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2 **First *in vitro* isolation of *Besnoitia besnoiti* from chronically infected**
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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, BHK₂₁, KH-R). The best growth of tachyzoites was observed in BHK₂₁ 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 BHK₂₁ 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.

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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 | <i>B. besnoiti</i> IFAT titer | <i>B. besnoiti</i> 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, periorcular and perioral hypotrichia and lichenification) (Table 1). Blood was taken from each animal from 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 knock-out (GKO) mice (C.129S7 (B6)-Irfngtm1Ts/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), BHK₂₁ (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 BHK₂₁ 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, BHK₂₁, NA42/13 and KH-R in 25 cm² 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

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* (Schaes 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 (Schaes 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 (Schaes 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 (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 | GGGTGCATTCCGAGAAGTGTG | 65 °C | ITS-1 | Cortes et al. (2006) |
| Bb-ITS1-R | TCCGTGATAGCAGAGTIGAGGAGG | 65 °C | ITS-1 | Cortes et al. (2006) |
| Bb-GS1F | TCGGCGACGGATCATTCAAGT | 54 °C | 18S-rDNA | This paper |
| Bb-GS1R | ATGCCCCCAACCGTCCCTAITTA | 54 °C | 18S-rDNA | This paper |
| Bb-GS2F | GGATTTCCGGCCCTATTTTG | 54 °C | 18S-rDNA | This paper |
| Bb-GS2R | CGCGTGACGCCAGAACA | 54 °C | 18S-rDNA | This paper |
| Bb-GS3F | TGACGGAAGGCCACCACAG | 54 °C | 18S-rDNA | This paper |
| Bb-GS3R | TCACCGGAACACTCAATC | 54 °C | 18S-rDNA | This paper |
| Bb-GS4F | GACTCAACACGGGAAACTCA | 54 °C | 18S-rDNA | This paper |
| Bb-GS4R | TCACCTACGGAAACCTT | 54 °C | 18S-rDNA | This paper |
| Bb-GS5F | CTGGTAGCGCTTCACACTCATTG | 54 °C | 18S-rDNA | This paper |
| Bb-GS5R | GTTTCAGCCTTCCGACCATACTCC | 54 °C | 18S-rDNA | This paper |
| Bb-GS6F | CTGCCAGTAGTCATATGCTGTCT | 54 °C | 18S-rDNA | This paper |
| Bb-GS6R | GCGCTGCTGCCTTCCTTAG | 54 °C | 18S-rDNA | This paper |

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

193 **Isolation attempt 2:** A second skin biopsy of one of the
194 sampled animals (Animal 70, Table 2) was first stored at

195 room temperature for 12 h, than stored at 4 °C for 30 h. 195
196 After transport to the Friedrich-Loeffler-Institut (FLI) 196
197 (without refrigeration), it was stored at 4 °C for another 197
198 2 days. Three days after sampling, the tissue was 198
199 processed and inoculated ip into a GKO mouse and 199
200 added to a Vero cell monolayer in parallel. In the Vero 200
201 cells, no significant replication was observed. No viable 201
202 parasites were seen until 7 days p.i. and cultivation was 202
203 stopped on day 57 p.i. The inoculated GKO mouse (K122) 203
204 fell ill 5 days p.i. and was sacrificed. Peritoneal washing 204
205 from this animal were transferred onto Vero cells and 205

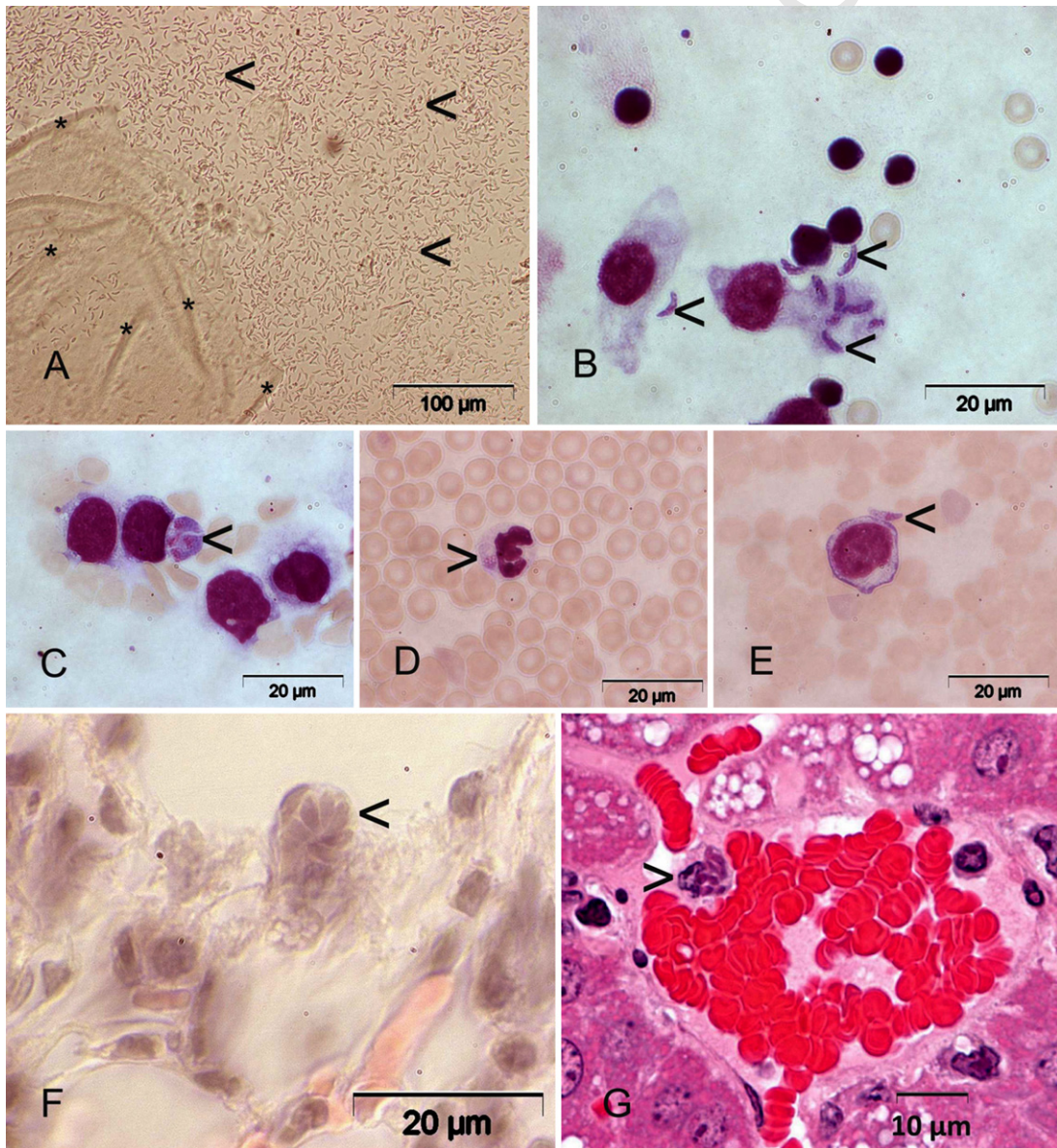


Fig. 1. *Besnoitia besnoiti* in a skin biopsy from an infected bull (A) and in tissues of an infected γ -interferon knockout mouse (B–G). (A) Numerous cystozoites (<) are released from tissue cysts (*) in bovine skin after squashing using a mortar and pestle. (B) Peritoneal washing of a *B. besnoiti* 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.

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

223 In Vero cells inoculated with peritoneal washings of
224 GKO mouse K122, no significant parasite replication was
225 observed and cultivation stopped 14 days p.i. The GKO
226 K123 mouse inoculated with the same material fell ill 5
227 days p.i. and was sacrificed. Peritoneal washings were
228 transferred onto Vero cells and inoculated after an
229 overnight storage at 4 °C ip into a third GKO mouse
230 (K124). Initially, no significant replication was observed
231 in the Vero cells, but on day 12 p.i., when the cell culture
232 was split, a few moving tachyzoites were detected. This
233 Vero cell culture was found infected with a few locations
234 of parasite-induced cytopathic effects on day 27 p.i. The
235 isolate was first cryopreserved 61 days p.i. and design-
236 ated Bb-GER1_{VERO}.

237 The GKO mouse K124 inoculated with peritoneal
238 washing of GKO mouse K123 fell ill (anorexia, ruffled
239 hair) 5 days p.i. Peritoneal fluid was inoculated ip into
240 two other GKO mice (K125/1; K125/2) and was added
241 onto monolayers of five different cell lines: Vero, MARC-
242 145, NA42/13, BHK₂₁ and KH-R cells. After 3 days in cell
243 culture, substantial parasite replication was observed in
244 all cell lines except Vero. Infected MARC-145, NA42/13,
245 BHK₂₁ and KH-R cultures were split and aliquots stored
246 in liquid nitrogen 4 days later. Parasites growing in the
247 KH cell line were further cultivated and stored in liquid
248 nitrogen 19 days p.i. for further studies. This isolate was
249 designated Bb-GER1_{KH}.

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

250
251

252 Peritoneal washings obtained during the fourth and the
253 fifth ip passage through GKO mice (K125/2, K126, K127)
254 were used for these experiments. The mice developed
255 clinical signs 5 days p.i. (K125/2, K126, K127). Equal
256 numbers of tachyzoites ($3-6 \times 10^5$) obtained from peri-
257 toneal washings of the animals were added to five different
258 cell lines: MARC-145, KH-R, NA42/13, BHK₂₁ and Vero.
259 After a cultivation period of 46.5 h (K125/2; Fig. 2,
260 Experiment A) or 48 h (K126/1, K126/2; Fig. 2, Experiments
261 B and C) extra- and intracellular parasites were counted.
262 The lowest replication occurred in Vero cells (0.14–0.43
263 tachyzoites per hour and inoculated parasite). In all other
264 cell lines, the parasite replication rate was higher. The
265 isolate replicated best in BHK₂₁ cells (6–20 tachyzoites per
266 hour and inoculated parasite).

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

267
268

269 Cystozoites (Fig. 1A) were obtained from bovine skin
270 tissue samples (Table 2, Animal 70) which were recovered
271 at slaughter. The samples were shipped overnight to the
272 FLI, where they were immediately processed upon arrival.
273 Parasites were counted and 6×10^4 or 37.5×10^4 cysto-
274 zoites added to two sets of five tissue culture flasks,
275 respectively, with monolayers of each of following five cell
276 lines: MARC-145, KH-R, NA42/13, BHK₂₁ and Vero. After a
277 cultivation period of 123 h, the number of parasites in the
278 supernatant and in the cells was determined (Fig. 2,
279 Experiments D and E). In cystozoite-inoculated cell
280 cultures, parasite replication was much slower than in
281 tachyzoite-inoculated cultures. Parasite replication was
282 best in NA42/13 cells (0.06–0.12 tachyzoites per hour and
283 inoculated parasite). The lowest replication (0.008–0.017
284 tachyzoites per hour and inoculated parasite) was
285 observed in MARC-145 and Vero cells.

3.4. Sequence comparison

286

287 Partial sequence of the 18S ribosomal RNA gene
288 (1725 bp), the entire sequence of the internal transcribed

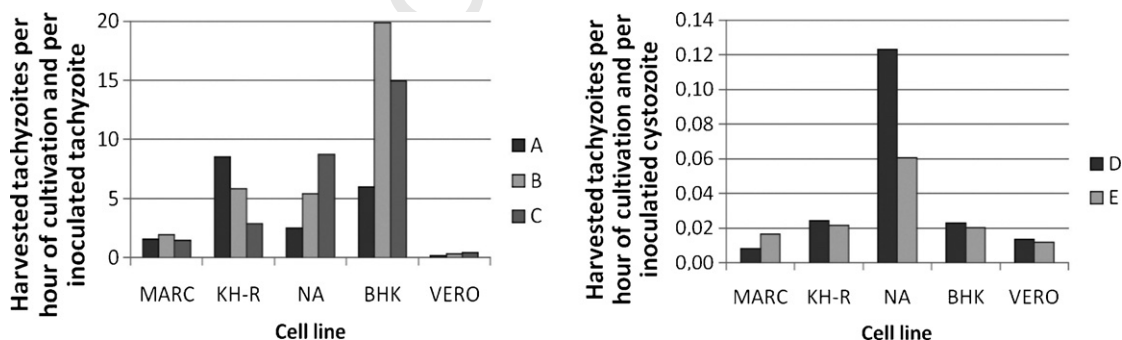


Fig. 2. Growth characteristics of the *Besnoitia besnoiti* isolate Bb-GER1 in five different cell lines: MARC-145 (MARC), KH-R (KH-R), NA42/13 (NA), BHK₂₁ (BHK) and Vero (VERO). Experiments A–C were performed with tachyzoites from the peritoneal cavity of infected γ -interferon knockout mice. Experiments D and E were performed with cystozoites from bovine skin.

289 spacer 1 (ITS1) (243 bp) and a partial sequence of the 5.8S
290 RNA gene (151 bp) were identical for Bb-GER1_{KH} and Bb-
291 GER1_{VERO} (FJ797432; GenBankTM).

292 When the sequence of the ITS1 rDNA was compared with
293 sequences deposited for *B. besnoiti* and other *Besnoitia* sp.,
294 identities of 100% (*B. besnoiti*, DQ227420.1, DQ227419.1,
295 DQ227418.1, AY833646.1), 100% (*B. tarandii*, AY665400.1),
296 99.6% (*B. bennetti*, AY665399.2, AY827839.1), 99.6% (*B.*
297 *besnoiti* from South Africa, AF076859, identical to the ITS1
298 of *B. caprae*, Ellis et al., 2000) or less than 80% (*B. darlingi*,
299 AF489696.1; *B. oryctofelisi*, AY182000.1; *B. akodonii*,
300 AY545987.1) were observed.

301 Comparison of the 18S ribosomal RNA gene sequence
302 was only possible with *B. besnoiti* strains from Spain
303 (DQ227419.1, DQ227418.1), Portugal (AY833646.1), Israel
304 (DQ227420.1) and South Africa (AF109678.1). Identities of
305 99.9–100% were observed with 18S ribosomal RNA gene
306 sequences of isolates from Spain, Portugal and Israel. An
307 identity of 99.5% was observed with the sequence available
308 for the South African isolate. The comparison of the 18S
309 ribosomal RNA gene sequence with sequences of the
310 related protozoa *Neospora caninum* and *Toxoplasma gondii*
311 revealed identities of 98.6–98.9% (U16159.1, M97703.1,
312 EF472967.1, X75429.1).

313 4. Discussion

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

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

337 To understand the epidemiology of *B. besnoiti* and the
338 reasons for the emerging importance of besnoitiosis in
339 France and other European countries, a detailed char-
340 acterization of the population structure of the causing
341 agents is necessary. Such a characterization can only be
342 achieved if many isolates from different countries become
343 available for analysis. There are reports on the *in vitro*
344 isolation of *B. besnoiti* in cell cultures, but only few
345 Q2 permanently growing isolates are existing worldwide
346 (Bigalke et al., 1967; Bigalke, 1968; Neuman, 1974; Göbel
347 et al., 1985; Shkap et al., 1987; Cortes et al., 2006;

348 Fernández-García et al., in press). For isolation purposes, a
349 variety of cell lines have been tested. Vero cells are often
350 used for the *in vitro* isolation and the long-term cultivation
351 of *B. besnoiti* (Bigalke et al., 1974; Neuman, 1974; Göbel
352 et al., 1985; Shkap et al., 1987; Cortes et al., 2006). In the
353 case reported here, however, the isolation and long-term
354 cultivation of *B. besnoiti* from a total number of 13 tissue
355 samples from eight cattle and from two of three peritoneal
356 washings of infected GKO mice failed in Vero cells in all but
357 one case, although numerous viable cystozoites (Table 2)
358 or tachyzoites were visible in many inocula. Cortes et al.
359 (2006) also reported a delayed adaptation of their isolates
360 to Vero cells when they used cystozoites to infect cell
361 cultures. Effective parasite growth with an easily visible
362 parasite-induced cytopathic effect in cell culture was only
363 reached after 30 and 40 days p.i. for both the Portuguese
364 isolates, Bb1Evora03 and Bb2Evora03, respectively (Cortes
365 et al., 2006). Recently, it was shown that *B. besnoiti* grew
366 rapidly in MARC-145 cells. This cell line was used for the
367 isolation and long-term cultivation of a *B. besnoiti* isolate
368 from Spain (BbSpain1) (Fernández-García et al., in press).
369 In a few publications various cell lines were compared for
370 *in vitro* isolation and permanent cultivation of *B. besnoiti*
371 (Neuman, 1974; Göbel et al., 1985; Shkap et al., 1987). In
372 our study, we employed cell lines (Vero, BHK₂₁, MARC-
373 145) which had successfully been used for the isolation or
374 the long-term cultivation of *B. besnoiti* by other working
375 groups. In addition, we also used the NA42/13 cell line and
376 primary embryonic bovine cells (KH-R), previously applied
377 to *in vitro* isolate *N. caninum* or *Hammondia* spp. (Schaes
378 et al., 2003, 2005; Basso et al., 2009).

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

396 In contrast to the results obtained with an isolate from
397 Spain (BbSpain1, Fernández-García et al., in press), rapid
398 replication was not seen in MARC-145 cells inoculated with
399 Bb-GER1 cystozoites. In cystozoite-infected cell cultures
400 cultivated for more than 5 days (123 h), the highest
401 replication rate was observed in NA42/13 cells. This
402 suggests that the transition from the cystozoite stage to
403 tachyzoites was supported best in this cell line. In contrast,
404 BHK₂₁ cells were superior to all other cell lines among the
405 tachyzoite-inoculated cell cultures. Interestingly, Neuman
406 (1974) also tried to use BHK₂₁ cells for *in vitro* isolation.
407 However, he observed only a slow replication and no long-
408 term cultivation of *B. besnoiti* was possible.

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 BHK₂₁, 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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