Parasitology International 69 (2019) 71–74

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

Besnoitia besnoiti seroprevalence in beef, dairy and bullfighting cattle in Catalonia (north-eastern Spain): A cross-sectional study

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ARTICLE INFO

Keywords:
Besnoitia
Beef
Dairy
Bullfighting
Cattle
Seroprevalence

ABSTRACT

Besnoitia besnoiti is the causative agent of bovine besnoitiosis, a chronic and debilitating disease of cattle that recently re-emerged and seems to be spreading in Europe. A cross-sectional serological study was carried out in different cattle herds in Catalonia, north-eastern Spain, to determine the seroprevalence of *B. besnoiti* in the region. A total of 791 serum samples (beef cattle $n = 338$, dairy cattle $n = 291$; bullfighting cattle $n = 162$) were tested. Sera were first screened for antibodies against *Besnoitia* using a commercial Enzyme-Linked Immunosorbent Assay (ELISA) applying a cut-off that was lower than that recommended by the manufacturer in order to reach highest sensitivity. Sera above the chosen cut-off of 15% positivity (PP) were further tested by the Indirect Fluorescent Antibody Test (IFAT) and respectively positive results were confirmed by a *B. besnoiti* tachyzoite-based immunoblot. A total of 504/791 (63.7%) sera showed ELISA values above the selected cut-off, and 91 of these samples also yielded positive results in IFAT (cut-off titre 1:200). By immunoblot, a positive result was obtained in 93.4% (85 out of the 91) of the IFAT-positive samples. Interestingly, all confirmed *Besnoitia*-seropositive cases corresponded exclusively to beef cattle from the Pyrenees area, resulting in a prevalence of 25.1% (85/338) at the animal level and of 46% (36/78) at the herd level in this cattle group. No specific antibodies against *Besnoitia* could be detected in dairy and bullfighting cattle. The obtained results suggested that *Besnoitia* infections are present in Catalonia, consequently, diagnosis of this parasitic infection should be included in the sanitary control and before trading and movement of animals.

1. Introduction

Bovine besnoitiosis is a disease caused by *Besnoitia besnoiti*, a tissue cyst-forming coccidium closely related to *Toxoplasma gondii* and *Neospora caninum*. After an initial febrile and oedematous stage that can be associated with orchitis and infertility in bulls, bovine besnoitiosis appears as a chronic debilitating skin disease that may have a fatal outcome; however, many infected animals present mild forms of the disease or remain asymptomatic [1,2]. Subclinically infected cattle are of major epidemiological importance, because they can unnoticed introduce the infection into naive farms and even into *Besnoitia*-free countries through animal trade, contributing to the dissemination of the disease [2–5]. The increased number of outbreaks during the last years in Europe, along with the associated economic impact has emphasized the importance of monitoring and control [1,2,6]. Consequently, the European Food Safety Authority (EFSA) has classified bovine besnoitiosis as a re-emergent disease in several European countries, including France, Portugal and Spain, which have been traditionally endemic for the disease [7]. To date, the only experimentally confirmed ways of transmission among cattle are mechanically through hematophagous insects and iatrogenically through contaminated needles [2,8]. Also a possible transmission by direct contact was assumed [1]. However, a horizontal transmission by ingestion of oocysts, as it occurs in most cyst-forming coccidia, has not been demonstrated for *B. besnoiti* so far. Thus, although an indirect life cycle was suspected, a definitive host which sheds oocysts after ingestion of infected tissues could not be conclusively identified [9]. At the present, a correct diagnosis of *B. besnoiti* infection and the establishment of the serological status of the herd represent the first measures in a control programme [10].

In Spain, since the first description in Navarra and Basque Country [11] some outbreaks of bovine besnoitiosis appeared throughout the country, including new areas in the Pyrenees in the province of Huesca

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https://doi.org/10.1016/j.parint.2018.12.001
Received 14 October 2018; Received in revised form 4 December 2018; Accepted 5 December 2018
Available online 06 December 2018
1383-5769/

(Aragon) [12]. Nevertheless, there is still a lack of epidemiological studies in other areas, such as Catalonia and the Catalan Pyrenees, despite the sporadic notification of clinical cases [13].

The aim of this study was to determine the seroprevalence of *B. besnoiti* in different cattle herds in Catalonia. To the authors' knowledge this is the first epidemiological study on *B.besnoiti* in Catalonia.

2. Materials and methods

The present survey was performed with serum samples collected during annual official eradication and control programs of cattle diseases during 2010 and 2011 from the Livestock Health Laboratory in Barcelona of the Department of Agriculture, Husbandry, Fishing and Natural Environment (Government of Catalonia). The minimum required sample size was calculated with 99.5% confidence level and an error margin of 5% using WinEpi software. The seroprevalence assumption used for calculation was 50% and was linked to a known bovine population of 555,829 according to Catalonia Government data (idesCat, 2010). Finally, 791 samples were randomly selected from 111 farms (78 beef cattle; 22 dairy cattle and 11 bullfighting cattle farms) from north and south Catalonia and kept frozen at −20 °C until processed at the laboratory. Data including age, sex, breed, herd type and farm geographical location were collected for each sampled animal. From 791 selected sera, 338 corresponded to beef cattle (302 Brown Pyrenean, known as Bruna dels Pirineus; 12 cross-breed, 11 Salers, 4 Simmental, 4 Spanish Brown Alpine, 3 Pyrenean, 2 Limousin), 291 to dairy cattle (Friesian) and 162 to bullfighting cattle (Lidia). Regarding animal sex, 767 animals were females and 24 were males. Analysed beef cattle derived from the Pyrenaic area (northern Catalonia) and were bred by natural mating, usually in extensive conditions during the warm months, being kept indoors during the cold season. On the opposite, analysed dairy cattle were wider distributed over Catalonia, always bred by artificial insemination and only some herds had access to pasture. Bullfighting cattle were considered as a different group because they were exclusively bred by natural mating and kept in extensive conditions during the whole year in the south of Catalonia.

Collected sera were first screened for antibodies against *Besnoitia* spp. using a commercial indirect enzyme-linked immunosorbent assay (ELISA) (PrioCHECK® *Besnoitia* Ab 2.0 ELISA, Prionics, Switzerland). Serum samples were tested at a 1:100 dilution and results were expressed as percentage of positivity (PP) relative to the reaction of the positive control. According to the manufacturer, values < 17 PP should be considered as negative, values between 17 and 23 PP as doubtful and values > 23 PP as positive. In this study, in order to increase the sensitivity of the test, a PP ≥ 15 was selected as cut-off for screening [3,14]. Samples showing ELISA values above the selected cut-off were further tested by the Indirect Fluorescent Antibody Test (IFAT), and positive results were confirmed by a *B. besnoiti* tachyzoite-based immunoblot. For IFAT, purified *B. besnoiti* Bb-Lisbon tachyzoites (originally isolated from a naturally infected cow in Portugal) cultured in human foreskin fibroblasts, fixed with cold acetone were used as antigen. Serum samples were two-fold diluted in PBS and a titre of 1:200 was set as cut-off [14,15]. Fluorescein isothiocyanate (FITC) labelled goat anti-bovine IgG (Southern Biotech, Birmingham, USA) was used as conjugate at a 1:100 dilution in PBS. The test was essentially performed as previously described [3]. Sera from naturally infected cattle, in which the infection was confirmed by histopathology and PCR were used as positive control. As negative control, sera from naïve cattle reacting also negative by ELISA and immunoblot were used. The immunoblot was performed as previously described [15] with few modifications. Briefly, 2×10^7 *B. besnoiti* Bb-Lisbon tachyzoites were electrophoresed in two 12% precast polyacrylamide gels (Criterion™ TGX Stain-Free™ precast gels for PAGE, Bio-Rad, USA) under non-reducing conditions and electrophoretically transferred to nitrocellulose membranes (Trans-Blot® Turbo™, Bio-Rad, USA). Cattle sera were tested at 1:200 dilution and peroxidase conjugated AffiniPure goat anti bovine IgG [H + L] antibodies (Dianova/Jackson Immuno Research, dilution 1:600) were used as detection antibodies. Reaction patterns were evaluated by comparison with the patterns observed in serum samples of naturally infected cattle, with infection status confirmed by both serology and PCR. Sera with reactivities to at least four of ten selected tachyzoite antigens (45, 40, 37, 34,135 30, 27, 22, 17, 16, and 15 kDa) were classified as positive. For this scoring system, a specificity of 100% and a sensitivity of 90% have been reported [15]. Sera from naïve cattle seronegative also by ELISA and IFAT were used as negative control.

Seroprevalence was estimated as the percentage of seropositive animals. “Cattle factors” such as age (≤2, 3–4 and ≥5 years) and herd type (beef, dairy or bullfighting cattle) were analysed statistically based on Pearson's chi-squared tests using IBM SPSS Statistics v.20.

3. Results

In ELISA, when the cut-offs suggested by the manufacturer were used, 305/791 (38.6%) samples yielded positive results, 138/791 (17.4%) samples were considered as doubtful and 348/791 (44.0%) as negative. Conversely, a total of 504/791 (63.7%) samples showed ELISA values ≥ 15 PP. When those 504 samples were tested by IFAT, only 91 of them showed a positive reaction (titre ≥ 1:200). All 91 samples positive in IFAT corresponded to samples with a PP > 23 in ELISA. By immunoblot, 93.4% (85 out of 91) of the positive samples in IFAT were confirmed as positive, giving a general prevalence of 10.7% (85/791). Considering only positive results confirmed by IFAT and immunoblot, following seroprevalences were obtained in the different cattle groups: beef cattle: 21.5% (85/338); dairy cattle: 0% (0/291); bullfighting cattle: 0% (0/162). Serological results for each herd type group are shown in Table 1. Regarding frequency of positive samples at the herd level, 36 out of 111 (32.4%) herds had at least one seropositive animal confirmed by immunoblot. Considering only beef cattle, 46% (36 out of 78) of the tested farms had positive animals.

Distribution of *B. besnoiti* seropositive animals by immunoblot according to age and herd type were summarised in Table 2. Statistical differences among different age (χ^2: 26.930; $p < .001$) and herd type (χ^2: 127.636; $p < .001$) groups were observed. The seroprevalence was higher in animals aged 3–4 and ≥ 5 years than in those aged ≤2 years, and it was higher in beef cattle than in dairy or bullfighting cattle (Table 2).

Table 1
Serological results for *B. besnoiti* in beef, dairy and bullfighting cattle from Catalonia.

	ELISA		IFAT			Immunoblot	
	n tested	*n* ≥ 15 PP (%)	*n* > 23 PP (%)	*n* tested	*n* positive (%)	*n* tested	*n* positive (%)
Beef	338	200 (59.2)	135 (39.9)	200	85 (42.5)	85	85 (100)
Dairy	291	175 (60.1)	116 (39.9)	175	5 (2.9)	5	0 (0)
Bullfighting	162	129 (79.6)	54 (33.3)	129	1 (0.78)	1	0 (0)
Total	791	504 (63.7)	305 (38.5)	504	91 (18)	91	85 (93.4)

n: sample size, %: proportion of seropositive animals for each technique; ELISA: Enzyme-Linked Immunosorbent Assay; IFAT: Indirect Fluorescent Antibody Test.

Table 2
Distribution of *B. besnoiti* seropositive animals by immunoblot, according to age and herd type.

	Herd type						
	Beef *(n = 338)*			Dairy *(n = 291)*		Bullfighting *(n = 162)*	
Age (years)	n	n Positive (%)	χ^2 *p*-Value	n	n Positive (%)	n	n Positive (%)
≤2	83	8 (9.64)	–	108	0	35	0
3–4	67	19 (28.36)	8.8 $p < .001$	112	0	43	0
≥	188	58 (30.85)	14.06 $p < .001$	71	0	84	0

n: sample size; χ^2: Chi-square statistic between the ≤2 age group and the other groups.

4. Discussion

The general seroprevalence obtained in Catalonia at the animal level was 10.7% (85/791), lower than that observed in other studies from Spain, carried out in known endemic areas of the Pyrenees [12,16] and Castilla la Mancha [17], which showed seroprevalences between 38 and 52% and 63%, respectively. However, the observed prevalence was higher (21.5%) when only the north Pyrenean region and/or beef cattle were considered. Precisely in this region is where the first clinical cases of bovine besnoitiosis in Catalonia were reported [13], suggesting that adequate conditions for the maintenance of the parasite might be given. In this study, seropositive animals were only detected within the beef cattle group, indicating that this group was more exposed to *Besnoitia*. The Brown Pyrenean breed represented 90% of the analysed beef cattle; however, if this cattle breed has a special genetic susceptibility to *B. besnoiti* infection is not known. According to other studies, the management system was considered a more significant risk factor for *Besnoitia* infection than breed or herd type [1]. In the Pyrenean region, beef cattle management usually includes an extensive valley-high mountain grazing system and natural mating. These factors might favour direct contact between infected and naive cattle within the same herd or even between different herds sharing grazing fields [6,11]. Also the contact with wild animals would be favoured [18], however, the role of wild ruminant species as reservoirs is still questioned [19]. On the other hand, results in bullfighting and dairy cattle were completely different. The bullfighting herds were located in the south of Catalonia, also bred in extensive conditions and by natural mating as beef cattle, but no seropositive animals could be confirmed. Alvarez-Garcia et al. tested by an in-house ELISA a group of bullfighting cattle from the Pyrenees region belonging to Aragon with similar results [20]. Since this was the first study on this population in Catalonia, further studies are required to better understand if the differences in seropositivity between beef and bullfighting cattle might be due to the fact that *Besnoitia* is still confined to the north Pyrenean region since the last decades [13], or if other factors such as potential Diptera vectors [1,21], cattle breed, or other still unknown factors are involved. Similarly, no seropositive animals could be confirmed among dairy cattle. In this group, all animals were of the Friesian breed and mainly adapted to an intensive herd management, sometimes with access to pasture. Besides, artificial insemination was used instead of natural mating, what would lower the risk of infection [8,22]. In Spain, most surveys reported seroprevalences from animals under extensive conditions [20,23]; however, bovine besnoitiosis was also described in dairy cattle [24]. Further reports, also confirmed positive results in indoor dairy cattle in other countries [25]. This could suggest that direct transmission among stabled animals [16] or vector transmission by endophilic Diptera such as *Stomoxys calcitrans* might play a role in some herds [8,26]. Therefore, more research on parasite transmission is needed to better understand the epidemiology of bovine besnoitiosis in this region. Age was considered a a risk factor for bovine besnoitiosis, as older animals had a greater chance of exposure to the parasite over time [2,10]. This could explain the increasing seropositivity with age that we observed in beef cattle when different age groups were assessed (Table 2). While several studies were unable to identify gender as a risk factor for infection, there are some reports stating that male cattle are more often serologically positive than female cattle and that clinical signs in bulls are more severe [1]. Recently, a study on breeding bulls showed that males were at a higher risk of *B. besnoiti* infection than females [27]. In the present study, female animals were overrepresented in the sample set, therefore no valid conclusions related to prevalence according to gender can be drawn.

Serological techniques are considered a good choice as a first epidemiological approach [10]. The ELISA technique is useful as a screening test and it allows detecting both clinically and subclinically infected cattle [3,28,29]. In an interlaboratory comparative study of serological tools for diagnosis of *B. besnoiti* infection in bovines, the commercial ELISA used in this study showed 100–91.9% sensitivity and 98.8–99.1% specificity (depending on the gold-standard criteria selected) when 15 PP was used as cut-off [14]. Some ELISAs used for screening may give a considerable number of false positive results depending on the serum panel, as it was already observed in some studies [3,30]. Similarly, in our study, the apparent seroprevalence by ELISA was reduced by using confirmatory techniques as only 85 out of 305 sera giving a positive result in ELISA (values > 23 PP) could be confirmed as positive by IFAT and immunoblot. Nevertheless, previous studies using the same commercial ELISA showed that *Besnoitia* infection could be occasionally confirmed by further serological methods and histopathology in some cattle with ELISA values as low as PP15 [3]. Accordingly, a cut-off PP15 was selected for screening in this study (instead of the higher cut-off PP 23 currently suggested by the manufacturer), in order to avoid losing real positive animals with low ELISA values. Nonetheless, no sample with ELISA values < 23 PP ended up being confirmed as positive by other serological tests. Several studies revealed that sera with high antibody titres to other tissue-cyst-forming apicomplexan parasites (i.e. *Neospora caninum* and *Sarcocystis* spp.) could cross-react with *B. besnoiti* antigens in ELISA [29–32], and in IFAT at low serum dilutions [15,33]; therefore, a confirmatory technique such as immunoblot is highly recommended. The cut-off titre of 1:200 that was selected for IFAT in our study showed very good results in different reports [14,15], including an interlaboratory comparative study of serological tools used in the diagnosis of *B. besnoiti* infection in bovines [14]. In that study, IFAT with a cut-off 1:200 showed the best performance with high sensitivities (100–91.9%) and specificities (95.4–97.4%), varying according to the gold-standard criteria selected. Besides, it should be taken into account, that serologic methods may lack sensitivity when testing acutely infected animals, as there is a diagnostic gap between infection and first detection of specific antibodies in serum [14,34].

This is the first epidemiological study on *Besnoitia* spp. based on serological diagnosis in Catalonia, north-eastern Spain. Probably, besnoitiosis is maintained as a subclinical disease like in other endemic regions, which could explain the sporadic description of clinical cases [13]. Nevertheless, the authors consider that detection of subclinically infected animals is important in order to reduce the risk for the dissemination of the disease both in the same herd and through animal trade between farms [2,4].

5. Conclusion

The obtained results suggested that *Besnoitia* infections are present in Catalonia. Therefore, proper diagnostic methods should be implemented during sanitary controls and before movement and trade of cattle from this region, in order to limit the spreading of this parasitic disease.

Acknowledgements

The authors are grateful to Florenci Vivas, head of Livestock Health Laboratory of Barcelona, and Iscle Selga, head of Animal Health Department of Generalitat de Catalunya, for kindly providing data and blood samples.

Conflict of interest

The authors declare that they have no conflict of interest.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

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