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

G. Schares a,g,h,*, W. Bassoa,b,c,g,h, M. Majzoubb,g,h, H.C.E. Cortesc,g,h, A. Rostaherd,g,h, J. Selmair e,g,h, W. Hermannsb,g,h, F.J. Conrathsa,g,h, N.S. Gollnickf,g,h

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, 60 y 118 (1900) La Plata, Argentina
c Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Buenos Aires, Argentina
d Institut of Veterinary Pathology, Ludwig-Maximilians-Universität, Munich, Germany
e Laboratório de Parasitologia, ICAM, Núcleo da Mitra, Universidade de Évora, Portugal
f Medizinische Kleintierklinik, Ludwig-Maximilians-Universität, Munich, Germany
g Inning am Holz, Germany
h Clinic for Ruminants, Ludwig-Maximilians-Universität, Munich, Germany

**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...
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. Peteshov 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 microscopic 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 mucus membranes of the vulva, periocular and perioral hypertrichia 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 knockout (GKO) mice (C129ST7 (B6)-Il10tm1Ts/J, The Jackson Laboratory, Bar Harbor, Maine, USA).

### Table 1

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

<table>
<thead>
<tr>
<th>ID of animal</th>
<th>No. of tissue samples</th>
<th>Breed①</th>
<th>Sex</th>
<th>Age (months)</th>
<th>Cystozoites in inoculated material</th>
<th>Cysts in conjunctival sclera</th>
<th>Cysts in vulva</th>
<th>B. besnoiti IFAT titer</th>
<th>B. besnoiti specific PCR</th>
<th>Histology</th>
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<td>1</td>
<td>L</td>
<td>Male</td>
<td>52</td>
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<td>No</td>
<td>NA</td>
<td>1:3200</td>
<td>Positive</td>
<td>ND</td>
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<td>62</td>
<td>3</td>
<td>Cha</td>
<td>Female</td>
<td>54</td>
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<td>1</td>
<td>Cha</td>
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<td>122</td>
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<tr>
<td>70</td>
<td>2</td>
<td>Cha</td>
<td>Male</td>
<td>21</td>
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<td>Yes</td>
<td>NA</td>
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<tr>
<td>92</td>
<td>2</td>
<td>L</td>
<td>Female</td>
<td>40</td>
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<td>Yes</td>
<td>Yes</td>
<td>1:3200</td>
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<tr>
<td>94</td>
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① L = Limousin and Cha = Charolais.
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 for-
malin-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
Giems’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

Table 2

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’–3’)</th>
<th>Annealing temperature</th>
<th>Region</th>
<th>Reference</th>
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<td>ITS-1</td>
<td>Cortes et al. (2006)</td>
</tr>
<tr>
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<td>ITS-1</td>
<td>Cortes et al. (2006)</td>
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<tr>
<td>Bb-G51F</td>
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<td>54 °C</td>
<td>18S-rDNA</td>
<td>This paper</td>
</tr>
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<td>18S-rDNA</td>
<td>This paper</td>
</tr>
<tr>
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<td>This paper</td>
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<td>Bb-G53F</td>
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<td>This paper</td>
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<td>Bb-G55F</td>
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<td>54 °C</td>
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<td>Bb-G55R</td>
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<td>This paper</td>
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<tr>
<td>Bb-G56F</td>
<td>CGCGGCTGACTACAGTTG</td>
<td>54 °C</td>
<td>18S-rDNA</td>
<td>This paper</td>
</tr>
</tbody>
</table>

| Bb-G56R       | GGCCTGCGCCTTCTT | 54 °C                 | 18S-rDNA | This paper                     |

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 (Finn-
zymes, 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 SequenaseTM DYEnamic Direct Cycle Sequen-
cing 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 Read IR 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 GenBankTM by a BLAST search.
The obtained consensus sequence was deposited in
GenBankTM 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

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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 i.p. 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

![Image](image.png)

**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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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 × 10⁵) obtained from peritoneal washings of the animals were added to five different cell lines: MARC-145, KH-R, NA42/13, BHK₂₁ 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 BHK₂₁ 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 × 10⁴ or 37.5 × 10⁴ 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, BHK₂₁ 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...
space 1 (ITS1) (243 bp) and a partial sequence of the 5.8S
RNA gene (151 bp) were identical for Bb-GER1,KH and Bb-
GER1VERO (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. tarsandi, AY665400.1).
99.6% (B. benetti, 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. oryctofelis, AY182000.1; B. akodonti,
AY549871.9) 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 ser-
ological 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 char-
acterization 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
permanent 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, Bb1Evoa03 and Bb2Evoa03, respectively (Cortes
et al., 2006). Recently, it was shown that B. besnoiti grew
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, BHK21, 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
in in vitro isolation N. caninum or Hammondia spp. (Schar-
es 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 (BbSpain1, Fernández-García et al., in press), rapid
replication was not seen in MARC-145 cells inoculated with
Bb-GER1 cystozoites. In cystozoite-infect 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,
BHK21 cells were superior to all other cell lines among
the tachyzoite-inoculated cell cultures. Interestingly, Neuman
(1974) also tried to use BHK21 cells for in vitro isolation.
However, he observed only a slow replication and no long-
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.

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