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# Serological survey of *Toxoplasma gondii* and *Besnoitia besnoiti* in a wildlife conservation area in southern Portugal

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ABSTRACT

*Toxoplasma gondii* and *Besnoitia besnoiti* are closely related apicomplexan protozoa. *T. gondii* is a zoonotic pathogen which may cause serious disease in man and warm-blooded animals, including wild species. *B. besnoiti* causes bovine besnoitiosis, an emergent disease in Europe, which is linked to important production losses. Unlike *T. gondii*, the life cycle of *B. besnoiti* remains a mystery, since the definitive host has not yet been identified. The aim of this work was to determine the seroprevalence of *T. gondii* in wildlife and feral cats from a hitherto unsampled area in southern Portugal and to identify likely candidates for definitive and/or other intermediate hosts of *B. besnoiti*. A total of 260 animals were screened for *T. gondii* and *B. besnoiti* by the modified agglutination tests, using the cut-off value of 1:20 and 1:80, respectively. The prevalence of *T. gondii* was 85.3% in Egyptian mongoose (*Herpestes ichneumon*; n = 34), 83.3% in wildcats (*Felis silvestris*; n = 6), 66.7% in stone martens (*Martes foina*; n = 6), 47.1% in genets (*Genetta genetta*; n = 17), 40% in foxes (*Vulpes vulpes*; n = 25), 39.2% in cats (*Felis catus*; n = 79), 33.3% in European polecats (*Mustela putorius*; n = 3), 21.4% in red deer (*Cervus elaphus*; n = 14), 7.7% in wild boars (*Sus scrofa*; n = 26), 2.8% in rabbits (*Oryctolagus cuniculus*; n = 36) and 0% in European otters (*Lutra lutra*; n = 2), European badgers (*Meles meles*; n = 6) and rodents (n = 5). None of the species tested was positive for *B. besnoiti*. Based on the present results, the monitoring of *T. gondii* in native animal populations may be of major importance for wildlife conservation strategies and human health protection, while the search for other hosts of *B. besnoiti* requires further investigations in wild and domestic species.


## 1. Introduction

*Toxoplasma gondii* Nicolle and Manceaux 1908 and *Besnoitia besnoiti* Marotel 1912 are closely related intracellular obligatory apicomplexan parasites, which belong to the cyst forming coccidia. This group of protozoa has typically a prey-predator life cycle, with a definitive carnivorous host and intermediate hosts harbouring parasite tissue cysts. *T. gondii* infects man and a wide range of warm-blooded animals, including domestic and wild species. Cats and other felids are the only known definitive hosts. *T. gondii* is considered an important zoonotic pathogen, which may lead to abortion in non-immune women and severe and fatal disease in immunocompromised individuals and foetuses. Toxoplasmosis is a major cause of abortion in sheep and goats, with obvious economic consequences in the livestock production. Clinical toxoplasmosis with fatal outcome was reported in several wildlife species, including red foxes (Dubey et al., 1990), red squirrels (Simpson et al., 2013) and marsupials (Canfield et al., 1990). Wild animals may become infected by the faecal-oral route, through the ingestion of sporulated oocysts in soil or contaminated water or, in the case of carnivores and omnivores, by the predation of birds and mammals containing *T. gondii* tissue cysts. Game animals pose a risk of infection for humans, through the consumption of contaminated raw or undercooked meat (Sacks et al., 1983) or by handling infected tissues (Dubey, 1991) during evisceration. Information on the prevalence of *T. gondii* in wildlife in Portugal is scarce and limited to the northern parts of the country (Lopes et al., 2011; Coelho et al., 2014). Concerning domestic species, *T. gondii* was recorded in 18.5% of goats and 17.1%–33.6% of sheep tested, but prevalence values were lower in pigs (7.1–9.8%), cattle (7.5%) and horses (13.3%) (Sousa et al., 2010; Lopes et al., 2013; Esteves et al., 2014).

Bovine besnoitiosis is considered an emergent disease of cattle in Europe (EFSA, 2010) causing important production losses in the cattle industry (Pols, 1960). While areas of endemicity have been recognized in Portugal, Spain, France and Italy, the disease seems to be spreading to neighbouring countries, representing a major veterinary concern (Cortes et al., 2014). In Portugal, recent data from a nationwide survey of *B. besnoiti* infection in cattle indicate a herd-prevalence of 5.1% (CI: 3.1–7.8%) and a mean within-herd prevalence of 33.0% (CI: 20.3–46.0%) (Waap et al., 2014). The life cycle of *B. besnoiti* remains a mystery. The existence of a heteroxenous life cycle involving a carnivorous definitive host

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and possibly other intermediate hosts has been postulated, based on the coccidian nature of *B. besnoiti* and the fact that felids were identified as the definitive host for other *Besnoitia* species (Wallace and Frenkel, 1975; Smith and Frenkel, 1984; Dubey et al., 2003; Dubey and Yabsley, 2010). However, so far, all attempts to find a carnivorous definitive host failed (Diesing et al., 1988; Basso et al., 2011). Blood sucking arthropods are frequently implied in the transmission, based on the cutaneous localization of tissue cysts, the higher incidence of besnoitiosis during the summer months, when insects are more active (Pols, 1960), and experimental evidence of subclinical infection through tabanids, tse-tse flies and stable flies (Bigalke, 1968).

Experimental studies carried out by others have shown that *B. besnoiti* is capable of infecting several animal species other than bovines, especially small ruminants (Pols, 1960), antelopes (McCully et al., 1966), rodents (Basso et al., 2011) and leporidae (Shkap et al., 1987; Basso et al., 2011). For this reason, the screening of *B. besnoiti* antibodies in a broad range of domestic and wildlife species, especially those in geographical areas were besnoitiosis is prevalent, could be useful to identify the most likely candidates for definitive and/or intermediate hosts.

A major drawback to the serological study of wildlife is the lack of diagnostic tests adapted to the species under study, due to the unavailability of host-specific secondary antibodies. The direct agglutination test, which avoids the use of secondary antibodies, has been widely employed to screen for exposure to *T. gondii* in different animal species and is commercially available (ToxoScreen DA, Biomériex). Sensitivity and specificity values for the direct agglutination test were determined in pigs (Se 83% and Sp 90%) (Dubey et al., 1995), sheep (Se 96% and Sp 89%) (Shaapan et al., 2008), cats (Se 98% and Sp 100%) (Macrì et al., 2009) and humans (Se 96% and Sp 99%). The B-MAT, a modified agglutination test using cell-culture derived *B. besnoiti* tachyzoites, was developed for the diagnosis of *B. besnoiti* infection in cattle. This test showed a high sensitivity, specificity and test agreement when compared with the indirect immunofluorescence test (IFAT) (Waap et al., 2011, 2014). The B-MAT is based on the same serological assay principle as the direct agglutination test for *T. gondii* and is therefore a promising tool for the screening of exposure to *B. besnoiti* in wildlife.

The objective of the study was to determine the presence of specific antibodies anti-*T. gondii* and anti-*B. besnoiti* in samples from wild animals and feral cats in a wildlife conservation area in the south of Portugal, using the DA test and the B-MAT, respectively.

## 2. Materials and methods

### 2.1. Sample origin

Serological tests were performed on serum (n = 209) and tissue samples (n = 51) from 181 wild animals and 79 feral cats. Samples were collected between 2010 and 2013 in the frame of the project LIFE Habitat Lince Abutre (LIFE08 NAT/P/000,227) and sent to the National Institute for Veterinary and Agrarian Research (INIAV) for the screening of diseases that may affect the Iberian lynx and black vulture populations as well as their habitats in southeast of Portugal. Data on sex and age were recorded by the veterinary practitioner responsible for sample collection whenever possible. Animals were classified as juvenile, subadult or adult, based on animal's size, coat features, and teeth characteristics. Geographic origin of animals was recorded at the level of the smallest administrative unit in Portugal (freguesia). Samples were obtained from a total of 24 freguesias belonging to 10 municipalities in the NUTS2 regions Alentejo and Algarve (Fig. 1). The Alentejo is characterised by a peneplain landscape with a gently undulating terrain, largely not exceeding 200 m above sea level. From this peneplain landscape emerges the Serra do Caldeirão (577) in the region Algarve, which is the most important mountain in the study area. The climate is typically Mediterranean Csa (Köppen climate classification), with warm to hot, dry summers and mild to cool, wet winters. Average monthly temperatures are above 22.0 °C during the warmest month and the average temperature in the coldest month ranges between 18 to −3 °C (Kottek et al., 2006). The average precipitation is <800 mm per year (www.ipma.pt). The population density ranges between 5–50 inhabitants/km$^2$ (INE, 2011) and the native fauna comprises mainly wild boars (*Sus scrofa scrofa*), red deer (*Cervus elaphus*), rabbits (*Oryctolagus cuniculus*) and several carnivorous species, including foxes (*Vulpes vulpes*), wildcats (*Felis silvestris*), the Iberian lynx (*Lynx pardinus*), Egyptian mongoose (*Herpestes ichneumon*), genets (*Genetta genetta*), stone martens (*Martes foina*), European otter (*Lutra lutra*), European polecats (*Mustela putorius*) and European badgers (*Meles meles*) (www.naturdata.com). The vegetation is composed by shrubs, native cork oaks, holm oaks, olive trees, eucalyptus trees and native trees. Information on sampling conditions (captured/ hunted/ road kill animals) was registered. Samples and animal records were kindly made available for the purposes of this study by the national veterinary authority DGAV.

Sampled species included, 79 domestic feral cats (*Felis catus*), 36 European rabbits, 34 Egyptian mongoose, 26 wild boars, 25 foxes, 17 common genets, 14 red deer, six stone martens, six wildcats, six European badgers, two European otters, three European polecats, one garden dormouse (*Eliomys quercinus*), one common rat (*Rattus norvegicus*), one western Mediterranean mouse (*Mus spretus*) and one wood mouse (*Apodemus sylvaticus*).

### 2.2. Preparation of lung extracts

Lung tissue samples were collected during necropsy. Lung extracts (LE) were prepared according to Ferroglio et al. (2000), with minor changes. Briefly, a sub-sample weighing approximately 2.5 g was collected from each lung fragment, washed in PBS and excess fluid was removed on filter paper. Fragments were sliced and transferred to 15 ml Falcon tubes containing 2 ml PBS. After 20 min at room temperature the tubes were shaken during 4 min and centrifuged at 800 g during 10 min. One and a half ml of each supernatant was transferred to Eppendorf tubes and centrifuged at 3500 g during 3 min to remove debris. The clear lung extracts were collected into new Eppendorf tubes and frozen at −20 °C until use.

### 2.3. Analysis of anti-T. gondii specific IgG antibodies

Serum samples and lung extracts were screened for immunoglobulin G antibodies to *T. gondii* at 1:20 dilution using the direct agglutination (DA) test kit (Toxo-Screen-bioMérieux), as prescribed by the manufacturer. The serological screening was performed at 1:20 dilution in order to increase sensitivity, since the test has not been validated for other species and because antibodies in lung extracts may be more diluted compared to serum. Positive and negative controls supplied with the kit, as well as an antigen control (PBS instead of serum) were included in each testing round. Agglutination of tachyzoites as a diffuse opacity covering at least half of the well was interpreted as a positive reaction and sedimentation as a button or ring was considered a negative reaction. Sera with an indistinct agglutination pattern were retested. Sera positive at 1:20 dilution were tested at 1:200 and 1:400 dilutions. Endpoint titres were determined for positive LE of cats and wild cats in order to assess the detection limit of *T. gondii* antibodies when using lung tissue.

### 2.4. Analysis of anti-B. besnoiti specific IgG antibodies

Serum samples and lung extracts were screened for immunoglobulin G antibodies to *B. besnoiti* at 1:80 dilution by the B-MAT, using whole formalin-fixed cell-culture derived tachyzoites as antigen and 2-beta-mercaptoethanol to prevent nonspecific agglutination, according to previously established procedures (Waap et al., 2011). The lower cut-off of 1:80 (instead of 1:160 in bovines) was used in order