

Large-scale serosurvey of *Besnoitia besnoiti* in free-living carnivores in Spain

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a b s t r a c t

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The disease bovine besnoitiosis is responsible for severe economic losses caused by the protozoan *Besnoitia besnoiti*. The identity of the definitive host (DH) of this parasite has yet to be determined, although it is presumed to be a carnivore. With the aim of advancing in the identification of *B. besnoiti* DH, a necessary step in implementing control strategies, the contact rate of 205 free-roaming carnivores with this parasite in Spain was studied. The study included 16 wolves (*Canis lupus*), 41 red foxes (*Vulpes vulpes*), 21 pine martens (*Martes martes*), eight stone martens (*M. foina*), 12 Eurasian badgers (*Meles meles*), 18 common genets (*Genetta genetta*), five Egyptian mongooses (*Herpestes ichneumon*), 28 European wildcats (*Felis silvestris silvestris*), 43 feral cats (*Felis silvestris catus*), and 13 other animals belonging to five other species. Serum samples were analysed by an indirect fluorescent antibody test (IFAT) and by two western immunoblots (WB, one with tachyzoite and the other with bradyzoite antigen). Twelve individuals (eight of which were cats) seroconverted by one or other of these techniques but no individual showed seroconversion by IFAT and one of the WBs. The results provided no evidence to support the idea that within the geographical regions covered by the analysis wild carnivores are implicated in the transmission of *B. besnoiti* in Spain.

1. Introduction

Bovine besnoitiosis is a disease caused by the protozoan *Besnoitia besnoiti* and on affected cattle farms causes severe economic losses due to mortality, weight loss, prolonged convalescence, definitive or transient sterility in males and a decline in milk production (Jacquet et al., 2010). It has been detected in Africa, the Middle East and Europe and, according to the European Food Safety

Authority, can be considered an emergent disease in European countries such as Spain, Portugal, France, Italy and Germany (European Food Safety Authority, 2010). Recent epidemiological data confirm the increased prevalence and geographical expansion of this disease (European Food Safety Authority, 2010) and in Spain it has been detected in cattle in several regions in the north of the country, where reported herd seroprevalences can be higher than 80% in some regions (Fernández-García et al., 2010).

Besnoitia besnoiti has a heteroxenous life cycle that has been defined as 'mysterious' (Jacquet et al., 2010), with both domestic (cattle) and wild (antelopes) bovids known to be intermediate hosts (Pols, 1960; Basson et al., 1970).

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Table 1Serologic survey for *Besnoitia besnoiti* in free-roaming carnivores in Spain. IFAT: indirect fluorescent antibody test. WB: western immunoblots.

Family	Species	n	Origin	Number of individuals positive by			
				IFAT ($\geq 1:100/\geq 1:200$)	Tachyzoite WB (≥ 3 bands/ ≥ 4 bands)	Bradyzoite WB (≥ 3 bands/ ≥ 4 bands)	IFAT+one WB
Canidae	Wolf <i>Canis lupus</i>	16	Asturias	0	0	0	0
	Red fox <i>Vulpes vulpes</i>	41	Asturias (5), Cantabria (4), Castilla-La Mancha (24), Castilla y León (2), unknown (3)	1/0	0	0	0
Mustelidae	Least weasel <i>Mustela nivalis</i>	1	Balearic Islands	0	0	0	0
	European polecat <i>Mustela putorius</i>	2	Asturias	0	0	0	0
	Pine marten <i>Martes martes</i>	21	Asturias (3), Balearic Islands (18)	0	0	2/0	0
	Stone marten <i>Martes foina</i>	8	Asturias (3), Castilla-La Mancha (2), Castilla y León (1), Valencia (2)	0	0	0	0
	Eurasian badger <i>Meles meles</i>	12	Andalusia (1), Asturias (6), Cantabria (2), Castilla-La Mancha (2), unknown (1)	0	0	0	0
	Eurasian otter <i>Lutra lutra</i>	4	Asturias (1), Cantabria (1), Castilla-La Mancha (1), unknown (1)	0	0	0	0
	American mink <i>Neovison vison</i>	1	Castilla y León	0	0	0	0
Viverridae	Common genet <i>Genetta genetta</i>	18	Andalusia (3), Asturias (3), Balearic Islands (8), Castilla-La Mancha (1), Valencia (3)	0	0	0	0
Herpestidae	Egyptian mongoose <i>Herpestes ichneumon</i>	5	Andalusia (4), Castilla-La Mancha (1)	0	0	0	0
Felidae	Iberian lynx <i>Lynx pardinus</i>	3	Andalusia	0	0	0	0
	European wildcat <i>Felis s. silvestris</i>	28	Asturias (3), Castilla-La Mancha (24), Castilla y León (1)	1/0	0	0	0
	Feral cat <i>Felis s. catus</i>	43	Andalusia (4), Balearic Islands (39)	4/1	3/1	2/1	0
Procyonidae	S. American coati <i>Nasua nasua</i>	1	Balearic Islands	0	0	0	0
	Northern raccoon <i>Procyon lotor</i>	1	Castilla-La Mancha	0	0	0	0
	Total	205		6/1	3/1	4/1	0

The definitive host (DH) has not been identified, although it has been suggested that domestic cats (*Felis silvestris catus*) or an undetermined wild carnivore may be involved in the parasite life cycle (Diesing et al., 1988; Basso et al., 2011). Peteshev et al. (1974, in European Food Safety Authority, 2010) reported that domestic cats and a wild cat (*F. silvestris lybica*) shed oocysts after the ingestion of tissues containing cysts. However, other attempts to demonstrate the role of cats and other carnivores as DH have been unsuccessful. For example, Diesing et al. (1988) found no oocysts in the faeces of 23 wild carnivores belonging to 12 different species (including domestic cats and dogs and wild carnivores) fed with tissue containing *B. besnoiti* cysts. In a more recent study, dogs and cats fed with *B. besnoiti* tachyzoites did

not seroconvert, although cats seroconverted after feeding on *B. besnoiti* tissue cysts (Basso et al., 2011). To date, the only serological study on wild carnivores failed to find antibodies by Western Blot in sera from two red foxes (*Vulpes vulpes*) and two Eurasian badgers (*Meles meles*) from France (Berhault, 2008). The determination of the role of wild carnivores as definitive or intermediate hosts (IH) for this parasite is essential if control practices are to be successfully implemented.

The aim of this work was to study the contact rate of 15 different species of wild carnivores and feral domestic cats with *B. besnoiti* as a first step to determining which species are candidates to be the DH for this parasite. We hypothesized that finding a high seroprevalence in a given carnivore

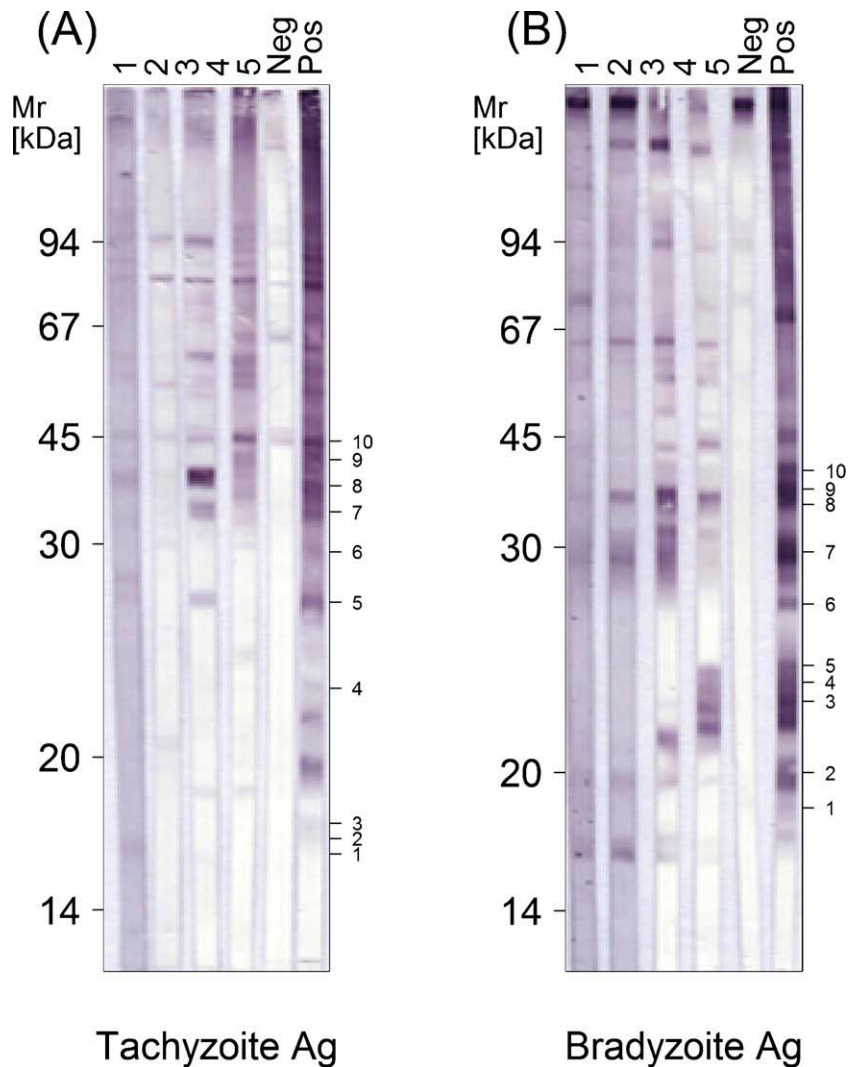


Fig. 1. Western blot results on animals for which a reaction with 3 (borderline) or 4 (positive) selected bands was recorded with antigens of *B. besnoiti* tachyzoites (Tachyzoite Ag) or bradyzoites (Bradyzoite Ag) relative to the reactions observed in controls (Neg, negative control; Pos, positive control). Locations of selected bands were based on studies in cattle (Schaeres et al., 2010) and are marked on the right side of each block. In the tachyzoite blot (A) reactions of sera of a wild cat (stripe 1) and feral cats (stripe 2, 3, 4) are shown. In the bradyzoite blot (B) reactions of sera of pine marten (stripe 1, 2) and feral cats (stripe 3, 4) are shown. Stripe 3, Tachyzoite Ag and stripe 4, bradyzoite display the reactions of an individual feral cat. The reciprocal IFAT titre of this cat was 50.

species would indicate that this species frequently came into contact with *B. besnoiti*, which would make this species a good candidate to be the DH. On the contrary, finding a low prevalence in such species may rule out a role in the life-cycle of this parasite.

2. Materials and methods

Two hundred and five serum samples obtained between 2004 and 2010 from free-living carnivores belonging to seven different families were used in this study and included 16 wolves (*Canis lupus*), 41 red foxes, 21 pine martens (*Martes martes*), eight stone martens (*M. foina*), 12 Eurasian badgers, 18 common genet (*Genetta genetta*), five Egyptian mongooses (*Herpestes ichneumon*), 28 European wildcats (*Felis silvestris silvestris*), 43 feral cats (i.e.

free-living domestic cats), and 13 other animals of other species. Samples came from seven different Spanish regions (see Table 1 for details).

An immunofluorescent antibody test (IFAT) and two western immunoblots (WBs; one with tachyzoite and the second with bradyzoite antigen) for the detection of *B. besnoiti*-specific antibodies were performed essentially as described by Schaeres et al. (2010). Sera were diluted 1:25, 1:50, 1:100 and 1:200 in IFAT and 1:100 in the WBs. FITC or POD labelled anti-cat-IgG(H+L) [goat] or anti-dog-IgG(H+L) [rabbit] were used as conjugates (both from Dianova, Hamburg, Germany). Anti-cat was used for cat, genet, mongoose and lynx, whilst anti-dog was used for wolf, fox, marten, polecat, weasel, otter, badger, racoon, coati and American mink. FITC and POD labelled conjugates were diluted 1:50 and 1:500, respectively.

A serum was regarded as positive if it gave in at least two of the three tests (IFAT and tachyzoite or bradyzoite WB) a positive result. In IFAT a serum was regarded as positive if the reciprocal IFAT titre was ≥ 200 . A reciprocal titre of 100 was regarded as borderline. In the WB a positive serological reaction was recorded if ≥ 4 of 10 bands selected per antigen (tachyzoite, bradyzoite) were recognized by a serum. If 3 of these 10 bands were recognized the reaction was recorded as borderline. These bands have previously been described by Schares et al. (2010) (Fig. 1).

95% Confidence Intervals (C.I.) were calculated using Quantitative Parasitology 3.0 (Rozsa et al., 2000). The Maximum Possible Prevalence was calculated using Win Episcope 2.0 (de Blas et al., 2000). Since we do not know the exact population of wild carnivores in Spain, we assumed it to be at least of 10,000 individuals.

3. Results and discussion

None of the 205 carnivores was seropositive (95% C.I. = 0.0–1.8) and in all only 12 (5.9%, 95% C.I. = 3.3–9.9) showed seroconversion or borderline reactions to any of the techniques (Table 1). The Maximum Possible Prevalence was 1.44%. Nine of these animals were felids and ten (eight feral cats and two pine martens) were sampled in Mallorca (Balearic Islands). Only one feral cat was seropositive in the tachyzoite and borderline in the bradyzoite WBs (Table 1). This cat, however, had a reciprocal IFAT titre of 50, which is below the established threshold.

To the best of our knowledge, this is the first large-scale serosurvey for antibodies against *B. besnoiti* in carnivores ever carried out in Spain or anywhere else in the world. None of the studied individuals can be considered seropositive to the parasite. Nevertheless, it is important to realize that none of the techniques used have ever been validated in wild carnivores. In addition, the actual seroprevalence may be underestimated because an infected definitive host may not seroconvert. For example, a cat experimentally infected with *Toxoplasma gondii* shed oocysts but did not seroconvert for at least 6 months (Dubey and Frenkel, 1972), and viable *T. gondii* has been isolated from seronegative naturally infected cats (Dubey et al., 2004). Similar findings have been reported for *Neospora caninum* in dogs (Schaes et al., 2001, 2005).

Among the studied species, most of the positive reactions to the techniques were observed in feral cats. Domestic cats are the DH for the *Besnoitia* species with known life cycles (*B. darlingi*, *B. wallacei* and *B. oryctofelis*; Dubey and Lindsay, 2003; Houk et al., 2011). Our results, although inconclusive, confirm that domestic or feral cats are still the best candidates as DH for *B. besnoiti*. However, there is also the possibility of a cross-reaction with other *Besnoitia* species using rodents as IH. In fact, feral cats from Mallorca (from where most of the positive samples were obtained) mainly fed upon rodents (Millán, 2010).

The samples analysed in the present communication were obtained during the course of different studies from a diversity of Spanish regions. Most of these regions are known to have an increasing number of cases of bovine besnoitiosis and include, for example, Cantabria, Castilla y León, Castilla-La Mancha and Valencia

(Fernández-García et al., 2010). No reports are available from Asturias, though cases have been detected in all bordering regions (Cantabria, Castilla y León, and Galicia). To date, nothing is known about the presence of this parasite in Andalusia (southern Spain) or in the Balearic Islands.

In conclusion, the analysis of more than 200 serum samples did not provide any evidence to support the hypothesis that wild carnivores are implicated in the transmission of *B. besnoiti* in Spain.

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