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# Evidence for bovine besnoitiosis being endemic in Italy—First *in vitro* isolation of *Besnoitia besnoiti* from cattle born in Italy☆

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ABSTRACT

Until 2009, bovine besnoitiosis had never been considered endemic in Italy and the only report on the disease in this country referred to animals imported from France shortly before. However, recently, an autochthonous outbreak of bovine besnoitiosis was reported in four herds located at the intersection of the borders between Emilia-Romagna, Toscana and Marche (Northern Apennine Mountains), which has led to an increased awareness concerning this disease. The present study describes a further outbreak of bovine besnoitiosis in Italy. The afflicted herd was a dairy herd with no evidence for contact with cattle from regions known to be endemic for bovine besnoitiosis. The farm investigation was initiated after a three-year old Holstein Friesian dairy cow with generalized thickening and lichenification of the skin was diagnosed with bovine besnoitiosis. The clinical diagnosis was confirmed by gross pathology, histopathology, serology and PCR. Bradyzoites released from tissue cysts obtained from the skin of this animal enabled the first *in vitro* isolation of *Besnoitia besnoiti* in Italy. This isolate was named Bb-Italy1. Sequencing of a 2118 bp spanning region including the complete internal transcribed spacer 1 and parts of the 18S and the 5.8S rRNA gene from DNA extracted from skin-derived zoites revealed a 99.9% identity to sequences known for other *B. besnoiti* isolated from cattle in Europe. Two GKO mice which had been inoculated intraperitoneally with bovine skin-derived bradyzoites became ill 7 days post inoculation. Parasitophorous vacuoles with multiplying zoites were observed in the cell culture inoculated with peritoneal fluids of these mice and a *B. besnoiti* infection in the mice and in the cell culture could be confirmed by real-time PCR. A serological investigation in the afflicted herd using immunoblots and an immunofluorescent antibody test (IFAT) revealed an overall herd seroprevalence of 9.7% (31/321), whereas within the female animals older than 2 years 17.0% (29/171) of the dams were tested positive. With one exception, an imported cow from Germany, all the seropositive animals were born in Italy. In connection with previously described autochthonous cases of bovine besnoitiosis the case described herein suggests that bovine besnoitiosis should be considered endemic in Italy.



☆ Nucleotide sequence data reported in this paper are available in the GenBank™ database under the accession number JF314861.

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## 1. Introduction

Bovine besnoitiosis is a parasitic disease of cattle caused by the cyst-forming coccidian *Besnoitia besnoiti* (Pols, 1960). The parasite targets mainly the skin, mucosal membranes, testes, the scleral conjunctiva, and endothelial cells of large vessels (Basson et al., 1970; Kumi-Diaka et al., 1981; Majzoub et al., 2010). Clinical signs in early stages of the disease include fever, lymphadenitis, subcutaneous edema, lameness, loss of body condition and testicular inflammation (Bigalke and Prozesky, 1994). During the chronic stage typical clinical signs are pinhead-sized papules in the scleral conjunctiva and in the mucous membranes of the genital organs of female animals (Majzoub et al., 2010; Nobel et al., 1981; Rostaher et al., 2010), as well as cutaneous lesions such as thickening and folding of the skin and alopecia. Severe necrotising orchitis may result in permanent infertility (Bigalke and Prozesky, 1994). The disease may be responsible for significant financial losses in affected farms due to reduction in meat and milk production, impairment of reproductive performance, early culling, and even death of affected cattle (Cortes et al., 2005; Gollnick et al., 2010b; Jacquiet et al., 2010).

In 1912, Besnoit and Robin described the histopathological lesions caused by *B. besnoiti* for the first time. Three years later bovine besnoitiosis was also reported from Portugal (Franco and Borges, 1915). It then took another 75 years until the disease was described in Spain (Juste et al., 1990). However, since the early 90s of the last century, *B. besnoiti* has spread from southern France over western France up to the Loire and into central regions of the country (Alzieu et al., 2007; Freudiger, 2008; Jacquiet et al., 2010; Legrand, 2003; Thomas, 2007), and has propagated within Portugal (Cortes et al., 2003, 2005) and Spain (Fernandez-Garcia et al., 2010; Irigoien et al., 2000). In 2008, bovine besnoitiosis was diagnosed for the first time in Germany in locally born cattle (Gollnick et al., 2009; Majzoub et al., 2010; Rostaher et al., 2010; Schares et al., 2009). Thus far *in vitro* isolation of *B. besnoiti* strains has only been reported three times in Europe (Cortes et al., 2006; Fernandez-Garcia et al., 2009; Schares et al., 2009). Molecular characterization of the 18S and the ITS1 RNA gene and parts of the 5.8S rRNA gene of these isolates from Portugal, Spain and Germany revealed an almost 100% identity (Schares et al., 2009).

Until recently, bovine besnoitiosis had not been considered endemic in Italy. Reports dealing with this disease were referred only to an animal imported shortly before from France (Agosti et al., 1994; Di Giancamillo et al., 1994; Vacirca et al., 1994; Mutinelli et al., 2011). However, as from 2009, autochthonous outbreaks of bovine besnoitiosis were detected in four herds (hereafter referred to as herds A, B, C and D) located at the intersection of the borders between Emilia-Romagna, Toscana and Marche (Northern Apennine Mountains).

The first evidence of disease was observed in a cow-calf operation (herd A). In September 2009, a 24-months old Chianina breeding bull, born and raised in the area was presented with severe generalized lichenification and induration of the skin, hyperkeratosis and alopecia (Gentile et al., 2010). A herd investigation in November 2009, revealed an overall *B. besnoiti* antibody seroprevalence of 41.2% (47 of 114). Eleven (23.4%) of 47 seropositive animals also showed the presence of cysts in the scleral conjunctiva and/or mucosa of the *vestibulum vaginae*, being therefore considered as clinically affected (Gollnick et al., 2010a).

Moreover, in November 2009, clinical examinations and blood sampling were conducted in a neighboring farm (herd B) whose animals had been grazing together with cattle of herd A in a Tuscan national park of about 3000 ha, during previous summers (Gollnick et al., 2010a). The herd investigation revealed a seroprevalence of 36.4% (8 of 22 subjects) and the presence of three animals with mild clinical symptoms defined by the presence of cysts in the scleral conjunctiva and/or mucosa of the *vestibulum vaginae* (Gollnick, personal observations, 2009).

Finally, further cases of skin disease due to bovine besnoitiosis were diagnosed in two other herds (herds C and D) which had been sharing the same afore mentioned grazing area together with the herds A and B (Gentile, personal observations, 2011; Manuali et al., 2011).

Unfortunately, bovine besnoitiosis is not an OIE-listed disease, and currently no systematic serological monitoring programs exist in affected regions. Nevertheless, the recent spread of the disease on the European continent is alarming which prompted the European Food Safety Authority (EFSA) in 2010 to classify the disease as an emerging disease in Europe (EFSA, 2010).

Here we present another autochthonous outbreak of bovine besnoitiosis in an Italian cattle herd (hereafter referred to as herd E) not correlated epidemiologically with those mentioned above. The herd investigation was preceded by the examination of a clinical case of bovine besnoitiosis, confirmed by gross pathology, histopathology, serology and PCR. Bradyzoites released from tissue cysts obtained from the skin of the affected cow were inoculated in a confluent MARC-145 cell culture and enabled the first *in vitro* isolation of *B. besnoiti* from Italian-born cattle.

## 2. Materials and methods

On May 25, 2010, a three-year-old non-pregnant Holstein cow, born and raised on the premises of a dairy farm (herd E) in the Emilia-Romagna region, was referred to the Faculty of Veterinary Medicine, University of Bologna, Italy with the tentative diagnosis “bovine besnoitiosis”. Following the thorough examination of the patient a herd survey was conducted on August 4, 2010. Permission to investigate the herd was granted by the owner in due consideration of minimal disruption of daily farm routine. Therefore, only blood sampling was conducted and clinical examinations of animals had to be omitted.

### 2.1. The clinical case

#### 2.1.1. Clinical examination and blood sampling

The Holstein cow was housed at the Teaching Hospital of the Faculty of Veterinary Medicine, University of Bologna, Italy. A complete clinical examination was performed (Divers and Peek, 2008) and samples for blood