1 **Application of conventional and real-time fluorescent ITS1 rDNA PCR for**

2 **detection of *Besnoitia besnoiti* infections in bovine skin biopsies**

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21 RUNNING TITLE: PCR and real-time PCR for *Besnoitia besnoiti*

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25 **We have developed ITS1 rDNA-sequence-based conventional and real-time**

26 **PCR (with an internal control) for sensitive specific and quantitative**

27 **detection of *Besnoitia besnoiti* infection in cattle. The assay, with**

28 **sensitivity equivalent to one *B. besnoiti*, also provides a tool to explore**

29 **parasite-host interaction and therapeutical aspects of *B. besnoiti***

30 **infections in experimental and natural infection.**

31

32   *Besnoitia besnoiti* is a cyst-forming coccidian parasite of cattle, mainly in

33   the sub-Saharan Africa, with high veterinary relevance (4,14). In Europe, it has

34   been recently reported in France (P. J Bourdeau, *et al.*, Abstr. IX European

35   Multicolloquium of Parasitology, pp 459-460, 2004), Spain (11,12) and Portugal

36   (5,6). The first clinical manifestations of the disease, consisting mainly of

37   respiratory disorders, are seldom recognised as *B. besnoiti* infection. The

38   subsequent chronic stage includes the formation of dermal lesions, dramatic

39   thickening, hardening and wrinkling of the skin, hyperkeratosis and alopecia and

40   leads to caquexia (1,3,14) and irreversible infertility in males (6).

41   Serological diagnosis of *B. besnoiti* infection using indirect

42   immunofluorescence, ELISA and western blot has been described (7,16,17).

43   However, detection of the parasite is exclusively based on visual observation of

44   cysts on the sub-conjuntiva (15) and on histopathology (2,10). The latter, based

45   on the morphological characteristics of the cyst wall (9), is specific and

46   conclusive but only applicable when the number of cysts is high. Here, we

47   describe a specific and sensitive conventional and a real-time ITS (internal

48   transcribed spacer) 1 rDNA PCR test which allows detection of the parasite in 8

49   mm diameter bovine skin biopsies through the amplification of parasite specific

50   DNA sequences.

51   Samples of DNA were extracted from skin using the DNAeasy™ tissue kit

52   system (Qiagen, Basel, Switzerland) with an additional step of three freezing-

53   thawing cycles prior to addition of ethanol in methodical step 4. Conventional

54   PCR was performed in a 25 µl mixture containing 2.5 µl 10xGene Amp™ PCR

55   buffer (Applied Biosystems, Basle, Switzerland), 0.2 mM each dATP, dGTP and

56   dCTP, 0.4 mM dUTP (Invitrogen, Dübendorf, Switzerland), 0.25 µM each *B.*

besnoitia-specific forward ITS1F (5'-TGACATTTAATAACAATCAACCCTT-3')
and reverse ITS1R1 (5'-GGTTTGTATTAACCAATCCGTGA-3') primers, 1.25
units of AmpliTaq™ DNA polymerase (Applied Biosystems) and 0.5 units of
heat-labile uracyl DNA glycosylase (UDG) (Roche Diagnostics, Basle,
Switzerland). To remove eventual dUTP containing carry-over contaminations
from previous diagnostic reactions, UDG and dUTP (instead of dTTP) was
included in the reaction mixture according to a method elaborated by Longo et
al. (13). For UDG-mediated decontamination prior to PCR, the reaction mixture
was initially incubated for 10 min at 20 °C. This incubation was followed by a 2
min incubation step at 95 °C to inactivate UDG and denature the DNA.
Subsequently, amplification was done in 45 cycles of denaturation at 94 °C for
30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 1 min; this was
followed by a final 15 min extension at 72°C and a 4 ºC hold at the completion of
the profile. As observed by agarose gel electrophoresis, the amplification
product of the conventional PCR had the expected size of 231 base pairs (bp)
(see Fig. 1).

To control for false-negative results, a recombinant PCR inhibition control
(13) was done with plasmid Bluescript KS plus (pBS+) (Stratagene) DNA using
chimeric primers containing the *B. besnoiti*-forward primer sequence plus a
sequence representing nt positions 986-1004 on the plasmid (chimeric forward
primer   BbICF: 5'-
TGACATTTAATAACAATCAACCCTTGAATCGGCCAACGCGCG-3)' and the
Besnoitia reverse primer sequence plus the reverse sequence from nt positions
1275-1293   on   the   pKS   (chimeric   reverse   primer   BbICR: 5'-
GGTTTGTATTAACCAATCCGTGATATAGTCCTGTCGGGTTTC-3').   These

82   chimeric primers produced a 355 bp pBS+ amplification product with the

83   *Besnoitia*-specific primer sequences incorporated at the ends. This amplification

84   product was then cloned into the pGEM™-Teasy vector (Promega) according to

85   the instructions of the manufacturer. About 10 molecules from the resulting

86   recombinant plasmid (subsequently referred to as inhibition control) were added

87   as a control to a duplicate from each sample reaction to monitor possible

88   inhibitory effects within the PCR (Fig. 1).

89   The real-time PCR in the LightCycler™ Instrument was performed with 1

90   µl of 1:10 diluted DNA sample (in absence and presence of inhibition control)

91   using the LightCycler DNA Master Hybridization Probes™ Kit (Roche

92   Diagnostics) in a standard reaction containing 0.25µM of each primer and

93   supplemented with 3 mM $MgCl_2$. After heat-activation of the Taq-polymerase

94   and simultaneous denaturation of DNA for 15 min at 95^oC, amplification was

95   done in 50 cycles (including denaturation: 95^oC, 15 s; annealing: 56^oC, 15 s;

96   extension: 72^oC, 30 s; ramp rates in all cycle steps were $20^oC/s$) with 1 µl of

97   1:10 diluted DNA samples. Fluorescence was measured after an increase of the

98   temperature to 82^oC at the end of each annealing phase in the "single" mode.

99   Fluorescence signals from the amplification products were quantitatively

100   assessed by applying the standard software (version 3.5.3) according to the

101   instructions for the LightCycler™ Instrument.

102   In order to determine the sensitivity of the conventional and the real-time

103   ITS1 rDNA PCR, amplification reactions on DNA equivalent to 10'000, 1'000,

104   100, 10, 1 and 0.1 in vitro propagated parasites (8) were performed. The

105   sensitivity of the amplification reactions was extremely high in that it consistently

106   allowed detection of 1 *B. besnoiti* cell by both conventional (not shown) and real-

107 time PCR (Fig. 2). The high specificity of the PCRs was demonstrated in that

108 exclusively *B. besnoiti* DNA was amplified from a panel of apicomplexan

109 parasite DNAs (*B. besnoiti, Neospora caninum, Toxoplasma gondii, Sarcocystis*

110 *neurona, S. cruzi, S. tenella, S. muris, S. spellei, S. miescheriana, S. zamari, S.*

111 *singapurencei, S. gigantea, S. moulei, S. capracanis, S. arieticanis, S. peeri*) as

112 well as from bovine genomic DNA (not shown).

113   Both, the conventional and the real-time ITS1 rDNA PCR were tested on

114 43 skin biopsies from *B. besnoiti*-infected and non-infected cattle from the South

115 of Portugal and selected after histopathological analysis (6) and indirect

116 immunofluorescence antibody test (IFAT) (16), defining three groups: (i) non-

117 infected animals as confirmed by negative IFAT and histopathology (21

118 animals), (ii) infected animals positive in IFAT and negative in histopathology (10

119 animals), and (iii) infected animals positive in both tests (12 animals). The latter

120 group contained one animal that exhibited macroscopic skin lesions. Only 3

121 samples (N^0 23, 35, and 37) were inhibitory i.e. negative in diagnostic PCR and

122 inhibitory in parallel inhibition control DNA reaction (Table 1). The analytical

123 features of inhibitory samples as well as non-inhibitory *B. besnoiti*-positive and

124 negative samples are exemplified in Fig 1. In contrast, none of the samples

125 inhibited the inhibition control reaction when tested by real-time PCR (Table 1).

126 The 12 samples that contained histologically detectable cysts (animals N^0 26,

127 29, 30, 31, 33, 34, 36, 39, 40, 41, 42, and 43) were positive in both diagnostic

128 PCR techniques. Significantly, 3/5 samples that were non-inhibitory by PCR,

129 and negative by histopathology (animals N^0 1, 16, 28, 32, and 38, see Table 1)

130 were positive by real time PCR (animals Nº 16, 28 and 32), emphasising the

131 great sensitivity of the PCR test. Interestingly all 3 animals had previously been

132   exposed to the parasite (titer $\geq$1:256 in IFAT, as previously described (16)). The

133   conventional PCR was somewhat less sensitive and only identified 2 of these

134   samples (animals N[0] 16 and 28, see Table 1) to be positive. Conversely, the 20

135   samples (N[0] 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 19, 20, 21, 22, and

136   25) that were non-inhibitory in PCR, and scored negative in IFAT-based

137   serology (see Table 1) were also negative in the two PCR tests.

138   In conclusion, the present study has demonstrated the practicability and

139   advantages of PCR-based diagnosis of *B. besnoiti* infections in bovine skin

140   samples, providing possible PCR-inhibitory effects of the samples are excluded.

141   The assays, particularly the real-time PCR are a useful improvement on current

142   procedures because they allow detection of *B. besnoiti* even in those skin

143   samples that were collected from sero-positive but subclinically infected animals.

144   As a quantitative assay, the real-time ITS1 rDNA PCR will be useful for

145   epidemiological, clinical and pharmacological studies, as well as for

146   investigations elucidating the consequences of immunological and (immuno-

147   )pathological effects on growth of the parasite in both natural and experimental

148   hosts.

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208

209 FIGURE LEGENDS

210

211 FIG. 1. Agarose gel-electrophoretic analysis (1% gels) of amplification products

212 from conventional *Besnoitia besnoiti* ITS1 rDNA PCR on skin biopsies (samples

213 21 to 30) from infected and non-infected cattle in absence (A) and presence (B)

214 of inhibition control DNA. Positive (P) and negative (N) PCR-controls are

215 included. On the left, the sizes of the amplification products are indicated in

216 base pairs (bp). Note that PCR-inhibition can be observed in sample 23.

217

218 FIG 2. Sensitvity of the real-time *Besnoitia besnoiti* ITS1 rDNA PCR. Results as

219 fluorescence signals, representing amplification reactions for 10'000, 1'000, 100,

220 10, 1 parasite(s) and a negative control (0 parasites) are presented. Dilutions of

221 DNA equivalent to < 1 cell (e.g. 0.1 cells) did not consistently result in a

222 detectable amplification reaction (not shown).

223

224 TABLE 1. Characterisitcs of animals included in this study

225

Animal no.	Histopathology/ Clin. manifest	IFAT[a] (titer)	Convent. PCR		Real-time PCR	
			Inhib.[b]	Result[c]	Inhib.[b]	Result[c]
1	-	1:1024	-	-	-	-
2	-	<1:128	-	-	-	-
3	-	<1:128	-	-	-	-
4	-	<1:128	-	-	-	-
5	-	<1:128	-	-	-	-
6	-	<1:128	-	-	-	-
7	-	<1:128	-	-	-	-
8	-	<1:128	-	-	-	-
9	-	<1:128	-	-	-	-
10	-	<1:128	-	-	-	-
11	-	<1:128	-	-	-	-
12	-	<1:128	-	-	-	-
13	-	<1:128	-	-	-	-
14	-	<1:128	-	-	-	-
15	-	<1:128	-	-	-	-
16	-	1:1024	-	+	-	+
17	-	<1:128	-	-	-	-
18	-	1:1024	-	-	-	-
19	-	<1:128	-	-	-	-
20	-	<1:128	-	-	-	-
21	-	<1:128	-	-	-	-
22	-	<1:128	-	-	-	-
23	-	<1:128	+	?	-	-
24	-	1:1024	-	-	-	-
25	-	<1:128	-	-	-	-
26	Cysts	1:1024	-	+	-	+
27	-	1:1024	-	-	-	-
28	-	1:1024	-	+	-	+
29	Cysts	1:1024	-	+	-	+
30	Cysts	1:1024	-	+	-	+
31	Cysts	1:1024	-	+	-	+
32	-	1:512	-	-	-	+
33	Cysts	1:512	-	+	-	+
34	Cysts	1:512	-	+	-	+
35	-	1:1024	+	?	-	-
36	Cysts	1:1024	-	+	-	+
37	-	1:1024	+	?	-	-
38	-	1:1024	-	-	-	-
39	Cysts	1:512	-	+	-	+
40	Cysts	1:1024	-	+	-	+
41	Cysts	1:1024	-	+	-	+
42	Cysts	1:1024	-	+	-	+
43	Cysts/disease	1:1024	-	+	-	+

226 [a]In the IFAT, sera with a titer $\geq$1:256 were scored positive
227 [b]PCRs with (+) or without (-) inhibition of amplification reaction
228 [c]Positive (+) or negative (-) PCR results or questionable (?) result due to PCR inhibition
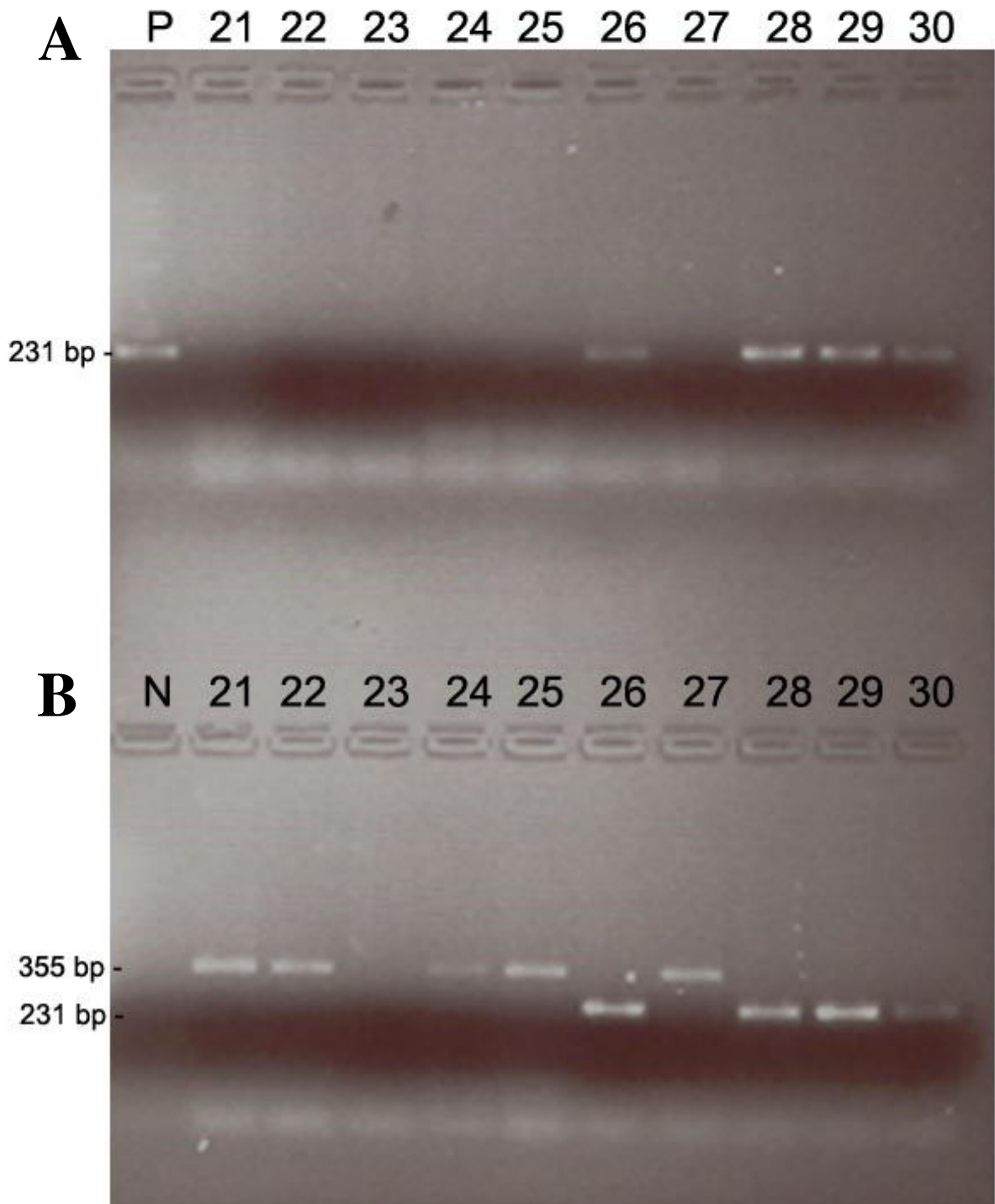229
230

Figure 1

Figure 2