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Abstract:

Detection of bovine Babesiosis in Mozambique by a novel hot-start PCR method

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Babesiosis is a tick borne disease (TBD) caused by parasites of the genus *Babesia*, with considerable worldwide economic, medical, and veterinary impact. Babesiosis and other TBDs were considered responsible for 50% of the deaths of cattle occurred in Mozambique in the first year after importation from neighbouring countries.

Here, we present the detection of *B. bigemina* and *B. bovis* in cattle from Mozambique and the development of a novel PCR method. For this study, blood samples were collected in one farm located near Maputo city. The samples were analyzed using a previously described nested PCR and a novel hot-start PCR method. Primers were selected for the hot-start PCR based on the putative gene of an undescribed aspartic protease named babesipsin, present in both *B. bovis* and *B. bigemina*. The combination of hot start polymerase and long primers (29-31bp) were in this study determinant for the

successful amplification and detection in only one PCR. The babesipsin hot-start PCR is a fast, simple, and sensitive method, with some advantages over current methods. A total of 117 field samples were tested by hot-start PCR, and 90 were positive for *B. bigemina* (77%), 82 were positive for *B. bovis* (70%), 61 were mixed infections (52%) and only 6 were negative (5.1%). The results confirm that this area of Mozambique is endemic for Babesiosis, and that this TBD should be regarded as a threat for imported cattle.

Keywords: *Babesia bigemina*; *Babesia bovis*; hot-start PCR; Molecular diagnosis; aspartic protease

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1. Introduction

In recent years, efforts have been made to rebuild the livestock population in Mozambique. Cattle have been imported mostly from the neighbouring countries Zimbabwe and South Africa. The success of this approach is being impaired by high mortality among the imported cattle, which is estimated at around 50% within the first year (Alfredo et al., 2005). Tick Borne Diseases (TBDs), particularly babesiosis, anaplasmosis and cowdriosis are arguably the major causes of mortality. Although the distribution and prevalence of TBDs in Mozambique is mostly unknown, seroprevalence of *Babesia bovis* (39%) and *Anaplasma marginale* (63%) was recently reported for Tete province (Alfredo et al., 2005).

Bovine babesiosis in Africa, and particularly in Mozambique, is caused by *B. bovis* and *Babesia bigemina* (Uilenberg, 2006). There are reports of *Babesia occultans* in South Africa (Gray and De Vos, 1981) and possibly Nigeria (Dipeolu and Amoo, 1984), but the prevalence and distribution of this benign form of cattle babesiosis is unknown. On the other hand, the tick vector responsible for the transmission of *B. occultans*, *Hyalomma marginatum rufipes*, was identified in Mozambique, suggesting that *B. occultans* may therefore be present. *In vitro* cultivation of *B. occultans* was accomplished (Niekerk and Zweygarth, 1996), but unfortunately there are no published sequences of *B. occultans* in the public databanks, and there is also no Polymerase chain reaction (PCR) detection method available. Nevertheless, molecular studies have to bear in mind the presence of this and possibly other unidentified species. Detection methods based on the 18S rRNA gene sequence or other genus conserved sequences, can originate false positives as these

59 sequences are expected to be more conserved with the sequences from unsequenced and
60 unidentified *Babesia* spp..
61 *B. bovis* was detected in Mozambique by serologic tests (Alfredo et al., 2005), but these
62 methods are less sensitive and specific in the detection of the carrier state of animals and
63 do not usually distinguish between past exposure and present infections. PCR based
64 techniques constitute an alternative method for the direct detection of *Babesia* in carrier
65 cattle. The carrier state occurs after acute or primary infections, in which the animals are
66 not clinically ill. Identification of carrier animals is important for the assessment of
67 infection risk, given that they serve as reservoirs for infection of ticks and, ultimately,
68 wider infection of the herd (Calder et al., 1996).
69 Several PCR based methods have been published that allow the detection of *B. bovis* and
70 *B. bigemina*. More specifically, two methods have been used by various authors for the
71 detection of *Babesia* from blood and ticks: the multiplex nested PCR (nPCR) for the
72 detection of *B. bovis* and *B. bigemina* (Figueroa et al., 1993; Almeria et al., 2001; Gayo et
73 al., 2003; Oliveira et al., 2005; Costa-Júnior et al., 2006) and the reverse line blot
74 hybridization assay (RLB) (Gubbles et al., 1999; Georges et al., 2001; Brígido et al.,
75 2004; Oura et al., 2004). Both methods appear to have similar sensitivities at 10^{-6}%
76 parasitemia (Gubbles et al., 1999; Costa-Júnior et al., 2006). However, it is interesting
77 that the non-multiplex nested PCR has an increased detection of 10^{-7}% parasitemia
78 (Oliveira-Sequeira et al., 2005). Here, we present the development of a faster and
79 sensitive method in the detection of *B. bovis* and *B. bigemina* in field samples, and in face
80 of the results the state of bovine babesiosis in Mozambique will be briefly addressed.

2. Materials and methods

2.1. *Blood samples from cattle*

A total of 117 blood samples were collected from cattle in the province of Maputo, Mozambique. The samples were collected in September near Umbeluzi in the Boane District, mainly from Friesian Cattle. Approximately 2–4 ml of blood was collected from the coccygeal vein into ethylenediaminetetraacetic acid (EDTA) buffered vacutainer tubes. Samples were kept at 4 ºC while being transported to the laboratory at the Faculty of Veterinary of Maputo. The blood was stored at -20 ºC until DNA extraction.

2.2. *DNA extraction*

DNA extraction was performed according to Centeno-Lima et al. (2003). Two hundred microlitres of EDTA buffered whole blood was added to 500 µl phosphate-buffered saline (PBS), vortexed for 10 s and then centrifuged at 16000 g for 5 min. The cells pellet was washed with PBS three more times or until the supernatant was clear. The pellet was then ressuspended with 100 µl of lysis buffer [50 mM KCl, 0.5% (v/v) Tween-20, 10 mM Tris-HCl (pH 8.0) and 10 µg of proteinase K added before use], incubated overnight in a water bath at 56 ºC and heated for 10 min at 100 ºC to inactivate proteinase K. Samples were stored at -20 ºC.

2.3. *PCR reactions*

2.3.1. *Primers*

The primers used in hot-start PCR amplification (Table 1), are localized within the putative aspartic proteinase babesipsin genes from both *B. bovis* and *B. bigemina*. The babesipsin putative gene sequences were identified in the Sanger Institute databases: in the *B. bovis* EST Sequencing Project (de Vries et al., 2006) and in the *B. bigemina* genome project.

The oligonucleotide primers were designed using the online GeneFisher program (Giegerich et al., 1996) with the following parameters: G+C content from 40 to 60%, melting temperature between 60 and 80 ºC, and primer size between 27 and 31 bp (Table 1). The expected size using babesipsin primers is 614 bp for *B. bigemina* and 426 bp for *B. bovis*. The amplification products span 2 partial exons and 1 intron.

The primer sequences described by Figueroa et al. (1993) were used in nPCR; BoF/R and BilA/B the outer primers, BoFN/RN and BilAN/BN the inner or nested primers (Table 1).

2.3.2. *Babesipsin hot-start PCR*

The babesipsin hot-start PCR reaction mixtures (20 µl) contained 16 mM $(NH_4)_2SO_4$, 67 mM Tris-HCl (pH8.8), 0.01% (v/v) Tween-20, 2.5 mM $MgCl_2$, 0.5 pmol each primer, 200 µM each dNTP, 1 U of Superhot Taq DNA polymerase (Bioron GmbH), and 1 µl total DNA. Hot-start PCR was carried out in a PTC-200 MJ Research thermocycler for 40

126 cycles. Each cycle consisted of 20 s of denaturation at 95 °C (1 min for the first cycle), 30
127 s of annealing at 69 ºC, and 45 s of extension at 72 ºC.
128 Twenty random PCR products were gel purified and sequenced in outsourcing at STAB
129 Vida, Portugal.
130
131 2.3.3. *nPCR*
132
133 The first PCR using the primers described by Figueroa et al. (1993), was carried out using
134 the same buffer as described for babesipsin hot-start PCR, 1 U DFS-Taq DNA
135 polymerase (Bioron GmbH), 0.5 pmol outer primers, 1 µl total DNA and the
136 thermocycler program described by Oliveira et al. (2005): 35 cycles (1 min at 95 ºC , 1
137 min at 60 ºC for *B. bovis* and 64 ºC for *B. bigemina*, 1 min 30 s at 72 ºC) and a final
138 extension step at 72 ºC for 5 min. The same conditions of the first PCR were used in
139 nPCR apart from using inner primers, 2 µl of the first PCR products as template and an
140 annealing temperature of 65 ºC for *B. bovis* and 70 ºC for *B. bigemina*.
141
142 2.4. *Analysis of PCR products*
143
144 Ten microlitres of PCR products were separated by electrophoresis in 1.2% agarose gel
145 containing ethidium bromide in 0.5 x TBE buffer (44.5 mM Tris-HCl, 44.5 mM Boric
146 Acid and 1 mM EDTA, pH 8.3). After electrophoresis, PCR products were visualized by
147 transillumination with UV light.
148

3. Results

3.1. *nPCR*

Of the 117 samples, 30 were analyzed by nPCR for comparison with the hot-start PCR method. The nPCR reactions using the primers described by Figueroa et al. (1993), resulted in amplification from field samples of the desired products, but showed some unspecific background amplification when using *B. bigemina* primers. The unspecific amplification is in the same range as the desired product, making difficult the interpretation of results, mainly for positive samples. In the first PCR no bands were visible (not even using hot start polymerase), possibly due to low levels of parasitemia. In the second or nPCR, 25 samples were positive for *B. bigemina* (83%) and 26 were positive for *B. bovis* (87%).

3.2. *Hot-start PCR*

The combinations using the babesipsin primers described in this study and genomic DNA (kindly provided by Dr. Varda Shkap from Kimron Veterinary Institute, Israel), allowed the amplification of single band products with the expected size (results not shown).

Hot-start PCR using field samples also allowed the amplification of single band products with the expected size (see Figure 1 and 2). Sequences of twenty random PCR products, for both *Babesia* spp., confirmed the specificity of amplification. There was no

amplification of PCR products when a regular *Taq* polymerase was used instead of the hot-start polymerase.

From the total 117 samples, 90 were positive for *B. bigemina* (77%) and 82 were positive for *B. bovis* (70%). 61 were reported as mixed infections (52%) and only 6 were negative (5.1%). In comparison with the 30 samples analyzed by the nPCR method, 23 were positive for *B. bigemina* (77%) and also 23 were positive for *B. bovis* (77%), but not always were the same samples positive for both, and only one sample was negative for both. Some samples were only positive using the hot-start PCR method, and some were only positive using the nPCR.

4. Discussion

In this study carried out at 30 km from Maputo, in Mozambique, almost the entire herd was positive (95%) for *B. bigemina* and/or *B. bovis*. The high detection of both *B. bigemina* (77%) and *B. bovis* (70%) infections in cattle from Mozambique is similar to another endemic region of the world. Oliveira et al. (2005) also observed that, in São Carlos, SP, Brazil, there was infection in high levels in cattle with *B. bigemina* (88%) and *B. bovis* (92%) using the nPCR as detection method. Nevertheless, other studies should be conducted in other areas in Mozambique to confirm the endemic status of Babesiosis in this country. Efforts to rebuild the stock population should consider the Babesiosis threat especially when importing cattle from countries that adopt stricter control measures.

Although there are several reported methods in the literature describing the detection of *B. bigemina* and *B. bovis*, only some have been tested with field samples, and only a few have been used globally: the nPCR (Figueroa et al., 1993; Almeria et al., 2001; Gayo et al., 2003; Oliveira et al., 2005; Costa-Júnior et al., 2006) and the RLB (Gubbles et al., 1999; Brígido et al., 2004; Oura et al., 2004). One of the goals of this work was to develop a method that could offer some advantages over the current ones, using cost effective methodologies, and equipments available in local molecular biology laboratories.

In this study the nPCR was a little more sensitive than the hot-start PCR (Table 2). This could be the result of the hot-start PCR being more sensitive to contaminants in the DNA samples, since in the second or nested PCR there is a ten fold dilution of the possible

PCR inhibitors. In any case, some samples were only positive using the hot-start PCR method, and some were only positive using the nPCR. This can be expected when the samples have low parasitemias, because the frequency of detection decreases with the decrease in parasitemia (Calder et al., 1996).

The nPCR requires two consecutive PCR, and therefore more preparation and running time than the new hot-start PCR. The RLB assay also requires more preparation and special equipment, but can be used in the detection of several species from different genera, and consequently can be a better method to have a broader view of TBDs infections.

The development of the hot-start PCR served the initial purposes. The method is sensitive, simple and fast, and no special equipment is needed. The hot-start PCR method allows the screening of a high number of samples in a short period of time and is therefore suited for epidemiological studies.

In the first PCR of the nPCR method, using BoF and BoR or BilA and BilB primers, there was no amplification, even when using hot start polymerase. When using the babesipsin longer primers with higher melting temperatures (Table 1) and a non hot-start polymerase, there was also no amplification. This appears to infer that only the combination of a hot-start polymerase and long primers (29-31 bp) allows the amplification from field samples that contain pathogen DNA in low concentrations. The use of hot-start polymerases and 30 bp primers with melting temperatures around 64 ºC can eventually be used in the development of new detection methods for pathogens that are present in low levels as in the case of the carrier state of *B. bovis* and *B. bigemina*.

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309

310 **Tables**

311

312 Table 1

313 Primers sequence and melting temperature (Tm)(Wallace et al., 1979).

Method/Parasite	Name	Primer sequence	Tm (ºC)
hot-start PCR	BovBA1F	5′-CCCGCTCTGGATACCGTAACCATAGGAGA-3′	64
B. bovis	BovBA1R	5′-ATACTGAGGATCCACTCCGCCTAGCATCA-3′	63
hot-start PCR	BigBA1F	5′-GGGAGATAAAAATCGGCACGCCCCCGCAA-3′	66
B. bigemina	BigBA1R	5′-GAGGATCTATGCCTCCTAACATTATCCGTGA-3′	62
nPCR	BoF	5′-CACGAGGAAGGAACTACCGATGTTGA-3′	60
B. bovis	BoR	5′-CCAAGGAGCTTCAACGTACGAGGTCA-3′	61
	BoFN	5′-TCAACAAGGTACTCTATATGGCTACC-3′	56
	BoRN	5′-CTACCGAGCAGAACCTTCTTCACCAT-3′	60
nPCR	BiIA	5′-CATCTAATTTCTCTCCATACCCCTCC-3′	58
B. bigemina	BiIB	5′-CCTCGGCTTCAACTCTGATGCCAAAG-3′	61
	BiIAN	5′-CGCAAGCCCAGCACGCCCCGGTGC-3′	69
	BiIBN	5′-CCGACCTGGATAGGCTGTGTGATG-3′	61

314

315 Table 2

316 Comparison of methods in the detection of cattle infected with *B. bigemina* and/or *B.*

317 *bovis* in Boane district, Mozambique.

Method / # samples	nPCR / 30	hotPCR / 30	hotPCR / 117
B. bigemina	25 (83%)	23 (77%)	90 (77%)
B. bovis	26 (87%)	23 (77%)	82 (70%)
Mixed infections	21 (70%)	17 (56%)	61 (52%)
No detection	0 (0%)	1 (3.3%)	6 (5.1%)

318

Figures

M b 1 2 3 4 5 6 7 8 9 10 b M

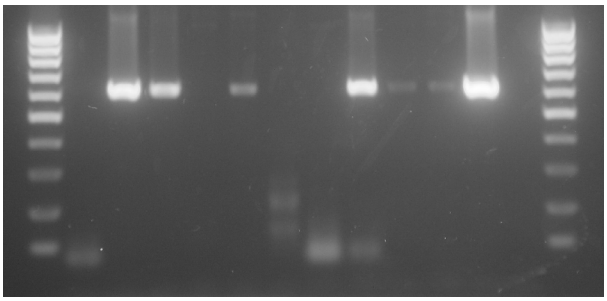

Figure 1. Hot-start PCR of *B. bigemina* DNA from field blood samples. DNA was subjected to hot-start PCR for the babesipsin 614 bp sequence amplification using the primers BigBA1F and BigBA1R. M: 100bp DNA ladder marker; b: negative control.

M b 1 2 3 4 5 6 7 8 9 10 b M

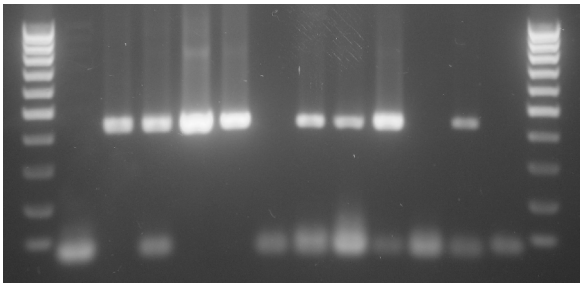

Figure 2. Hot-start PCR of *B. bovis* DNA from field blood samples. DNA was subjected to hot-start PCR for the babesipsin 426 bp sequence amplification using the primers BovBA1F and BovBA1R. M: 100bp DNA ladder marker; b: negative control.

Table1

Table 1

Primers sequence and melting temperature (Tm)(Wallace et al., 1979).

Method/Parasite	Name	Primer sequence	Tm (ºC)
hot-start PCR	BovBA1F	5′-CCCGCTCTGGATACCGTAACCATAGGAGA-3′	64
B. bovis	BovBA1R	5′-ATACTGAGGATCCACTCCGCCTAGCATCA-3′	63
hot-start PCR	BigBA1F	5′-GGGAGATAAAAATCGGCACGCCCCCGCAA-3′	66
B. bigemina	BigBA1R	5′-GAGGATCTATGCCTCCTAACATTATCCGTGA-3′	62
nPCR	BoF	5′-CACGAGGAAGGAACTACCGATGTTGA-3′	60
B. bovis	BoR	5′-CCAAGGAGCTTCAACGTACGAGGTCA-3′	61
	BoFN	5′-TCAACAAGGTACTCTATATGGCTACC-3′	56
	BoRN	5′-CTACCGAGCAGAACCTTCTTCACCAT-3′	60
nPCR	BiIA	5′-CATCTAATTTCTCTCCATACCCCTCC-3′	58
B. bigemina	BiIB	5′-CCTCGGCTTCAACTCTGATGCCAAAG-3′	61
	BiIAN	5′-CGCAAGCCCAGCACGCCCCGGTGC-3′	69
	BiIBN	5′-CCGACCTGGATAGGCTGTGTGATG-3′	61

Table 2

Comparison of methods in the detection of cattle infected with *B. bigemina* and/or *B. bovis* in Boane district, Mozambique.

Method / # samples	nPCR / 30	hotPCR / 30	hotPCR / 117
B. bigemina	25 (83%)	23 (77%)	90 (77%)
B. bovis	26 (87%)	23 (77%)	82 (70%)
Mixed infections	21 (70%)	17 (56%)	61 (52%)
No detection	0 (0%)	1 (3.3%)	6 (5.1%)

Figure1

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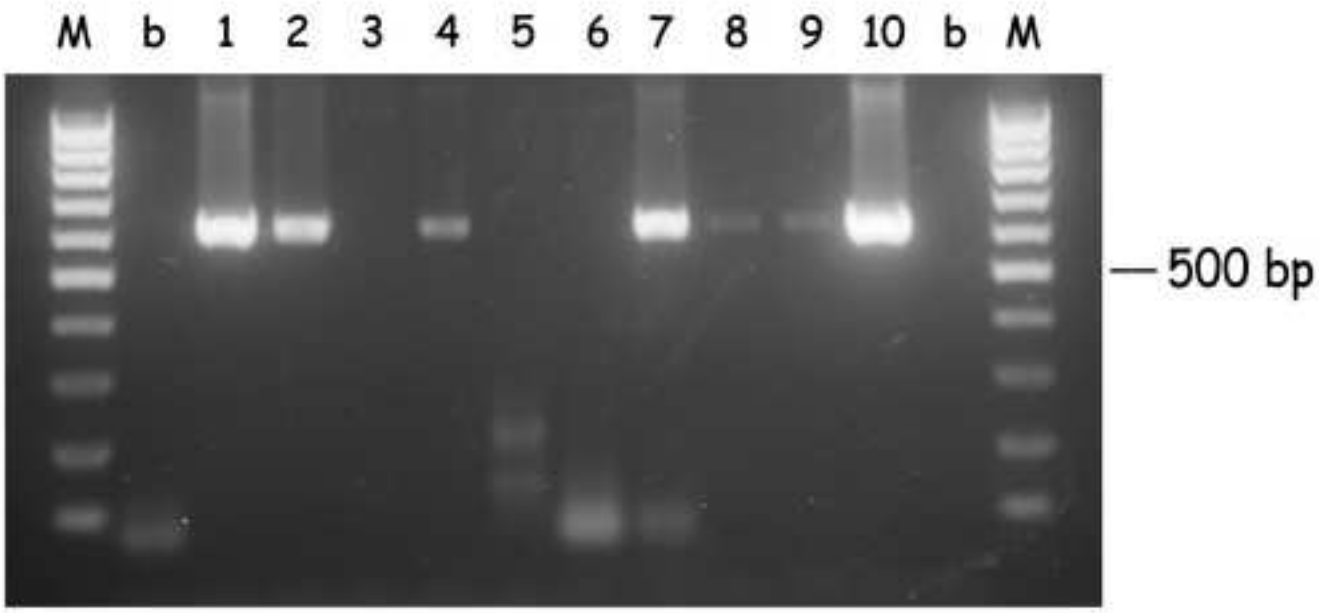

Figure2

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