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Short communication

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

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Abstract

Besnoitia besnoiti, an apicomplexan protozoan parasite, is the causative agent of bovine besnoitiosis. This infection may dramatically affect body condition, and, in males, lead to irreversible infertility. While identification of clinical cases and their histopathological confirmation is relatively simple to carry out, finding subclinical forms of infection is more difficult, thus a more sensitive test for the identification of the etiological agent may be an appropriate diagnostic tool. We have developed the ITS1 rDNA-sequence-based conventional and real-time PCR which are highly sensitive and specific for the detection of *B. besnoiti* infection in cattle. A recombinant internal positive control was introduced to assess possible sample-related inhibitory effects during the amplification reaction and, in order to prevent false-positive results, a pre-PCR treatment of potentially contaminating dU-containing PCR product with uracyl-DNA-glycosylase (UDG) was followed.

Keywords: *Besnoitia besnoiti*; PCR; Real-time PCR; Diagnosis; Cattle

Besnoitia besnoiti is a cyst-forming coccidian parasite of cattle, mainly in the sub-Saharan Africa, with high veterinary relevance (Bigalke et al., 1967; Pols, 1960). In Europe, it has been recently reported in France (Bourdeau et al., 2004), Spain (Irigoien et al., 2000; Juste et al., 1990) and Portugal (Cortes et al., 2003, 2005). 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 (Basson et al., 1970; Bigalke, 1960; Pols, 1960) and irreversible infertility in males (Cortes et al., 2005).

Serological diagnosis of *B. besnoiti* infection using indirect immunofluorescence, ELISA and western blot has been described (Cortes et al., 2006a; Shkap et al., 1984, 2002). However, detection of the parasite is exclusively based on visual observation of cysts on the sub-conjuntiva (Sannusi, 1991) and on histopathology (Besnoit and Robin, 1912; Franco and Borges, 1915a,b). The latter, based on the morphological

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characteristics of the cyst wall (Dubey et al., 2003), is specific and conclusive but only applicable when the number of cysts is high.

Here, based on the fact that ITS1 is a valuable species-specific marker for members of the Toxoplasmatinae (Tenter et al., 2002), 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 bovine skin biopsies through the amplification of parasite specific DNA sequences.

DNA was prepared from skin biopsies 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. For convenience and to favour swift regeneration, 8 mm diameter biopsies, sufficient for 200 tests, were collected from the neck. Conventional PCR was performed in a 25 μl mixture containing 2.5 μl 10× Gene 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 ITS1R (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. (1990). 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 °C 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 (Longo et al., 1990) 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

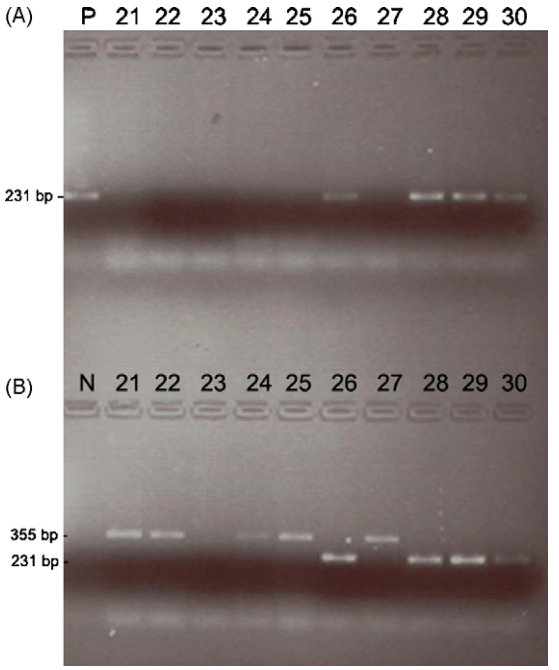

Fig. 1. Agarose gel-electrophoretic analysis (1% gels) of amplification products from conventional *Besnoitia besnoiti* ITS1 rDNA PCR on skin biopsies (samples 21–30) from infected and non-infected cattle in absence (A) and presence (B) of inhibition control DNA. Positive (P) and negative (N) PCR-controls are included. On the left, the sizes of the amplification products are indicated in base pairs (bp). Note that PCR-inhibition can be observed in sample 23.

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 QuantTect SYBR Geen PCR Kit (Qiagen) in a standard reaction containing 0.25 μM 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