Babesia bovis and *B. bigemina* DNA detected in cattle and ticks from Zimbabwe by polymerase chain reaction

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ABSTRACT

From blood collected from 94 cattle at 12 locations in the eastern and northeastern areas of Zimbabwe, DNA was extracted and analysed by polymerase chain reaction with primers previously reported to be specific for *Babesia bigemina* and *Babesia bovis.* Overall, DNA of *Babesia bigemina* was detected in the blood of 33/94 (35 %) cattle and DNA from *B. bovis* was detected in 27/58 (47 %) of cattle. The prevalence of DNA of *B. bigemina* was significantly higher in young animals (<2 years) (23/46) than in animals over 2 years of age (10/48; χ^2= 8.77; P <0.01 %). Although tick sampling was not thorough, *Boophilus decoloratus* could be collected at 7/9 sites sampled and *Boophilus microplus* at 4/9 sites. Of the 20 *B. decoloratus* allowed to oviposit before PCR analysis, 1 (5 %) contained DNA that could be amplified with primers for *B. bigemina* while 12 (60 %) were positive with primers for *B. bovis.* Of the *B. microplus* allowed to oviposit, 11/16 (69 %) were positive for *B. bovis* DNA by PCR and 2/16 (12 %) were positive for *B. bigemina.*

Key words: *Babesia bigemina, Babesia bovis, Boophilus decoloratus, Boophilus microplus,* cattle, PCR, ticks, Zimbabwe.

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INTRODUCTION

Babesia bigemina is the aetiological agent of African redwater, which is an important cause of morbidity and mortality in cattle in Zimbabwe[17]. The organism is known to have been present in the country since the 1890s, when it caused considerable mortality in imported cattle[11]. In the early 1980s, serological surveys showed that *B. bigemina* infections were very common in Zimbabwe[16,18], and the principal vector of the organism in southern Africa, *Boophilus decoloratus*[3] was found to be widely distributed in the country[16,18].

Babesia bovis is the agent of Asiatic redwater, which is endemic along the east coast of Africa[3]. Its vector is *Boophilus microplus,* which was introduced into Zimbabwe from Mozambique in the mid-1970s, and subsequently this tick and Asiatic redwater spread from the eastern border areas into the northeastern and central areas of Zimbabwe[19]. Following successive years of drought between 1981 and 1984, *Boophilus microplus* was reported to have disappeared from Zimbabwe owing to the unfavourable climatic conditions and interspecific competition with *B. decoloratus*[19]. Recent reports have indicated, however, that *B. microplus* may be found in the eastern, northeastern and northwestern areas of Zimbabwe, and clinical cases of Asiatic redwater have been described[10]. Furthermore, antibodies reactive with *B. bovis* have been found in cattle from the northeast of the country[15].

Although clinical cases of redwater are common in Zimbabwe, distinguishing between *B. bovis* and *B. bigemina* infections is often not easy[3]. It is generally impossible to reliably distinguish infections based on history, clinical signs and macroscopic findings. Microscopic examination of the parasites in stained blood smears can enable the differentiation of the aetiological agents, as *B. bigemina* are generally 'large' intra-erythrocytic organisms, while *Babesia bovis* are 'small'. There is considerable variation in the size of the organisms, however, and it is then often difficult to accurately differentiate the organisms microscopically[3]. Although serological tests have been described that detect antibodies reactive with *Babesia* spp., they do not consistently detect infections in carrier animals, and their specificity is limited by the cross-reactivity of *Babesia* spp. antigens[1,5]. Recently, polymerase chain reaction (PCR)-based detection methods have been described that have been shown to be extremely sensitive and specific in the detection of *B. bigemina* and *B. bovis* organisms[5,6].

To provide further data on the prevalence of *B. bovis* and *B. bigemina* infections in Zimbabwe, we tested blood from cattle by PCR for DNA of the organisms. In addition, *Boophilus* spp. were collected from cattle and analysed by PCR for the presence of *B. bovis* and *B. bigemina.*

MATERIALS AND METHODS

Tick and blood samples

Whole blood was collected in EDTA from the jugular veins of 94 cattle at 12 locations in the eastern and northeastern areas of Zimbabwe (Fig. 1). The samples were stored on ice until they were returned to the laboratory (<2 days) and placed at –20 °C. Blood samples (stored at –20 °C) from cattle with known *B. bigemina* and *B. bovis* infections (supplied by the Department of Infectious Diseases and Immunology of the Faculty of Veterinary Science, University of Utrecht, and J Turton of the Veterinary Research Laboratories, Harare) were used as controls and, following serial dilutions in phosphate-buffered saline (PBS; pH 7.2), tested to determine the sensitivity of the PCR analyses.

Engorged female *Boophilus* ticks found on cattle at some of the survey sites were removed and placed in 70 % ethyl alcohol or incubated over a saturated sodium chloride solution at 28 °C for at least 3 weeks. Each tick was macerated in 0.3 ml sterile PBS using a sterile pestle and mortar and the tick suspensions stored at –20 °C until DNA was extracted from the ticks for PCR analyses (see below).

DNA extraction

After thawing at room temperature, 250 µl of the blood samples were added to

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Table 1. **Primers used to amplify the DNA of *Babesia bovis* and *B. bigemina.***

Target organism	Nucleotide sequence	Product length	Reference
B. bigemina	5'-CATCTAATTTCTCTCCATACCCCTCC-3' 5'-CCTCGGCTTCAACTCTGATGCCAAAG-3'	278 bp	5
B. bovis	5'-CACGAGGAAGGAACTACCGATGTTGA-3' 5'-CCAAGGAGCTTCAACGTACGAGGTCA-3'	350 bp	6
Nested	5'-TCAACAAGGTACTCTATATGGCTACC-3' 5'-CTACCGAGCAGAACCTTCTTCACCAT-3'	289 bp	6

1 ml of saponin lysis buffer (0.015 % saponin, 35 mM NaCl and 1 mM EDTA) and centrifuged (12 000 × g for 10 min). After 2 washes in saponin lysis buffer to remove the haemoglobin, the pellet was resuspended in 100 μl Tris buffer (10 mM Tris-HCl, 50 mM KCl, pH 8.0) and digested with Proteinase K (10 μg) (Sigma) for 1 hr at 56 °C followed by 10 min incubation at 95 °C. Phenol/ chloroform extraction of tick DNA was carried out as described previously[8] except that ticks were boiled in PBS instead of water.

PCR

Extracted DNA (5 μl), or PBS for negative controls, was added to 95 μl of reaction buffer (10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1 % Triton X-100 (Promega Corporation), 1.5–2.0 mM $MgCl_2$, 0.2 μM of each dNTP, 2.5 U *Taq* polymerase (Promega Corporation) and 0.5 μM of the relevant primers (Table 1). The primers for *B. bigemina*[5] and *B. bovis*[6] have been described previously. The reaction mixture was overlaid with paraffin oil and placed in a Perkin-Elmer thermocycler for DNA amplification. After denaturation at 90 °C for 7 min the reaction mixtures were subjected to 35 cycles of denaturation (1 min at 94 °C), annealing (1 min at 55 °C) and primer extension (1.5 min at 72 °C). To detect *B. bovis* DNA, a nested PCR was performed on 5 μl of the amplified products and 95 μl of the reaction mixture described above, except the MgCl was used at 1.5 mM. The nested PCR consisted of 20 amplification cycles as described above. The PCR products obtained were visualised by UV transillumination after electrophoresis of 30 μl aliquots of the reaction mixtures through 1.5 % agarose-ethidium bromide gels. The sizes of the PCR products were determined by comparison with lambda DNA digested with *Pst*I.

RESULTS

By PCR, 278 base pair (bp) DNA fragments of *B. bigemina* could be amplified from the blood of cattle known to be infected with strains of the organism from Nigeria (parasitaemia 15 %) and Zimbabwe (parasitaemias of 15 % and 0.001 %). Following serial dilutions of these bloods, parasitaemias of 0.001 % could be reliably detected in all 3 samples. In serial dilutions of a sample from Zimbabwe (parasitaemia 0.001 %) a parasitaemia of 0.000001 % could be detected. *Babesia bovis* DNA fragments of 289 bp could be amplified by nested PCR in blood from cattle infected with organisms from Australia (parasitaemia 15 %) and from Zimbabwe (parasitaemia 5 %). With serial dilutions of these bloods, parasitaemias of 0.001 % could be detected in all samples. The primers for *B. bigemina* failed to amplify DNA of *B. bovis* and *vice versa.* Similarly, previous experiments in our laboratory have shown that the primers also do not react with DNA of *Anaplasma* spp., *Theileria* spp. or *Boophilus* spp. not infected with *B. bigemina* or *B. bovis.*

The blood samples collected emanated from 3 commercial farms (Table 2) and from communal land cattle at dip tanks at 9 locations. The cattle sampled at the dip tanks were local Mashona breed cattle (70), while those sampled in Mount Hampden were Limousins (12) and those in Mount Selinda (5) and Chipinge (4) were Friesians. When PCR analyses were carried out on blood samples from these animals, 33/94 (35 %) were found to contain DNA that could be amplified using primers for *B. bigemina.* Positive animals were detected in 11/12 (92 %) of the sites sampled. Nested PCRs with primers specific for *B. bovis* were performed on the DNA from 58 blood samples and 27/58 (47 %) were found to be positive. Prevalences varied from 25 % to 100 % at the 8/12 (67 %) sites where positive animals were found.

Of the 58 blood samples tested by PCR with primers for DNA of *B. bigemina* and with primers for *B. bovis,* samples positive for both organisms (15/58; 26 %) were obtained from cattle in Chikwakwa (1/5; 20 %), Chipinge (2/4; 50 %), Chirau (1/5; 20 %), Chiweshe (3/3; 100 %), Hwedza (5/9; 56 %), Rusape (2/10, 20 %) and Shamva (1/2; 50 %). Cattle that were negative for one or both organisms were from Chinamora (1), Chisumbanje (4), Mount Hampden (5), Mount Selinda (4) and Manzvire (6). Most of the cattle found to contain DNA of both *B. bigemina* and *B. bovis* were under 2 years of age (13/15; 87 %).

Although different age groups of cattle were not necessarily tested from all sites, the overall age/prevalence data for cattle tested by PCR for DNA of *B. bigemina* showed that there were significantly more positive young animals (under 2 years of age) (23/46) than animals over 2 years (10/48; $\chi^2 = 8.77$; $P < 0.01$ %). For cattle positive by PCR for DNA of *B. bovis,* there were no significant differences in the prevalences of positive young animals (19/36) and those greater than 2 years of age (8/22; $\chi^2 = 1.48$; $P > 0.10$ %).

While thorough tick collections were not carried out, *B. decoloratus* was collected at 7/9 sites sampled and *B. microplus* at 4/9 sites (Table 3). None of the 18 ticks that were preserved in alcohol had DNA

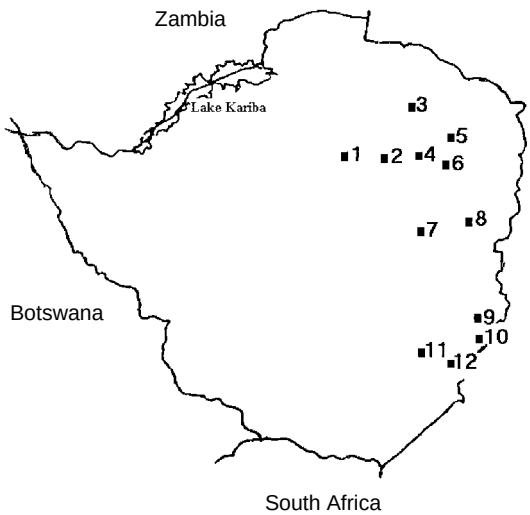

Fig. 1. **Map of Zimbabwe showing sites where blood and ticks were collected from cattle.** 1: **Chirau;** 2: **Mount Hampden;** 3: **Chiweshe;** 4: **Chinamora;** 5: **Shamva;** 6: **Chikwaka;** 7: **Hwedza;** 8: **Rusape;** 9: **Chipinge;** 10: **Mount Selinda;** 11: **Manzvire;** 12: **Chisumbanje.**

Table 2. **Prevalences of DNA of *Babesia bigemina* and *B. bovis* in whole blood from cattle in Zimbabwe.**

Site	Number (%) of blood samples positive for	
	B. bigemina	***B. bovis***
Chikwakwa	2/7 (29 %)	4/5(80 %)
Chipinge*	2/4 (50 %)	3/4 (75 %)
Chinamora	1/9 (11 %)	0/1 (0 %)
Chirau	1/5 (20 %)	3/5 (60 %)
Chisumbanje	3/4 (75 %)	1/4 (25 %)
Chiweshe	4/7 (57 %)	3/3 (100 %)
Hwedza	6/16 (38 %)	7/9 (78 %)
Manzvire	5/6 (83 %)	0/6 (0 %)
Mt Hampden*	0/12 (0 %)	0/5 (0 %)
Mt Selinda*	5/5 (100 %)	0/4 (0 %)
Shamva	1/7 (14 %)	2/2 (100 %)
Rusape	2/12 (17 %)	4/10 (40 %)
Totals	33/94 (35 %)	27/58 (47 %)

*Commercial farms.

Table 3. **Numbers of ticks collected into alcohol/collected and allowed to oviposit and numbers PCR positive for DNA of *Babesia bigemina*** (in brackets) **or *B. bovis*** (in square brackets)**.**

Site	***Boophilus decoloratus***	***Boophilus microplus***
Chikwakwa	0 (0) [0] / 9 (1) [5]	0 (0) [0] / 0 (0) [0]
Chipinge*	0 (0) [0] / 0 (0) [0]	0 (0) [0] / 0 (0) [0]
Chinamora	ND	ND
Chirau	0 (0) [0] / 8 (0) [5]	0 (0) [0] / 0 (0) [0]
Chisumbanje	0 (0) [0] / 2 (0) [1]	0 (0) [0] / 7 (1) [6]
Chiweshe	ND	ND
Hwedza	6 (0) [0] / 0 (0) [0]	0 (0) [0] / 0 (0) [0]
Manzvire	0 (0) [0] / 1 (0) [1]	0 (0) [0] / 9 (1) [5]
Mt Hampden*	ND	ND
Mt Selinda*	0 (0) [0] / 0 (0) [0]	0 (0) [0] / 0 (0) [0]
Shamva	2 (0) [0] / 0 (0) [0]	4 (0) [0] / 0 (0) [0]
Rusape	4 (0) [0] / 0 (0) [0]	2 (0) [0] / 0 (0) [0]
Totals	12 (0) [0] / 20 (1) [12]	6 (0) [0] / 16 (2) [11]

*Commercial farms; ND = not done.

of *B. bigemina* or *B. bovis* that could be detected by PCR analysis. Of the 20 *B. decoloratus* allowed to oviposit before PCR analysis, 1 (5 %), from Chikwakwa, contained DNA that could be amplified with primers for *B. bigemina,* while 12/20 (60 %) were positive with primers for *B. bovis.* Of the *B. microplu*s allowed to oviposit, 11/16 (69 %) were positive for *B. bovis* DNA by PCR were positive for *B. bigemina.* A *B. decoloratus* from Chikwakwa and a *B. microplus* from Chisumbanje and Manzvire were positive for both B. *bigemina* and *B. bovis* by PCR.

DISCUSSION

Using PCR primers previously shown to be specific and sensitive in the detection of *B. bigemina*[5] and *B. bovis*[6], we were able to consistently detect DNA of these organisms in our control experiments. Furthermore, the primers for *B. bigemina* and *B. bovis* were found to be specific for each organism. We wish to note that we were unable to determine the reactivity of the primers against *B. occultans* and an unnamed *Babesia* sp., which have as yet only been described to occur in South Africa[3], although kinetes morphologically similar to those of *B. occultans* have been described in ticks from Nigeria[4]. In serial dilutions of the control blood we were able to detect low levels of parasitaemia that were comparable to those described previously[5,6].

In blood collected from cattle in Zimbabwe we detected 289 bp DNA fragments of *B. bigemina* in 35 % of the samples, with positive cattle being found at all but 1 of the 12 sites where sampling was undertaken. These findings confirm an earlier report that *B. bigemina* infections are common in cattle in Zimbabwe[18]. In this study, cattle in 262/274 (96 %) of the sites sampled had antibodies reactive with *B. bigemina* in indirect fluorescent antibody assays (IFAT) and, in 58 % of the communal lands sites sampled, over 80 % of the sera were serologically positive for *B. bigemina.* Serological surveys using IFAT[21] or enzyme-linked immunosorbent assays[7,23] elsewhere in Africa have shown prevalences of antibodies in cattle to *B. bigemina* of 10 to 88 %. Antibodies against *B. bigemina* can be detected in cattle sera for up to 2 years[22], and in animals continually exposed to natural challenge, titres tend to increase with age[23]. Latent *B. bigemina* infections seldom persist for more than a year and, after infection, cattle remain infective for ticks for only up to 2 months[9]. Our findings, then, that the prevalence of the DNA of *B. bigemina* was significantly higher in young cattle (≤2 years) compared to older animals, indicates that cattle in Zimbabwe are commonly infected with the organism early in life.

We were also able to detect 289 bp DNA fragments of *B. bovis* in 47 % of the cattle sampled. This finding is consistent with that of a recent serological survey[15], which showed that antibodies reactive with *B. bovis* are prevalent in cattle in the eastern and northeastern areas of Zimbabwe. It thus appears that, despite earlier reports that *B. microplus,* the major vector of *B. bovis,* had disappeared from the country[19], the prevalence of infections with *B. bovis* is high in cattle in the northeastern and eastern areas of Zimbabwe. Previous studies have shown that cattle infected with *B. bovis* remain carriers of the organism for long periods[3], and the lack of significant differences in the prevalences of *B. bovis* infections in the young and older animals we studied is consistent with this finding. Similarly, the prolonged carrier status of cattle infected with *B. bovis* might explain our findings of significantly higher prevalences of *B. bovis* infections compared to *B. bigemina* infections, which are reported to be relatively transient ($\chi^2 = 1.97$; $P = 16$ %)[9].

Boophilus decoloratus is reported to be the principal vector of *B. bigemina* infections in southern Africa[3]. Infection of engorging females results in transmission of *B. bigemina* to subsequent nymphal and adult stages of the next generation that may then transmit the infection to susceptible cattle[3]. In our study, although exhaustive tick collections were not carried out, we were able to collect *B. decoloratus* at almost all sites sampled (7/9), which confirms earlier reports that the tick is widely distributed in Zimbabwe[16,18]. To the best of our knowledge, field tick infection rates of *B. decoloratus* with *B. bigemina* have yet to be reported from Africa. In our study we could detect DNA of *B. bigemina* in only a low percentage of *B. decoloratus,* mainly 5 % of ticks allowed to oviposit and 0 % of ticks preserved in alcohol

before PCR analysis. Of note are our findings of high prevalences of DNA of *B. bovis* (60 %) in *B. decoloratus*, which indicates that these ticks may become infected with the organism. We note, however, that there is no epidemiological evidence that *B. decoloratus* is a vector of *B. bovis*, and studies have shown the tick not to be a vector of *B. bovis*[20].

The other recognised vector of *B. bigemina* in southern Africa is *B. microplus*. By PCR analysis, *B. microplus* collected in our study showed relatively high prevalences of *B. bigemina* DNA (2/16; 12 %) compared to data from Australia, where infection rates of up to 0.3 % have been reported using tick transmission studies[12,13]. The Australian studies also showed that *B. microplus* transmit *B. bigemina* more readily than *B. bovis*, and it is possible then that *B. microplus* is a significant vector of *B. bigemina* in Zimbabwe.

In southern Africa, the only known vector of *B. bovis* is *B. microplus*[3]. Engorging female ticks become infected with *B. bovis* only in the final 24 hours of rapid engorgement on infected cattle and transmit the infection transovarially, with a relatively low percentage of eggs (10 %) being infected with the organism[2]. One infected larva can transmit the infection to susceptible cattle hosts[3]. Data from the Zimbabwe National Tick Survey reported by Katsande *et al.*[10] showed that *B. microplus*, the vector of *B. bovis*, occurred in the east and northeastern areas of Zimbabwe. This was contrary to earlier reports that this tick had disappeared from the country[19]. In our limited study we were able to identify *B. microplus* at 4/9 (44 %) sites where ticks were collected. The available evidence therefore suggests that *B. microplus* can periodically spread widely into Zimbabwe, and the factors influencing this migration appear to warrant further investigation. Although we are unaware of reports from Africa of field infection rates of adult *B. microplus* with *B. bovis*, studies in Australia have shown rates of up to 0.04 %[12,14]. Although our tick sampling was not exhaustive, a high percentage of the *B. microplus* collected and allowed to oviposit, were positive (11/16; 69 %) for DNA of *B. bovis*. As in the case with *B. bigemina*, we were unable to demonstrate *B. bovis* DNA in ticks preserved in alcohol, and further studies are indicated to determine the most appropriate methods to preserve ticks before PCR analysis for *Babesia* DNA.

In conclusion, our study has shown a high prevalence of *B. bigemina* and *B. bovis* infections in cattle in the eastern and northeastern parts of Zimbabwe. The study has also provided further evidence that *B. microplus*, the vector of *B. bovis*, has the ability to periodically spread into Zimbabwe. The demonstrated high prevalences of infections of cattle in Zimbabwe with *B. bigemina* and *B. bovis* suggest that enzootic stability, or a situation closely approaching enzootic stability, is present in the areas studied. In these areas it appears that animals become infected at a relatively young age when non-specific immune reactions prevent them from suffering from clinical signs of disease and mortality[3]. Such data should be valuable to authorities deciding on national tick control policies in the region.

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