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Research paper

Bovine chronic besnoitiosis in a calf: Characterization of a novel *B. besnoiti* isolate from an unusual case report

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ABSTRACT

Bovine besnoitiosis, caused by the apicomplexan *Besnoitia besnoiti*, is a chronic and debilitating disease characterized by cutaneous and systemic manifestations that primarily affects adult beef cattle. Previous studies have reported that clinical besnoitiosisis is rare in calves. However, we isolated *B. besnoiti* from a chronically infected calf for the first time. The identity of the *Besnoitia* species was determined after parasite isolation and molecular genotyping. According to the results obtained *in vitro* the new isolate, named as Bb-Spain3, was characterized in a reproducible *in vitro* model and was categorized as a low invader and low prolific isolate with a slower lytic cycle compared to Bb-Spain 1 isolate. Specific traits that differentiate isolates obtained from adult animals from those infecting calves were not found. Next, we described the first case report of chronic besnoitiosis in a female calf less than 6 months-old with a low body condition. The disease was confirmed by the presence of specific anti-*B. besnoiti* antibodies and parasite detection in the skin. At post-mortem examination, tissue samples were collected for histological, immunohistochemical and molecular analyses. DNA-parasite was detected in 31 different calf's tissues, being the most highly parasitized tissues the skin and the respiratory and reproductive tracts. In addition, the parasite was also present in heart, eyes, lymph nodes and brain. The high parasite load, a wide intra-organic parasite distribution and the presence of both viable and degenerated cysts, were indicative of a rapid progression of the disease. This case report underlines the need to include the inspection of young animals in besnoitiosis control.

1. Introduction

Bovine besnoitiosis, caused by the cyst forming apicomplexan parasite *Besnoitia besnoiti* (Besnoit and Robin, 1912), is a chronic and a debilitating disease that primarily affects beef cattle and has a negative impact on productive and reproductive parameters, welfare and causes occasional deaths. The European Food Safety Authority in 2010 (European Food Safety Authority, 2010) was alerted on the re-emergence of this disease in Europe from areas where the disease was traditionally endemic (French Pyrenees, the Alentejo region in Portugal and the northeastern part of Spain). At present, the disease has already reached Ireland and Eastern countries (Beck et al., 2013; Hornok et al., 2014; Vanhoudt et al., 2015; Ryan et al., 2016). Unfortunately, there are still gaps in the epidemiology of the disease, and this lack of knowledge hampers its control, which solely relies on diagnosis and management measures in the absence of either drugs or vaccines. In particular, the definitive host and transmission routes remain to be elucidated (Álvarez-García et al., 2013).

It has been postulated that *B. besnoiti* could have a heteroxenous life cycle, where cattle, antelopes, red deer and roe deer act as intermediate hosts (Arnal et al., 2017; Gutiérrez-Expósito et al., 2016). In the intermediate host, two different asexual parasitic stages develop. First, the tachyzoites invade the vascular endothelium of blood vessels and are responsible for the acute stage of the disease. During the acute stage of the disease, the infected animals develop non-specific clinical signs that may go unnoticed, such as fever, nasal and ocular discharge, depression, lameness, orchitis and subcutaneous oedema. Next, tachyzoites switch into bradyzoites as a mechanism of immune response evasion, which pack inside thick-walled cysts mostly found in subcutaneous tissues and mucous membranes during the chronic stage of the disease. Consequently, chronically infected cattle develop hyperkeratosis, alopecia, atrophy of the testes, as well as pathognomonic tissue cysts in the sclera conjunctiva or the *vestibulum vaginae* (Álvarez-García et al., 2014b; Gollnick et al., 2015).

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Parasite and host dependent factors may determine the outcome of the infection (Álvarez-García et al., 2014b), such as the isolate. Recently, Frey et al. (2016) has studied and compared seven *B. besnoiti* isolates, from six countries and two continents, and reported different *in vitro* characteristics (invasion and proliferation) but no obvious difference between them with regard to virulence. Gutiérrez-Expósito et al. (2016) using microsatellite analysis, could only find variation in a unique MS marker in one isolate (Bb- Italy2) that differed from 9 other, homogeneus, *B. besnoiti* isolates. This may be due to the fact that all compared isolates had been obtained from adult cattle clinically affected. Until now, *B. besnoiti* isolates have not been obtained from affected young animals, which may allow us to address if there are specific traits that differentiate isolates obtained from adult animals from those infecting younger animals.

Whether animal age has influence in the infection remains to be clarified. There are only two recent reports of the disease in calves. Hornok et al. (2014) reported the first case of besnoitiosis in Hungary and found three seropositive calves with respiratory disorders. More recently, Ryan et al. (2016) detected scleral tissue cysts in 41.9% of the calves present in an affected dairy herd in Ireland. The calves tested by ELISA proved to be seronegative. In the past, several authors (Bigalke, 1968; Alzieu, 2007) have concluded that the clinical incidence of besnoitiosis is very low in animals under one year of age. Indeed, clinical signs are more often observed among 2–4-year-old adults and rarely occur in calves under 6 months of age (Janitschke et al., 1984); this finding is also supported by recent reports demonstrating an increase of prevalence rates with age (Fernández-García et al., 2010; Gutiérrez-Expósito et al., 2014).

In the present study, firstly we obtained and characterized the first *B. besnoiti* isolate from a calf origin in an *in vitro* system. Next, we described the first case report of chronic bovine besnoitiosis in a calf younger than 6 months of age. After the post-mortem examination of the animal, an exhaustive tissue collection was carried out, and data on lesions and intra-organic parasite distribution were obtained by means of histopathological and PCR techniques.

2. Material and methods

2.1. Case report and herd samplings

In August 2013, local veterinarians reported three suspicious clinical cases of chronic bovine besnoitiosis located in Central Spain (north-eastern part of Toledo province), where the presence of the disease had not previously been confirmed. The affected animals were an adult Limousin cow and a calf from a closed beef cattle herd. This herd was composed of 176 animals raised under extensive conditions where natural mating was the rule. In addition, a Charolais cow in a nearby farm that practised similar management measures was also affected.

The adult animals showed compatible clinical signs of chronic besnoitiosis, such as hyperkeratosis, skin folding in the neck and limbs, nodules and cracks in the udders and tissue cysts that could be seen by the naked eye in the sclera conjunctivae (Supplementary figure). The 4-month-old Limousin calf also showed signs of alopecia in the periorbital region and muzzle (Fig. 1). Two months before, the animal had displayed non-specific clinical signs compatible with the acute stage of bovine besnoitiosis, such as fever, depression and anorexia.

Sera from these animals were collected and examined for specific antibodies by ELISA. Biopsies from the skin in the tarsal region in the calf and from the *vestibulum vaginae* in the adult cow were collected and squashed between trichinelloscopy plates to visualize tissue cysts by direct microscopic examination. The serological analysis was repeated in the calf at 7 and 8 months of age. Subsequently, it was donated to the Veterinary Medical Teaching Hospital (Complutense University of Madrid) for further examination.

Due to poor body condition, the calf was sacrificed at 8 months of age. The animal was sedated with xylazine hydrochloride (Rompun®; Bayer) and immediately euthanized by an intravenously overdose of embutramide and mebezonium iodide (T61®; Intervet) in the ruminant medical facilities at the Veterinary Medical Teaching Hospital. All experimental procedures complied with current EU legislation (Directive 2003/65/CE and 2010/63/EU).

A complete and systematic post-mortem study was carried on the carcass, and tissue samples from 35 different locations were taken (Table 1). Tissues were fixed in neutral buffered 10% formalin solution for histopathology and immunohistochemistry studies and frozen at −80ºC for PCR analyses. In addition, an epiglottis sample was also collected for parasite isolation in cell culture.

2.2. Parasite isolation and in vitro assays

The presence of *B. besnoiti* tissue cysts in the calf was confirmed in samples from the epiglottis after tissue compression on a trichinelloscopy plate and visualization under the microscope (Nikon eclipse 50*i*). The tissue was homogenized using a Potter Elvehjem homogenizer (Sigma-Aldrich®) in a solution consisting of PBS (phosphate buffer saline) with 2% antibiotic (Penicillin/Streptomycin + Amphotericin B (Lonza®)). Bradyzoites released from the tissue cysts were observed at 40× in a light microscope, and aliquots of 4×10^7 bradyzoites were inoculated onto fresh monolayers of Marc-145 cells in T75 flasks (Nunc®; Thermofisher Scientific). All isolates were maintained on this type of cell, employing the same procedure described by Fernández-García et al. (2009). Invaded bradyzoites were observed as soon as 48 h post infection (hpi). Tachyzoites were maintained and passaged every 3–5 days in confluent cell cultures. This newly obtained isolate was named Bb-Spain 3. Tachyzoites from Bb-Spain 1 and the newly obtained Bb-Spain 3 isolates were grown in Marc-145 cells and purified as described by Frey et al. (2016). In order to avoid adaptation of the parasites to the cell line employed, isolates with a low passage number were included in the experiments (Bb-Spain 1 from 10 to 16 and Bb-Spain 3 from 6 to 10 passage number). Bb-Spain 1 was included as an internal control of reproducibility in invasion and proliferation assays. All isolates used for *in vitro* assays tested negative to *Mycoplasma* spp. infection by PCR (Venor™GeM Mycoplasma Detection Kit; Minerva Biolabs) and bovine viral diarrhea virus (BVDV) by quantitative real-time PCR (qPCR) (Hoffmann et al., 2006). Foetal calf serum used in all the experiments was previously checked for the absence of either specific IgG against *B. besnoiti*, *Neospora caninum* and *Toxoplasma gondii* by IFAT or BVDV RNA by qPCR (Fernández-García et al., 2009).

2.2.1. Parasite invasion rate

For invasion assays, 10^5 Marc-145 cells/well were grown to confluence in P24 cell culture plates (Nunc®; Thermofisher Scientific) incubated at 37 ˚C with 5% CO2. The next day, 10^3 tachyzoites of each isolate (infection rate 1:100) were added to the cell cultures. Four washes with PBS were performed at 4, 6, 8 and 24 hpi to discard non-invaded tachyzoites. One ml of fresh culture medium was added, and the plates were incubated at 37 ˚C with 5% CO2. In addition, non-washed plates were kept until fixation. After 72 hpi, IFAT (see Section 2.6.) was carried out in order to count the total number of invasion events (parasitophorous vacuoles and/or lysis plaques) per well according to the procedure established by Frey et al. (2016). Three replicates of invasion assays were repeated in three independent experiments for each isolate.

2.2.2. Proliferation kinetics, tachyzoite yield and doubling time determination

For the proliferation assay, P24 cell culture plates with confluent Marc-145 cells (10^5 cells/well), maintained with DMEM 10% FCS (HyClone®; Thermofisher Scientific), were used. The monolayers were infected with 10^6 purified tachyzoites/well (infection rate 10:1) suspended in 1 ml DMEM 5% FCS. After 4 h, the wells were washed three times with DMEM and 5% FCS, and the infected monolayers were