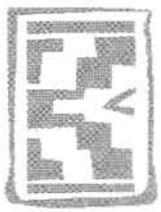

PCR-based detection of the transovarial transmission of Uruguayan *Babesia bovis* and *Babesia bigemina* vaccine strains

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ABSTRACT

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Bovine babesiosis is responsible for serious economic losses in Uruguay. Haemovaccines play an important role in disease prevention, but concern has been raised about their use. It is feared that the attenuated *Babesia bovis* and *Babesia bigemina* vaccine strains may be transmitted by the local tick vector *Boophilus microplus*, and that reversion to virulence could occur. We therefore investigated the possibility that these strains could be transmitted via the transovarial route in ticks using a *Babesia* species-specific polymerase chain reaction (PCR) assay. DNA was extracted from the developmental stages of the tick vector that had fed on calves immunized with the haemovaccine. It was possible to detect *Babesia* DNA not only in adult ticks, but also in their eggs and larvae. In addition, it was shown that calves infested with larvae derived from eggs laid by ticks fed on acutely infected calves, were positive for *Babesia* using PCR. Caution should therefore be shown with the distribution of the haemovaccine in marginal areas. It is still advisable that suitable tick control measures be used to prevent transovarial transmission and the potential risk of attenuated *Babesia* reverting to virulence.

Keywords: *Babesia bigemina, Babesia bovis, Boophilus microplus*, bovine babesiosis, PCR, tick transovarial transmission, Uruguayan vaccine strains

INTRODUCTION

Babesia bovis and *Babesia bigemina* are the intraerythrocytic protozoal parasites causing bovine babesiosis. Transmission to cattle occurs via several species of hard ticks, of which only *Boophilus microplus* is present in Uruguay (Castro & Trenchi 1955). Enzootic instability exists for both of these *Babesia* species as this country is situated in a marginal area for the development of *B. microplus*. This instability results in significant annual losses amounting to approximately USD 25 million (Avila 1999). Disease control consists of the use of a haemovaccine that contains attenuated strains of *B. bovis* and *B. bigemina*. This vaccine has been produced at DILAVE (Veterinary Laboratory Direction, Montevideo) since 1980. There has been great concern, however, that transovarial transmission of these attenuated strains could occur. Should this happen, then it is possible that reversion to virulence within the tick vector could take place and lead to further disease spread.

The purpose of this study was, firstly, to investigate whether such transovarial transmission could occur by using a *Babesia* species-specific polymerase

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chain reaction (PCR) on all the developmental stages of the tick vector. Secondly, an attempt was made at determining whether transovarially-infected larvae were capable of transmitting *Babesia* to cattle.

MATERIALS AND METHODS

Babesia vaccine strains

The *B. bovis* vaccine strain had been attenuated by 27 syringe passages obtained from 3-month-old splenectomized calves during the acute phases of infection. Attenuation of the *B. bigemina* strain had been achieved after eight syringe passages during the chronic phases of infection in non-splectomized 5-month-old calves. These haemovaccine strains were given intravenously (i.v.) in the first trial to three of the five experimental calves (calves 1–3). In the second trial, it was given i.v. to two of the three tick-donor calves (calves 6 and 7). The dosages used were 10^7 *B. bovis* infected erythrocytes (I.E.) and 10^5 *B. bigemina* I.E., administered 20 to 30 days after the first tick infestation.

Babesia field strains

The *Babesia* field strains were derived from an outbreak of babesiosis in the Cerro Largo district in 1994, where the affected cattle had been positively diagnosed by light microscopy of Giemsa-stained blood smears. Morphologically, both *B. bovis* and *B. bigemina* were present, although this was not confirmed by PCR. Blood from these animals was stored in DMSO at –196 °C.

Boophilus microplus strain

The Mozo *B. microplus* tick strain had been obtained in 1974 from naturally infested animals from a *Babesia*-free Uruguayan farm and has since then been maintained at DILAVE by continual passage on *Babesia*-free calves. Larvae were placed on the backs of calves two to three times weekly.

This strain of tick was used to infest all the calves two to three times weekly over a 1–3 month period with 50 mg (about 1 000) *B. microplus* larvae. In the trial to determine transmissibility of infection via the offspring of ticks feeding on infected calves, 150 000 larvae were used per animal.

Experimental animals

Five 1-year-old intact Hereford calves from a tick-free area were kept in separate crates for 3 months throughout the first phase of the experiment. They were marked and identified as calves 1, 2, 3, 4 and 5. All these calves tested negative for the presence of antibodies against *Babesia* prior to the experiment, using the indirect fluorescent antibody test (IFAT). Calf 4 was infected with *B. bigemina*/*B. bovis* field strains while calf 5 served as a negative control. Haemovaccine, containing *B. bovis* and *B. bigemina*, was obtained from DILAVE and administered to the remaining calves. Frequent haematocrit and body temperature readings were recorded.

In the second phase of the trial, nine calves (calves 6–14) from a tick-free area were used. Two of these were infected with the *B. bovis*/*B. bigemina* haemovaccine (calves 6 and 7) and one with *B. bovis*/*B. bigemina* field strains (calf 8). These three calves (named the tick donors) were then used as carriers of *Babesia* parasites for the purpose of infecting ticks. The larvae derived from eggs laid by these ticks were allowed to feed on the remaining six calves i.e. calves 9–14 (named the tick recipients). These were divided into two groups, viz. calves 9–11 in group 1 and calves 12–14 in group 2. These groups were used to determine the relative infectivity of *Babesia* spp. derived from either the acute (week 1–4) or chronic (week 5–13) stages of infection in the donor calves.

Evaluation of infection in calves

The calves were monitored over a 3-month period by light microscopy of Giemsa-stained blood smears prepared by standard procedures. Both central and/or peripheral blood samples were used in the preparation of the smears from each animal after every one to two days during the first three to four weeks post infection, and thereafter approximately weekly. In addition, temperature and haematocrit readings were taken and blood was collected for PCR analysis.

Tick vector samples for PCR analysis

Detached engorged ticks were collected every 3–5 days. These were then washed in water and randomly divided into two groups. One group was placed in Petri dishes in a 26 °C walk-in incubator (90 % humidity), and the other was placed in a –20 °C freezer for PCR analysis. Eggs from ticks that had been incubated for 15 days were collected and stored at –20 °C for later analysis by PCR. The remaining eggs were left for further incubation and the resultant larvae were collected and stored at –20 °C for PCR analysis.

DNA purification from blood

Briefly, 500 µℓ blood collected in EDTA (5 mg/mℓ) was mixed with 500 µℓ buffer 1 (20 mM NaCl, 20 mM disodium ethylenediaminetetraacetate (EDTA), 20 mM Tris HCl, pH 7.5 and 0.5 % Triton X) and left on ice for 30 min. After centrifugation at 12 000 rpm in a microfuge for 10 min, the supernatant fraction was removed and 500 µℓ 1 X SSC (0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) was added to the pellet. The suspension was again mixed and centrifuged at 12 000 rpm for 10 min. Following the removal of the supernatant fraction, 500 µℓ Buffer 2 (50 mM Tris, pH 7.5, 50 mM EDTA, 10 mM NaCl and 1 % sodium dodecyl sulphate (SDS)) was added to the pellet together with 10 µℓ proteinase K (20 mg/mℓ). This mixture was then incubated at 56 °C overnight. The following day, phenol-chloroform-isoamyl alcohol (P:C:I; 25:24:1 v/v) was added, and the sample mixed and then centrifuged at 12 000 rpm in a microfuge for 15 min. The aqueous phase was transferred into a new Eppendorf tube and a one-third volume of ammonium acetate (10 M) and two volumes of cold 100 % ethanol were added to precipitate the DNA. The mixture was centrifuged in the microfuge at 12 000 rpm for 10 min and the DNA was washed with 70 % ethanol and air-dried (Sambrook, Fritsch & Maniatis 1989). The DNA obtained was re-suspended in 20–50 µℓ Tris-EDTA (TE), pH 8, depending on pellet size.

DNA isolation from engorged ticks

Engorged ticks were ruptured and the pooled contents from three ticks were transferred into an Eppendorf tube. The same extraction buffer and DNA purification protocol as for the blood samples were used, except for two P:C:I extractions.

DNA isolation from eggs and larvae

Approximately 50 mg eggs or larvae were crushed in a glass tissue grinder. Except for minor variations, the same extraction buffer and procedure was used as for the blood samples. Samples were incubated on ice for approximately 30 min and centrifuged in a microfuge at 5 000 rpm for 5 min. No SSC step was included.

The second extraction procedure was performed on the supernatant as for the blood samples, except that 30 µℓ proteinase K (20 mg/mℓ) was added. After overnight incubation at 56 °C, these samples were frozen and thawed four times. The remaining protocol was used as described but with two P:C:I extractions.

PCR analysis

Primers sets (Table 1) designed for *B. bovis* detection (Azambuja, Gayo & Solari 1994) using PCR-PLAN software, were derived from the BBMER60 sequence which encodes a 60 kDa merozoite surface protein. Sequence data was obtained from GENBANK (Suarez, Palmer, Jasmer, Hines, Perryman & Mcelwain 1991; Palmer, Mcelwain, Perryman, Davis, Reduker, Jasmer, Shkap, Pipano, Goff & Mcguire 1991). The primers used for *B. bigemina* detection were as described by Figueroa, Chieves, Johnson & Buening (1992).

PCR tests were performed on 4 µℓ (0.8 ug) of the DNA samples. These were placed into a mixture containing 50 pmol of each primer (Bov N#1 and Bov N#2, or Big #IA and Big #IB), 40 nmol of a deoxynucleoside triphosphate (dNTP) mix (10 nmol each), 1.5 U of Taq polymerase and PCR buffer (50 mM KCl, 10 mM Tris-HCl pH 8.5, 1.5 mM MgCl2, 0.001 % gelatin) in a final reaction volume of 12.5 µℓ. For the hemi-nested PCR, 0.5 µℓ of the first PCR amplicon-containing solution together was added to reagents as above but with different primers (Bov N#1 and Bov #S, or Big #IAN and Big #IBN) and the water volume adjusted accordingly. The thermocycling parameters used for primary *B. bovis* PCR (Bov N#1 and Bov N#2) were 94 °C for

TABLE 1 DNA sequences of the oligonucleotide primers used for the species-specific *Babesia* PCR assays and the predicted amplicon sizes

Babesia spp.	Primer	Sequence 5' - 3'	Primer pair	Amplicon size (bp)
B. bovis	Bov N#1	TCGAACCCTGCCAAGAACAGCG		
	Bov N#2	CGAGGTCAAGCTACCGAGCAGAACC	Bov N#1 + Bov #S	452
	Bov #S	TCACCATGTCAGCATAACGACGTGC	Bov N#1 + Bov N#2	423
B. bigemina	Big #IA	CATCTAATTTCTCTCCATACCCCTCC		
	Big #IB	CCTCGGCTTCAACTCTGATGCCAAAG	Big #IA + Big #IB	278
	Big # IAN	CGCAAGCCCAGCACGCCCCGTGC		
	Big # IBN	CCGACCTGGATAGGCTGTGTGATG	Big # IAN + Big # IBN	170

TABLE 2 Results of Giemsa-stained smears and PCR assays on blood obtained over a 3-month period from haemovaccine immunised (calves 1–3), field strain infected (calf 4) and uninfected calves (calf 5)

Day	Giemsa					PCR									
						B. bovis					*B. bigemina*				
	Calf 1	Calf 2	Calf 3	Calf 4	Calf 5	Calf 1	Calf 2	Calf 3	Calf 4	Calf 5	Calf 1	Calf 2	Calf 3	Calf 4	Calf 5
1	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
4	–	–	–	–	–	–	ND	–	–	–	–	ND	–	–	–
5	–	–	–	–	–	+	+	+	+	–	+	+	–	–	–
6	–	–	–	–	–	+	+	+	+	–	+	+	–	–	–
8	–	–	–	–	–	+	+	+	+	–	+	+	+	–	–
11	–	–	+	+	–	+	+	+	+	–	+	+	+	–	–
12	+	+	+	+	–	ND	+	ND	+	–	ND	+	ND	–	–
13	+	+	–	–	–	+	+	+	+	ND	+	+	+	–	ND
14	+	–	+	+	–	ND	ND	ND	+	ND	ND	ND	ND	–	ND
16	–	–	–	–	–	+	+	+	+	ND	+	+	+	–	ND
19	–	–	–	–	–	+	–	ND	+	ND	+	+	ND	–	ND
22	–	–	–	–	–	+	+	+	+	–	+	+	+	–	–
26	–	–	–	–	–	+	+	+	+	ND	+	+	+	–	ND
34	–	–	–	–	–	+	+	–	–	–	+	+	–	–	–
40	–	–	–	–	–	+	+	+	–	ND	+	–	+	–	ND
49	–	–	–	–	–	+	+	ND	–	–	+	+	ND	–	–
57	–	–	–	–	–	+	+	ND	–	ND	+	+	+	–	ND
62	–	–	–	–	–	+	+	+	–	ND	+	+	+	–	ND
70	–	–	–	–	–	+	+	+	–	ND	+	+	+	–	ND
77	–	–	–	–	–	+	+	+	–	ND	+	+	–	–	ND
82	–	–	–	–	–	+	+	+	ND	–	–	+	–	ND	–
89	–	–	–	–	–	+	+	+	ND	ND	–	+	–	ND	ND
97	–	–	–	–	–	+	–	+	–	ND	–	+	–	–	ND
105	–	–	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND
112	–	–	–	–	–	ND	ND	ND	ND	ND	ND	ND	ND	ND	ND

– = negative
\+ = positive
ND = not done

TABLE 3 Results from Giemsa-stained smears and PCR assays from blood samples taken from calves infested with hatched larvae derived from ticks taken from acutely or chronically *Babesia*-infected donor calves

Day[3]	Infected with larvae from ticks from acute phase of infection[1]									Day[3]	Infected with larvae from ticks from chronic phase of infection[2]								
	Giemsa			PCR							Giemsa			PCR					
				B. bovis			*B. bigemina*							*B. bovis*			*B. bigemina*		
	Calves 9–11										Calves 12–14								
	9	10	11	9	10	11	9	10	11		12	13	14	12	13	14	12	13	14
1	–	–	ND	ND	ND	ND	ND	ND	ND	1	–	–	–	–	–	–	–	–	–
3	–	–	–	ND	ND	ND	ND	ND	ND	5	–	–	–	–	–	–	–	–	–
4	–	–	–	ND	ND	ND	ND	ND	ND	13	–	–	+	–	–	+	–	–	+
6	–	–	–	–	ND	ND	–	ND	ND	14	–	–	ND	–	–	ND	–	–	ND
7	–	–	–	ND	ND	ND	ND	ND	ND	20	–	–	ND	–	–	ND	–	–	ND
8	–	–	–	+	+	+	ND	ND	+	21	–	–	ND	–	–	ND	–	–	ND
9	–	–	–	+	+	–	+	–	–										
10	–	–	–	+	–	–	+	–	+										
11	–	–	–		+	+	ND	+	ND										
12	+	–	–	+	–	+	+	+	–										
13	+	–	–	+	–	–	+	+	ND										
14		–	ND	ND	–	ND	ND	–	–										
17		–	ND	ND	ND	ND	ND	ND	ND										
21		–	ND	ND	ND	ND	ND	ND	+										
27		–	–	ND	–	–	ND	–	ND										

– = negative + = positive
ND = not done

1 Calves 10 and 11 received larvae derived from ticks that had fed on calves in the acute phase of infection with *Babesia* haemovaccine strains. Calf 9 received larvae derived from ticks from calf acutely infected with field strain *Babesia* spp.

2 Calves 12 and 13 received larvae derived from ticks that had fed on calves in the chronic stage of infection with *Babesia* haemovaccine strains. Calf 14 received larvae derived from ticks from the calf in the chronic stages of infection with field strain *Babesia spp.*

3 Days post larval infestation

40 s, 63 °C for 30 s and 72 °C for 40 s, for 25 cycles. The predicted amplicon size was 452 base pairs (bp) (Table 1). For the heminested *B. bovis* PCR (Bov N#1 and Bov #S), the same temperatures were used but each step was performed for 30 s for each cycle. The predicted amplicon size was 423 bp. The thermocycling parameters for primary *B. bigemina* PCR (Big #IA and Big #IB) were: 94 °C for 40 s, 56 °C for 30 s and 72 °C for 40 s, for a total of 25 cycles. The predicted amplicon size was 278 bp. For the nested *B. bigemina* PCR (Big #IAN and Big #IBN), the same temperatures were used but each step was performed for 30 s. The predicted amplicon size was 170 bp. PCR amplicons were analysed by electrophoretic separation of DNA fragments in 1 % agarose gels and visualised using ethidium bromide staining and transillumination with UV light.

RESULTS

Blood analyses during the first trial

The three vaccinated calves (calves 1–3) were positive on examination of Giemsa-stained blood smears for only a short period, viz. from approximately day 11 until day 14 post vaccination (Table 2). This window period coincided with an increase in body temperature and a decrease in packed cell volume (not shown). The wild type infected positive control (calf 4) was positive on a Giemsa-stained blood smear over the same period of time as the vaccine infected animals (Table 2). It was treated with diminazene on days 10 and 12 post inoculation. The non-infected calf (calf 5) remained negative to *B. bovis* and *B. bigemina* for the whole trial period as expected. PCR analysis was performed prior to inoculation until trial termination. *Babesia bovis* and *B. bigemina* could on average be detected in the haemovaccine-infected calves 1–3 from day 5 post inoculation until the end of the trial, 3 months later (Table 2). The wild type infected calf (calf 4) was PCR positive only for *B. bovis* from day 5 post inoculation and was PCR negative a month later. The negative control calf (calf 5) remained negative for both *Babesia* spp. throughout the trial period.

PCR analysis of *Boophilus microplus* adult ticks, eggs and larvae

Babesia organisms could be detected in engorged adult ticks derived from the haemovaccinated calves from as early as day 4 post inoculation (calf 3) until the last day of testing on day 16 post inoculation (calves 1–3). As expected, no *Babesia* organisms were found in the ticks from the negative control (calf 5). The eggs derived from engorged adult ticks taken from the vaccinated calves were also found to be positive for *B. bovis* and/or *B. bigemina* (calves 1 and 3) from as early as day 8 and as long as 3 months (the time period of adult ticks on infected donor calves). Larvae showed a similar PCR positive incidence although in the case of calf 3, *B. bovis* could be detected in larvae from ticks obtained as early as day 4 post inoculation. Eggs and larvae from ticks from the wild type infected calf 4 were, as was the calf itself, PCR positive (days 12, 16 and 22) for *B. bovis* only. The different tick stages from the negative control (calf 5) were all PCR negative.

Blood analyses during the second trial

The two calves (calves 10 and 11) infected with larvae derived from ticks from the vaccine-infected donors (calves 6 and 7) in the acute phase of infection, remained negative on examination of Giemsa-stained smears (Table 3), but were PCR positive for *B. bovis* from day 8 after infestation with larvae. The other calf (calf 9), where the donor (calf 8) was infected with *Babesia* spp. field strains, was PCR positive on days 8 (*B. bovis*) and 9 (*B. bigemina*) post infestation with larvae but only on day 12 using Giemsa-stained blood smears. Unfortunately this calf died soon afterwards despite treatment with diminazene and a blood transfusion. By comparison, calves 12 and 13 that had been infected by larvae derived from engorged ticks feeding on vaccinated donors (calves 6 and 7) during the chronic phase of infection, remained negative on examination of Giemsa smears and PCR throughout the trial. Calf 14, however, which had been infected by larvae derived from ticks from the *Babesia* field strain-infected donor (calf 8) during the chronic phase of infection, was Giemsa-smear and PCR positive (*B. bovis*) on day 13. It unfortunately succumbed the following day before any treatment could be implemented, following a sudden decline in the haematocrit reading.

DISCUSSION

Several authors have described the increased sensitivity of PCR over microscopy for *Babesia* detection (Fahrimal, Goff & Jasmer 1992; Figueroa *et al.* 1992; Persing, Mathiesen, Marshall, Telford, Spielman, Thomford & Conrad 1992; Böse, Jorgensen, Dalgliesh, Friedhoff & De Vos 1995). In this trial, *Babesia* organisms could be detected from day 5 post infection using PCR, whilst on examination of

blood smears this could only be done from day 11. It was also shown that *B. bovis* and *B. bigemina* organisms were still detectable after 3 months post inoculation using PCR but not by microscopy, where a positive result was only detectable up to day 14. The positive control animal became *B. bovis*-negative a month after inoculation, probably because of diminazene treatment 12 days previously. The wild type inoculum used for infecting this calf (calf 4), however, had been selected using microscopy alone and may have only contained *B. bovis*, probably explaining the negative PCR results for the presence of *B. bigemina*. The occasional negative PCR results obtained elsewhere could be possibly ascribed due to cyclical variations in parasitaemia.

Engorged ticks that had detached from these infected calves, were tested for *B. bovis* and/or *B. bigemina* by PCR and were found positive within 1 week after infection, or after 2 weeks in the case of the positive control—except for ticks derived from the negative control calf. Of note is that organisms were also found in the tick progeny, indicating that transovarial transmission of not only the wild type but also the vaccine strain had in fact occurred.

Much has been published on the transovarial transmission of different *Babesia* vaccine strains, mostly with reference to *B. bovis*. Substantial differences in findings are described depending on amongst others, the isolate examined and the number of passages through calves during the attenuation process. The Ka *B. bovis* Australian vaccine strain, for example, was shown to be tick transmissible (Timms, Stewart & De Vos 1990). Other vaccine strains, on the other hand, appear to have lost their ability to infect *B. microplus* ticks (Mangold, Aguirre, Cafrune, Echaide & Guglielmone 1993; Mason, Potgieter & Van Rensburg 1986; O'Sullivan & Callow 1966). This phenomenon was ascribed to a reduction or loss in the infectivity of blood-passaged *Babesia* strains for their tick hosts (O'Sullivan & Callow 1966; Dalgliesh, Stewart & Duncalfe 1981; Mason *et al.* 1986).

Stewart (1978) studied the differences in the life cycles of a repeatedly needle-passaged *B. bovis* and an unmodified strain and came to the conclusion that with continuous blood-passage, the selection of parasites incapable of penetrating the tick gut epithelium takes place. We have here described the use of PCR for studying the transovarial transmission of a vaccine strain of *B. bovis* and *B. bigemina*. This is, as far as we know, the first published PCR-based study to determine transovarial transmission of *Babesia* organisms.

The second trial showed that larvae derived from the eggs laid by ticks that had fed on calves infected by the vaccine or field strains, could infect susceptible cattle. In the case of vaccinated tick donors, infection of susceptible animals only occurred after ticks were collected from donors in the acute phase of infection.

The use of a vaccine containing *Babesia* spp. that are transmissible by ticks in enzootic areas where such vector ticks are established, can be effective in creating enzootic stability by maintaining or increasing the infection rates in tick populations. However in Uruguay, which is situated in a marginal area for the development of *Boophilus microplus*, this would not be the case. On the contrary, issuing a tick-transmissible vaccine could potentially lead to a reversion to virulence, which has been demonstrated to occur after only one tick passage (Timms *et al.* 1990). Taking into account that the Uruguayan vaccine strains were transmitted during the acute phase of infection, strong recommendations will be given to farmers to apply tick control after haemovaccination of cattle.

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