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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 and edema in

☆ 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*.

Animal	No. of tissue samples	Breed[a]	Sex	Age (months)	Cystozoites in inoculated material	Cysts in conjunctival sclera	Cysts in vulva[b]	*B. besnoiti* IFAT titer	*B. besnoiti* specific PCR	Histology[c]
31	1	L	Male	52	Yes	No	NA	1:3,200	Positive	ND
62	3	Cha	Female	54	Yes	No	Yes	1:6,400	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	1:3,200	Positive	Positive
94	2	L	Female	65	Yes	Yes	Yes	1:3,200	Positive	Positive
168	1	L	Female	44	No	Yes	Yes	1:12,800	Positive	Positive
169	1	L	Male	35	No	Yes	NA	1:6,400	Positive	Positive

[a] L = Limousin and Cha = Charolais.
[b] NA = Not applicable.
[c] ND = Not done.

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 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 (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