, North and East Africa and Europe[5,36]. There are at least 3 genetically distinct entities in this group: the Asian, North American (California) and Spanish isolates, respectively[18]. In the United States, most reported *B. gibsoni* infections are those of the Asian genotype[2,14,20,22]. The Spanish isolate was named *Theileria annae*[37], but is now regarded as *Babesia annae*[10] and the Californian isolate has been named *Babesia conradae*[19].

Clinical disease associated with *B. gibsoni* may range from being a mild to a severe infection[4,22]. The disease may follow a hyper-acute, acute or chronic course. The acute course is characterised by fever, lethargy, anaemia, thrombocytopenia, lymphadenopathy and splenomegaly[14,25]. The hyper-acute state is characterised by shock and extensive tissue damage[6,8]. The chronic form has been reported in Australia and the USA[13,18]. Although it is difficult to diagnose chronic *Babesia* infections, PCR can be a useful tool in confirming such infections[30].

Definitive diagnosis of *Babesia* infections in dogs depends on the demonstration of infected erythrocytes on Romanowsky-stained blood smears[31]. 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[32,33]. Also new technologies that include Polymerase Chain Reaction (PCR) and Reverse Line Blot (RLB) can be used to confirm *Babesia* infections[23,24].

Dogs with clinical babesiosis normally improve within 24 hours of treatment with an anti-babesial drug[15]. The drugs of choice in South Africa against *B. c. rossi* are diminazene aceturate and/or imidocarb 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. c. rossi* infections[27]. 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[3].

MATERIALS AND METHODS

Case history

A 3-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 a 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. 1) which also revealed thrombocytopenia and a marked reticulocytosis.
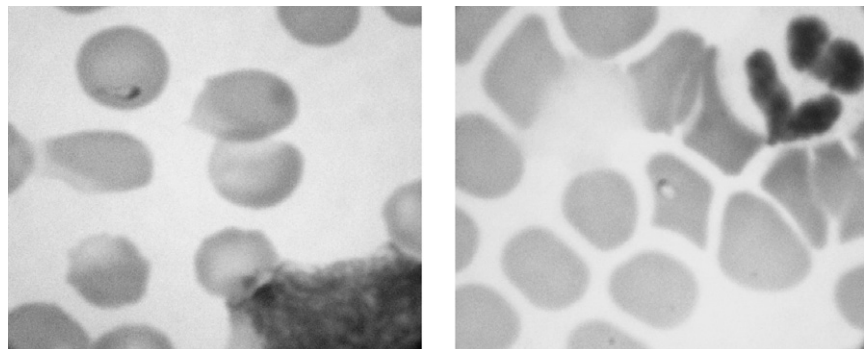
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Fig. 1: **Peripheral blood smears showing infected erythrocytes.**

The dog was treated by subcutaneous injection of diminazene aceturate (Berenil RTU®) at a dose of 3.5 mg/kg.

The dog was returned to the clinic 2 days later. 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 diproprionate (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 10 days and atovaquone (13.3 mg/kg) twice daily for 10 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.

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 2 weeks before treatment with azithromycin and atovaquone began. The 2 other samples were taken on Day 1 and Day 40 after completion of treatment with azithromycin and atovaquone.

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.

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 460–540 bp from the 18S rRNA gene spanning the V4 region[11,23]. 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 (20 s)–67 °C (30s)–72 °C (30 s), with lowering of annealing step after every 2nd cycle by 2 °C (touchdown PCR). The reaction was then followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s and extension at 72 °C for 30 s.

Reverse Line Blot hybridisation

PCR-amplified products were tested with the RLB, as previously described[23]. 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[24].

Sequencing

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

Phylogenetic analysis

Phylogenetic analysis was done to confirm the relationship between our positive *B. gibsoni* sample and other *Babesia* sp. (Fig. 2). Sequence data were assembled to a total length of 413 bp 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 2 parameter model of Kimura and the Jukes and the Cantor correction model for multiple base changes were used to construct similarity matrices[16,17]. Neighbour-joining[28] and the maximum parsimony methods were used for the construction of phylogenetic trees using Mega 3.0 software package[21]. The above methods were used in combination with the bootstrap method[7].

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. 1), PCR and RLB. Sequence analysis (413 bp) revealed that the sequence had a 100% homology with at least 2 full sequences (*c.* 1600 bp) of 2 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. 2). These procedures also showed that there were no significant changes in topology of trees or in bootstrap values using either the neighbour joining or the maximum parsimony methods. Haematology results showed that the dog had a low PCV, a reduced erythrocyte count and a thrombocytopenia (Table 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.

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 parasitising 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 5 occasions that the dog was examined. This was true for up to 1 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[4,29]. 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[3].

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[1,6]. Thrombocytopenia with variable leukocyte change is also a common feature of dogs infected with *B. gibsoni*[4,6,9,34,35].

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.

Babesia 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 2 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[6].

Bull terrier-type dog breeds have a higher incidence of sub-clinical *B. gibsoni* infection than other breeds[4,20]. American Pit-bull terriers are reported to be the most commonly *B. gibsoni*-infected breed in the USA, but the reasons for this remain unclear[4,20,22]. 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[18,35]. Both *H. leachi* and *R. sanguineus* are endemic to South Africa [12].

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. The dog reported in this case report was lost to further clinical follow up, as the dog had
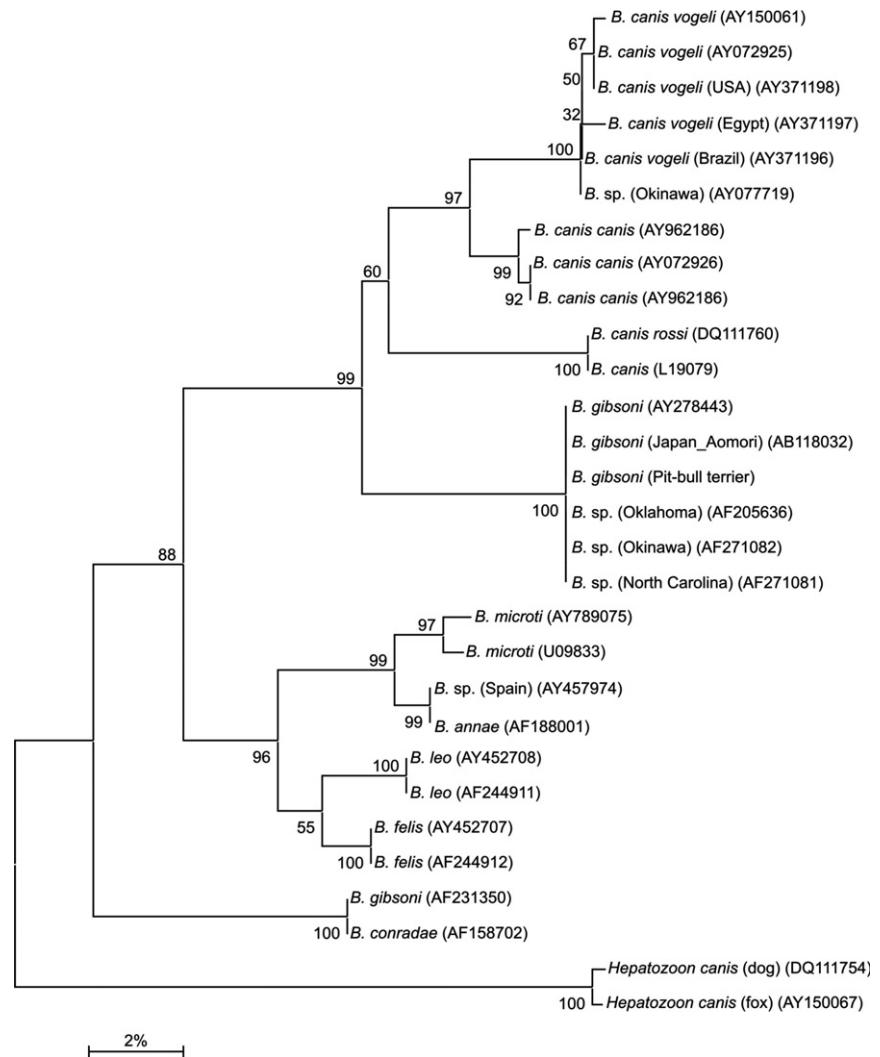

Fig. 2: **Neighbour-joining tree, based on the Kimura 2-parameter distance calculation, showing the phylogenetic relationship of *B. gibsoni* (Pit-bull terrier) to other *Babesia* sp. Relationships are presented as an unrooted tree with branch lengths being proportional to the estimated genetic distance between the strains. The scale bar represents the percentage nucleotide difference. *Hepatozoon canis* was used as an outgroup.**

Table 1: **Haematological report indicating full blood counts 2 weeks prior to treatment of the dog with a combination of atovaquone and azithromycin.**

Parameter	Pit-bull terrier	Normal range
Red blood cell count	4.2	5.5–8.5
Haemoglobin	9.55	12–18
Haematocrit (PCV)	29.00	37–55
Mean cell volume (MCV)	69.00	60–77
Mean cell haem cons (MCHC)	33.00	32–36
White cell count	7.32	6–15
Platelet counts	83.00	200–500
Differential counts		
Segmented neutrophils (%)	64.00	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	3–11.5
Band neutrophils (abs)	0.15	0–0.03
Lymphocytes (abs)	1.24	1–4.8
Monocytes (abs)	1.10	0.1–1.4
Eosinophils (abs)	0.07	0–1.2
Basophils (abs)	0.07	0–0.02

allegedly died of unknown causes.

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.

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