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First *in vitro* isolation of *Besnoitia besnoiti* from chronically infected cattle in Germany☆

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ABSTRACT

Besnoitia besnoiti was *in vitro* isolated during the first recorded outbreak of bovine besnoitiosis in Germany. Molecular characterization of the new isolate, named Bb-GER1, revealed almost 100% identity with other *B. besnoiti* isolates obtained in Portugal, Spain, Israel or South Africa, when partial sequences of the 18S ribosomal RNA gene, of the internal transcribed spacer 1 and of the 5.8S RNA gene were compared. Cystozoites obtained from skin tissue of one bull were infectious for γ-interferon knockout (GKO) mice by intraperitoneal (ip) inoculation. Tachyzoites were detected in the peritoneal cavity, spleen, liver and lung of the mice 5 days post-infection. The parasite could be maintained in GKO mice by ip inoculation for at least 5 passages. Peritoneal washings containing tachyzoites were obtained from infected mice and used to infect five cell lines (Vero, MARC-145, NA42/13, BHK_{21}, KH-R). The best growth of tachyzoites was observed in BHK_{21} cells, but replication occurred to a smaller extent also in MARC-145, NA42/13 and KH-R cells. Subsequent comparative analyses revealed that after direct infection of these cell lines with cystozoites derived from bovine skin, the growth was best in NA42/13 cells. Considerable replication was also observed in the BHK_{21} and KH-R cell lines. Our observations on the growth characteristics of Bb-GER1 partially contrast those for other isolates. The preferential growth in particular cell lines may be characteristic for particular *B. besnoiti* isolates. A potential association between growth properties and differences in virulence remains to be established. This is the first *in vitro* isolation of *B. besnoiti* from cattle in Germany.

1. Introduction

Besnoitia besnoiti is a cyst-forming apicomplexan parasite closely related to *Toxoplasma gondii* and *Neospora caninum*. It is the cause of bovine besnoitiosis, a severe but usually non-fatal disease with significant economic impact in many countries of Africa, Asia and Europe. Bovine besnoitiosis is characterized by pyrexia

☆ Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession number FJ797432.

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Table 1
Diagnostic results for animals from which bovine skin samples had been collected for *in vitro* isolation of *Besnoitia besnoiti*.

ID of animal	No. of tissue samples	Breed[a]	Sex	Age (months)	Cystozoites in inoculated material	Cysts in conjunctival sclera	Cysts in vulva	*B. besnoiti* IFAT titer	*B. besnoiti* specific PCR	Histology
31	1	L	Male	52	Yes	No	NA	1:3200	Positive	ND
62	3	Cha	Female	54	Yes	No	Yes	1:6400	Positive	Positive
63	1	Cha	Female	122	No	Yes	Yes	1:400	Negative	Negative
70	2	Cha	Male	21	Yes	Yes	NA	1:12,800	Positive	Positive
92	2	L	Female	40	No	Yes	Yes	1:3200	Positive	Positive
94	2	L	Female	65	Yes	Yes	Yes	1:3200	Positive	Positive
168	1	L	Female	44	No	Yes	Yes	1:12,800	Positive	Positive
169	1	L	Male	35	No	Yes	NA	1:6400	Positive	Positive

[a] L = Limousin and Cha = Charolais.

and edema in acutely infected cattle. In chronically infected cattle the alopecic skin can become severely lichenified and hyperpigmented (Levine, 1985). Bulls may develop orchitis and permanent infertility (Bigalke, 1968). Bovine besnoitiosis has not yet been reported from European countries north of the Alps. However, in France there is evidence that the disease has spread from the southern endemic areas to the north of the country recently (Alzieu et al., 2007). *B. besnoiti* can be transmitted mechanically by tabanids and biting muscids (Bigalke, 1968). Its definitive host is not known. Peteshev and Galzuo (1974) reported that cats shed *Besnoitia*-like oocysts after they had fed on tissues from cattle naturally infected with *B. besnoiti*. However, these findings could not be confirmed by other investigators and further attempts to identify a definitive host of *B. besnoiti* failed (Diesing et al., 1988).

Recently, a case of bovine besnoitiosis was observed in an extensively managed beef herd in Southern Germany, close to the city of Munich (Rostaher et al., submitted for publication). Besnoitiosis was confirmed by clinical, cytological, histological, electron microscopical and serological examinations and by detection of specific DNA using the polymerase chain reaction (PCR). The aim of the present study was to *in vitro* isolate *B. besnoiti* from this German herd in order to further characterize this isolate.

2. Materials and methods

2.1. Source of samples

An extensively managed beef herd, in which the first case of bovine besnoitiosis in Germany had recently been observed (Rostaher et al., submitted for publication) was examined for signs of clinical besnoitiosis. Eight animals were selected with obvious clinical signs indicating besnoitiosis (tissue cysts in the scleral conjunctiva or on the mucous membranes of the vulva, periocular and perioral hypotrichia and lichenification) (Table 1). Blood was taken from each animal form either the jugular or the tail vein. In addition, skin samples were collected from the lateral thigh region of these animals using a sterile biopsy punch (diameter 6 mm or 8 mm) after trimming and local anesthesia by subcutaneous application of 5 ml 2% (w/v) procaine hydrochloride.

2.2. Processing of skin samples for inoculation into cell cultures and mice

To remove accidental surface contaminations, the external parts of the samples were removed and the cores squashed using a mortar and pestle in 1 ml Dulbecco's Modified Eagle Medium (DMEM) supplemented with 2% fetal calf serum (FCS), 1% antibiotic solution (10,000 IU Penicillin and 10,000 μg Streptomycin/ml solution) and 1% amphotericin B (250 μg/ml). The suspensions were examined by light microscopy (400× magnification) to confirm the presence of cystozoites and inoculated into cell cultures or intraperitoneally (ip) into γ-interferon knockout (GKO) mice (C.129S7 (B6)-Ifngtm1Ts/J, The Jackson Laboratory, Bar Harbor, Maine, USA).

2.3. Cell cultivation

The following cell lines were inoculated with cystozoites or tachyzoites: Vero (African green monkey, epithelial kidney cells, permanent), MARC-145 (rhesus monkey, fetal kidney cells, permanent), NA42/13 (mouse, neuroblastoma cells, permanent), BHK_{21} (baby hamster kidney cells, permanent) and KH-R (embryonic calf heart cells, primary, finite). All cell lines except KH-R were maintained in DMEM, 2% FCS, 1% antibiotic solution and 1% amphotericin B. KH-R cells were cultivated in DMEM, 10% FCS and 1% amphotericin B. Depending on the growth rate, Vero, MARC-145 and BHK_{21} cells were split 1–2 times every 2 weeks. NA42/13 and KH-R cells were split every 4 weeks.

2.4. Comparison of parasite growth in different cell lines

Monolayers of Vero, MARC-145, BHK_{21}, NA42/13 and KH-R in 25 cm^2 cell culture flasks were inoculated with parasites from murine peritoneal washings or bovine skin samples. Inoculation doses were determined by the examination of 10 μl aliquots of suspensions containing cystozoites or tachyzoites in a Neubauer chamber. After the end of the cultivation period, the parasites present in the supernatant and in the cell layer were also counted in a Neubauer chamber. To count parasites inside the cell layer, 2 ml PBS supplemented with 0.01% (w/v) sodium dodecyl sulfate were added, the cells removed by a rubber policeman, the tachyzoites released by aspiration through a series of needles with 19 G, 21 G, 24 G and 27 G and processed as described above. From cell cultures inoculated with

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peritoneal washings of mice (K126/1 and K126/2) or with cystozoites, the supernatant was removed 24 h later and replaced by fresh medium. Parasites removed during the change of medium were counted and their numbers added to the final count.

2.5. Examination of tissue samples and blood

Samples of brain, heart, lung, liver, spleen, kidney and striated muscle from inoculated GKO mice and tissue samples from diseased cattle were fixed in 10% neutral buffered formaldehyde for histological studies. All formalin-fixed tissue samples were routinely processed and embedded in paraffin. Sections were cut to 5 μm thickness and stained with hematoxylin and eosin (H&E). Blood smears from the inoculated mice were stained according to Giemsa's stain.

2.6. Indirect Fluorescent Antibody Test (IFAT)

For IFAT, purified *B. besnoiti* Bb1Evora03 tachyzoites cultivated in Vero cells (Cortes et al., 2006) were used as antigen. The assay was performed essentially as described for *N. caninum* (Schares et al., 1998). Serum dilution started at 1:50. Rabbit anti-bovine IgG (H + L) FITC (Jackson ImmunoResearch Laboratories, West Grove, USA) was used as a conjugate at a dilution of 1:50.

2.7. DNA isolation

DNA was extracted from bovine skin samples, GKO mouse tissues and *in vitro*-grown tachyzoites with a commercial kit (NucleoSpin® Tissue, Macherey-Nagel, Düren, Germany) according to the manufacturer's instructions.

2.8. PCR

In addition to the primers JS4, Tim2, Tim3 and Tim11 previously used in other studies to detect tissue cyst-forming coccidia (Schares et al., 2005, 2008a), the primers listed in Table 1 were employed. For the specific detection of *B. besnoiti*, the primer pair Bb-ITS1-F and Bb-ITS1-R was used (Table 2). The remaining primers listed in Table 2 served sequencing purposes. PCR primers were used at a final concentration of 0.5 μM and dNTPs at a final concentration of 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. 1 μl of genomic DNA was used as template. Water PCR Reagent (Sigma–Aldrich, Taufkirchen, Germany) served as a negative control and DNA from cell cultured *B. besnoiti* (Bb1Evora03) tachyzoites was used as a positive control (Schares et al., 2008b). 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 (1 min at 95 °C), annealing (1 min at 54 °C if no other temperature is stated in Table 1) and extension (72 °C, 1 min), followed by a final extension step at 72 °C for 5 min. The amplification products were visualized after electrophoresis in 1.5% agarose gels stained with ethidium bromide. A 100 bp DNA ladder (Invitrogen GmbH, Karlsruhe, Germany) was used as a size standard.

2.9. Sequencing and DNA examination

Amplicons were sequenced using a kit with 7-deaza-dGTP (Thermo Sequenase™ DYEnamic Direct Cycle Sequencing Kit, GE Healthcare, Munich, Germany) and infrared dye (IRD) 700 and 800-5′-labelled primers. The sequences of the IRD-labelled primers were the same as those used for PCR. Each sample was analyzed in a DNA sequencer with a dual laser detection system (Long Readir LI-COR 4200 DNA Sequencer, MWG Biotech, Ebersberg, Germany). Sequences were assembled using the Lasergene 7.0 software 161 (DNASTAR Inc., Madison, USA) and compared with sequences of *B. besnoiti* in GenBank™ by a BLAST search. The obtained consensus sequence was deposited in GenBank™ under the accession number: FJ797432.

3. Results

3.1. Isolation of *B. besnoiti*

Isolation attempt 1: Thirteen skin biopsies were taken from 8 animals (Table 1) of the herd in which the first case

Table 2
List of primers.

Name	Sequence (5′–3′)	Annealing temperature	Region	Reference
Bb-ITS1-F	GGGTGCATTCGAGAAGTGTG	65 °C	ITS-1	Cortes et al. (2006)
Bb-ITS1-R	TCCGTGATAGCAGAGTGAGGAGG	65 °C	ITS-1	Cortes et al. (2006)
Bb-GS1F	TCGGCGACGGATCATTCAAGT	54 °C	18S-rDNA	This paper
Bb-GS1R	ATGCCCCCAACCGTCCCTATTA	54 °C	18S-rDNA	This paper
Bb-GS2F	GGATTTCGGCCCTATTTTG	54 °C	18S-rDNA	This paper
Bb-GS2R	CGCGTGCAGCCCAGAACA	54 °C	18S-rDNA	This paper
Bb-GS3F	TGACGGAAGGGCACCACCAG	54 °C	18S-rDNA	This paper
Bb-GS3R	TCACCGGAACACTCAATC	54 °C	18S-rDNA	This paper
Bb-GS4F	GACTCAACACGGGGAAACTCA	54 °C	18S-rDNA	This paper
Bb-GS4R	TCACCTACGGAAACCTT	54 °C	18S-rDNA	This paper
Bb-GS5F	CTGGTAGCGCTTCACACTTCATTG	54 °C	18S-rDNA	This paper
Bb-GS5R	GTTTCAGCCTTGCGACCATACTCC	54 °C	18S-rDNA	This paper
Bb-GS6F	CTGCCAGTAGTCATATGCTTGTCT	54 °C	18S-rDNA	This paper
Bb-GS6R	GCGCCTGCTGCCTTCCTTAG	54 °C	18S-rDNA	This paper

of bovine besnoitiosis in Germany had been observed (Rostaher et al., submitted for publication). Samples were processed 6–14 h later. Vero cell cultures were inoculated with cystozoites (Fig. 1A). Viable parasites were seen until day 7 post-inoculation (p.i.). However, no significant replication of parasites was observed, i.e. no parasite-induced focal cytopathogenic effect was detected. Later on, parasites were not seen any more and the isolation attempt was stopped 2 months p.i.

Isolation attempt 2: A second skin biopsy of one of the sampled animals (Animal 70, Table 2) was first stored at room temperature for 12 h, than stored at 4 °C for 30 h. After transport to the Friedrich-Loeffler-Institut (FLI) (without refrigeration), it was stored at 4 °C for another 2 days. Three days after sampling, the tissue was processed and inoculated ip into a GKO mouse and added to a Vero cell monolayer in parallel. In the Vero cells, no significant replication was observed. No viable parasites were seen until 7 days p.i. and cultivation was stopped on day 57 p.i. The inoculated GKO mouse (K122) fell ill 5 days p.i. and was sacrificed. Peritoneal washing from this animal were transferred onto Vero cells and

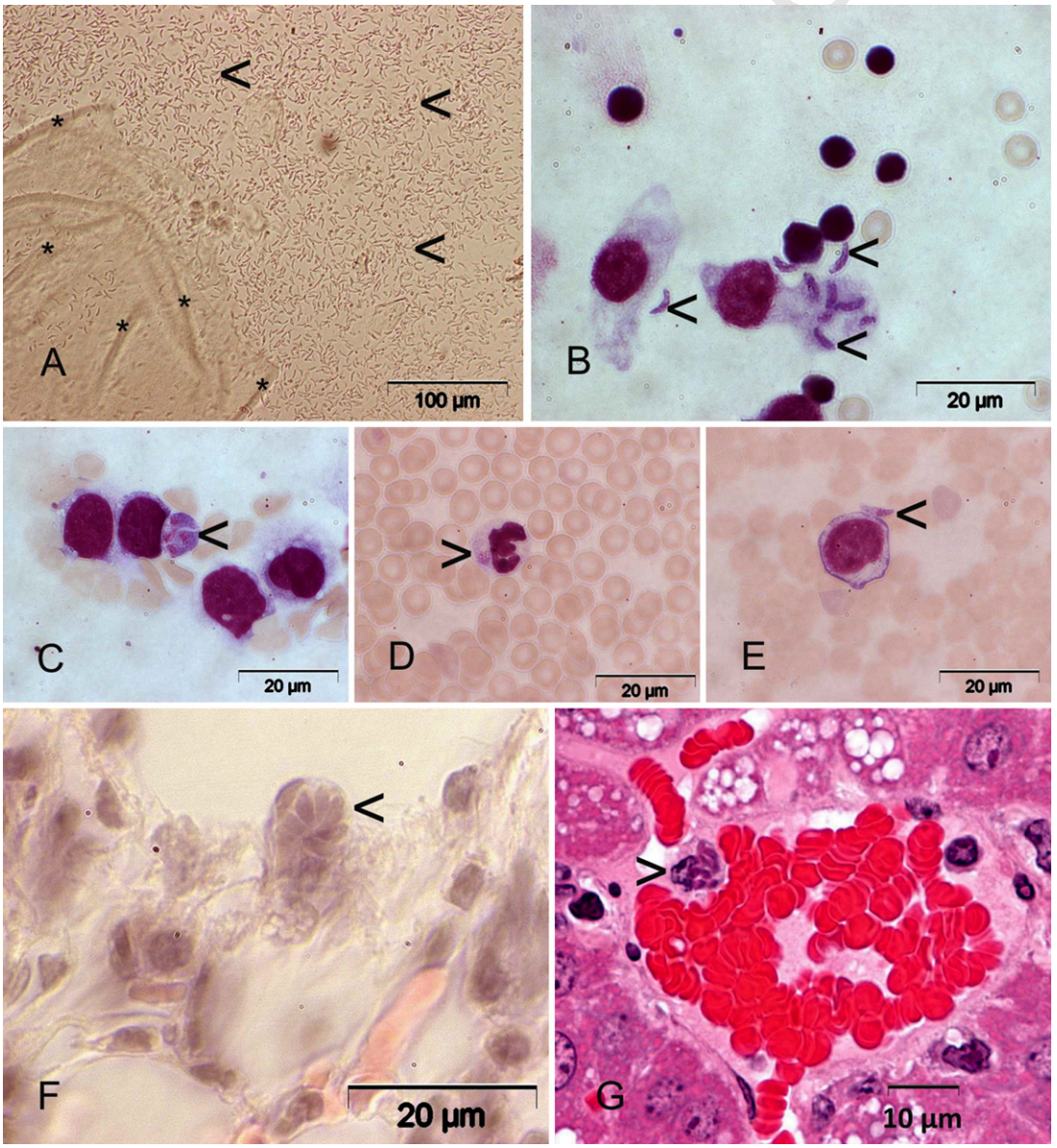

Fig. 1. *Besnoitia besnoiti* in a skin biopsy from an infected bull (A) and in tissues of an infected γ-interferon knockout mouse (B–G). (A) Numerous cystozoites (<) are released from tissue cysts (*) in bovine skin after squashing using a mortar and pestle. (B) Peritoneal washing of a *B. besnoiti* infected γ-interferon knockout mouse with numerous extra- and intracellular tachyzoites (<), Giemsa stained. (C and D) Parasites were observed in blood smear of a GKO mouse (Giemsa stained), either in monocytes (C, <), or in neutrophil granulocytes (D, >) or extracellular (E, <) 5 days post-infection (K122). (F) Parasitophorous vacuole containing *B. besnoiti* tachyzoites arranged as a rosette in a lung section of an infected GKO mouse (K122), H&E staining. (G) Cluster of tachyzoites (<) in a blood vessel of the liver of a GKO mouse (K122), H&E staining.

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inoculated ip into another GKO mouse (K123). Numerous intra- and extracellular parasite stages were visible in the peritoneal washing of this mouse (Fig. 1B). *B. besnoiti* DNA was found in all sampled organs of this mouse (brain, heart, lung, liver, spleen, kidney, skeletal muscle) by PCR using the primer pair Bb-ITS1-F and Bb-ITS1-R. Infected monocytes and neutrophilic granulocytes as well as extracellular parasites were detected in Giemsa-stained blood smears of GKO mouse K122 (Fig. 1C and D). Histological examination of tissues of this animal revealed red hepatization of the lungs, multifocal necrosis and mixed cell infiltrations in the liver tissue. Extra- and intracellular parasites were observed in lungs, spleen and liver sections—especially associated with blood vessels (Fig. 1F and G). In brain sections, perivascular cuffings were detected but no parasites observed.

In Vero cells inoculated with peritoneal washings of GKO mouse K122, no significant parasite replication was observed and cultivation stopped 14 days p.i. The GKO K123 mouse inoculated with the same material fell ill 5 days p.i. and was sacrificed. Peritoneal washings were transferred onto Vero cells and inoculated after an overnight storage at 4 °C ip into a third GKO mouse (K124). Initially, no significant replication was observed in the Vero cells, but on day 12 p.i., when the cell culture was split, a few moving tachyzoites were detected. This Vero cell culture was found infected with a few locations of parasite-induced cytopathic effects on day 27 p.i. The isolate was first cryopreserved 61 days p.i. and designated Bb-GER1$_{VERO}$.

The GKO mouse K124 inoculated with peritoneal washing of GKO mouse K123 fell ill (anorexia, ruffled hair) 5 days p.i. Peritoneal fluid was inoculated ip into two other GKO mice (K125/1; K125/2) and was added onto monolayers of five different cell lines: Vero, MARC-145, NA42/13, BHK$_{21}$ and KH-R cells. After 3 days in cell culture, substantial parasite replication was observed in all cell lines except Vero. Infected MARC-145, NA42/13, BHK$_{21}$ and KH-R cultures were split and aliquots stored in liquid nitrogen 4 days later. Parasites growing in the KH cell line were further cultivated and stored in liquid nitrogen 19 days p.i. for further studies. This isolate was designated Bb-GER1$_{KH}$.

3.2. Comparison of parasite growth in different cell lines infected with tachyzoites

Peritoneal washings obtained during the fourth and the fifth ip passage through GKO mice (K125/2, K126, K127) were used for these experiments. The mice developed clinical signs 5 days p.i. (K125/2, K126, K127). Equal numbers of tachyzoites (3–6 × 10^5) obtained from peritoneal washings of the animals were added to five different cell lines: MARC-145, KH-R, NA42/13, BHK$_{21}$ and Vero. After a cultivation period of 46.5 h (K125/2; Fig. 2, Experiment A) or 48 h (K126/1, K126/2; Fig. 2, Experiments B and C) extra- and intracellular parasites were counted. The lowest replication occurred in Vero cells (0.14–0.43 tachyzoites per hour and inoculated parasite). In all other cell lines, the parasite replication rate was higher. The isolate replicated best in BHK$_{21}$ cells (6–20 tachyzoites per hour and inoculated parasite).

3.3. Comparison of parasite growth in different cell lines infected with cystozoites

Cystozoites (Fig. 1A) were obtained from bovine skin tissue samples (Table 2, Animal 70) which were recovered at slaughter. The samples were shipped overnight to the FLI, where they were immediately processed upon arrival. Parasites were counted and 6 × 10^4 or 37.5 × 10^4 cystozoites added to two sets of five tissue culture flasks, respectively, with monolayers of each of following five cell lines: MARC-145, KH-R, NA42/13, BHK$_{21}$ and Vero. After a cultivation period of 123 h, the number of parasites in the supernatant and in the cells was determined (Fig. 2, Experiments D and E). In cystozoite-inoculated cell cultures, parasite replication was much slower than in tachyzoite-inoculated cultures. Parasite replication was best in NA42/13 cells (0.06–0.12 tachyzoites per hour and inoculated parasite). The lowest replication (0.008–0.017 tachyzoites per hour and inoculated parasite) was observed in MARC-145 and Vero cells.

3.4. Sequence comparison

Partial sequence of the 18S ribosomal RNA gene (1725 bp), the entire sequence of the internal transcribed

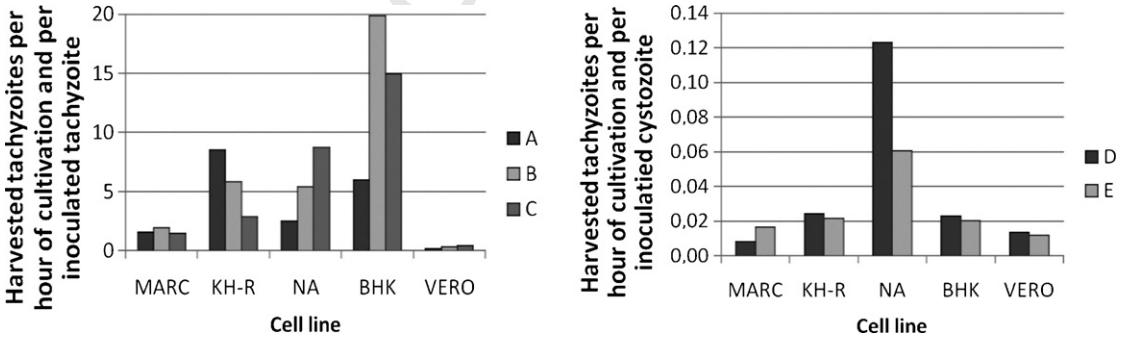

Fig. 2. Growth characteristics of the *Besnoitia besnoiti* isolate Bb-GER1 in five different cell lines: MARC-145 (MARC), KH-R (KH-R), NA42/13 (NA), BHK$_{21}$ (BHK) and Vero (VERO). Experiments A–C were performed with tachyzoites from the peritoneal cavity of infected γ-interferon knockout mice. Experiments D and E were performed with cystozoites from bovine skin.

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spacer 1 (ITS1) (243 bp) and a partial sequence of the 5.8S RNA gene (151 bp) were identical for Bb-GER1$_{KH}$ and Bb-GER1$_{VERO}$ (FJ797432; GenBank™).

When the sequence of the ITS1 rDNA was compared with sequences deposited for *B. besnoiti* and other *Besnoitia* sp., identities of 100% (*B. besnoiti*, DQ227420.1, DQ227419.1, DQ227418.1, AY833646.1), 100% (*B. tarandii*, AY665400.1), 99.6% (*B. bennetti*, AY665399.2, AY827839.1), 99.6% (*B. besnoiti* from South Africa, AF076859, identical to the ITS1 of *B. caprae*, Ellis et al., 2000) or less than 80% (*B. darlingi*, AF489696.1; *B. oryctofelisi*, AY182000.1; *B. akodoni*, AY545987.1) were observed.

Comparison of the 18S ribosomal RNA gene sequence was only possible with *B. besnoiti* strains from Spain (DQ227419.1, DQ227418.1), Portugal (AY833646.1), Israel (DQ227420.1) and South Africa (AF109678.1). Identities of 99.9–100% were observed with 18S ribosomal RNA gene sequences of isolates from Spain, Portugal and Israel. An identity of 99.5% was observed with the sequence available for the South African isolate. The comparison of the 18S ribosomal RNA gene sequence with sequences of the related protozoa *Neospora caninum* and *Toxoplasma gondii* revealed identities of 98.6–98.9% (U16159.1, M97703.1, EF472967.1, X75429.1).

4. Discussion

Besnoitiosis is endemic in southern of Europe (in parts of Portugal, Spain and France), in sub-Saharan Africa and in Asia. A northward spread of the disease has recently been reported for France (Alzieu et al., 2007). Apart from areas in France where besnoitiosis is endemic (eastern Pyrenees, Massif Central, and Alps), also western parts up to the Loire Valley and central regions of France are afflicted by the occurrence of sporadic besnoitiosis (Alzieu et al., 2007).

Recently, a first case of besnoitiosis was observed in an extensively managed beef herd in Southern Germany, close to the city of Munich (Rostaher et al., submitted for publication). Besnoitiosis was confirmed by clinical, cytological, histological, electron microscopical and serological examinations and by the detection of specific DNA in skin biopsies by PCR (Rostaher et al., submitted for publication). Here we report on the *in vitro* isolation of *B. besnoiti* from cattle from the affected herd. The respective tissues were obtained from eight animals which had been born and raised in Germany, showed clinical signs of besnoitiosis and tested positive for *Besnoitia* by PCR and IFAT (titers higher than 1:100). *B. besnoiti* was isolated from the animal with the highest IFAT titer (1:12,800) (Table 2).

To understand the epidemiology of *B. besnoiti* and the reasons for the emerging importance of besnoitiosis in France and other European countries, a detailed characterization of the population structure of the causing agents is necessary. Such a characterization can only be achieved if many isolates from different countries become available for analysis. There are reports on the *in vitro* isolation of *B. besnoiti* in cell cultures, but only few permanently growing isolates are existing worldwide (Bigalke et al., 1967; Bigalke, 1968; Neuman, 1974; Göbel et al., 1985; Shkap et al., 1987; Cortes et al., 2006; Fernández-García et al., in press). For isolation purposes, a variety of cell lines have been tested. Vero cells are often used for the *in vitro* isolation and the long-term cultivation of *B. besnoiti* (Bigalke et al., 1974; Neuman, 1974; Göbel et al., 1985; Shkap et al., 1987; Cortes et al., 2006). In the case reported here, however, the isolation and long-term cultivation of *B. besnoiti* from a total number of 13 tissue samples from eight cattle and from two of three peritoneal washings of infected GKO mice failed in Vero cells in all but one case, although numerous viable cystozoites (Table 2) or tachyzoites were visible in many inocula. Cortes et al. (2006) also reported a delayed adaptation of their isolates to Vero cells when they used cystozoites to infect cell cultures. Effective parasite growth with an easily visible parasite-induced cytopathic effect in cell culture was only reached after 30 and 40 days p.i. for both the Portuguese isolates, Bb1Evora03 and Bb2Evora03, respectively (Cortes et al., 2006). Recently, it was shown that *B. besnoiti* growed rapidly in MARC-145 cells. This cell line was used for the isolation and long-term cultivation of a *B. besnoiti* isolate from Spain (BbSpain1) (Fernández-García et al., in press). In a few publications various cell lines were compared for *in vitro* isolation and permanent cultivation of *B. besnoiti* (Neuman, 1974; Göbel et al., 1985; Shkap et al., 1987). In our study, we employed cell lines (Vero, BHK$_{21}$, MARC-145) which had successfully been used for the isolation or the long-term cultivation of *B. besnoiti* by other working groups. In addition, we also used the NA42/13 cell line and primary embryonic bovine cells (KH-R), previously applied to *in vitro* isolate *N. caninum* or *Hammondia* spp. (Schares et al., 2003, 2005; Basso et al., 2009).

In cell cultures inoculated with cystozoites, replication was more than 100 times slower than in those inoculated with tachyzoites obtained from the peritoneal cavity of mice. Obviously, the transition from slowly replicating bradyzoites to fast multiplying tachyzoites took several days in all used cell lines. For *T. gondii* it is known that particular types of cells (e.g. astrocytes, muscle cells) support stage conversion from tachyzoites to bradyzoites, i.e. as yet unknown conditions in these cells favor the growth of bradyzoites (Ferreira da Silva et al., 2008). It seems likely that *B. besnoiti* cystozoites also need specific as yet unknown cell types or other factors to transform into tachyzoites. In experimentally infected GKO mice, the transition from cystozoite to tachyzoite was faster than in cell cultures, i.e. the available cell types or conditions in the mouse supported the transition from cystozoite to tachyzoite more efficiently.

In contrast to the results obtained with an isolate from Spain (BbSpainB1, Fernández-García et al., in press), rapid replication was not seen in MARC-145 cells inoculated with Bb-GER1 cystozoites. In cystozoite-infected cell cultures cultivated for more than 5 days (123 h), the highest replication rate was observed in NA42/13 cells. This suggests that the transition from the cystozoite stage to tachyzoites was supported best in this cell line. In contrast, BHK$_{21}$ cells were superior to all other cell lines among the tachyzoite-inoculated cell cultures. Interestingly, Neuman (1974) also tried to use BHK$_{21}$ cells for *in vitro* isolation. However, he observed only a slow replication and no long-term cultivation of *B. besnoiti* was possible.

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In our experiments, also MARC-145, NA42/13 and KH-R cells produced considerable numbers of parasites when they were infected with GKO-mouse-derived tachyzoites. In Vero cells, almost no replication was observed. This finding is in accord with the almost complete failure in our study to isolate *B. besnoiti* via Vero cells. Only Vero cells inoculated with tachyzoites obtained from the peritoneal cavity of a GKO mouse supported the growth of the parasite. However, similar to observations of Cortes et al. (2006), a cytopathic effect induced by multiplying parasites was observed only after 27 days p.i. Interestingly, *B. besnoiti* from other sources were able to replicate fast in Vero cells, irrespective of the stage or source used for inoculation, i.e. cystozoites from cattle from South Africa (Göbel et al., 1985) or tachyzoites isolated from gerbils previously infected with cystozoites from naturally infected cattle from Israel (Shkap et al., 1987). It is not yet clear whether *B. besnoiti* from different parts of the world exhibit different characteristics regarding *in vitro* cultivation.

To examine whether Bb-GER1 can be distinguished from other isolates, a major part of the 18S and the complete ITS1 RNA gene were sequenced. Comparison of the sequences obtained for Bb-GER1 (FJ797432) with other sequences in GenBank (AF076859, AF109678, DQ227419, DQ227418, DQ227420, AY833646) revealed 99.5–100% identity with other *B. besnoiti* isolates. However, molecular comparison of *B. besnoiti* sequences with those available for other *Besnoitia* species, specific for goats (*B. caprae*), donkeys or horses (*B. bennetti*), and caribous or reindeers (*B. tarandi*) also revealed a high degree of identity (>99%). These findings are in accord with observations of other investigators (Ellis et al., 2000; Dubey et al., 2005). The rRNA genes are therefore of limited use to differentiate at the species level within the genus *Besnoitia*. Further studies are needed to identify genetic loci by which a differentiation of *B. besnoiti* isolates becomes possible. Such loci may also allow to correlate the genotype of *B. besnoiti* isolates with particular phenotypes (e.g. host cell prevalence or virulence).

5. Conclusions

B. besnoiti was isolated from the first known outbreak of bovine besnoitiosis in Germany. Molecular characterization of the 18S and the ITS1 RNA gene and parts of the 5.8S rRNA gene of this isolate revealed an almost 100% identity with those of *B. besnoiti* obtained in Portugal, Spain, Israel and South Africa. *In vitro*-isolation via cystozoites of Bb-GER1 was achieved in NA42/13, BHK21, and KH-R cells. Isolation via tachyzoites of Bb-GER1 obtained after several GKO mouse passages and subsequent *in vitro* cultivation succeeded in BHK_{21}, KH-R and NA42/13 cells. It remains to be established whether the preferential growth in certain cell lines is characteristic for particular isolates and if these differences are associated with differences in their virulence.

Uncited references

Bigalke (1962) and Dubey et al. (2004).

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References

Alzieu, J.-P., Dorchies, P., Schelcher, F., Gottstein, B., 2007. L'extension de la besnoitiose bovine en France. Le Point Vétérinaire 276, 37–43.

Basso, W., Schares, S., Bärwald, A., Herrmann, D.C., Conraths, F.J., Pantchev, N., Vrhovec, M.G., Schares, G., 2009. Molecular comparison of *Neospora caninum* oocyst isolates from naturally infected dogs with cell culture-derived tachyzoites of the same isolates using nested polymerase chain reaction to amplify microsatellite markers. Vet. Parasitol. 160, 43–50.

Bigalke, R.D., 1962. Preliminary communication on the cultivation of *Besnoitia besnoiti* (Marotel, 1912) in tissue culture and embryonated eggs. J. Afr. Vet. Assoc. 33, 523–532.

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

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., Schoeman, J.H., McCully, R.M., 1974. Immunization against bovine besnoitiosis with a live vaccine prepared from a blue wildebeest strain of *Besnoitia besnoiti* grown in cell cultures. 1. Studies on rabbits. Onderstepoort J. Vet. Res. 41, 1–5.

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

Diesing, L., Heydorn, A.O., Matuschka, F.R., Bauer, C., Pipano, E., de Waal, D.T., Potgieter, F.T., 1988. *Besnoitia besnoiti*: studies on the definitive host and experimental infections in cattle. Parasitol. Res. 75, 114–117.

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.

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.

Fernández-García, A., Risco-Castillo, V., Pedraza-Díaz, S., Aguado-Martínez, A., Alvarez-García, G., Gómez-Bautista, M., Collantes-Fernández, E., Ortega-Mora, L.M., in press. First isolation of *Besnoitia besnoiti* from a chronically infected cow in Spain. J. Parasitol.

Ferreira da Silva, M.F., Barbosa, H.S., Gross, U., Lüder, C.G., 2008. Stress-related and spontaneous stage differentiation of *Toxoplasma gondii*. Mol. Biosyst. 4, 824–834.

Göbel, E., Widauer, R., Reimann, M., Munz, E., 1985. Ultrastructure of the asexual multiplication of *Besnoitia besnoiti* (Marotel, 1912) in Vero- and CRFK-cell cultures. Zbl. Vet. Med. B 32, 202–212.

Levine, N.D., 1985. Veterinary Protozoology. Iowa State University Press, Ames, IA.

Neuman, M., 1974. Cultivation of *Besnoitia besnoiti* Marotel, 1912, in cell culture. Tropenmed. Parasitol. 25, 243–249.

Peteshev, V.M., Galzuo, i.G.P.A.P., 1974. Koshki—definitivnye khoziaeva besnoitii (*Besnoitia besnoiti*) [Cats—definitive hosts of Besnoitia (*Besnoitia besnoiti*)]. Izvest. Akad. Nauk. Kazakh. Ser. Biol. 1, 33–38.

Rostaher, A., Mueller, R.S., Majzoub, M., Schares, G., Gollnick, N.S., submitted for publication. Bovine besnoitiosis in Germany. Vet. Dermatol.

Schares, G., Peters, M., Wurm, R., Bärwald, A., Conraths, F.J., 1998. The efficiency of vertical transmission of *Neospora caninum* in dairy cattle analysed by serological techniques. Vet. Parasitol. 80, 87–98.

Schares, G., Meyer, J., Bärwald, A., Conraths, F.J., Riebe, R., Bohne, W., Rohn, K., Peters, M., 2003. A *Hammondia*-like parasite from the European fox (*Vulpes vulpes*) forms biologically viable tissue cysts in cell culture. Int. J. Parasitol. 33, 229–234.

Please cite this article in press as: Schares, G., et al., First *in vitro* isolation of *Besnoitia besnoiti* from chronically infected cattle in Germany. Vet. Parasitol. (2009), doi:10.1016/j.vetpar.2009.04.033

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., 2008a. 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., Herrmann, D.C., Beckert, A., Schares, S., Hosseininejad, M., Pantchev, N., Vrhovec, G.M., Conraths, F.J., 2008b. Characterization of a repetitive DNA fragment in *Hammondia hammondi* and its utility for the specific differentiation of *H. hammondi* from *Toxoplasma gondii* by PCR. Mol. Cell. Probes 22, 244–251.

Shkap, V., Pipano, E., Greenblatt, C., 1987. Cultivation of *Besnoitia besnoiti* and evaluation of susceptibility of laboratory animals to cultured parasites. Vet. Parasitol. 23, 169–178.

Please cite this article in press as: Schares, G., et al., First *in vitro* isolation of *Besnoitia besnoiti* from chronically infected cattle in Germany. Vet. Parasitol. (2009), doi:10.1016/j.vetpar.2009.04.033