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Research paper

The role of wild ruminants as reservoirs of *Besnoitia besnoiti* infection in cattle

Daniel Gutiérrez-Expósito[a], María. C Arnal[b], David Martínez-Durán[b], Javier Regidor-Cerrillo[a], Miguel Revilla[b], Daniel L Fernández de Luco[b], Alejandro Jiménez-Meléndez[a], Rafael Calero-Bernal[c], Miguel Angel Habela[c], Ignacio García-Bocanegra[d], Antonio Arenas-Montes[d], Luis. M Ortega-Mora[a], Gema Álvarez-García[a,*]

[a] SALUVET, Animal Health Department, Faculty of Veterinary Sciences, Complutense University of Madrid, Ciudad Universitaria s/n, 28040 Madrid, Spain
[b] Animal Pathology Department, Faculty of Veterinary, University of Zaragoza, Miguel Servet 177, 50013 Zaragoza, Spain
[c] Animal Health Department, Faculty of Veterinary Sciences, University of Extremadura, 10071 Cáceres, Spain
[d] Animal Health Department, Faculty of Veterinary Sciences, University of Córdoba-Agrifood Excellence International Campus (ceiA3), Córdoba, Spain

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ABSTRACT

Bovine besnoitiosis, a parasitic disease caused by *Besnoitia besnoiti*, has been reported mainly in beef cattle raised under extensive pastoral systems and is considered to be re-emerging in Western Europe. Horizontal transmission probably occurs either by means of blood sucking arthropods or as a consequence of direct contact between infected and non-infected cattle. However, the role that wild ruminants (e.g., red deer (*Cervus elaphus*) and roe deer (*Capreolus capreolus*)) may play in the parasite life cycle as putative reservoirs remains elusive. Thus, we investigated the presence of *Besnoitia* spp. infection in 2608 wild ruminants located in areas where bovine besnoitiosis is present and identified the *Besnoitia* species detected. First, a serosurvey was conducted in red deer ($n = 309$), roe deer ($n = 417$), Pyrenean chamois (*Rupicapra p. pyrenaica*, $n = 383$) and Iberian wild goat (*Capra pyrenaica hispanica*, $n = 288$) from two areas of Aragon, northeastern Spain, where bovine besnoitiosis is endemic. Second, red deer ($n = 820$), roe deer ($n = 37$), fallow deer (*Dama dama*, $n = 166$), Iberian wild goat ($n = 86$) and European mouflon (*Ovis orientalis musimon*, $n = 102$) from southwestern Spain, where new outbreaks have recently been reported, were also sampled. The presence of *Besnoitia* spp.-specific antibodies was confirmed by western blot in one red deer and one roe deer from the Pyrenees, and *Besnoitia* spp. DNA was detected by ITS1-PCR in the seropositive red deer. *Besnoitia* genotyping based on 6 microsatellite (MS) analyses was carried out in red deer samples and compared with *B. besnoiti* genotypes from 7 *in vitro* isolates and 3 infected bovines, *B. tarandi* (1 isolate) and *B. bennetti* (from tissues of an infected donkey) for *Besnoitia* spp. assignation. Multilocus MS analysis of *B. besnoiti*, *B. tarandi* and *B. bennetti* showed specific genotypes for each species. A restricted genetic diversity with two genotypes by variation in a unique MS marker was revealed among the 7 *B. besnoiti* isolates. Incomplete *Besnoitia* spp. genotype of 3 MS markers from red deer samples entirely matched the *B. besnoiti* genotypes. Accordingly, this work gives clues for the presence of *B. besnoiti* infection in red deer from Western Europe. Further molecular genotyping is needed to confirm that red deer may act as an intermediate host of *B. besnoiti*, although the low prevalences that were found indicate that wild ruminant species do not pose a significant risk of transmitting the infection to cattle.

1. Introduction

Genus *Besnoitia* belongs to the family Sarcocystidae and is included in the subfamily Toxoplasmatinae, together with the closely related protozoa *Neospora caninum* and *Toxoplasma gondii* (Ellis et al., 2000). Four out of 10 *Besnoitia* species reported to date (*Besnoitia besnoiti*, *Besnoitia tarandi*, *Besnoitia bennetti* and *Besnoitia caprae*) infect ungulates (bovids, subarctic cervines, equids and goats, respectively), which act as intermediate hosts. *B. besnoiti* and *B. tarandi* were first reported in the beginning of the twentieth century (Besnoit and Robin, 1912; Hadween, 1922). *Besnoitia besnoiti* (reported in cattle and antelope) is widely distributed in Africa,

* Corresponding author.
E-mail addresses: gemaga@vet.ucm.es, gemalgar@yahoo.es (G. Álvarez-García).

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Asia and southwestern Europe, where the disease is considered to be re-emergent (EFSA, 2010). In contrast, *Besnoitia* spp. infections detected in several wild ruminant species were attributed to *B. tarandi* (reviewed by Leighton and Gajadhar, 2001). In particular, the disease has occasionally been reported in *Bovidae* (musk ox) as well as *Cervidae* species (reindeer, caribou and mule deer) from different subarctic regions from Alaska (USA) and Canada (Choquette et al., 1967; Wobeser, 1976; Lewis, 1989; Glover et al., 1990; Lewis, 1992; Ducrocq et al., 2012) and in reindeer in Scandinavia and Russia (Nikolaevskii, 1961; Rehbinder et al., 1981; Hilali et al., 1990; Dubey et al., 2004).

The disease caused by both *Besnoitia* species (*B. besnoiti* and *B. tarandi*) is chronic, debilitating and characterized by the development of subcutaneous tissue cysts responsible for skin lesions such as hyperkeratosis and alopecia as well as ocular conjunctiva cysts (Álvarez-García et al., 2014). However, many biological and epidemiological aspects of both parasites remain obscure, including the complete life cycles, transmission routes and the possibility of interaction between sylvatic and domestic life cycles. Regarding *B. besnoiti*, domestic cats or an undetermined wild carnivore has been suggested to act as the definitive host, and wild ruminants have been postulated as putative reservoirs (Álvarez-García et al., 2013; Basso et al., 2011; Gutiérrez-Expósito et al., 2013; Olias et al., 2011). Indeed, in previous studies (Fernández de Luco et al., 2000) reported *Besnoitia* spp. infection in a roe deer from Central Pyrenees, where bovine besnoitiosis is endemic (Gutiérrez-Expósito et al., 2014) and later on Gutiérrez-Expósito et al. (2013) detected specific antibodies against *Besnoitia* spp. in a red deer and a roe deer from Eastern Pyrenees. Nevertheless, the role of wild ruminants as parasite reservoirs as well as the identity of the *Besnoitia* species circulating among European wild ruminant populations needs to be clarified. *Besnoitia besnoiti* and *B. tarandi* seem to be separated geographically, according to previous reports (Olias et al., 2011) and cross-infections have never been described.

Studies on *Besnoitia* spp. genetic variability is a challenge because the genome is still not sequenced, and 61 nucleotide sequences are available in databases. These sequences mainly correspond to Internal Transcribed Spacer 1 (ITS-1) and subunits ribosomal RNA sequences, which showed 99.6-100% identity in *Besnoitia* spp. species affecting large mammals (Ellis et al., 2000; Schares et al., 2009). A recent genetic tool based on microsatellite (MS) markers differentiated *B. besnoiti*, *B. tarandi* and *B. bennetti* species, and variations among four *B. besnoiti* isolates have also been described (Madubata et al., 2012).

The aim of the present work was to determine the spread of *Besnoitia* spp. infection in wild ruminants located in areas where bovine besnoitiosis is present and to determine whether these species may act as intermediate hosts of *B. besnoiti*. Thus, a wide serosurvey was carried out in areas where the disease is endemic (Gutiérrez-Expósito et al., 2014), and outbreaks of bovine besnoitiosis have recently been reported (Álvarez-García et al., 2013). Moreover, the presence of *Besnoitia* spp. was confirmed by PCR in a seropositive red deer, and genotyping of *Besnoitia* species was carried out by means of multilocus MS analysis.

2. Material and methods

2.1. Sampled areas

Mountainous areas where bovine besnoitiosis has been endemic for a long period of time (areas A and B) and where new outbreaks have recently been reported (area C) were selected for the study (Fig. 1). Area A covers the whole of the central Spanish Pyrenees (Huesca Province, Aragon), where the true prevalence was recently reported to be 50% in beef cattle (Gutiérrez-Expósito et al., 2014). Area B comprises a mountainous region located in the eastern foothills of the Iberian System (Maestrazgo of Teruel Province, Aragon) with 26% (342/1307) true prevalence in beef cattle (unpublished data). In these areas, beef cattle are usually kept indoors in late autumn and winter, whereas during the spring and summer, the beef cattle cohabit in communal pastures with wild ruminants and are exposed to blood-sucking arthropods such as tabanids. The representative wild ruminants in area A are red deer, roe deer and Pyrenean chamois, and in area B, the representative wild ruminants are roe deer and Iberian wild goats. In area C, the disease is considered emerging due to new outbreaks in beef cattle herds during the last few years (EFSA, 2010; unpublished results). This region covers extensive semi-natural silvopastoral woodlands known as “dehesas”, as well as Mediterranean forest, where a mixture of different livestock species (beef cattle, sheep, goats and Iberian pigs) share the habitat with several wild animal species (Gaspar et al., 2007). Moreover, this area is close to the Portuguese Alentejo region where bovine besnoitiosis is endemic (Franco and Borges, 1915; Waap et al., 2014). The most representative wild ruminant species in this area are red deer, roe deer, fallow deer and mouflon.

2.2. Samples from wild ruminants

Red deer (*Cervus elaphus*, $n = 309$), roe deer (*Capreolus capreolus*, $n = 347$) and Pyrenean chamois (*Rupicapra p. pyrenaica*, $n = 383$) were sampled in area A, Iberian wild goat (*Capra pyrenaica hispanica*, $n = 288$) and roe deer ($n = 70$) were sampled in area B, whereas red deer ($n = 820$), roe deer ($n = 37$), fallow deer (*Dama dama*, $n = 166$), Iberian wild goat ($n = 86$) and European mouflon (*Ovis orientalis musimon*, $n = 102$) were sampled in area C (Fig. 1; Table 3). Sampling was biased towards the hunting season since most of the samples were collected from September to February. Blood was collected in plastic tubes (Vacutainer®, Becton-Dickinson, NJ, USA) from the heart or the thoracic cavity, and serum was obtained after centrifugation and stored at −20 °C until analysis. Moreover, additional tissue samples conserved at −80 °C from a few animals were also available.

All serum samples were first analyzed by ELISA, and subsequently, positive and doubtful results were confirmed by western blot (Gutiérrez-Expósito et al., 2013). Available tissue samples from western blot-positive animals were further analyzed by ITS-1 PCR to confirm the presence of the parasite, and positive samples were analyzed by MS genotyping. The tissue samples used were from (i) red deer: bronchus, nasal turbinate, sclera conjunctiva and subcutaneous lymph node; and (ii) roe deer: skin.

2.3. Parasites and cell culture

Bb Spain 1 isolate was propagated as a source of antigen for ELISA and western blot. Briefly, tachyzoites were grown in a Marc-145 cell monolayer with Dulbeccoís modified Eagle medium (DMEM) (Thermo Fisher Scientific, MA, USA) supplemented with 5% fetal calf serum (Thermo Fisher Scientific, MA, USA), scraped from the cell culture flask after 72 h post-inoculation and purified following a previously described procedure (Fernández-García et al., 2009). Tachyzoites were counted using a Neubauer chamber (Blaubrand®, Germany), pelleted by centrifugation at 1350*g* for 15 min at 4 °C and frozen at −80 °C until use.

2.4. Serology

Roe deer, red deer and fallow deer serum samples were analyzed by an in-house ELISA previously standardized for *Cervidae* species (100% sensitivity; 95% CI: 100–100 and 98% specificity; 95% CI: 94.8–100) (Gutiérrez-Expósito et al., 2013). For Iberian wild goat, chamois and mouflon serum samples, a secondary antibody protein