



First serosurvey of *Besnoitia* spp. infection in wild European ruminants in Spain



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ABSTRACT

Besnoitia besnoiti has been reported to affect cattle, wildebeest, kudu and impala, and *B. tarandi* other wild ruminants (caribou, reindeer, mule deer and musk ox), causing similar characteristic clinical signs and lesions. However, both *Besnoitia* species have been reported in different geographical areas and the link between the sylvatic and domestic life cycles of *Besnoitia* spp. in wild ruminants and cattle remains unknown. The aim of this study was to evaluate the presence of specific antibodies against *Besnoitia* spp. in wild ruminants in Spain. A wide panel of sera from red deer (*Cervus elaphus*) ($n = 734$), roe deer (*Capreolus capreolus*) ($n = 124$), chamois (*Rupicapra pyrenaica*) ($n = 170$) and mouflon (*Ovis musimon*) ($n = 20$) collected from different locations of Spain was analyzed. Beef cattle were present in all sampled areas and, interestingly, bovine besnoitiosis has been widely reported in some of them (e.g., Pyrenees and Central Spain). Sera samples were first examined with an Enzyme-Linked Immunosorbent Assay (ELISA). For red deer and roe deer, the ELISA was standardized with positive and negative control sera from several Cervidae species (100% Se and 98% Sp). Chamois and mouflon sera samples were tested with a previously reported ELISA validated for bovine sera (97% Se and 95% Sp) using protein G as a conjugate. Positive results by ELISA were confirmed *a posteriori* with a tachyzoite-based Western blot. Sixty-one sera samples from red deer and 17 sera samples from roe-deer were seropositive or doubtful by ELISA. All samples from mouflon were seronegative and 15 sera samples from chamois were considered doubtful. *B. besnoiti* exposure was only confirmed clearly by Western blot in one red deer and one roe deer from the Spanish Pyrenees where the disease is traditionally endemic.

This is the first serological report of *Besnoitia* spp. infection carried out in European wild ruminants and the results show that specific antibodies are present at least in red deer and roe-deer. Thus, wild ruminants from endemic regions of bovine besnoitiosis should be further studied because they may be putative reservoirs of the parasite.

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1. Introduction

Parasites of the genus *Besnoitia* are classified in the subfamily Toxoplasmatinae, family Sarcocystidae, phylum Apicomplexa. This genus currently comprises 10 species: *B. akadoni*, *B. bennetti*, *B. besnoiti*, *B. caprae*, *B. darlingi*,

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(G. Álvarez-García).

B. jellisoni, *B. neotomofelis*, *B. oryctofelisi*, *B. tarandi* and *B. wallacei*; four of these species (*B. caprae*, *B. besnoiti*, *B. bennetti* and *B. tarandi*) have been reported to affect ungulates (goats, bovids, equids and wild ruminants, respectively) (Dubey and Yabsley, 2010; Kiehl et al., 2010; Namazi et al., 2011; Olias et al., 2011).

B. besnoiti (Marotel, 1912) is responsible for bovine besnoitiosis, a re-emergent, chronic and debilitating disease in Europe traditionally endemic in some regions of France, Portugal and Spain (European Food Safety Authority, 2010). Cattle and wild bovids act as the intermediate host (IH) for this parasite. The life cycle of this parasite consists of two asexual and infective stages, the fast-replicating tachyzoites and the slow-dividing bradyzoites gathered inside tissue cysts (Bigalke, 1981).

Wild ruminants (reindeer, caribou, mule deer, roe deer and muskox) have been described to act as the intermediate host for *B. tarandi*, a species that was first reported in reindeer (*Raginifer tarandus tarandus*) and caribou (*Raginifer tarandus caribou*) by Hadwen (1922) in Alaska (USA) through microscopical observation. To date, this disease has also been described in Canada, Sweden, Finland, Russia and Spain (Levine, 1961; Choquette et al., 1967; Wobeser, 1976; Ayroud et al., 1995; Leighton and Gajadhar, 2001; Dubey et al., 2004; Ducrocq et al., 2012). In Spain, *Besnoitia* spp. tissue cysts were visualized microscopically in a skin biopsy from a roe deer (Luco et al., 2000).

Clinical signs consist of a progressive thickening, hardening and folding of the skin, hyperkeratosis and alopecia; these signs are observed during the chronic stage of infections with both *Besnoitia* spp. (Bigalke, 1981). In addition, pathognomonic tissue cysts in the upper respiratory tract, testes, scleral conjunctiva and mucous membranes of the *Vestibulum vaginae* and vagina can be observed (Jacquet et al., 2010).

Many biological and epidemiological aspects of the *Besnoitia* spp. that infect ungulates remain unknown, including the complete life cycle, the definitive host (DH) and the role of wild animals as reservoirs for the infection (Diesing et al., 1988; Olias et al., 2011). Some authors have suggested that both domestic and wild cats could act as definitive hosts for *B. besnoiti* and for other *Besnoitia* spp. (*B. darlingi*, *B. wallacei*, *B. oryctofelisi* and *B. neotomofelis*) (Wallace and Frenkel, 1975; Dubey, 1977; Rommel, 1978). However, these observations have not been reproduced by others (Diesing et al., 1988; Basso et al., 2011). Therefore, the definitive host and the link between the domestic and putative sylvatic life cycle of the parasite remain to be elucidated (Millán et al., 2012). Moreover, recent molecular characterization studies have reported close relationships

among *Besnoitia* species that affect ungulates (Namazi et al., 2011). This finding is in accordance with reports of Ellis et al. (2000) who found no difference between the ITS1 region sequences of *Besnoitia* species and isolates affecting cattle, wildebeest and goats. On the other hand, serological tools currently available are not able to differentiate *Besnoitia* spp. affecting ungulates, and strong serological cross reactions have been recently reported between *B. besnoiti* and *B. tarandi*, and between *B. besnoiti* and *B. bennetti* (Gutiérrez-Expósito et al., 2012; Ness et al., 2012).

Thus, the aim of the present work was to evaluate the presence of specific antibodies against *Besnoitia* spp. in European wild ruminants to elucidate the putative role of these animals in the life cycle of *Besnoitia* spp.

2. Materials and methods

2.1. Serum samples and experimental design

A total of 1048 wild ruminant serum samples were collected between 2005 and 2012 from different locations in northern, central and southern Spain. The species sampled included red deer (*Cervus elaphus*, $n=734$), roe deer (*Capreolus capreolus*, $n=124$), chamois (*Rupicapra pyrenaica*, $n=170$) and mouflon (*Ovis musimon*, $n=20$). Both male and females from different age groups were sampled (Table 1). Sampled areas included endemic regions of bovine besnoitiosis and previously *Besnoitia*-free regions where the disease has been recently reported (Table 2, Fig. 1). Interestingly, most wild ruminants share pastures with cattle under extensive management and are also exposed to bloodsucking arthropods in all sampled areas. Blood samples were collected from thoracic fluid or by intra-cardiac puncture and kept at -20°C until further analysis. All sera samples were first analyzed by ELISA in an initial screening. Subsequently, positive and doubtful results were confirmed *a posteriori* using tachyzoite-based Western blot under non-reducing conditions (Fernández-García et al., 2009a; Schares et al., 2010; García-Lunar et al., 2013).

2.2. Cell culture and antigen purification

Tachyzoites of *B. besnoiti* (BbSp-1 isolate) (Fernández-García et al., 2009b) were grown in a Marc-145 cell monolayer with DMEM supplemented with 5% fetal calf serum and purified following a previously described procedure (Fernández-García et al., 2009b). These zoites were used in both ELISA and Western blot informed by the results

Table 1
Sex and age data of sampled wild ruminants.

Species	Sex			Age (years)			
	Male	Female	Unknown	<1	1–4	>4	Unknown
Red deer (<i>Cervus elaphus</i>)($n=734$)	229	335	170	52	257	45	380
Roe deer (<i>Capreolus capreolus</i>)($n=124$)	64	31	29	5	86	5	28
Chamois (<i>Rupicapra pyrenaica</i>)($n=170$)	84	66	20	0	35	79	56
Mouflon (<i>Ovis musimon</i>)($n=20$)	8	8	4	1	2	5	12
Total	385	440	223	58	380	134	476

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