Molecular survey of Besnoitia spp. (Apicomplexa) in faeces from European wild mesocarnivores in Spain.

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Abstract

Numerous studies have unsuccessfully tried to unravel the definitive host of the coccidian parasite Besnoitia besnoiti. Cattle infections by B. besnoiti cause a chronic and debilitating condition called bovine besnoitiosis that has emerged in Europe during the last two decades, mainly due to limitations in its control associated to the absence of vaccines and therapeutical tools. Although the exact transmission pathway of B. besnoiti is currently unknown, it is assumed that the parasite might have an indirect life cycle with a carnivore as definitive host. Current lack of studies in wildlife might underestimate the importance of free-living species in the epidemiology of B. besnoiti. Thus, the aim of the present study is to assess the presence of Besnoitia spp. in free-ranging mesocarnivores in Spain. DNA was searched by PCR on faeces collected from wild carnivores as a first approach to determine which species could be considered as potential candidates for definitive hosts in further research. For this purpose, a total of 352 faecal samples from 12 free-living wild carnivore species belonging to the Canidae, Felidae, Herpestidae, Mustelidae, Procyonidae, and Viverridae families were collected in seven Spanish regions. PCR testing showed that Besnoitia spp. DNA was present in four faecal samples from red foxes collected in western Spain, an area with the greatest density of extensively reared cattle and associated to high incidence of bovine besnoitiosis in the country. To date, this is the first report of a Besnoitia besnoiti-like sequence (99.57% homology) from carnivore faeces in a worldwide context. Red foxes might contribute to the epidemiology of B. besnoiti, although further studies, mostly based on bioassay, would be needed to elucidate the accuracy and extent of these interesting findings.

Introduction

Besnoitia besnoiti (Apicomplexa) is the aetiological agent of a chronic and debilitating disease of cattle called bovine besnoitiosis characterized by a low body score, non-specific systemic clinical signs and skin lesions. Reproductive failure is a major concern since males may develop infertility, sterility or even may die during the acute phase of the infection (González-Barrio et al., 2020). Bovine besnoitiosis causes considerable economic losses in many countries in Africa, the Middle East and Europe. In these regions, the disease has been increasingly spreading over the last two decades (EFSA, 2010; Álvarez-García et al., 2013), mainly due to control drawbacks including the absence of vaccines and therapeutical tools. Several surveys confirmed the increased prevalence and geographical expansion of this disease in cattle, in areas of Western and Northern Europe (Álvarez-García et al., 2016). A few studies carried out in Spain reported high seroprevalence rates in beef cattle herds, ranging from 36 to 87% in Urbasa Andía mountains and the Pyrenees in North Spain (Cortes et al., 2014; Álvarez-García et al., 2014; Gutiérrez-Expósito et al., 2014).

Despite the fact that its biological cycle is not fully known, it is assumed that *B. besnoiti* might have an indirect life cycle with a carnivore as definitive host able to shed oocysts after ingestion of tissues harbouring mature cysts (Jaquiet et al., 2010; Cortes et al., 2014. Although domestic cattle act as intermediate host of the parasite, roe deer (*Capreolus capreolus*) is also an intermediate host in Spain (Arnal et al., 2017) and specific antibodies against *B. besnoiti* have been also described in wild ruminants (roe deer and red deer [*Cervus elaphus*]) in North Eastern Spain, regions where bovine besnoitiosis is endemic (Gutiérrez-Expósito et al., 2013, 2016); despite this fact, their role in the epidemiology of bovine besnoitiosis is of scarce importance (Gutiérrez-Expósito et al., 2016). In contrast, specific antibodies were not detected in small ruminants (Gutiérrez-Expósito et al., 2017). Similarly, the lack of antibodies against *Besnoitia* spp. in wild carnivore species from Spain in which results provided no evidence to support the idea that within the geographical regions covered by the analysis wild carnivores were implicated in the epidemiology of *B. besnoiti* (Millán et al., 2012).

Domestic and wild felines have been suggested as definitive hosts for *B. besnoiti* , however, experimental infections in these species failed to confirm their suitability as potential definitive hosts (Diesing et al., 1988; Basso et al., 2011). The putative role of a sylvatic life cycle, involving other Carnivora species, in the epidemiology of the disease has not been fully elucidated (see Table 1).

The aim of the present survey is to assess the presence of *Besnoitia* spp. DNA in faeces from wild mesocarnivores in Spain as a first step to determine which species might be considered as potential definitive host candidates in further investigations.

Material and Methods

Sample collection

A total of 352 faecal samples from 12 free-living carnivore species belonging to the Canidae ($n = 193$), Felidae ($n = 15$), Herpestidae ($n = 1$), Mustelidae ($n = 131$), Procyonidae ($n = 1$), and Viverridae ($n = 11$) families were collected in seven Spanish regions between December 2013 to October 2017 (Table 2, Figure 1). The sampling was mainly focused on areas with higher densities of extensively reared cattle and where positive cases of bovine besnoitiosis had been previously reported (Nieto-Rodríguez et al., 2016), as is the case of Central and Western Spain (Figure 1). Samples were obtained from road- and hunter-killed animals, from accidentally found carcasses, camera-trap surveys, or animals entering rescue shelters (see Calero-Bernal et al., 2020). Faeces were collected directly from the rectum of each animal and placed in individual plastic cups with records of the date, location and species, and kept frozen before 12 h after collection.

DNA extraction and molecular detection of **Besnoitia*spp.***

Total DNA was extracted from an aliquot of ˜200 mg of fresh faecal material using the QIAamp® DNA Stool Mini Kit (QIAGEN, Hilden, Germany) following the manufacturer's instructions. Purified DNA samples (200 µl) were stored at –20 °C until downstream PCR-based diagnostic and subtyping analyses were conducted. A water extraction control was routinely included in each sample batch processed for DNA extraction. The products of the DNA extraction process were tested for the specific detection of *Besnoitia* spp. by ITS-1 rDNA

PCR (Cortes et al., 2007). The forward primer ITS1F (50-TGACATTTAATAACAATCAACCCTT-30) and the reverse primer ITS1R (50-GGTTTGTATTAACCAATCCGTGA-30) were added at a concentration of 10 µM, and the rest of reagents were incorporated in the mixture (final volume: 25 µl), as indicated by Frey et al. (2013). The amplified products were visualized after electrophoresis on a 1.5% agarose gel containing 0.1 µl/ml GelRed Nucleic Acid Gel Stain (Biotium, Fremont, USA). DNA extraction and PCR were performed in separate laboratories under biosafety level II conditions (BIO II A Cabinet, TELSTAR, Madrid, Spain) to avoid cross contamination. The positive control was DNA extracted from *in vitro* cultured tachyzoites of *B. besnoiti* , and PCR grade water was used as the negative control.

Sequence and phylogenetic analyses

In order to confirm the specificity of the amplicons yielded, positive PCR products were subjected to direct sequencing at the Center for Genomic Technologies of the Complutense University of Madrid (Spain). Briefly, amplicons were sequenced in both directions with the same internal primer pair used for PCR amplification, Big Dye chemistries and an ABI 3730xl sequencer analyzer (Applied Biosystems, Foster City, CA). Raw sequencing data in both forward and reverse directions were manually edited by Bioedit Software, and BLAST tool (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to compare resulting nucleotide sequences with sequences retrieved from the National Center for Biotechnology Information (NCBI) database.

The evolutionary relationships among the identified*Besnoitia* -positive samples were inferred by a phylogenetic analysis using the Neighbor-Joining method (Saitou & Nei, 1987) in MEGA 6. The evolutionary distances were computed using the Kimura 2-parameter method and modelled with a gamma distribution. The reliability of the phylogenetic analyses at each branch node was estimated by the bootstrap method using 1000 replications. Representative sequences of different*Besnoitia* spp. isolates were retrieved from the NCBI database and included in the phylogenetic analysis for reference and comparative purposes. Representative sequences obtained in the present study were deposited in GenBank under the accession numbers MW035607 to MW035610.

Results and discussion

PCR and sequencing results showed that *Besnoitia* spp. DNA was present in four faecal samples (1.13%) analysed from 352 wild carnivores. Those positive samples corresponded to four red foxes from Castilla y León and Extremadura in western Spain. To date, this is the first finding of a *B. besnoiti* -like sequence from a carnivore in Europe, and from any carnivore species in a worldwide context.

To the best of our knowledge, this is the first large-scale molecular survey for *Besnoitia* spp. DNA in free-living carnivores ever carried out globally. The survey benefits from the inclusion of 12 different species of free-living carnivores and a national coverage, paying special attention to regions where bovine besnoitiosis is present (except in North West Spain) in conjunction with greater densities of extensive cattle production.

Serological, molecular and parasitological techniques have been used in an attempt to elucidate the role of several animal species as potential definitive host of *B. besnoiti* , but they failed to find it in wild and domestic carnivores, in addition to mammals, reptiles and birds (see Table 1). Several studies on felines, including the domestic cat (*Felis catus*), have attempted to clarify its role as a definitive host (see Table 1). Rommel (1975) and Peteshev et al. (1974) reported inconclusive results to confirm domestic cats as definitive hosts of*B. besnoiti* in experimental studies. Despite detecting oocysts in the faeces, authors could not achieve further characterization for fully confirmation of their identity as *B. besnoiti.* Other authors did not find *B. besnoiti* oocysts in the faeces of *per os*challenged cats over a 3 to 20 weeks observation period (Diesing et al., 1988; Basso et al., 2011). Several serological studies have been also carried out to detect antibodies of *B. besnoiti* in felines (Table 1). In European felines (Millán et al., 2012) antibodies were found in eight feral cats (*Felis silvestris catus*) by any of the serological techniques used (indirect fluorescent antibody test [IFAT] and by two western immunoblots [WB, one with tachyzoite and the other with bradyzoite antigen]), but no individual showed positivity/contact by IFAT and one of the WBs. These animals originated from areas where no cases of bovine besnoitiosis had been detected until year 2010. The results suggested their unlikely implication in the parasite transmission. In a recent study in Namibian wildlife, antibodies have been detected in a lion, *Panthera leo* (Seltmann et al., 2020). On the other hand, only one study has managed to detect by molecular techniques *Besnoitia* spp. DNA in pond bat (*Myotis dasycneme*) faeces in the Netherlands (Hornok et al., 2015). The authors hypothesized that *B. besnoiti* -like sequence might have originated from French cattle via bloodsucking dipterans (*Stomoxys calcitrans, Tabanus* spp.). In this regard, bats frequently use cattle stables for roosting, where they can prey on the mechanical vectors of *B. besnoiti* .

In the present survey, *Besnoitia* spp. DNA has been demonstrated in four individual faecal samples from red foxes from Ávila, Badajoz and Salamanca provinces (Table 2) in western Spain. All four fox-derived*Besnoitia* spp. sequences were equivalent to positions 527–737 of reference sequence KX013107 (a bovine isolate of the parasite previously reported in Spain), differing from it by a single di-nucleotide site (a G/C double peak) at position 706. An additional ambiguous position (an A/G double peak) was also detected at position 711 of reference sequence KX013107 in one (GenBank accession number MW035609) of the four generated sequences. The topology of the produced phylogenetic tree clearly clustered all these sequences with other *Besnoitia*species in large mammals (*B. besnoiti* , *B. bennetti* ,*B. caprae* and *B. tarandi*) from European countries (Belgium, Finland, Italy, Germany, Portugal and Spain), Israel and Iran. In separate phylogenetic cluster, other species of *Besnotia*spp. (*B. neotomofelis* , *B. oryctofelisi* , *B. akodoni* and *B. darlingi*) infecting small mammals from Argentina, Brazil and USA (Figure 2) are placed. These results are in agreement with those described by Olias et al. (2011), in which *ITS-1*region shows the most informative nucleotide variances and phylogenetically clearly split small mammalian from large mammalian*Besnoitia* species. Of note, all foxes with *Besnotia* spp. PCR-positive faecal samples were caught within Western Spain (Figure 1), where the highest number of bovine besnoitiosis clinical cases were found in a previous survey (Nieto-Rodríguez et al., 2016).

This is the first molecular evidence of *B. besnoiti* in a European mesocarnivore. The red fox is present in a wide range of habitats in the Iberian Peninsula (Macdonald & Reynolds 2004) with densities of 0.7–2.5 foxes/Km^2, depending on environmental conditions (Sarmento et al., 2009). In addition, this wild canid is a highly adaptable omnivorous mammal distributed across all continents on the northern hemisphere. Numerous studies on the red fox diet show it as a generalist predator, feeding mainly on prey which are abundant and easily accessible. Red foxes feed most frequently on small mammals as rodents and wild rabbits, but utilize also other food items such as carrion, birds, reptiles, amphibians, invertebrates, fruit and vegetables (Díaz-Ruiz et al., 2013).

Taking into account that in our study the faecal samples were collected from regions where beef cattle are usually raised in extensive production systems (Figure 1) and bovine besnoitiosis is widespread (Nieto-Rodríguez et al., 2016), there are three possible explanations for this interestingfindings; i) our first hypothesis is that red foxes may have a role in the transmission of the parasite as definitive host: the red fox is considered to be one of the most widespread generalist vertebrate predators in the world (Macdonald & Reynolds 2004). Therefore, predation on small mammals as rodents and wild rabbit, intermediate hosts in others species of *Besnoitia* spp., it could make us think that red fox might have a role as a definitive host in other species of *Besnoitia,* and that the sequences found represents a novel *Besnoitia* genotype/species closely related to*B. besnoiti* ; ii) our second hypothesis is that there has been consumption of carrion infected with *B. besnoiti,* and foxes are acting as passive carriers without developing the infection; iii) and the third and last hypothesis is that the red fox could act as an accidental or paratenic host, in which the accidental ingestion of the hypothetical *B. besnoitia* oocysts from the contaminated soil would be excreted or digested and we would find parasite DNA in the fox faeces.

Although we have found *Besnoitia* spp. DNA in red fox faeces and subsequently confirmed it by Sanger sequencing, present survey has several limitations. First, no serological analysis has been performed on these species, sampling was carried out in most cases on road- and hunter-killed animals, from accidentally found carcasses, and camera-trap surveys. Thus, fresh, good quality blood samples were unavailable for serological testing, in addition to the difficulty of finding validated techniques in wildlife for detecting this parasite (González-Barrio & Ruiz-Fons, 2019). Second, no additional parasitological techniques (e.g. floatation) were used due to the retrospective nature of this study and the insufficient amount of remaining faecal material for performing complementary techniques. Finally, identification of *Besnoitia*spp. was accomplished on a single locus. Low quantity and quality of genomic DNA from faeces prevented us of conducting multilocus microsatellite analyses. However, on the other hand, fox has been the unique species out of the 12 studied in which *B. besnoiti* has been identified, suggesting that this species might play a role in the epidemiology of the disease.

To conclude, the low prevalence rate found here suggests that the role of the red fox in the epidemiology of bovine besnoitiosis could be of limited relevance in Spain. Additional epidemiological and experimental studies with a similar approach, may help in the search for the definitive host of this parasite, where the main hypothesis is that the definitive host is a predator or scavenger from Africa where the disease originated.

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Conflict of interest

The authors have no conflict of interest to declare.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure caption.

Figure 1 . Spatial distribution of sample collection. Each black dot represents the province of sampling. The current geographic distribution and census of beef cattle raised under extensive husbandry conditions for each province is represented by grayscale. The number of fecal samples per province is shown. The white dot indicates that*Besnoitia* spp. DNA has been found.

Figure 2 . Phylogenetic relationships among *Besnoitia*spp. sequences identified in this study and known *Besnoitia* spp. isolates, as inferred by a neighbour-joining analysis of ITS ribosomal RNA gene partial sequences, based on genetic distances calculated by the Kimura two-parameter model. Nucleotide sequences determined in this study are identified with dark green filled circles. Bootstrap values lower than 50% are not displayed. *Toxoplasma gondii* was used as outgroup taxa.

Table 1 . Summary of the available studies reporting investigations to elucidate possible definitive host of *Besnoitia besnoiti* .

Table 2 . Wild carnivore species examined, region, number of samples tested and number of samples in which DNA from *Besnoitia*spp. has been detected in Spain. In bold the species and number of faeces samples in which DNA from *Besnoitia* spp. has been found and confirmed by sequencing.

Hosted file

`Table 1.pdf` available at https://authorea.com/users/397046/articles/510080-molecular-survey-of-besnoitia-spp-apicomplexa-in-faeces-from-european-wild-mesocarnivores-in-spain

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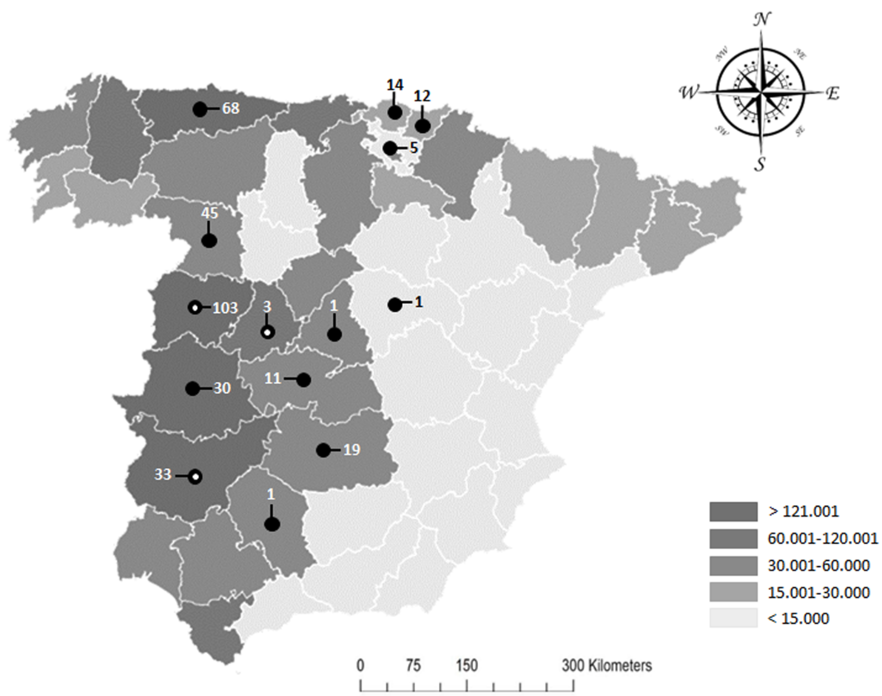
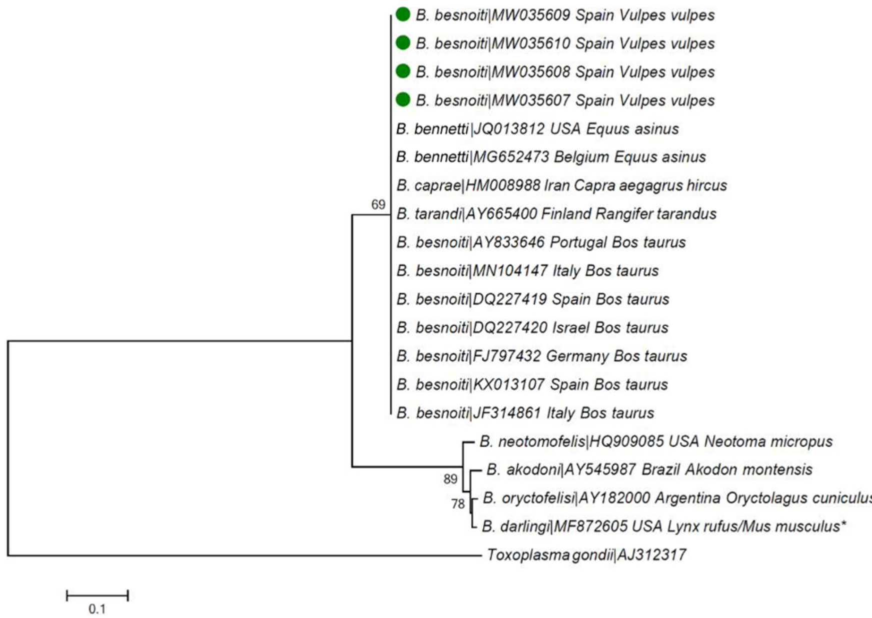
`Table 2.pdf` available at https://authorea.com/users/397046/articles/510080-molecular-survey-of-besnoitia-spp-apicomplexa-in-faeces-from-european-wild-mesocarnivores-in-spain




B. besnoiti|MW035609 Spain Vulpes vulpes
B. besnoiti|MW035610 Spain Vulpes vulpes
B. besnoiti|MW035608 Spain Vulpes vulpes
B. besnoiti|MW035607 Spain Vulpes vulpes
B. bennetti|JQ013812 USA Equus asinus
B. bennetti|MG652473 Belgium Equus asinus
B. caprae|HM008988 Iran Capra aegagrus hircus
69
B. tarandi|AY665400 Finland Rangifer tarandus
B. besnoiti|AY833646 Portugal Bos taurus
B. besnoiti|MN104147 Italy Bos taurus
B. besnoiti|DQ227419 Spain Bos taurus
B. besnoiti|DQ227420 Israel Bos taurus
B. besnoiti|FJ797432 Germany Bos taurus
B. besnoiti|KX013107 Spain Bos taurus
B. besnoiti|JF314861 Italy Bos taurus
B. neotomofelis|HQ909085 USA Neotoma micropus
89
B. akodoni|AY545987 Brazil Akodon montensis
78
B. oryctofelisi|AY182000 Argentina Oryctolagus cuniculus
B. darlingi|MF872605 USA Lynx rufus/Mus musculus*
Toxoplasma gondii|AJ312317
0.1