Veterinary Parasitology 178 (2011) 208–216

Contents lists available at ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

Quantitative real time polymerase chain reaction assays for the sensitive detection of *Besnoitia besnoiti* infection in cattle

G. Schares[a,*], A. Maksimov[a], W. Basso[a,b,c,d], G. Moré[b,c], J.P. Dubey[e], B. Rosenthal[e], M. Majzoub[f], A. Rostaher[g], J. Selmair[h], M.C. Langenmayer[f,i], J.C. Scharr[i], F.J. Conraths[a], N.S. Gollnick[i]

[a] *Friedrich-Loeffler-Institut, Federal Research Institute for Animal Health, Institute of Epidemiology, Wusterhausen, Germany*
[b] *Laboratorio de Inmunoparasitología, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, La Plata, Argentina*
[c] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina*
[d] *Institute of Parasitology, University of Zürich, Zürich, Switzerland*
[e] *Animal Parasitic Disease Laboratory, Agricultural Research Service, USDA, Beltsville, Maryland, USA*
[f] *Institute of Veterinary Pathology, Ludwig Maximilian University, Munich, Germany*
[g] *Centre for Clinical Veterinary Medicine, Ludwig Maximilian University, Munich, Germany*
[h] *Inning am Holz, Germany*
[i] *Clinic for Ruminants with Ambulatory and Herd Health Services, Ludwig Maximilian University, Munich, Germany*

ARTICLE INFO

Article history:
Received 24 September 2010
Received in revised form 6 January 2011
Accepted 17 January 2011

Keywords:
Besnoitiosis
Besnoitia besnoiti
Besnoitia bennetti
Besnoitia tarandi
Real time PCR
Diagnosis

ABSTRACT

Bovine besnoitiosis, an economically important disease in cattle in some countries of Africa and Asia, is emerging in Europe. The definitive host of *Besnoitia besnoiti*, the causative agent of bovine besnoitiosis, is unknown and the transmission of the parasite is not completely understood. Sensitive and quantitative DNA detection methods are needed to determine whether serologically positive animals are infectious and to examine the role of vectors (e.g. haematophagous insects) in the transmission of the parasite. To this end, we established two different 5′-nuclease quantitative assays to detect *B. besnoiti* infection in cattle and to estimate the parasite load in samples (BbRT1 and BbRT2). These PCRs are based on the sequence of the internal transcribed spacer region 1 (ITS-1) of the ribosomal RNA gene. Tests with serial dilutions of *B. besnoiti* genomic DNA in a buffer containing 100 ng/μl bovine DNA revealed a detection limit of 0.01 pg genomic *B. besnoiti* DNA. Reliable quantification was possible in samples containing ≥1 pg *B. besnoiti* genomic DNA with a coefficient of variation of ≤2%. To estimate the diagnostic sensitivity of the tests, skin biopsies and scrapings from the mucous membrane of the vestibulum vaginae (vaginal scrapings) were taken from cattle with clinical signs of chronic besnoitiosis. Regardless of the real time PCR assay used, 90.7% (39/43) of these animals were positive in at least one of two samples (skin or vaginal scrapings). Antibody titers, as determined by an immunofluorescent antibody test, and the threshold cycle values of the real time PCR obtained for skin samples and vaginal scrapings, were significantly correlated. The specificity of the PCRs was confirmed using genomic DNA from related parasites, including genomic DNA of *Besnoitia* spp., *Neospora caninum*, *Toxoplasma gondii*, *Hammondia hammondi*, *Hammondia heydorni*, *Isospora* spp., *Sarcocystis* spp., *Eimeria bovis*, *Cryptosporidium parvum*, and *Trypanosoma brucei brucei*. Since the sequence of the ITS-1 region of *B. besnoiti* is identical with that of Besnoitia species isolated from donkeys (*Besnoitia bennetti*), and reindeer (*Besnoitia tarandi*), both real time PCRs detected also DNA of these parasites. One of the *B. besnoiti* real time PCRs, BbRT1, but not BbRT2, cross-reacted

* Corresponding author at: Federal Research Institute for Animal Health, Institute of Epidemiology, Seestraße 55, D-16868 Wusterhausen, Germany. Tel.: +49 33979 80193; fax: +49 33979 80222.
E-mail address: gereon.schares@fli.bund.de (G. Schares).

0304-4017/$ – see front matter
doi:10.1016/j.vetpar.2011.01.038

with *Besnoitia darlingi*, *Besnoitia oryctofelisi*, and *Besnoitia neotomofelis* when large amounts of genomic DNA (10 ng) were used. The other *B. besnoiti* real time PCR assay (BbRT2) was specific for *B. besnoiti*, *B. bennetti* and *B. tarandi*, but did not react when 10 ng DNA of other related parasite species from the genus Besnoitia or other genera were subjected to analysis.

1. Introduction

Besnoitia spp. are cyst-forming apicomplexan parasites closely related to *Toxoplasma gondii*, *Hammondia hammondi*, *Neospora caninum*, and *Hammondia heydorni*. *Besnoitia besnoiti* is the causative agent of bovine besnoitiosis, a severe but often non-fatal disease with a significant economic impact in some countries of Africa and Asia. The mortality of bovine besnoitiosis is usually low. Bovine besnoitiosis is spreading in Europe, as recently reported by the European Food Safety Authority (http://www.efsa.europa.eu/en/scdocs/scdoc/1499.htm). In addition to *B. besnoiti*, there are several other species within the genus Besnoitia infecting goats, equids, reindeer, caribou, opossums, rabbits, rodents, and lizards (Dubey et al., 2003; Dubey and Yabsley, 2010). The validity of some reported species has been questioned. The complete life cycles of only four species within the genus Besnoitia (*Besnoitia darlingi*, *Besnoitia wallacei*, *Besnoitia oryctofelisi*, and *Besnoitia neotomofelis*) are known, and the domestic cat serves as definitive host for these species (Dubey et al., 2002, 2003; Dubey and Yabsley, 2010). As judged by DNA sequences coding for the ribosomal RNA, *B. besnoiti*, *Besnoitia bennetti*, *Besnoitia tarandi* and *Besnoitia caprae* are phylogenetically very closely related (identities approximately 99%) (Ellis et al., 2000; Dubey et al., 2004, 2005). Despite these evident phylogenetic affinities, these parasites differ in their host ranges, a fact which supports their recognition as distinct species. Besnoitia parasites isolated from cattle were not infectious for horses and parasites of this genus isolated from horses failed to infect cattle (Pols, 1960; Bigalke, 1970). Gerbils (*Meriones unguiculatus*) and rabbits are less susceptible to *B. tarandi* and *B. bennetti* than to *B. besnoiti* infections (Dubey et al., 2004, 2005; Bigalke, 1968). Njenga et al. (1993) reported that *B. caprae* was not infectious for cattle or rabbits.

B. besnoiti infection in cattle is characterized by two subsequent phases, the acute and the chronic phase. Acutely infected cattle may have pyrexia, nasal and ocular discharge, salivation, stiff gait, and in severe cases clinically apparent subcutaneous edema. In chronically infected cattle, the skin may become severely lichenified and alopecic. Bulls can develop orchitis, which may result in temporary or permanent infertility (Bigalke, 1968). However, most infected cattle remain asymptomatic (Bigalke, 1968). Detection of acutely infected cattle is difficult since clinical signs are often not characteristic. Antibodies, as well as parasite stages in tissues, may appear only in a later stage of the infection. The presence of tissue cysts containing bradyzoites in the scleral conjunctiva, the mucous membrane of the vestibulum vaginae or the skin, which may be identified macroscopically during clinical examination or microscopically by histological or cytological techniques, enables a first diagnosis (Cortes et al., 2006; Rostaher et al., 2010). Diagnosis can later on be confirmed by the detection of specific antibodies in a variety of serological tests.

Conventional PCRs and SYBR®Green-based real time PCRs have been developed for diagnostic and research purposes, e.g. to identify drugs which may be suitable to treat infected intermediate hosts (Cortes et al., 2006, 2007) or to control parasite purification (Elsheikha et al., 2006). However, quantitative PCR assays are not only needed for these purposes, but also for experimental or field studies aiming to elucidate the role of chronically infected animals or vectors in transmission of the infection.

The aims of our study were thus to develop a 5′-nuclease assay for the specific and quantitative detection of *B. besnoiti*, and to validate this assay for diagnostic purposes in chronically *B. besnoiti*-infected cattle. To this end, two primer/probe combinations were selected and comparatively validated in the present study.

2. Material and methods

2.1. Parasites

Tachyzoites of *B. besnoiti* (Bb1Evora03), *N. caninum* (NC-1) and *T. gondii* (Me49) were cultivated in Vero cells, isolated and purified as reported previously (Schares et al., 2010). *B. tarandi* (BE-21 clone G7m-454, reindeer, Finland), *B. bennetti* (Michigan, US), *B. darlingi* (Michigan, US), *B. neotomofelis* (Texas, US), *B. oryctofelisi* (Argentina) were collected as previously described (Dubey et al., 2002, 2003, 2004; Elsheikha et al., 2004, 2005; Dubey and Yabsley, 2010). Bradyzoites of *Sarcocystis cruzi* (Argentina) and *Sarcocystis aucheniae* (Argentina) were obtained from infected tissues from naturally infected cattle and lamas, respectively (Moré et al., 2008; Moré et al., unpublished). *Sarcocystis miescheriana* DNA (Switzerland) was isolated from the myocardium of a pig with acute naturally acquired sarcocystosis (Caspari et al., 2010). Oocysts of H. *heydorni*, *H. hammondi*, *Cystoisospora* spp., *Eimeria bovis* (all from Germany) were obtained by sucrose flotation from the feces of dogs, cats and cattle as reported previously (Schares et al., 2005). Oocysts of *Cryptosporidium parvum* (Switzerland) were collected from a naturally infected calf (kindly provided by Dr. C. Lippuner, Institute of Parasitology Zürich, Switzerland). *Trypanosoma brucei brucei* (Uganda; CP2469; 5×10^7), purified from the blood of infected mice using DEAE cellulose (Lanham & Godfrey, 1970) were kindly provided by Dr. P.-H. Clausen, Free University, Berlin, Germany.

2.2. Tissues, blood and sera from cattle chronically infected with B. besnoiti

Tissues and blood from chronically infected female cattle ($n = 43$; 4.0–155.6 months old; mean age, 56.7 months)

were obtained from a herd in Germany where clinical bovine besnoitiosis was first diagnosed in September 2008 (Rostaher et al., 2010). Tissue cysts (about 0.5 mm in diameter) were observed in the scleral conjunctiva of one or both eyes and/or in the mucous membrane of the vestibulum vaginae in all 43 animals at clinical examination in November 2008 (Schares et al., 2009). Blood was taken from a jugular or the tail vein and either collected in 5 ml tubes supplemented with EDTA (for DNA extraction) or without supplementation (for serum collection). Serum was obtained and conserved at −20 °C until use.

At least two tissue biopsies (one for DNA extraction, one for histology) were taken from the left hind limb (femoral region) of these animals using biopsy punches of 0.8 cm diameter. This site was selected because it was readily accessible when the animals were restrained in a cattle crush. The maximal distance between the locations where the biopsies were taken was 5 cm. The tissue biopsies of all animals were further examined using PCR and histological techniques.

2.3. DNA extraction

DNA was extracted from tissue biopsies using a commercial kit (NucleoSpin Tissue, Macherey and Nagel, Düren, Germany). According to the instructions of the manufacturer, 25 mg aliquots of the tissue biopsies were processed after an over-night incubation with proteinase K. DNA from isolated parasites provided by other laboratories was extracted with two commercial kits (*B. besnoiti*, *N. caninum*, *T. gondii*, *T. brucei brucei*: NucleoSpin Tissue, Macherey and Nagel, Düren, Germany; *B. oryctofelisi*, *B. bennetti*, *B. tarandi*, *B. darlingi*, *B. neotomofelis*, *S. cruzi*, *S. aucheniae*: DNeasy Tissue Kit, QIAGEN, Hilden, Germany). The digestion buffer of the respective kit served as a negative processing control.

EDTA blood was centrifuged (800 × *g*, 10 min) and the interphase between plasma and blood cells (buffy coat, about 500 μl) collected in a separate tube. The red blood cells were lysed by the addition of 5 ml erythrocyte lysis buffer (Geys' buffer, pH 7.0; 7.3 g NH_4Cl; 0.2 g KCl; 1.15 g $Na_2HPO_4{\cdot}2H_2O$; 0.16 g KH_2PO_4; 0.1 g $MgCl_2{\cdot}6H_2O$; 0.1 g $CaCl_2$ in 1 l distilled water). After centrifugation (800 × *g*, 10 min), the pellet was washed three times with 2–3 ml erythrocyte lysis buffer. DNA was extracted from half of the final pellet using a commercial kit (NucleoSpin Blood, Macherey and Nagel, Düren, Germany) according to the instructions of the manufacturer. The digestion buffer of the kit served as a negative processing control. DNA from oocysts (10^4–10^5) of *H. hammondi*, *H. heydorni*, *Isospora* spp., *Sarcocystis* spp., *E. bovis*, and *C. parvum* was extracted as previously described (Schares et al., 2005).

2.4. Conventional PCRs

PCR primers were used at a final concentration of 0.5 μM and dNTPs at 250 μM each (Amersham Biosciences, Piscataway, USA). DyNAzyme II DNA polymerase (Finnzymes, Espoo, Finland) was added at 1 U/25 μl with the provided buffer. The reaction mix was supplemented with bovine serum albumin at a concentration of 20 μg/ml. Water PCR Reagent (Sigma–Aldrich, Taufkirchen, Germany) served as a negative control. The reactions were performed in a thermal cycler (Eppendorf Mastercycler, Personal Thermal Cycler, Hanover, Germany). Amplification products were visualized after electrophoresis in 1.5% agarose gels stained with ethidium bromide. A 100 bp DNA ladder (Invitrogen GmbH, Karlsruhe, Germany) served as a size standard.

The presence of genomic DNA of coccidian parasites (*Besnoitia* spp., *T. gondii*, *H. hammondi*, *N. caninum*, *H. heydorni*, *Isospora* spp., *Sarcocystis* spp., *E. bovis*, *C. parvum*), was confirmed using COC-1 and COC-2 primers, which are specific for the coccidian 18S rRNA gene, under published cycling conditions (Ho et al., 1996; Schares et al., 2005). The presence of *T. brucei brucei* was confirmed by kinetoplastid-specific primers (KIN1, KIN2) using previously published cycling conditions (Desquesnes et al., 2002). Two ng/μl of genomic DNA were used as template. DNA from cell cultured *B. besnoiti* (Bb1Evora03) tachyzoites was used as a positive control (Schares et al., 2008) except in the kinetoplastid PCR, where no positive control was available.

For the specific detection of *B. besnoiti*, the primer pair Bb-ITS1-F/Bb-ITS1-R was used (Cortes et al., 2006; Schares et al., 2009). DNA from cell cultured *B. besnoiti* (Bb1Evora03) tachyzoites was used as a positive control (Schares et al., 2008). The reactions were performed in a thermal cycler (Eppendorf Mastercycler, Personal Thermal Cycler, Hanover, Germany) with an initial denaturation step of 95 °C for 5 min, followed by 35 cycles of denaturation (95 °C, 0.5 min), annealing (65 °C, 0.5 min) and extension (72 °C, 0.5 min), followed by a final extension step at 72 °C for 5 min.

2.5. Real time PCR

Suitable primers and probes (Table 1) for two 5′-nuclease quantitative real time PCR assays (BbRT1 and BbRT2) were selected with software tools available at https://ecom.mwgdna.com. For this purpose the sequences of *B. besnoiti*, *B. bennetti*, *B. tarandi*, *B. caprae*, *B. akodoni*, *B. oryctofelisi*, *B. jellisoni*, *N. caninum*, *H. heydorni*, *T. gondii*, and *H. hammondi* were downloaded from GenBank™. Locations of primers and probes are displayed in Fig. 1A and B.

Table 1
Primers, probes and their final concentrations in the real time PCR assays.

Assay	Names of probes and primers	Sequences of probes and primers	Modifications of probes	Final concentration
BbRT1	Bb11	CAA TCA ACC CTT GAA TCC CT		600 nM
	Bb12	AGC ACA CTT CTC GAA TGC AC		300 nM
	Bb11–12	CCC TCG AAA CGA GAG ATG CAA GC	5′-FAM, 3′-BHQ1	100 nM
BbRT2	Bb3	CAA CAA GAG CAT CGC CTT C		500 nM
	Bb6	ATT AAC CAA TCC GTG ATA GCA G		500 nM
	Bb3–6	TCC AAC ACC GTT TAA CTA AAC CAA CGA TCT G	5′-FAM, 3′-BHQ1	100 nM

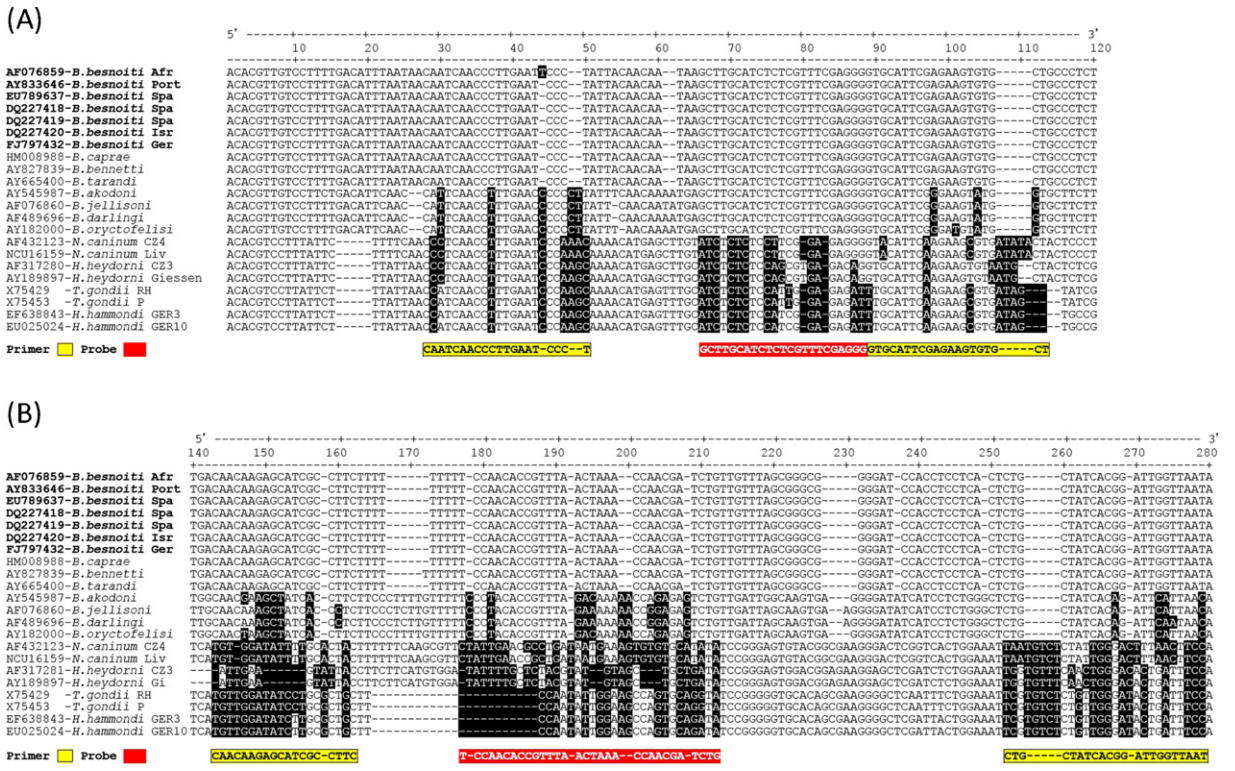

Fig. 1. Location of primers and probes (Table 1) of two real time PCR assays, BbRT1 (A) and BbRT2 (B) within the ITS-1 region of the rRNA gene. The sequence of the ITS-1 region of *B. besnoiti* isolates from Africa (Afr), Portugal (Port), Spain (Spa), Israel (Isr) and Germany (Ger) are aligned relative to those of other *Besnoitia* species and those of *N. caninum*, *H. heydorni*, *T. gondii* and *H. hammondi* by using Clustal V (DNAStar). Deletions and substitutions within the sequences relative to the primer and probe sequences are indicated by a black background.

Reactions were performed in a final volume of 20 μl using a commercial master-mix (iQ supermix, Biorad) and a CFX384 instrument (Biorad Laboratories GmbH, Munich, Germany). Primers and probes (Table 1) were purchased from MWG-Biotech (Ebersberg, Germany). Optimal cycling conditions were determined using standard concentrations for primers (500 nM) and probes (100 nM) (data not shown). Subsequently, primer and probe concentrations were optimised by titration experiments (data not shown). Optimal concentrations are displayed in Table 1. The cycling conditions were 95.0 °C (5 min, initial denaturation), followed by 45 cycles in which the samples were first incubated at 95 °C for 10 s and then at 58.0 °C (BbRT1) or 62.5 °C (BbRT2) for 30 s. After each cycle the light emission by the fluorophore was measured. Real time PCR results were analyzed using the CFX manager software Version 1.6 (Biorad Laboratories GmbH, Munich, Germany).

2.6. Experiments to estimate analytical sensitivity and specificity of real time PCR assays

To estimate the analytical sensitivity of the assays, DNA was extracted from cell culture-derived tachyzoites. DNA was serially diluted in TE buffer solution containing 100 ng/μl bovine DNA at concentrations of 200–0.002 ng/ml. Five microlitres of these dilutions were used as template DNA per analysis. The lowest amount of template DNA at which at least one replicate tested positive was regarded as the detection limit of the respective assay.

The analytical specificity was determined using 10 ng DNA extracted from tachyzoites or bradyzoites or 5 μl DNA extracted from oocysts of parasite species related to *B. besnoiti*.

2.7. Immunofluorescent antibody test (IFAT) and histological examination

IFAT and histological examinations were performed essentially as previously described (Schares et al., 2010; Majzoub et al., 2010).

2.8. Statistics

Linear regression was used to model the standard curves in BbRT1 and BbRT2 with the CFX manager software Version 1.6 (Biorad Laboratories GmbH, Munich, Germany) using the threshold cycles (CT) determined at a threshold of 50 RFU (relative fluorescence units) in both assays. The amplification efficiency for BbRT1 and BbRT2 was calculated with the same program. Mean, standard deviation (StdDev.) and coefficient of variation were calculated using EXCEL 2007 (Microsoft Deutschland GmbH, Unterschleißheim, Germany).

Linear regression to determine potential correlations between the CT values of the real time PCR assays and

Table 2
Analytical sensitivity of the real time PCR assays using genomic DNA of different *Besnoitia* species diluted in 100 ng/μl bovine DNA (no. of positive replicates/no. of replicates).

Parasite	Assay	Amount of DNA per assay							
		10^4 pg	10^3 pg	10^2 pg	10^1 pg	1 pg	10^{-1} pg	10^{-2} pg	10^{-3} pg
B. besnoiti	BbRT1	ND	6/6	6/6	6/6	6/6	6/6	5/6	0/6
	BbRT2	ND	6/6	6/6	6/6	6/6	6/6	5/6	0/6
B. bennetti	BbRT1	ND	4/4	4/4	4/4	2/4	0/4	0/4	ND
	BbRT2	ND	4/4	4/4	4/4	1/4	0/4	0/4	ND
B. tarandi	BbRT1	ND	4/4	4/4	4/4	4/4	0/4	0/4	ND
	BbRT2	ND	4/4	4/4	4/4	4/4	0/4	0/4	ND
B. darlingi (TxBe-1)	BbRT1	0/4	0/4	ND	ND	ND	ND	ND	ND
	BbRT2	0/4	0/4	ND	ND	ND	ND	ND	ND
B. darlingi (Michigan isolate)	BbRT1	4/4	2/4	0/4	0/4	ND	ND	ND	ND
	BbRT2	0/4	0/4	ND	ND	ND	ND	ND	ND
B. oryctofelisi	BbRT1	1/4	0/4	ND	ND	ND	ND	ND	ND
	BbRT2	0/4	0/4	ND	ND	ND	ND	ND	ND
B. neotomofelis	BbRT1	4/4	0/4	0/4	0/4	ND	ND	ND	ND
	BbRT2	0/4	0/4	ND	ND	ND	ND	ND	ND

$\log_{10}$-transformed IFAT titers was performed with the computer program Statistica 7.0 (StatSoft, Tulsa, OK, USA). IFAT titers < 50 were recorded as 25 for this analysis.

3. Results

3.1. Analytical sensitivity and specificity

Serial dilutions of genomic *B. besnoiti* DNA in a buffer containing 100 ng/μl bovine genomic DNA were used to determine the analytical sensitivity of the newly developed real time PCR assays. For both assays, BbRT1 and BbRT2, the detection limit was 0.01 pg when *B. besnoiti* genomic DNA was tested (Table 2 and Fig. 2).

The analytical specificity of the real time PCR was confirmed by testing genomic DNA from other parasite species. The presence of sufficient genomic parasite DNA in the respective samples was confirmed with coccidian- or kinetoplastid-specific primers (Fig. 3). In the real time PCR assays, reactions were only observed with *B. bennetti* and *B. tarandi* (BbRT1, BbRT2) or with *B. darlingi*, *B. oryctofelisi*, and *B. neotomofelis* (BbRT1). With genomic DNA from *B. bennetti* and *B. tarandi*, the detection limit was 1 pg in both the assays, BbRT1 and BbRT2. DNA aliquots (10 ng, 1 ng) of one of the two *B. darlingi* isolates (Michigan) and DNA aliquots (10 ng) of *B. neotomofelis* or *B. oryctofelisi* DNA reacted also in the BbRT1 (CT values > 39), but not in the BbRT2 assay (Table 2).

3.2. Quantification of genomic B. besnoiti DNA

Five or six replicates of 10-fold serial dilutions of *B. besnoiti* DNA (1000–0.01 pg) were used to determine the amplification efficiency (E) and the coefficient of determination (R^2) for both assays, BbRT1 and BbRT2. The regression models revealed R^2 values of 0.978 (BbRT1) and 0.980 (BbRT2). The amplification efficacy was calculated as 102.1% for BbRT1 and 95.7% for BbRT2 (Fig. 2).

The coefficients of variation for CT values observed for different DNA concentrations were ≤2% for DNA concentrations ≥1 pg in both assays. At lower DNA concentrations, the values of the coefficients of variation were ≤5% with the exception of 6.8% for the results obtained with BbRT2 at a DNA concentration of 0.01 pg.

3.3. Diagnostic sensitivity in cattle with clinical signs of chronic bovine besnoitiosis

Samples of 43 cattle with demonstrable tissue cysts in scleral conjunctiva and in the mucous membrane of the vestibulum vaginae were used to assess the diagnostic sensitivity of the assays. DNA was extracted and analyzed

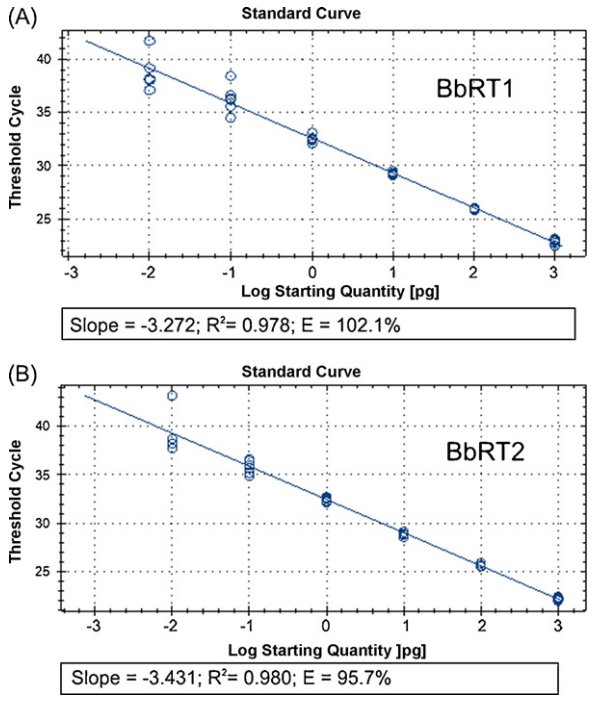

Fig. 2. Standard curves obtained for the threshold cycle (CT) values obtained in two Besnoitia real time PCRs, BbRT1 (A) and BbRT2 (B) for various amounts of genomic *Besnoitia besnoiti* DNA (1000 pg, 100 pg, 10 pg, 1 pg, 0.1 pg and 0.01 pg) diluted in 100 ng/μl bovine DNA. Characteristics of the regression models (slope of the standard curve; R^2 value) and the amplification efficiency (E) are displayed in each graph.

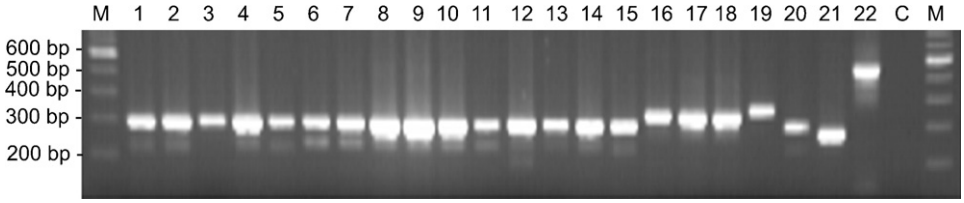

Fig. 3. Coccidia-specific PCR to confirm the presence of sufficient DNA of various parasite species which were used to confirm the specificity of two *Besnoitia* real time PCRs. (1) *Besnoitia besnoiti*, (2) *B. tarandi*, (3) *B. bennetti*, (4) *B. darlingi* (Michigan isolate), (5) *B. darlingi* (Be-1), (6) *B. oryctofelisi*, (7) *B. neotomofelis*, (8) *Neospora caninum*, (9) *Toxoplasma gondii*, (10) *Hammondia hammondi*, (11) *H. heydorni*, (12) *Isospora canis*, (13) *I. burrowsi*, (14) *I. felis*, (15) *I. rivolta*, (16) *Sarcocystis cruzi*, (17) *S. aucheniae*, (18) *S. aucheniae*, (19) *S. miescheriana*, (20) *Eimeria bovis*, (21) *Cryptosporidium parvum*, (22) *Trypanosoma brucei brucei*. C,negative control; M, marker.

by real time and conventional PCR (Table S1; supplementary data).

BbRT2 revealed a slightly higher diagnostic sensitivity than BbRT1 for skin samples (65.1% [28/43] vs 60.5% [26/43]), vaginal scrapings (83.7% [36/43] vs 76.7% [33/43]), and buffy coat (9.3% [4/43] vs 7% [3/43]). Overall, in both real time PCR assays 90.7% (39/43) of the animals with demonstrable tissue cysts tested positive in at least 1 of the three samples.

By conventional PCR, 46.5% (20/43) of skin samples, 60.5% (26/43) of the vaginal scrapings and 7% (3/43) of the buffy coats tested positive.

Skin samples positive by conventional PCR showed mean CT values of 27.55 (6.68 StdDev.) or 27.15 (7.04 StdDev.) in both real time PCR assays, BbRT1 and BbRT2. Histologically positive skin samples revealed mean CT values of 21.13 (3.93 StdDev.) or 20.25 (3.85 StdDev.) in both real time PCR assays.

Vaginal scrapings positive in the conventional PCR showed mean CT values of 29.5 (6.36 StdDev.) or 30.04 (7.33 StdDev.) by both BbRT1 and BbRT2.

Buffy coat samples positive in both real time PCR assays were only observed in those animals which were positive in all other tests including the histological examination. One

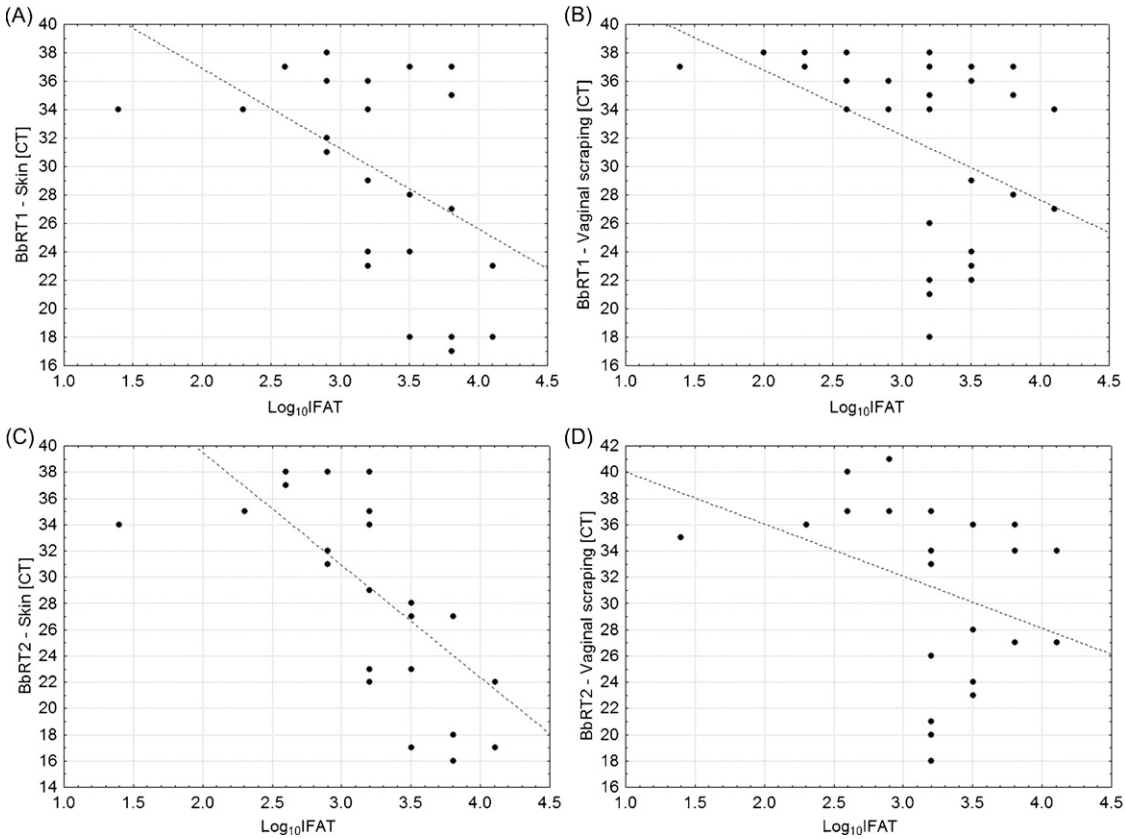

Fig. 4. Serological and real time PCR results in cattle with chronic *B. besnoiti* infection. Scatterplots and linear regression lines are shown to compare log_{10}-transformed IFAT titers (log_{10}IFAT) and the threshold cycles (CT) obtained in two real time PCR assays, BbRT1 (A and B) and BbRT2 (C and D) after analyzing skin (A and C) and vaginal tissues (B and D).

Table 3
Linear regression models constructed to explain $\log_{10}$-transformed IFAT titers using CT (threshold cycle) values determined for skin (Skin) and vaginal (Vag) tissue in cattle examined using *Besnoitia besnoiti* real time PCR assays, BbRT1 and BbRT2.

Model	Variable	R^2	Corrected R^2	*F*-Ratio	*p*-value
1	BbRT1 Skin	0.223	0.190	6.9	0.015
2	BbRT1 Vag	0.182	0.156	6.9	0.013
3	BbRT1 Skin + BbRT1 Vag	0.313	0.232	3.9	0.041
4	BbRT2 Skin	0.352	0.327	14.1	0.001
5	BbRT2 Vag	0.145	0.120	5.8	0.022
6	BbRT2 Skin + BbRT2 Vag	0.465	0.416	9.6	0.001

buffy coat sample was only positive by BbRT2. In this case (animal No. 33, Table S1; supplementary data), all other diagnostic tests on samples from this animal except the histological examination were positive.

3.4. Correlation of antibody levels with real time PCR results for skin and vaginal scrapings

In both real time PCR assays, the CT values obtained for skin and vaginal scrapings were statistically significantly ($p < 0.05$) correlated with the $\log_{10}$-transformed IFAT titer of the corresponding animal, i.e. fewer cycles were required to exceed the real time PCR thresholds in animals possessing the highest IFAT titers (Fig. 4). The correlations (Table 3) were characterized by coefficients of determination (R^2 values) of 0.223 (BbRT1, skin), 0.352 (BbRT2, skin), 0.182 (BbRT1, vaginal scraping), and 0.145 (BbRT2, vaginal scraping) if single variables were used for modeling. When the CT values for both, skin and vaginal scrapings, were included, the coefficients of determination were much higher, ranging from 0.313 (BbRT1) to 0.465 (BbRT2) (Table 3).

Two clinically positive animals with IFAT titers < 50 tested positive by PCR: the vaginal scraping of animal No. 29 was positive in the BbRT2 assay, while skin and vaginal scraping of animal No. 43 were found positive in all three PCRs (Table 3).

4. Discussion

We established two 5′-nuclease assays to quantify *B. besnoiti*-DNA in tissues and blood of infected cattle. Although the detection of parasite DNA does not imply that the parasites are alive and infectious, these assays can be used in experimental and epidemiological studies to characterize infected cattle regarding their parasite load in skin and in other organs and to examine the role of transmission by vectors, e.g. by insects.

Until now, only conventional PCR and SYBR®Green based real time PCR assays were available for the detection of *B. besnoiti* DNA. Because 5′-nuclease assays combine the sensitivity of the real time PCR with an increased specificity afforded by use of a probe, this type of PCR may be superior to the protocols used thus far. As our assays are based on the ITS-1 sequence of *B. besnoiti* and since the ITS-1 sequence of *B. besnoiti* is identical with that of other Besnoitia species infecting ungulates, namely *B. bennetti*, *B. tarandi*, and *B. caprae*, these real time PCRs are also useful in epidemiological and experimental studies on these Besnoitia species. We found, however, that the sensitivity for the detection of *B. bennetti* and *B. tarandi* with the real time PCR assays reported herein was lower than the sensitivity determined for *B. besnoiti* genomic DNA. Differences in the copy numbers of the ITS-1 region between *B. besnoiti*, *B. bennetti* and *B. tarandi* could perhaps explain this result. For *T. gondii* rDNA, a copy number of 110 has been estimated (Guay et al., 1992), but the copy numbers of the ITS-1 region in other Sarcocystidae are not known (Torres-Machorro et al., 2010).

The BbRT2 real time PCR assay was specific for *Besnoitia* spp., infecting ungulates and did not detect other tested *Besnoitia* spp., (*B. darlingi*, *B. oryctofelisi* and *B. neotomofelis*). In case of BbRT1, we observed in addition to reactions with Besnoitia from ungulates also weak cross-reactions with DNA from *B. darlingi*, *B. oryctofelisi*, and *B. neotomofelis* if high DNA concentrations were examined. The comparison of the *B. darlingi* and *B. oryctofelisi* sequences with those of the BbRT1 primers and the probe revealed only differences in the primer region. This might have been the reason for the low-level cross-reactivity. In the case of the BbRT2 real time PCR assay, no cross-reactivity to either *B. darlingi*, *B. oryctofelisi*, or *B. neotomofelis* was observed; both, the primer and probe regions, were different from sequences of *B. darlingi* and *B. oryctofelisi*. Unfortunately, ITS-1 sequences for *B. neotomofelis* were not available for this analysis.

To test the suitability of the assays for estimating DNA concentrations, and to assess the sensitivity of the real time PCR assays, 10-fold serial dilutions of *B. besnoiti* genomic DNA were analyzed. For both assays, the coefficients of determination ranged between 0.978 and 0.980 and the amplification efficiencies (E) calculated for both assays were within the range of 90%–105%, indicating that the results of both assays were reliable and reproducible and were thus suitable to estimate DNA concentrations (Pestana et al., 2010). The optimal analytical sensitivity for *B. besnoiti* of both assays was a DNA concentration of 0.01 ng which might be equivalent to the DNA of 0.1 parasites assuming the DNA content of a single *B. besnoiti* organism is similar to that estimated for *N. caninum* (Ellis et al., 1999).

To confirm the diagnostic sensitivity of this PCR, we used different samples from 43 *B. besnoiti*-infected cattle in which macroscopic tissue cysts were identified in the scleral conjunctiva and/or the vaginal mucosa during thorough clinical examination. The sensitivity of both real time PCR assays was better than that of the conventional PCR. The sensitivity varied depending on the type of sample. The highest sensitivity was observed in vaginal scrapings and skin biopsy samples, whereas the sensitivity for DNA extracted from the buffy coat collected from EDTA blood samples after centrifugation was very low. This

was expected since we sampled only animals with signs of chronic bovine besnoitiosis while a parasitaemia has so far been reported only from animals that had recently become infected (Bigalke, 1968).

An important finding was that vaginal samples more often tested positive than did skin samples of these same animals, irrespective of the PCR technique (real time or conventional PCR) used. This may be attributable to the fact that vaginal tissues were sampled at sites where tissue cysts were macroscopically visible. On the contrary, skin biopsies were taken in a defined region (femoral region) in order to assure a comparability of samples, independently of the presence of typical alterations on this site. The examination of about one third of these skin samples revealed a false-negative result. This may be also explained by an uneven distribution of tissue cysts in the skin biopsies, which could increase the likelihood that no tissue cyst was present in some of the 25 mg aliquots used for DNA extraction. It remains to be examined, whether the sensitivity of the method could be improved by homogenizing the entire skin biopsy sample prior to taking an aliquot for DNA extraction. Thus, the targeted sampling as performed in case of vaginal tissues might be responsible for the higher diagnostic sensitivity of the PCRs at this sampling site. Several authors previously reported tissue cysts in the mucous membrane of the vagina (Nobel et al., 1977) or the vestibulum vaginae (Majzoub et al., 2010; Rostaher et al., 2010). However, this is the first study providing evidence that the vestibulum vaginae may be a preferred site where bovine besnoitiosis can be detected by molecular diagnosis.

Another important finding was that fewer cycles of PCR tended to be necessary in animals possessing the greatest concentration of antibodies (expressed as a significant correlation between CT values and IFAT titers). This was true for skin biopsies and vaginal scrapings. In addition, $\log_{10}$-transformed IFAT titers could be statistically modeled using both the CT values obtained for skin biopsies and vaginal scrapings. This may suggest that higher parasite concentrations induce stronger antibody responses, but the causal pathway cannot be demonstrated with this correlation alone. In both assays, the CT values obtained for skin samples showed a better correlation with the IFAT titers (i.e. revealed higher R^2 values) than the CT values obtained for vaginal scrapings. This was expected since the targeted sampling of the vaginal mucosa biased the real time PCR results. The CT values do therefore not reflect the tissue cyst concentration at this site in an optimal way.

In summary, we established two 5′-nuclease *B. besnoiti* real time PCR assays for the quantitative detection of *B. besnoiti* in chronically infected cattle. Due to a high level of identity in the ITS-1 region of the rDNA, these PCRs can also be used for epidemiological and experimental studies on *B. bennetti*, *B. tarandi* and *B. caprae*. One of the two assays, BbRT2, was superior in terms of specificity for *Besnoitia* spp., infecting ungulates.

Conflict of interest statement

All authors declare that they or their institutions have no financial and personal relationship with other people or organizations that could inappropriately influence their work.

Acknowledgements

We are indebted to Dr. Christian Bauer (Institute of Parasitology, Giessen, Germany), Dr. Peter-Henning Clausen (Institute of Parasitology and Tropical Veterinary Medicine), Dr. C. Lippuner (Institute of Parasitology, Zürich, Switzerland) and Dr. Majda Globokar Vrhovec (VetMedLabor GmbH, Division of IDEXX Laboratories, Ludwigsburg, Germany) for providing parasite isolates. We acknowledge the technical help of Andrea Bärwald, Lilo Minke, Susann Schares, Diana Herold and Dr. Birgit Rohlederer.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vetpar.2011.01.038

References

Bigalke, R.D., 1968. New concepts on the epidemiological features of bovine besnoitiosis as determined by laboratory and field investigations. Onderstepoort J. Vet. Res. 35, 3–137.

Bigalke, R.D., 1970. Studies on equine besnoitiosis. J. Parasitol. 56, 29.

Caspari, K., Grimm, F., Kühn, N., Caspari, N.C., Basso, W., 2010. First report of naturally acquired clinical sarcocystosis in a pig breeding stock. Vet. Parasitol., doi:10.1016/j.vetpar.2010.11.019.

Cortes, H.C., Reis, Y., Waap, H., Vidal, R., Soares, H., Marques, I., Pereira, d.F., Fazendeiro, I., Ferreira, I., Caeiro, M.L., Shkap, V., Hemphill, V., Leitao, A., 2006. Isolation of *Besnoitia besnoiti* from infected cattle in Portugal. Vet. Parasitol. 141, 226–233.

Cortes, H.C., Reis, Y., Gottstein, B., Hemphill, A., Leitao, A., Muller, N., 2007. Application of conventional and real-time fluorescent ITS1 rDNA PCR for detection of *Besnoitia besnoiti* infections in bovine skin biopsies. Vet. Parasitol. 146, 352–356.

Desquesnes, M., Ravel, S., Cuny, G., 2002. PCR identification of *Trypanosoma lewisi*, a common parasite of laboratory rats. Kinetoplastid. Biol. Dis. 1, 2.

Dubey, J.P., Lindsay, D.S., Rosenthal, B.M., Sreekumar, C., Hill, D.E., Shen, S.K., Kwok, O.C., Rickard, L.G., Black, S.S., Rashmir-Raven, A., 2002. Establishment of *Besnoitia darlingi* from opossums (*Didelphis virginiana*) in experimental intermediate and definitive hosts, propagation in cell culture, and description of ultrastructural and genetic characteristics. Int. J. Parasitol. 32, 1053–1064.

Dubey, J.P., Sreekumar, C., Lindsay, D.S., Hill, D., Rosenthal, B.M., Venturini, L., Venturini, M.C., Greiner, E.C., 2003. *Besnoitia oryctofelisi* n. sp. (Protozoa: Apicomplexa) from domestic rabbits. Parasitology 126, 521–539.

Dubey, J.P., Sreekumar, C., Rosenthal, B.M., Vianna, M.C., Nylund, M., Nikander, S., Oksanen, A., 2004. Redescription of *Besnoitia tarandi* (Protozoa: Apicomplexa) from the reindeer (*Rangifer tarandus*). Int. J. Parasitol. 34, 1273–1287.

Dubey, J.P., Sreekumar, C., Donovan, T., Rozmanec, M., Rosenthal, B.M., Vianna, M.C., Davis, W.P., Belden, J.S., 2005. Redescription of *Besnoitia bennetti* (Protozoa: Apicomplexa) from the donkey (*Equus asinus*). Int. J. Parasitol. 35, 659–672.

Dubey, J.P., Yabsley, M.J., 2010. *Besnoitia neotomofelis* n. sp (Protozoa: Apicomplexa) from the southern plains woodrat (*Neotoma micropus*). Parasitology, doi:10.1017/S00311822010000636.

Ellis, J.T., Holmdahl, O.J., Ryce, C., Njenga, J.M., Harper, P.A., Morrison, D.A., 2000. Molecular phylogeny of Besnoitia and the genetic relationships among Besnoitia of cattle, wildebeest and goats. Protist 151, 329–336.

Ellis, J.T., Mcmillan, D., Ryce, C., Payne, S., Atkinson, R., Harper, P.A.W., 1999. Development of a single tube nested polymerase chain reaction assay for the detection of *Neospora caninum* DNA. Int. J. Parasitol. 29, 1589–1596.

Elsheikha, H.M., Fitzgerald, S.D., Rosenthal, B.M., Mansfield, L.S., 2004. Concurrent presence of *Sarcocystis neurona* sporocysts *Besnoitia dar-*

lingi tissue cysts, and *Sarcocystis inghami* sarcocysts in naturally infected opossums (*Didelphis virginiana*). J. Vet. Diagn. Invest. 16, 352–356.

Elsheikha, H.M., Mackenzie, C.D., Rosenthal, B.M., Marteniuk, J.V., Steficek, B., Windsor, S., Saeed, A.M., Mansfield, L.S., 2005. An outbreak of besnoitiosis in miniature donkeys. J. Parasitol. 91, 877–881.

Elsheikha, H.M., Rosenthal, B.M., Murphy, A.J., Dunams, D.B., Neelis, D.A., Mansfield, L.S., 2006. Generally applicable methods to purify intracellular coccidia from cell cultures and to quantify purification efficacy using quantitative PCR. Vet. Parasitol. 135, 223–234.

Guay, J.M., Huot, A., Gagnon, S., Tremblay, A., Levesque, R.C., 1992. Physical and genetic mapping of cloned ribosomal DNA from T*oxoplasma gondii*: primary and secondary structure of the 5S gene. Gene 114, 165–171.

Ho, M.S.Y., Barr, B.C., Marsh, A.E., Anderson, M.L., Rowe, J.D., Tarantal, A.F., Hendrickx, A.G., Sverlow, K., Dubey, J.P., Conrad, P.A., 1996. Identification of bovine Neospora parasites by PCR amplification and specific small-subunit rRNA sequence probe hybridization. J. Clin. Microbiol. 34, 1203–1208.

Lanham, S.M., Godfrey, D.G., 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-Cellulose. Exp. Parasitol. 28, 521–534.

Majzoub, M., Breuer, W., Gollnick, N., Rostaher, A., Schares, G., Hermanns, W., 2010. Ein Ausbruch von Besnoitiose bei Rindern in Deutschland. Wien. Tierärztl. Mschr. 97, 9–15.

Moré, G., Basso, W., Bacigalupe, D., Venturini, M.C., Venturini, L., 2008. Diagnosis of *Sarcocystis cruzi*, *Neospora caninum*, and *Toxoplasma gondii* infections in cattle. Parasitol. Res. 102, 671–675.

Njenga, J.M., Bwangamoi, O., Mutiga, E.R., Kangethe, E.K., Mugera, G.M., 1993. Preliminary findings from an experimental study of caprine besnoitiosis in Kenya. Vet. Res. Commun. 17, 203–208.

Nobel, T.A., Neumann, M., Klopfer, U., Perl, S., 1977. Kystes de *Besnoitia besnoiti* dans les organes genitaux de la vache. Bull. Acad. Vet. Fr. 50, 569–574.

Pestana, E.A., Belak, S., Diallo, A., Crowther, J.R., Viljoen, G.J., 2010. Early, Rapid and Sensitive Veterinary Molecular Diagnostics - Real Time PCR Applications. Springer, New York.

Pols, J.W., 1960. Studies on bovine besnoitiosis with special reference to the aetiology. Onderstepoort J. Vet. Res. 28, 265–356.

Rostaher, A., Mueller, R.S., Majzoub, M., Schares, G., Gollnick, N.S., 2010. Bovine besnoitiosis in Germany. Vet. Dermatol. 21, 329–334.

Schares, G., Pantchev, N., Barutzki, D., Heydorn, A.O., Bauer, C., Conraths, F.J., 2005. Oocysts of *Neospora caninum*, *Hammondia heydorni*, *Toxoplasma gondii* and *Hammondia hammondi* in faeces collected from dogs in Germany. Int. J. Parasitol. 35, 1525–1537.

Schares, G., Vrhovec, M.G., Pantchev, N., Herrmann, D.C., Conraths, F.J., 2008. Occurrence of *Toxoplasma gondii* and *Hammondia hammondi* oocysts in the faeces of cats from Germany and other European countries. Vet. Parasitol. 152, 34–45.

Schares, G., Basso, W., Majzoub, M., Cortes, H.C., Rostaher, A., Selmair, J., Hermanns, W., Conraths, F.J., Gollnick, N.S., 2009. First in vitro isolation of *Besnoitia besnoiti* from chronically infected cattle in Germany. Vet. Parasitol. 163, 315–322.

Schares, G., Basso, W., Majzoub, M., Rostaher, A., Scharr, J.C., Langenmayer, M.C., Selmair, J., Dubey, J.P., Cortes, H.C., Conraths, F.J., Gollnick, N.S., 2010. Comparative evaluation of immunofluorescent antibody and new immunoblot tests for the specific detection of antibodies against *Besnoitia besnoiti* tachyzoites and bradyzoites in bovine sera. Vet. Parasitol. 171, 32–40.

Torres-Machorro, A.L., Hernandez, R., Cevallos, A.M., Lopez-Villasenor, I., 2010. Ribosomal RNA genes in eukaryotic microorganisms: witnesses of phylogeny? FEMS Microbiol. Rev. 34, 59–86.