Isolation and characterization of a *Babesia* species from *Rhipicephalus evertsi evertsi* ticks picked off a sable antelope (*Hippotragus niger*) which died of acute babesiosis

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ABSTRACT

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Transmission of a *Babesia* species to susceptible cattle by *Rhipicephalus evertsi evertsi* ticks picked off a sable which died of acute babesiosis is described. The parasite was initially isolated by feeding *R. e. evertsi* ticks on a susceptible, splenectomised bovine which developed parasitaemia. Blood stabilate from the parasitaemic bovine produced a fatal babesiosis in a spleen- intact bovine. Clinical signs shown by the affected animals corresponded with those of acute babesiosis. Parasitological examination, the immunofluorescence antibody test and the polymerase chain reaction test revealed that the parasite transmitted by the ticks initially and the blood stabilates prepared from affected animals was *Babesia bigemina*. This parasite was morphologically identical to that observed in Giemsa-stained blood smears prepared from the dead sable.

Keywords: *Babesia*, *Hippotragus niger*, protozoa, *Rhipicephalus evertsi evertsi*, sable antelope, ticks

INTRODUCTION

Babesiosis is a tick-borne disease that affects a wide range of domestic animals including cattle, sheep, goats, horses, swine, dogs and cats, as well as nearly 70 species of wild animals and man. In Zimbabwe, the *Babesia* species of economic importance are those affecting cattle, namely *Babesia bigemina* Smith & Kilbourne, 1893 and *B. bovis* Babes, 1888. A serological survey in 1983 indicated that *B. bigemina* occurred throughout the country together with its major vector *Boophilus decoloratus* Koch, 1844, but *B. bovis*, with its vector *Boophilus microplus* Canestini, 1887, was only limited to the eastern part of the country (Norval, Fivaz, Lawrence & Daillecourt 1983). However, a recent tick survey has shown that *B. microplus* exists in other parts of the country and cases of *B. bovis* have also been diagnosed in these areas (Katsande, Mazhowu, Turton & Munodzana 1996). Though babesiosis is mainly transmitted transovarially through the one host ticks it can also be transmitted transstadially by two host and three host ticks (Levine 1973). In southern Africa, *Rhipicephalus evertsi evertsi* Neumann, 1987 and *R. appendiculatus* Neumann, 1901 have also been incriminated as vectors of *B. bigemina* (De Bruijn 1984; Soulsby 1986).

Wild bovidae such as giraffe (*Giraffa camelopardalis*), sable antelope (*Hippotragus niger*) and waterbuck (*Kobus ellipsiprymnus*) also harbour *Babesia* species (Wallach & Boever 1983). Most of these animals carry the parasites as asymptomatic infections with clinical babesiosis appearing when the animals are stressed. The clinical signs observed in the latter are similar

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to those seen in domestic animals and include anaemia, haemoglobinuria, pale to icteric mucous membranes, depression and, in some cases, death. The *Babesia* in wild hosts are also transmitted primarily by the ixodid ticks. *Boophilus decoloratus* has been collected from wild ungulates [kudu (*Tragelaphus strepsiceros*), sable antelope, impala (*Aepyceros melampus*) and giraffe] on commercial ranches on the highveld of Zimbabwe whereas *B. microplus* has only been collected from domestic hosts (Mason and Norval 1980). *Rhipicephalus e. evertsi*, a two host tick, was also found on giraffe, eland (*Taurotragus oryx*), kudu, sable, waterbuck, tsessebe antelope (*Damaliscus lunatus lunatus*), wildebeeste (*Connochaetes taurinus*), impala and zebra (*Equus burchelli*) on small game parks on the Zimbabwe highveld (Norval 1981).

In this paper a babesia parasite isolated from immature *R. e. evertsi* picked off a sable which died of acute babesiosis is described. The sable was heavily infested with *R. e. evertsi* adult and immature ticks. This tick was the only species present on the animal. Incision of the bladder revealed the presence of haemoglobinuria and the spleen was enlarged. A Giemsa stained blood smear prepared from the dead sable revealed intraerythrocytic piroplasms. Engorged nymphs collected from the sable were allowed to moult to adults in the laboratory. The paper also describes the isolation of the parasite from the ticks in cattle as well as its parasitological, immunological and molecular characteristics and the clinical disease it produced.

MATERIALS AND METHODS

Animals

Calves used in the experiment originated from farms that practised strict tick control. They were kept in tickproof pens at the Veterinary Research Laboratory, Harare, during the experiment. Animals 321, 090 and 4859 were Friesian crosses and animal 4870 was a Tuli. Animals 321 and 4870 were splenectomised whereas 090 and 4859 were spleen-intact. Before the experiment, they were negative for haemoparasites in peripheral blood smears and for *B. bigemina* and *B. bovis* on the indirect fluorescent antibody test (IFAT). The four animals were between 6 and 9 months of age.

Ticks

Engorged nymphs of *R. e. evertsi* were collected from the external meatus of the ear of the dead sable. They were kept in a cooling incubator at 26°C in tubes plugged with cotton wool and stored in glass jars over saturated potassium chloride solution to maintain a relative humidity of 80% until they moulted to adults.

Isolation of the parasite

Adult R *e. evertsi* ticks (moulted from nymphs collected from sable) were allowed to feed on the ears of a susceptible calf, animal 321. On day 13 post infection (PI), jugular blood was collected from the animal into ethylenediamine tetracetic acid (EDTA) sodium, an anticoagulant, at a concentration of 1,5 mg EDTA per mℓ of blood. The blood was cryopreserved with 10% dimethylsulphoxide (DMSO) by the addition of an equal volume of 20% DMSO to the blood drop wise. The blood stabilate was kept at −20°C overnight and transferred to liquid nitrogen the following day until use.

Ten mℓ of stabilate from animal 321 were administered intravenously into animal 090. On day 18 PI a stabilate was prepared from jugular blood and cryopreserved as described above.

Animals 4859 and 4870 received intravenous inoculations of 19 mℓ and 20 mℓ of stabilate from animal 090, respectively.

Monitoring and assessment of infection

Clinical

Rectal temperatures and the general condition of the animals were monitored daily. A temperature of ≥ 39,5°C was considered a fever.

Haematological

Haemoglobin (Hb) and packed cell volume (PCV) levels were assessed every 3 d (Anon 1984).

Parasitological

Peripheral blood smears were prepared daily, fixed in absolute methanol and stained with 10% Giemsa for 30 min for identification of haemoparasites. Parasitaemia was expressed as a percentage of infected red blood cells to the total number of these cells counted.

Morphometric analysis

Parasite dimensions were measured on Giemsa-stained peripheral blood smears using a light microscope with a calibrated ocular micrometer.

Immunological

Sera harvested from jugular blood on days −3, 5, 7, 9 and 12 PI was examined for antibody titres to *Babesia bigemina* and *B. bovis* by the IFAT as described by De Vos & Jorgensen (1991). Antigen slides were prepared by coating glass slides with a cell monolayer of infected erythrocytes. After drying, they were protected with masking tape and stored at −70°C. Before the test, slides were removed from the deep

freeze, placed in an incubator briefly and the tape removed to expose the antigen film. Test sera was diluted in phosphate buffered saline (PBS). Sera was screened at dilutions of 1 in 30. Negative and positive controls were included on each slide. The slides were marked with a diamond marker. Eight microlitres of diluted test sera was added to the antigen by means of filter paper discs. The slides were then incubated in a humid chamber at 37 °C for 30 min. After incubation, the filter paper discs were washed off. The slides were further washed in PBS for 10 min and distilled water for 5 min. Excess water was removed by shaking and wiping the edges of the slides with filter paper. Test sera was covered with conjugate and incubated at 37 °C for 30 min. The conjugate was flushed off by immersion in PBS for 10 min and distilled water for 5 min. After removing excess moisture, the slides were mounted using a drop of 50 % glycerol in PBS and examined using a fluorescent microscope (Leitz).

Molecular characterization

The polymerase chain reaction test (PCR) was used to detect species—specific parasite DNA in whole blood samples collected on days 8 and 14 PI. Whole blood was collected into sterile vacutainer tubes containing EDTA. Primers used were derived from pure B. bigemina (Figueroa, Chieves, Johnson & Buening 1992) and B. bovis (Suarez, Palmer, Jasmer, Hines, Perryman & McElwain 1991).The nucleotide sequence of the primers is supplied in Table 1.

DNA was purified from blood by saponin lysis. Two hundred and fifty microlitres of blood was added to 1 mℓ of saponin lysis buffer [0,22 % NaCl (w/v), 0,015 % saponin (w/v), 1 mM EDTA], centrifuged for 10 min at 12 000 rpm and the pellet washed twice with the saponin lysis buffer. The pellet was resuspended in 100 µℓ buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8). Ten micrograms proteinase K was added and the samples were incubated for 1 h at 56 °C followed by a 10 min incubation at 95 °C. Five microlitres of the DNA preparation was used in one PCR reaction.

The PCR was adapted from Figueroa, Chieves, Johnson & Buening (1993). Reaction conditions were changed in order to amplify Zimbabwean strains of Babesia. PCRs were perfomed using thermostable DNA polymerase of Thermus aquaticus (Taq). Five microlitres of purified DNA was added to 95 µℓ of PCR mix containing 0,5 µM sense and antisense primer, 1 x PCR buffer [10 mM Tris-HCl (pH 9,0) at 25 °C; 50 mM KCl; 0,1 % Triton X-100 (Promega)], 1,5–2,0 mM $MgCl_2$, 0,2 µM dNTP and 2,5 units of Taq DNA polymerase (Promega). One and a half mM $MgCl_2$ was used for amplification of B. bigemina DNA and 2,0 mM $MgCl_2$ was used for amplification of B. bovis DNA. The reaction mix was overlaid with 60 µℓ of paraffin oil. Amplification was carried out in an automated thermal cycler (Perkin Elmer) for 7 min at 90 °C, followed by 35 cycles of 1 min at 94 °C (denaturation), 1 min at 55 °C (annealing) and 1,5 min at 72 °C (primer extension). A nested PCR was perfomed for additional amplification of 289 bp of B. bovis, if the first PCR did not result in a visible product. Five micoliters of the first amplification solution was added to 95 µℓ of the nested PCR solution, containing the same chemical concentrations as the first PCR reaction mix. The $MgCl_2$ concentration used was 1,5 mM. The nested PCR was perfomed in 20 cycles in an automated thermocycler at the same temperature profile that was used in the first PCR.

Positive controls used for specificity and sensitivity were B. bovis DNA (Rusape, Zimbabwe) and B. bigemina DNA (Raymondale, Zimbabwe) prepared from whole blood of 1 % parasitaemia for B. bovis and 15 % parasitaemia for B. bigemina. The sensitivity of the nested PCR was also determined by using blood purified parasite DNA diluted with distilled water.

The PCR products were analysed using gel electrophoresis on a 1,5 % agarose-ethidium bromide gel on 0,5 x TAE buffer (1 x TAE buffer, 0,04 M Tris-acetate and 1,0 mM EDTA). Length of PCR products was determined by comparison with lambda DNA digested with PstI.

TABLE 1 Nucleotide sequence of primers used in PCR

Target specificity	Sequence	Product length	Reference
B. bigemina	First PCR		
	5'CATCTAATTTCTCTCCATACCCCTCC 5'CCTCGGCTTCAACTCTGATGCCAAAG	278 bp	Figueroa et al. 1992
B. bovis	First PCR		
	5'ACGAGGAAGGAACTACCGATGTTGA 5'CCAAGGAGCTTCAACGTACGAGGTCA	350 bp	Suarez et al. 1991
	Nested PCR		
	5'TCAACAAGGTACTCTATATGGCTACC 5'CTACCGAGCAGAACCTTCTTCACCAT	289 bp	Suarez et al. 1991

Precautions to avoid carry over of amplification products included separation of pre and post PCR procedures. In each run at least one negative control of distilled water was included.

Treatment

An animal which met one or more of the following criteria was treated with diminazene aceturate at a dose rate of 3,5 mg/kg intramuscularly:

- PCV ≤ 15 %
- PCV ≥ 20 % and parasitaemia ≥ 3 %
- parasitaemia > 5 %

RESULTS

The results are summarized in Table 2.

Tick transmission of parasite

Of the total number of ticks fed on animal 321, 50 engorged and dropped off. Animal 321 presented a fever (temperature of 39,5 °C) on days 8 and 11 post tick infestation only. The calf had haemoglobinuria, was anaemic and had a piroplasm parasitaemia of 10 % on day 13 post tick infestation. A blood stabilate was prepared from it on the same day. After preparation of the stabilate the calf was treated with diminazene aceturate. The following day, no parasites were detected in peripheral blood smears.

Blood transmission of parasite

On day 6 PI, animal 090 had a piroplasm parasitaemia of 0,2 % which rose to 5 % on day 8 PI. This initial parasitaemia was accompanied by a fever. No parasites were detectable in peripheral blood on days 11–14 PI and the animal temperature during that period was also normal. On day 15 PI, piroplasms re-appeared in peripheral blood. The following day the animal had a fever, haemoglobinuria, respiratory distress, looked pale and was recumbent. On day 18 PI, animal 090 died with a parasitaemia of 10 %. A blood stabilate was prepared from it before its death.

Characterization of the parasite

Animal 4859 only showed a mild reaction to the stabilate prepared from animal 090. It had no fever throughout the experiment. Hb (minimum: 7,1 gm/dℓ) and PCV (minimum: 18 %) dropped slightly (cf. Table 2). Piroplasm parasitaemia appeared on day 6 PI to day 12, but only at a low level (range: 0,1–0,2 %). After infection, the animal tested negative to both *B. bigemina* and *B. bovis* on the IFAT. However, PCR detected *B. bigemina* on days 8 and 14 PI but not *B. bovis* (cf. Fig. 1, 2 and 3). The first PCR for *B. bovis* could not detect the *B. bovis* positive control but the nested PCR did. Polymerase chain reaction test detected *B. bigemina* on day 14 PI when no parasites were detectable on light microscopy. The calf recovered without treatment.

Animal 4870 developed a severe reaction to the stabilate, with a fever as from day 6 PI. Haematological parameters decreased quite markedly, the lowest being on day 11 PI when the Hb and PCV levels dropped to 4,5 gm/dℓ and 11 %, respectively. Parasitaemia appeared as from day 6 PI, reaching a peak of 15 % on day 8 PI. The animal was positive for *B. bigemina* on the IFAT and PCR but was negative for *B. bovis* in both tests (cf. Fig. 1, 2 and 3). This calf also showed clinical signs of acute babesiosis and was treated with diminazene aceturate.

Parasite description

Morphometric analysis of the parasite in Giemsa stained peripheral blood smears revealed an intra-erythrocytic organism with some polymorphism. The

TABLE 2 Summary of the clinical responses to tick challenge and stabilate challenge

Animal no.	Condition	Challenge	Fever	Days to parasitaemia	Peak parasitaemia (%)	Lowest PCV (%)	Lowest Hb (gm/dℓ)	Clinical reaction
321	No spleen	Tick transmission	+	9	10	ND	ND	Severe (treated, recovered)
090	Spleen intact	10 mℓ stabilate (321)	+	6	10	ND	ND	Severe (died)
4859	Spleen intact	19 mℓ stabilate (090)	−	6	0,2	18	7,1	Mild (recovered)
4870	No spleen	20 mℓ stabilate (090)	+	6	15	11	4,5	Severe (treated)

ND = Test not done
− = No fever developed
+ = Developed temperature of 39,5 °C or more

majority of the parasites were in the form of large paired pyriforme bodies (piroplasms) located at an acute angle to one another within the centre of the erythrocyte. These pyriforme bodies were longer than the radius of the red blood cells and most of their distal ends were pointed. The parasite measured 2,0–4,8 µm in length and 0,6–1,8 µm in width ($\bar{x}$: 3,4 x 1,4 µm).

DISCUSSION

Rhipicephalus e. evertsi has been incriminated as a vector for *B. bigemina* (Theiler 1950; Hoogstraal 1956; Neitz 1956). In a tick survey done by Norval (1981), the tick was found to occur in the absence of domestic animals in most of the small game parks on the high veld of Zimbabwe. It was abundant on commercial farms and ranches with large populations of undipped alternate hosts (domestic equines and/or wild ungulates). The majority of wild hosts for this tick are ungulates of which the giraffe, eland, kudu, sable, waterbuck, tsessebe, wildebeeste, impala and zebra were the most frequently infested. To a large extent, the distribution of *R. e. evertsi* overlaps with

that of *Boophilus decoloratus* (Mason & Norval 1980; Norval 1981). In Zimbabwe, no relationship has been established between the occurrence of *R. e. evertsi* and that of tick-borne diseases of cattle. *Rhipicephalus e. evertsi* is however known to be the major tick vector for *B. equi* in horses (Theiler 1950; Hoogstraal 1956; Neitz 1956).

There are over 40 species of wild bovidae in Africa and most of these harbour *Babesia* and/or *Theileria* species. These parasites have not been well characterized in most of the wild animals except for those in buffalo, eland and deer (Fowler 1986). *Babesia bigemina* has been reported to occur in cattle, water buffalo (*Bubalis bubalis*), African buffalo (*Syncerus caffer*) and certain deer in Africa (Nyindo 1992). Clinically recovered cattle are known to be asymptomatic carriers of *B. bigemina*, but the role of wildlife in the epidemiology of this parasite is still unknown. Blood smears of 124 sable antelope from South Africa and Zimbabwe revealed that seven were positive for a *Babesia* sp (*B. irvinesmithi*), 70 were positive for theilerial piroplasms, one had schizonts of *Cytauxzoon* sp. and one was positive for *Anaplasma* sp (Thomas, Wilson & Mason 1982). Attempts to
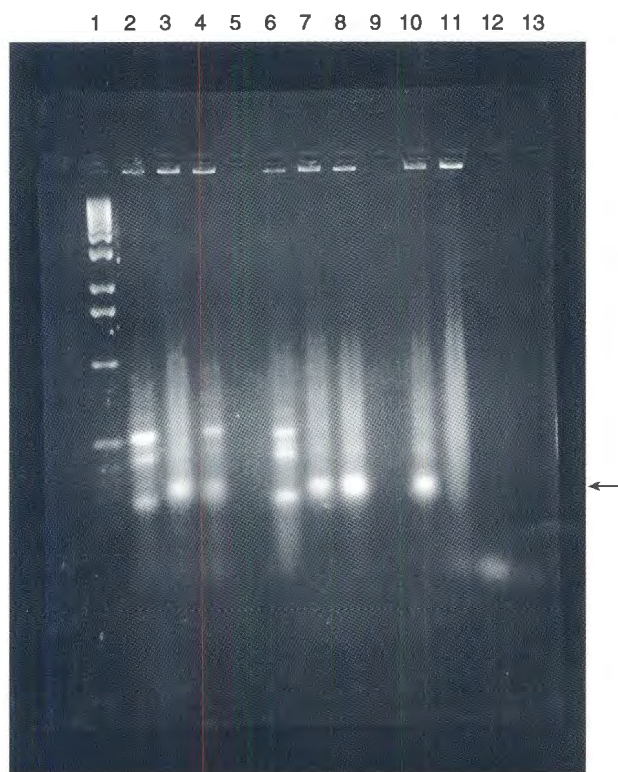

FIG. 1   *B. bigemina* PCR assay

Lanes: 1, 1 kB ladder GIBCO BRL; 2 day 0 (4859); 3 day 8 (4859); 4, day 14 (4859); 5, blank; 6, day 0 (4870); 7, day 8 (4870); 8, day 14 (4870); 9, blank; 10, *B. bigemina* (Raymondale, Zimbabwe); 11, bovine DNA 1 µg; 12, blank; 13, blank
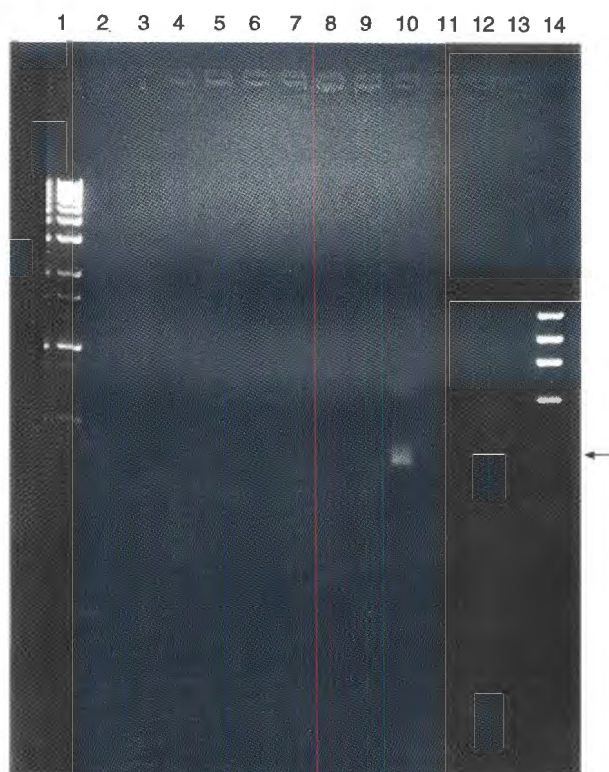

FIG. 2   *B. bovis* first PCR assay

Lanes: 1, 1 kB ladder GIBCO BRL; 2 day 0 (4859); 3 day 8 (4859); 4, day 14 (4859); 5, blank; 6, day 0 (4870); 7, day 8 (4870); 8, day 14 (4870); 9, blank; 10, *B. bovis* (Rusape, Zimbabwe); 11, bovine DNA 1 µg; 12, blank; 13, blank; 14, øx 174 RF DNA Hae III Dig

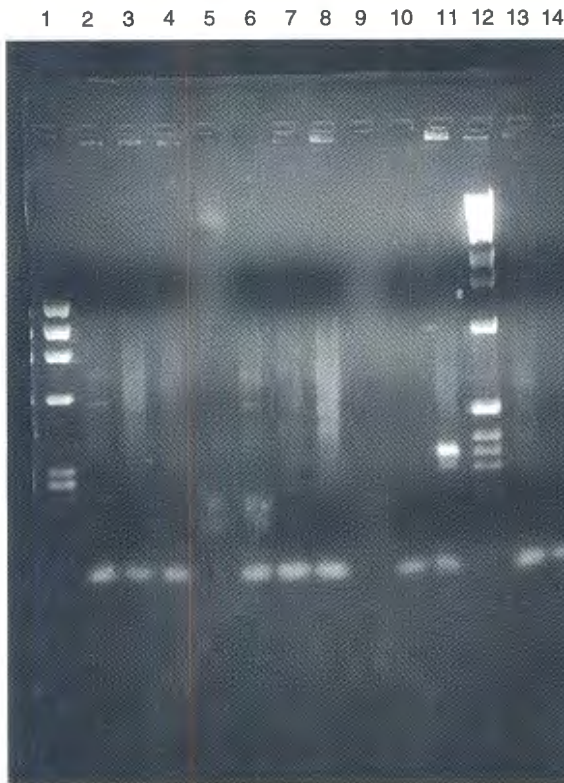1 2 3 4 5 6 7 8 9 10 11 12 13 14

FIG. 3 *B. bovis* nested PCR assay

Lanes: 1, Nx 174 RF DNA Hae III Dig; 2 day 0 (4859); 3 day 8 (4859); 4, day 14 (4859); 5, blank; 6, day 0 (4870); 7, day 8 (4870); 8, day 14 (4870); 9, blank; 10, bovine DNA 1Fg; 11, *B. bovis*; 12, 1 kB ladder GIBCO BRL blank; 13, blank; 14, blank

transmit the *Babesia irvinesmithi* from sable to splenectomized sable and cattle and unsplenectomized cattle by subinoculating infected blood intravenously were unsuccessful. In the same experiment, attempts to transmit the *Babesia* sp. with the larval progeny of *B. decoloratus* from sable to a splenectomized bovine were also unsuccessful. However, in the present experiment, the adult progeny of *R. e. evertsi* that developed from the nymphal stage collected from the sable were able to transmit *Babesia* to a susceptible, splenectomized bovine.

Clinical signs and haematological changes observed in the animals were consistent with babesiosis. Morphology has been a useful criterion in the speciation of *Babesia* species. The *Babesia* observed in this study was a large babesia (2–4,8 µm in length), morphologically identical to that described for *B. bigemina* (Soulsby 1986). Confirmation that the parasite was *B. bigemina* was obtained by positive IFAT and PCR.

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