Application of conventional and real-time fluorescent ITS1 rDNA PCR for detection of *Besnoitia besnoiti* infections in bovine skin biopsies

HELDER C. E. CORTES[1*], YARA REIS[2], BRUNO GOTTSTEIN[3], ANDREW HEMPHILL[3], ALEXANDRE LEITÃO[2] AND NORBERT MÜLLER[3*]

[1*] Laboratório de Parasitologia, Núcleo da Mitra, ICAM, Universidade de Évora, Apartado 94, 7000-554 Évora, Portugal

[2] Instituto de Investigação Científica Tropical, CIISA, Faculdade de Medicina Veterinária, Av. da Universidade Técnica, 1300-447 Lisboa, Portugal.

[3] Institute of Parasitology, Länggass-Str. 122, P.O. Box 8466, CH-3001 Berne, Switzerland.

*Corresponding authors: Phone: 00351 266760860, Fax: 00351 266 760912 Electronic mail address: hcec@uevora.pt (HCEC); Phone: 0041 31 631 2474; Fax. 0041 31 631 2477; email: nmueller@ipa.unibe.ch (NM)

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

We have developed ITS1 rDNA-sequence-based conventional and real-time PCR (with an internal control) for sensitive specific and quantitative detection of *Besnoitia besnoiti* infection in cattle. The assay, with sensitivity equivalent to one *B. besnoiti*, also provides a tool to explore parasite-host interaction and therapeutical aspects of *B. besnoiti* infections in experimental and natural infection.

Besnoitia besnoiti is a cyst-forming coccidian parasite of cattle, mainly in the sub-Saharan Africa, with high veterinary relevance (4,14). In Europe, it has been recently reported in France (P. J Bourdeau, *et al*., Abstr. IX European Multicolloquium of Parasitology, pp 459-460, 2004), Spain (11,12) and Portugal (5,6). The first clinical manifestations of the disease, consisting mainly of respiratory disorders, are seldom recognised as *B. besnoiti* infection. The subsequent chronic stage includes the formation of dermal lesions, dramatic thickening, hardening and wrinkling of the skin, hyperkeratosis and alopecia and leads to caquexia (1,3,14) and irreversible infertility in males (6).

Serological diagnosis of *B. besnoiti* infection using indirect immunofluorescence, ELISA and western blot has been described (7,16,17). However, detection of the parasite is exclusively based on visual observation of cysts on the sub-conjuntiva (15) and on histopathology (2,10). The latter, based on the morphological characteristics of the cyst wall (9), is specific and conclusive but only applicable when the number of cysts is high. Here, we describe a specific and sensitive conventional and a real-time ITS (internal transcribed spacer) 1 rDNA PCR test which allows detection of the parasite in 8 mm diameter bovine skin biopsies through the amplification of parasite specific DNA sequences.

Samples of DNA were extracted from skin using the DNAeasy™ tissue kit system (Qiagen, Basel, Switzerland) with an additional step of three freezing-thawing cycles prior to addition of ethanol in methodical step 4. Conventional PCR was performed in a 25 µl mixture containing 2.5 µl 10xGene Amp™ PCR buffer (Applied Biosystems, Basle, Switzerland), 0.2 mM each dATP, dGTP and 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 ℃. This incubation was followed by a 2 min incubation step at 95 ℃ to inactivate UDG and denature the DNA. Subsequently, amplification was done in 45 cycles of denaturation at 94 ℃ for 30 s, annealing at 58 ℃ for 30 s, and extension at 72 ℃ for 1 min; this was followed by a final 15 min extension at 72℃ 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

chimeric primers produced a 355 bp pBS+ amplification product with the *Besnoitia*-specific primer sequences incorporated at the ends. This amplification product was then cloned into the pGEM™-Teasy vector (Promega) according to the instructions of the manufacturer. About 10 molecules from the resulting recombinant plasmid (subsequently referred to as inhibition control) were added as a control to a duplicate from each sample reaction to monitor possible inhibitory effects within the PCR (Fig. 1).

The real-time PCR in the LightCycler™ Instrument was performed with 1 µl of 1:10 diluted DNA sample (in absence and presence of inhibition control) using the LightCycler DNA Master Hybridization Probes™ Kit (Roche Diagnostics) in a standard reaction containing 0.25µM of each primer and supplemented with 3 mM $MgCl_2$. After heat-activation of the Taq-polymerase and simultaneous denaturation of DNA for 15 min at 95°C, amplification was done in 50 cycles (including denaturation: 95°C, 15 s; annealing: 56°C, 15 s; extension: 72°C, 30 s; ramp rates in all cycle steps were 20°C/s) with 1 µl of 1:10 diluted DNA samples. Fluorescence was measured after an increase of the temperature to 82°C at the end of each annealing phase in the “single“ mode. Fluorescence signals from the amplification products were quantitatively assessed by applying the standard software (version 3.5.3) according to the instructions for the LightCycler™ Instrument.

In order to determine the sensitivity of the conventional and the real-time ITS1 rDNA PCR, amplification reactions on DNA equivalent to 10’000, 1’000, 100, 10, 1 and 0.1 in vitro propagated parasites (8) were performed. The sensitivity of the amplification reactions was extremely high in that it consistently allowed detection of 1 *B. besnoiti* cell by both conventional (not shown) and real-

time PCR (Fig. 2). The high specificity of the PCRs was demonstrated in that exclusively *B. besnoiti* DNA was amplified from a panel of apicomplexan parasite DNAs (*B. besnoiti*, *Neospora caninum, Toxoplasma gondii, Sarcocystis neurona, S. cruzi, S. tenella, S. muris, S. spellei, S. miescheriana, S. zamari, S. singapurencei, S. gigantea, S. moulei, S. capracanis, S. arieticanis, S. peeri*) as well as from bovine genomic DNA (not shown).

Both, the conventional and the real-time ITS1 rDNA PCR were tested on 43 skin biopsies from *B. besnoiti*-infected and non-infected cattle from the South of Portugal and selected after histopathological analysis (6) and indirect immunofluorescence antibody test (IFAT) (16), defining three groups: (i) non-infected animals as confirmed by negative IFAT and histopathology (21 animals), (ii) infected animals positive in IFAT and negative in histopathology (10 animals), and (iii) infected animals positive in both tests (12 animals). The latter group contained one animal that exhibited macroscopic skin lesions. Only 3 samples (N^0 23, 35, and 37) were inhibitory i.e. negative in diagnostic PCR and inhibitory in parallel inhibition control DNA reaction (Table 1). The analytical features of inhibitory samples as well as non-inhibitory *B. besnoiti*-positive and negative samples are exemplified in Fig 1. In contrast, none of the samples inhibited the inhibition control reaction when tested by real-time PCR (Table 1). The 12 samples that contained histologically detectable cysts (animals N^0 26, 29, 30, 31, 33, 34, 36, 39, 40, 41, 42, and 43) were positive in both diagnostic PCR techniques. Significantly, 3/5 samples that were non-inhibitory by PCR, and negative by histopathology (animals N^0 1, 16, 28, 32, and 38, see Table 1) were positive by real time PCR (animals Nº 16, 28 and 32), emphasising the great sensitivity of the PCR test. Interestingly all 3 animals had previously been

exposed to the parasite (titer $\geq$1:256 in IFAT, as previously described (16)). The conventional PCR was somewhat less sensitive and only identified 2 of these samples (animals N^0 16 and 28, see Table 1) to be positive. Conversely, the 20 samples (N^0 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 17, 19, 20, 21, 22, and 25) that were non-inhibitory in PCR, and scored negative in IFAT-based serology (see Table 1) were also negative in the two PCR tests.

In conclusion, the present study has demonstrated the practicability and advantages of PCR-based diagnosis of *B. besnoiti* infections in bovine skin samples, providing possible PCR-inhibitory effects of the samples are excluded. The assays, particularly the real-time PCR are a useful improvement on current procedures because they allow detection of *B. besnoiti* even in those skin samples that were collected from sero-positive but subclinically infected animals. As a quantitative assay, the real-time ITS1 rDNA PCR will be useful for epidemiological, clinical and pharmacological studies, as well as for investigations elucidating the consequences of immunological and (immuno-)pathological effects on growth of the parasite in both natural and experimental hosts.

We are deeply thankful to Michael Parkhouse for critical reviewing and providing valuable suggestions about the manuscript. This work was supported by CIISA, ICAM, and the ”Hans-Sigrist”-Foundation, and was part of the EU research collaboration COST Action 854.

REFERENCES

1. **Basson, P. A., R. M. McCully, and R. D. Bigalke**. 1970. Observations on the pathogenesis of bovine and antelope strains of *Besnoitia besnoiti* (Marotel, 1912) infection in cattle and rabbits. Onderstepoort J.Vet.Res. **37**:105-126.

2. **Besnoit, C. and V. Robin**. 1912. Sarcosporidioses cutanée chez une vache. Rec.Vet. **37**:649.

3. **Bigalke, R. D.** 1960. Preliminary observation on the mechanical transmission of cyst organisms of *Besnoitia besnoiti* (Marotel, 1912) from a chronically infected bull to rabbits by *Glossina brevipalpis* Newstead, 1910. J.S.Afr.Vet.Assoc. **31**:37-44.

4. **Bigalke, R. D., J. W. van Niekerk, P. A. Basson, and R. M. McCully**. 1967. Studies on the relationship between *Besnoitia* of blue wildebeest and impala, and *Besnoitia besnoiti* of cattle. Onderstepoort J.Vet.Res. **34**:7-28.

5. **Cortes, H., M. L. Ferreira, J. F. Silva, R. Vidal, P. Serra, and V. Caeiro**. 2003. Contribuição para o estudo da besnoitiose bovina em Portugal. Revista Portuguesa de Ciências Veterinárias **98, nº 545**:43-46.

6. **Cortes, H., A. Leitão, R. Vidal, M. J. Vila-Vicosa, M. L. Ferreira, V. Caeiro, and C. A. Hjerpe**. 2005. Besnoitiosis in bulls in Portugal. Vet Rec. **157**:262-264.

7. **Cortes, H., S. Nunes, Y. Reis, D. Staubli, D. Vidal, H. Sager, A. Leitão, and B. Gottstein**. 2006. Improved immunodiagnosis of *Besnoitia*

besnoiti-infection in cattle by the use of ELISA and Western blot. Vet Parasitol. **in press**.

8. **Cortes, H. C. E., Y. Reis, H. Waap, R. Vidal, H. Soares, I. Marques, I. Pereira da Fonseca, I. Fazendeiro, M. L. Ferreira, V. Caeiro, V. Shkap, A. Hemphill, and A. Leitão**. 2006. Isolation of *Besnoitia besnoiti* from infected cattle in Portugal. Vet Parasitol. **In press**.

9. **Dubey, J. P., V. Shkap, E. Pipano, L. Fish, and D. L. Fritz**. 2003. Ultrastructure of *Besnoitia besnoiti* tissue cysts and bradyzoites. J.Eukaryot.Microbiol. **50**:240-244.

10. **Franco, E. E. and I. Borges**. 1915. Nota sobre a sarcosporidiose bovina. Revista de Medicina Veterinária **Ano XIV**:255-268.

11. **Irigoien, M., E. Del Cacho, M. Gallego, F. Lopez-Bernad, J. Quilez, and C. Sanchez-Acedo**. 2000. Immunohistochemical study of the cyst of *Besnoitia besnoiti*. Vet.Parasitol. **91**:1-6.

12. **Juste, R. A., L. A. Cuervo, J. C. Marco, and L. M. Oregui**. 1990. La besnoitiosis bovina: desconocida en España? Medicina Veterinária **7**:613-618.

13. **Longo, M. C., M. S. Berninger, and J. L. Hartley**. 1990. Use of uracil DNA glycosylase to control carry-over contamination in polymerase chain reactions
1. Gene **93**:125-128.

14. **Pols, J. W.** 1960. Studies on Bovine besnoitiosis with special reference to the aetiology. Onderstepoort J.Vet.Res. **28**:265-356.

15. **Sannusi, A.** 1991. A simple field diagnostic smear test for bovine besnoitiosis. Vet.Parasitol. **39**:185-188.

16. **Shkap, V., A. Reske, E. Pipano, L. Fish, and T. Baszler**. 2002. Immunological relationship between *Neospora caninum* and *Besnoitia besnoiti*. Vet.Parasitol. **106**:35-43.

17. **Shkap, V., H. Ungar-Waron, E. Pipano, and C. Greenblatt**. 1984. Enzyme linked immunosorbent assay for detection of antibodies against *Besnoitia besnoiti* in cattle. Trop.Anim Health Prod. **16**:233-238.

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 ≥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

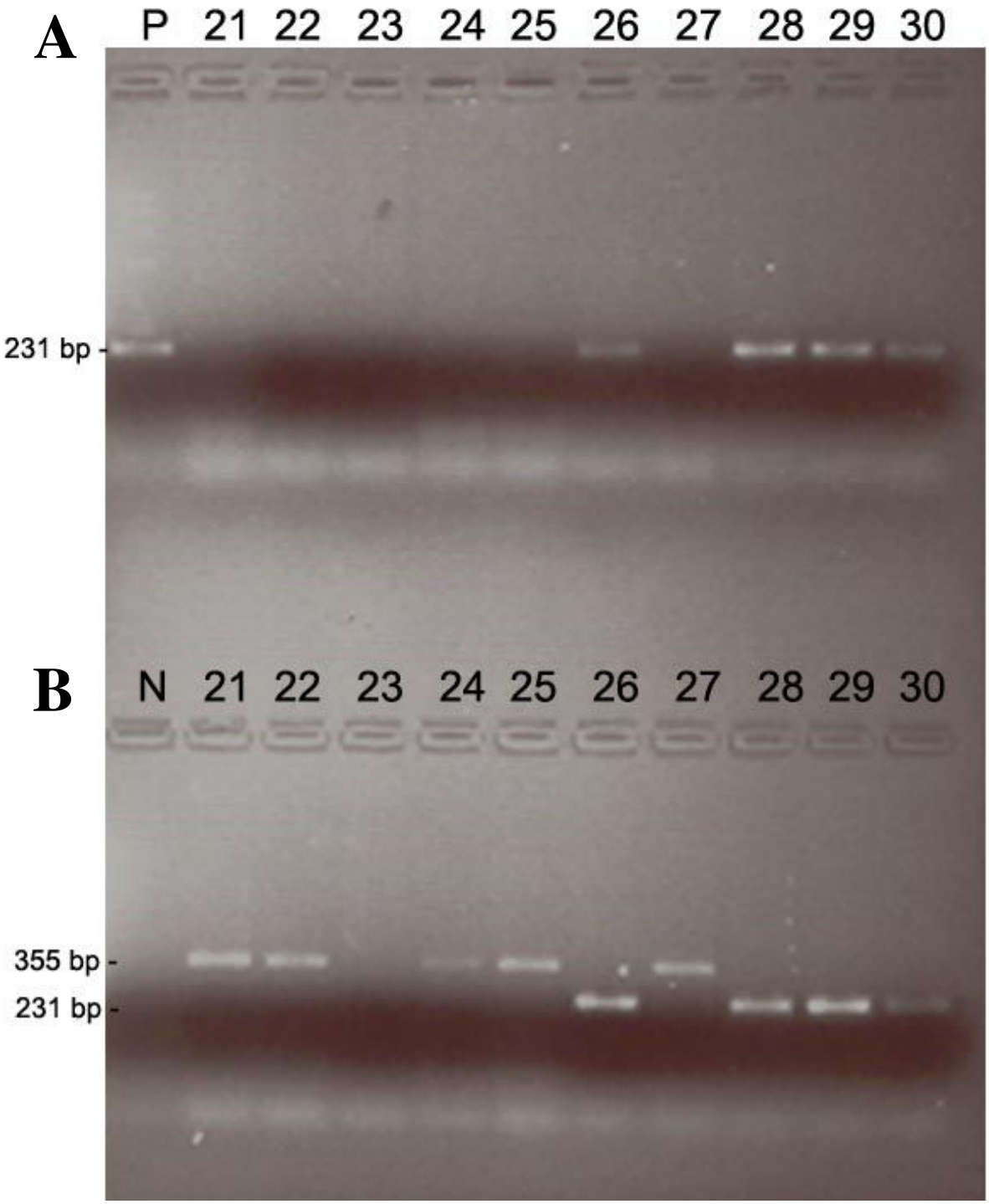
A
P 21 22 23 24 25 26 27 28 29 30
231 bp
B
N 21 22 23 24 25 26 27 28 29 30
355 bp
231 bp

Figure 2