Serological study of *Neospora caninum* in dogs and wildlife in a nature conservation area in southern Portugal

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SUMMARY

A serological survey was performed to evaluate the presence of specific antibodies against *Neospora caninum* in dogs and native animals in a wildlife conservation area in southern Portugal. The study involved 463 animals, including dogs (*n* = 286), European rabbits (*Oryctolagus cuniculus*; *n* = 32), Egyptian mongoose (*Herpestes ichneumon*; *n* = 34), wild boars (*Sus scrofa*; *n* = 26), foxes (*Vulpes vulpes*, *n* = 25), common genets (*Genetta genetta*; *n* = 17), red deer (*Cervus elaphus*; *n* = 14), wildcats (*Felis silvestris*; *n* = 6), four mustelid species (*n* = 17) and rodents (*n* = 6). Samples from dogs were analysed by Indirect Fluorescent Antibody Test (IFAT). Samples from wild animals were screened by the modified agglutination test and positive and doubtful results were confirmed by IFAT. The seroprevalence of *N. caninum* in dogs was 32·5% [95% confidence interval (95% CI) 27·3–38·1]. Among wild animals, exposure to *N. caninum* was confirmed only in foxes (12%, 95% CI 4·2–30) and rabbits (25%, 95% CI 13·3–42·1). This is the first evidence of natural exposure to *N. caninum* in foxes and rabbits in Portugal, and our results suggest that rabbits may play a role as reservoirs of infection to dogs, foxes and other wildlife carnivores. The relevance of this finding in the sylvatic cycle of *N. caninum* needs further studies, since infection may affect wildlife species and cattle grazing in the same areas.

Key words: *Neospora caninum*, dogs, rabbits, foxes, wildlife, seroprevalence.

INTRODUCTION

Neospora caninum is an obligate intracellular cyst-forming coccidian parasite with a worldwide distribution. *Neospora caninum* is a major cause of abortion in cattle and causes severe neuromuscular disease in dogs (Dubey, 2003; McInnes *et al.* 2006). The disease is responsible for considerable economic losses to the beef and dairy industries, which are mainly linked to the lower reproductive performance of affected cattle (Thurmond and Hietala, 1997; Dubey, 2003), but also to reduced milk production, premature culling and decreased weight gain (Thurmond and Hietala, 1996; Barling *et al.* 2000; Hernandez *et al.* 2001). Prevalence rates determined in seroepidemiological studies carried out at farm level may reach 100% (Dubey, 1999).

Neospora caninum has a heteroxenous prey–predator life cycle. The definitive carnivorous host (DH) sheds unsporulated oocysts into the environment following predation of intermediate hosts (IH) containing *N. caninum* tissue cysts and IH become infected after ingestion of sporulated oocysts in water or food, or in the case of carnivores and omnivores, also through the consumption of infected tissues. In addition, *N. caninum* can be transmitted transplacentally in several host species (Dubey, 2003). Up to now, the only confirmed DH are canids from the genus *Canis*, namely dogs (*Canis familiaris*) (McAllister *et al.* 1998), coyotes (*C. latrans*) (Gondim *et al.* 2004*b*), dingoes (*C. lupus dingo*) (King *et al.* 2010) and grey wolves (*C. lupus lupus*) (Dubey *et al.* 2014).

A wide range of domestic and wildlife species were shown to be susceptible to *N. caninum* infection, but their role in domestic and sylvatic transmission cycles still needs to be elucidated (Almería, 2013). Little is known about clinical neosporosis in wildlife. Disease in non-domestic carnivores was described only in a European pine marten and in blue and red foxes, in young animals, with similar neurological and dermatological presentations as in domestic dogs (Donahoe *et al.* 2015). *Neospora caninum* infection in artiodactyls is associated with stillbirths and systemic disease in very young animals (Donahoe *et al.* 2015).

Dogs become infected when fed with unfrozen, raw or undercooked beef. Other possible infection sources include feeding on abortion tissues from bovines, predation of small wildlife mammals and feeding on animal carcasses or offal from game animals left in the field (Gondim *et al.* 2004*a*). Though transplacental infection is considered the

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main mechanism for parasite transmission in cattle, the presence of dogs as a source of environmental contamination with oocysts, was found to be crucial to maintain the life cycle (Dubey, 2003; Dubey *et al.* 2007). Dogs should therefore be regarded as a potential risk factor, not only for cattle but also for natural populations sharing the same habitat. In Portugal, this is especially important in regards to nature conservation areas located in the region Alentejo, which has the largest inventory of grass-fed, extensively raised cattle in the country. *Neospora caninum* was identified for the first time as a cause of abortion in Portuguese cattle farms in 2001 (Thompson *et al.* 2001) and isolated from an aborted fetus in 2002 (Canada *et al.* 2002). Subsequent studies in intensive dairy farms, showed a prevalence of 28% in randomly sampled farms and 46% in farms with abortion problems (Canada *et al.* 2004).

The serological screening of *N. caninum* in dogs is an indirect means to evaluate environmental contamination, since seropositive animals probably already shed oocysts. The prevalence of antibodies to *N. caninum* in wildlife will mirror the impact of environmental contamination and reflect the susceptibility of native IH species to infection, therefore providing further evidence on the circulation of *N. caninum* between domestic and wild ecosystems (Almería, 2013).

This study aimed to evaluate the exposure to *N. caninum* in dogs and wild animals in a wildlife conservation area in the southeast of Portugal.

MATERIALS AND METHODS

Sample characterization

Serological tests were performed on serum samples from 286 dogs and serum ($n = 126$) and lung extracts ($n = 51$) from 177 wild animals. Wildlife species included 32 European rabbits (*Oryctolagus cuniculus*), 34 Egyptian mongoose (*Herpestes ichneumon*), 26 wild boars (*Sus scrofa*), 25 foxes (*Vulpes vulpes*), 17 common genets (*Genetta genetta*), 14 red deer (*Cervus elaphus*), six wildcats (*Felis silvestris*), six stone martens (*Martes foina*), six wildcats (*F. silvestris*), six European badgers (*Meles meles*), two European otters (*Lutra lutra*), six European polecats (*Mustela putorius*), one garden dormouse (*Eliomys quercinus*), one common rat (*Rattus norvegicus*), three western Mediterranean mice (*Mus spretus*) and one wood mouse (*Apodemus sylvaticus*). Sampling was performed between 2010 and 2013 in the frame of the project LIFE Habitat Lince Abutre (LIFE08 NAT/P/000227). Samples were obtained in a wildlife conservation area in the southeast of Portugal involving a total of 24 *freguesias* (the smallest administrative unit in Portugal) belonging to 13 municipalities in the NUTS2 regions

Alentejo and Algarve (Fig. 1). The sampling area is largely characterized by a peneplain landscape, generally not exceeding 200 m above sea level. 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 range between 18 and −3 °C in the coldest month (Kottek *et al.* 2006), while the average precipitation is <800 mm per year (www. ipma.pt). Data on sex, age and sampling conditions of wildlife species (captured/hunted/road kill animals) were recorded whenever possible. Samples were sent to the National Institute for Veterinary and Agrarian Research (INIAV) for the diagnosis of several diseases that may affect the Iberian lynx and black vulture populations in this particular area. Samples and animal data were kindly provided for the purposes of this study by the national veterinary authority Direcção-Geral de Alimentação e Veterinária (DGAV).

Neospora caninum *production and purification*

Neospora caninum tachyzoites used to prepare the antigen suspension for the *N. caninum* modified agglutination test (N-MAT) and to coat Indirect Fluorescent Antibody Test (IFAT) slides were propagated by continuous passage in Vero cell (African Green Monkey kidney epithelial cells) cultures using Dulbecco's Minimal Essential Medium (DMEM) supplemented with 100 UI mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, 2 mM l-glutamine, 20 mM HEPES and fetal calf serum (10% to initiate cultures and 2% for maintenance) at 37 °C in closed 75 cm^2 cell culture flasks. Tachyzoites were harvested after 3–4 days of infection. At this stage, most of the cell layer was confluent and *N. caninum* tachyzoites intracellular. The cell monolayer was scraped from the cell culture flask into the supernatant and the resulting suspension was passed through a 21 G syringe to release tachyzoites from cells, centrifuged at 770 $\bm{g}$ for 15 min, at 4 °C, and the pellet was resuspended in phosphate-buffered saline (PBS). Tachyzoites were separated from host cell debris, using columns made of Whatman CF 11 cellulose powder, as described by Dempster (1984). The purified parasites were used immediately for N-MAT antigen or IFAT slide preparation.

N-MAT *antigen and IFAT slide preparation*

The N-MAT antigen was prepared as described by Packham *et al.* (1998) with minor changes (Waap *et al.* 2011). Briefly, cell-free parasites were washed once in 20 mL PBS and the pellet was resuspended in 2–3 mL of 37% formaldehyde diluted in PBS to 6% formaldehyde. Parasites were fixed overnight at 4 °C and then washed three times in 20 mL PBS to
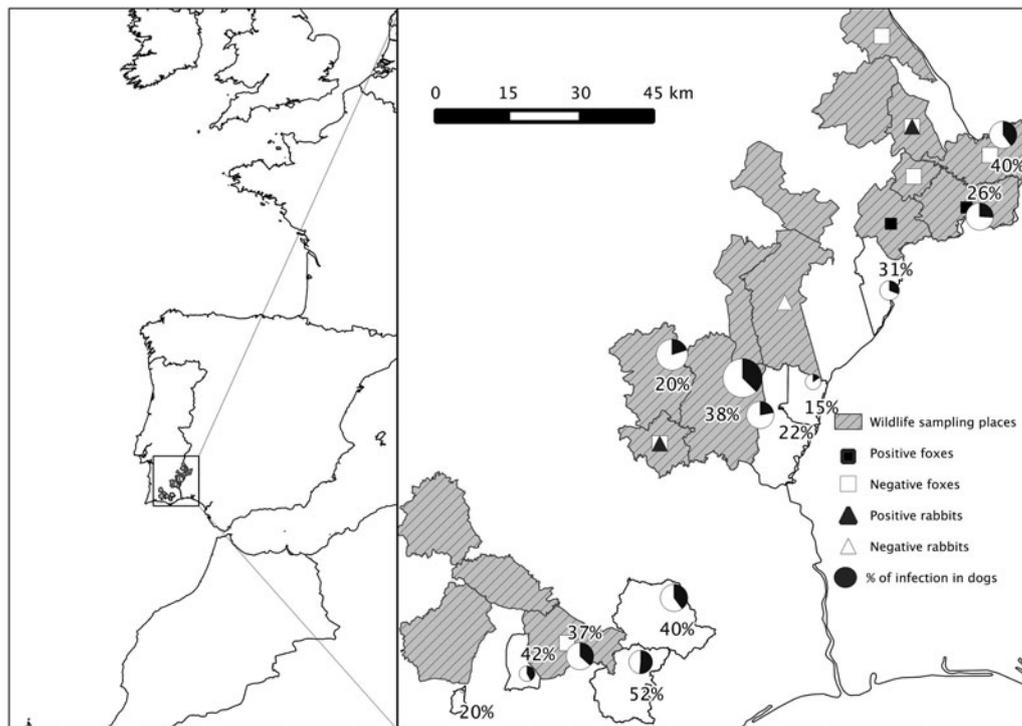
Fig. 1. Survey area: wildlife sampling places, sample prevalence of *Neospora caninum* in dogs and location of positive and negative foxes and rabbits. © EuroGeographics for the administrative boundaries.

remove formaldehyde. The fixed tachyzoites were counted on a Neubauer chamber and resuspended to a final concentration of $35-45 \times 10^6$ tachyzoites mL^{-1} in filtered (0·2 μM pore size) alkaline buffer (pH 8·7), containing 7·02 g NaCl, 3·09 g H$_3$BO$_3$, 24 mL 1 N NaOH, 50 mg eosin Y, 1·0 g sodium azide and 4 mg mL^{-1} bovine albumin (fraction V) per litre.

IFAT slide preparation was based on the procedure proposed by Shkap *et al.* (2002). Briefly, purified tachyzoites were washed and suspended in a 4% formaldehyde solution in PBS, on ice, during 30 min. The fixed tachyzoites were washed three times to remove formaldehyde and the pellet was suspended in 1 mL PBS. Parasites were counted and diluted in PBS to a final concentration of 2×10^6 tachyzoites mL^{-1}, distributed in 6 μL drops on slides, dried at 37 °C and fixed in cold acetone (-20 °C) for 10 min.

*Screening of anti-*N. caninum-*specific IgG antibodies in dogs*

Serum samples from dogs were screened for specific anti-*N. caninum* antibodies by the IFAT at three dilutions (1:50, 1:100 and 1:200) using rabbit antidog IgG-FITC secondary antibody (Sigma Aldrich) diluted at 1:750 in 0·01% Evans blue solution. Positive and negative IFAT controls (canine origin, VMRD, Inc) were included in each testing round. Bovine serum albumin was added to the serum and conjugate diluting buffers in order to reduce non-specific binding of antibodies. Slides

were mounted with 50% glycerol in PBS (pH 8·5) and viewed under a Zeiss fluorescence microscope with a 40× objective. Complete peripheral fluorescence was regarded as a positive reaction, while partial, apical or absent fluorescence was considered a negative reaction.

*Analysis of anti-*N. caninum-*specific IgG antibodies in wildlife species*

Lung tissue samples were collected during necropsy and processed as described by Ferroglio *et al.* (2000) to prepare lung extracts for serological analysis. Lung extracts were shown to be suitable for the screening of specific antibodies against several pathogens in wildlife, including *Encephalitozoon* (Waller *et al.* 1980), *Francisella tularensis* (Mörner *et al.* 1988), canine parvovirus (Duarte *et al.* 2012) and *Toxoplasma gondii* (Jakubek *et al.* 2012; Waap *et al.* 2016).

Serum samples and lung extracts were screened for IgG antibodies to *N. caninum* at 1:50, 1:150 and 1:450 dilutions using N-MAT according to previously established procedures (Packham *et al.* 1998). Each testing round included positive and negative control sera from cattle and dogs tested by IFAT and ELISA, as well as an antigen control (PBS instead of serum). Agglutination of tachyzoites as a diffuse opacity covering at least half of the well was considered a positive reaction and sedimentation as a button or ring a negative reaction. Positive and doubtful results, characterized by an indistinct agglutination pattern, were tested by the IFAT at

1:50, 1:100 and 1:200 dilutions, in order to confirm the serological status of animals. Sera from foxes were assayed with rabbit antidog IgG-FITC secondary antibody (Sigma Aldrich) and rabbits were tested with donkey antirabbit IgG secondary antibody, Alexa Fluor 488 (Thermo Fisher Scientific). Proteins A and G can bind with strong affinity to immunoglobulin of several species and are commonly applied in ELISA tests for multi-species diagnosis (Zhang *et al.* 2010; Schaefer *et al.* 2011) Therefore, confirmation of positive and doubtful results in the other wildlife species was attempted with Alexa Fluor 488 proteins A and G conjugates (Thermo Fisher Scientific).

Data analysis

The 95% confidence intervals (CI) for prevalence data were calculated by Wilson's score method assuming a perfect test using EpiTools Epidemiological Calculator (Sergeant, 2016). Geographical distribution maps were constructed using Quantum Geographic Information System (QGIS) software 2.0.1.

RESULTS

The overall seroprevalence in dogs at the established cut-off of 1:50 was 32·5% (CI 27·3–38·1). Considering the three dilutions used for titration of sera, 22 dogs were positive at the cut-off dilution 1:50, 17 dogs had an endpoint titre at 1:100 dilution and 54 had endpoint titres ≥1:200 (Table 1). Overall, reading of results at 1:50 dilution was difficult, due to considerable non-specific binding of antibodies,

visible as a strong apical fluorescence, which frequently extended to more than half of the tachyzoite membranes.

In wildlife animals tested by the N-MAT, a distinct agglutination pattern indicating the presence of antibodies to *N. caninum* was observed in two foxes and 15 rabbits, while six rabbits, two foxes, two wild boars and two Egyptian mongoose were considered doubtful. Positive reactivity was only detected in sera; all lung extracts tested negative. Two foxes and one rabbit had an N-MAT endpoint titre at 1:50 dilution, and 14 rabbits had endpoint titres ≥1:450 (Table 1). Confirmation of N-MAT positive and doubtful results by IFAT showed the presence of antibodies to *N. caninum* in both positive foxes and eight seropositive rabbits, while one of the doubtful fox sera and one of the doubtful rabbit sera were also found to be positive. One fox and one rabbit had an IFAT titre of 1:50, two rabbits had a titre of 1:100 and two foxes and five rabbits had titres ≥200 (Table 1). Seropositive foxes came from two out of eight *freguesias* sampled, and seropositive rabbits came from two out of three *freguesias* (Fig. 1). At the positive sampling sites, antibodies to *N. caninum* were found in 1 of 12 and 2 of 6 foxes and 3 of 12 and 5 of 17 rabbits. Positive foxes included one adult female and one juvenile and one subadult male. No data on sex and age were available for rabbits. Only animals with a positive reaction in both assays were considered seropositive; therefore, the percentage of infected individuals in the sample was considered to be 12% (CI 4·2–30%) in foxes and 25% (CI 13·3–42·1%) in rabbits. Evaluation of the protein A/G system for confirmation of doubtful results in the two Egyptian mongoose and two wild

Table 1. Number (*n*) of lung extracts (LE) and sera from dogs and wildlife animals tested, percentage of infection in the total sample (%) and NAT and IFAT titres in confirmed results

Family	Species	LE (*n*)	Sera (*n*)	%	NAT titres			IFAT titres		
					1:50	1:150	1:450	1:50	1:100	1:200
Canidae	Dog (*Canis familiaris*)	–	286	32·5	–	–	–	22	17	54
	Fox (*Vulpes vulpes*)	7	18	12·0	3	0	0	1	0	2
Leporidae	European rabbit (*Oryctolagus cuniculus*)	–	32	25·0	1	0	14	1	2	5
Felidae	Wild cat (*Felis silvestris*)	3	3	0						
Mustelidae	European badger (*Meles meles*)	6	–	0						
	European otter (*Lutra lutra*)	2	–	0						
	Stone marten (*Martes foina*)	2	4	0						
	European polecat (*Mustela putorius*)	2	1	0						
Herpestidae	Egyptian mongoose (*Herpestes ichneumon*)	5	29	0						
Viverridae	Common genet (*Genetta genetta*)	4	13	0						
Suidae	Wild boar (*Sus scrofa*)	–	26	0						
Gliridae	Garden dormouse (*Eliomys quercinus*)	1	–	0						
Muridae	Common rat (*Rattus norvegicus*)	1	–	0						
	Western Mediterranean mouse (*Mus spretus*)	3	–	0						
	Wood mouse (*Apodemus sylvaticus*)	1	–	0						
Cervidae	Red deer (*Cervus elaphus*)	–	14	0						

boar sera included titration of Alexa Fluor 488 proteins A and G conjugates (Thermo Fisher Scientific) and titration of control sera. The binding of the conjugate was assessed with *N. caninum* slides and positive and negative dog sera, and with *T. gondii* slides and positive and negative fox, pig, wild boar and Egyptian mongoose sera. However, except for dogs and foxes, the fluorescent signal was too weak to establish the seropositivity of sera (data not shown). Based on these results, the protein A/G system was found to be unsuitable to confirm infection in Egyptian mongoose and wild boars and this species was therefore considered to be negative.

DISCUSSION

The aim of this study was to determine the presence of specific antibodies anti-*N. caninum* in samples from dogs and wild animals in a nature conservation area in the southeast of Portugal.

Several serological techniques have been employed to screen for anti-*N. caninum* antibodies in different animal species, including the IFAT, usually using the cut-off of 1:50 in dogs (Björkman and Uggla, 1999; Silva *et al.* 2007; Uzeda *et al.* 2007; Yakhchali *et al.* 2010; Robbe *et al.* 2016; Wang *et al.* 2016), the N-MAT and various ELISAs. The competitive cELISA and N-MAT are the most commonly used tests in wildlife, because these techniques do not require the use of species-specific secondary antibodies (Almería, 2013). Though the assay principle of the cELISA makes this test attractive to be used in all species, validation data are absent and several authors have stated a lack of test agreement with IFAT results (Capelli *et al.* 2006; Sobrino *et al.* 2008).

In this study, we opted to use the IFAT for the serological survey in dogs. The IFAT has been widely employed for *N. caninum* serology in dogs and is generally accepted as the reference test (Björkman and Uggla, 1999; Capelli *et al.* 2006; Silva *et al.* 2007). Several authors have also claimed that the IFAT is more specific than the ELISA, because the use of crude tachyzoite antigen as coating antigen in soluble extract-based ELISAs may be a cause of cross-reactivity with *T. gondii* and other related apicomplexan parasites (Björkman and Hemphill, 1998; Silva *et al.* 2007). Though many authors consider the dilution 1:50 the most appropriate cut-off for seropositivity in dogs (Björkman and Uggla, 1999; Silva *et al.* 2007; Uzeda *et al.* 2007; Yakhchali *et al.* 2010; Robbe *et al.* 2016; Wang *et al.* 2016), in this study, interpretation of results was difficult, with a high percentage of sera producing strong apical reactions, in many instances with incomplete peripheral fluorescence of fixed tachyzoites. Others, when using the IFAT for the serological screening of *N. caninum*

in different species made similar observations (Paré *et al.* 1995; Mineo *et al.* 2001; Galvão *et al.* 2015). Apical, unspecific fluorescence may arise from serological cross-reactivity with other apicomplexan species (Paré *et al.* 1995), due to the presence of conserved apical complex antigens (Conrad *et al.* 1993). Nevertheless, according to other authors, the IFAT for *N. caninum* shows little cross-reactivity with frequent apicomplexan parasites of dogs, such as *T. gondii*, *Sarcocystis* spp., *Babesia canis* and *Hammondia heydorni* (Dubey and Lindsay, 1993; Trees *et al.* 1993; Dubey *et al.* 1996; Gondim *et al.* 2015), but the extent to which antibodies to other protozoa (e.g. *Cystoisospora* spp., *Cryptosporidium* spp.) may cross-react is unknown. Since reading of results depends on the subjective assessment of the observer, this emphasizes even more the need for reassessment of cut-offs and harmonization of IFAT reagents and slide preparation procedures.

Wildlife species were assayed by a serial testing strategy, using the N-MAT as the first screening assay and the IFAT to confirm results. The use of two tests to determine the infection status of wild animals is generally recommended, due to the lack of validated tests for wildlife species. The choice of the N-MAT was based on the higher sensitivity, compared with the IFAT, possibly resulting from a better exposure of epitopes of suspended whole formalin-fixed tachyzoites, while the IFAT has proven to be more specific (Packham *et al.* 1998).

Information on *N. caninum* prevalence in dogs in Portugal is available from only one study (Maia *et al.* 2014), which recorded an overall prevalence of 7·9% in dogs by cELISA. The seroprevalence found in the regions Alentejo (5·5%) and Algarve (8·8%) was substantially lower than in the present survey. This may be explained by differences in sample size, time and place of sampling, as well as the serological screening assay used. Prevalence data from other European countries vary likewise substantially. Similar infection rates between 32 and 32·7% were recorded in Italy (Robbe *et al.* 2016) and Romania (Gavrea *et al.* 2012), and rates up to 43·6% were reported from Spain (Collantes-Fernández *et al.* 2008; Regidor-Cerrillo *et al.* 2010), while studies carried out in Austria (Wanha *et al.* 2005), Czech Republic (Václavek *et al.* 2007), Serbia (Kuruca *et al.* 2013) and Poland (Ploneczka and Mazurkiewicz, 2008; Goździk *et al.* 2011) found a lower prevalence, ranging between 4·9 and 21·7%.

Infection of dogs in both urban and rural environments is likely to occur through feeding on raw or poorly cooked beef, and vertical transmission from bitches to successive litters has also been described (Dubey *et al.* 2007). However, dogs in rural/nature conservation areas are more probable to predate potentially infected small mammals and birds and to have access to aborted fetal tissues from cattle and wild ruminants, carcasses from wild animals

and hunting offal, which may explain the high infection rate found in the present survey.

Concerning the wildlife species tested in this study, anti-*N. caninum*-specific antibodies were detected only in foxes and rabbits. This is the first evidence of exposure to *N. caninum* in wild animals in Portugal. Unfortunately, it was not possible to establish a correlation between the seropositivity found in dogs, foxes and rabbits, mainly because most sampling areas of these species were not coincident. This may be explained by the difficulty to match the sampling from hunted, captured or road kill wild animals with the sampling from dogs in the same areas. Spatial analysis is also hampered by the low seroprevalence of *N. caninum* in wildlife and because some species of wild animals have a wide home range. Spatial analysis to correlate infection in dogs and wild animals will therefore require a greater geographical coverage and a more representative sample size. The percentage of infection found in foxes in the present study (12%) is higher than that reported in most studies carried out in Europe. Thus, studies using the IFAT recorded infection rates between 0·9 and 4·4% in foxes in Britain (Hamilton *et al.* 2005), Czech Republic (Bártová *et al.* 2016), Ireland (Murphy *et al.* 2007) and Spain (Sobrino *et al.* 2008), while studies carried out by ELISA showed a prevalence of 1·5% in Hungary (Jakubek *et al.* 2007) and absence of infection among 221 foxes sampled in different parts of Sweden (Jakubek *et al.* 2001). However, in some areas, exposure of foxes to *N. caninum* may be high, as shown by the prevalence of 69·8% determined by the N-MAT in the Spanish Pyrenees (Marco *et al.* 2008). Despite the seroprevalence and the occasional finding of *N. caninum*-like oocysts in feces, red foxes were not shown to be definitive hosts (Dubey *et al.* 2017). Nonetheless, a study on the feeding habits of foxes in Britain showed that foxes feed mainly on medium-sized mammals, primarily rabbits, corresponding to 74% of mass ingested (Baker *et al.* 2006), and there is evidence that foxes select rabbits when they are abundant, feeding on small mammals and fruits/seeds when lagomorphs are scarce (Díaz-Ruiz *et* al. 2013). The infection rate of 25% found in the present survey suggests therefore that rabbits could be the main source of infection with *N. caninum* to foxes and that fluctuation of the rabbit population may account for the variable seroprevalence found in different areas. In fact, though Almería *et al.* (2007) did not detect antibodies in any of 251 wild rabbits tested in Spain, rabbits were shown to be natural IH of *N. caninum* by molecular methods, with an infection prevalence of 10·5% (Hughes *et al.* 2008). In addition, antibodies to *N. caninum* were found in 1·2 and 1·85% of animals tested by the IFAT in rabbit farms in Northern Italy and Northern Egypt, respectively (Ibrahim *et al.* 2009;

Machacova *et al.* 2015). The lower prevalence in farmed rabbits can be explained by the fact that caged animals in industrial units have fewer opportunities to become infected with *N. caninum* oocysts.

Though seropositivity to *N. caninum* was described in several Mustelidae, Herpestidae, wild boars, wild cats and wild ruminants (Almería, 2013; Donahoe *et al.* 2015), none of these species in the present survey were positive. Since the prevalence of infection can be low, the number of animals analysed may not be representative enough and further investigations on these species are needed, in order to assess their role in the sylvatic cycle of *N. caninum* in the area studied.

Conclusion

Our results show a high prevalence *N. caninum* in dogs in a wildlife conservation area in Portugal and suggest that rabbits could be a reservoir of infection to dogs, foxes and other wildlife carnivores. The high seroprevalence in dogs and foxes at 1:50 dilution may be caused by a too low cut-off and should be interpreted carefully. Prevalence of *N. caninum* in dogs and wildlife will need to be taken into account to define adequate prevention measures to reduce transmission to cattle. Asides cattle management practices aimed at reducing vertical transmission in affected farms, prevention strategies should address more incisively horizontal transmission, i.e. discourage feeding dogs with fresh raw meat and restricting as much as possible the access of dogs to ruminants, calving areas and livestock feed and water. Additionally, hunters should take special care in regards to the safe disposal of tissues and organs from hunted animals, in order to prevent infection of dogs and wild carnivores.

CONFLICT OF INTEREST

None.

REFERENCES

Almería, S. (2013). *Neospora caninum* and Wildlife. *ISRN Parasitology* **2013**, 947347.

Almería, S., Vidal, D., Ferrer, D., Pabón, M., Fernández-de-Mera, M. I., Ruiz-Fons, F., Alzaga, V., Marco, I., Calvete, C., Lavin, S., Gortazar, C., López-Gatius, F. and Dubey, J. P. (2007). Seroprevalence of *Neospora caninum* in non-carnivorous wildlife from Spain. *Veterinary Parasitology* **143**, 21–28.

Baker, P., Furlong, M., Southern, S. and Harris, S. (2006). The potential impact of the red fox (*Vulpes vulpes*) predation in agricultural landscapes in lowland Britain. *Wildlife Biology* **12**, 39–50.

Barling, K. S., McNeill, J. W., Thompson, J. A., Paschal, J. C., McCollum, F. T., Craig, T. M. and Adams, L. G. (2000). Association of serologic status for *Neospora caninum* with postweaning weight gain and carcass measurements in beef calves. *Journal of the American Veterinary Medical Association* **217**, 1356–1360.

Bártová, E., Slezáková, R., Nágl, I. and Sedlák, K. (2016). *Neospora caninum* and *Toxoplasma gondii* antibodies in red foxes (*Vulpes vulpes*) in the Czech Republic. *Annals of Agricultural and Environmental Medicine* **23**, 84–86.

Björkman, C. and Hemphill, A. (1998). Characterization of *Neospora caninum* iscom antigens using monoclonal antibodies. *Parasite Immunology* **20**, 73–80.

Björkman, C. and Uggla, A. (1999). Serological diagnosis of *Neospora caninum* infection. *International Journal for Parasitology* **29**, 1497–1507.

Canada, N., Meireles, C. S., Rocha, A., Sousa, S., Thompson, G., Dubey, J. P., Romand, S., Thulliez, P. and Correia da Costa, J. M. (2002). First Portuguese isolate of *Neospora caninum* from an aborted foetus from a dairy herd with endemic neosporosis. *Veterinary Parasitology* **110**, 11–15.

Canada, N., Carvalheira, J., Meireles, C. S., Correia da Costa, J. M. and Rocha, A. (2004). Prevalence of *Neospora caninum* infection in dairy cows and its consequences for reproductive management. *Theriogenology* **62**, 1229–1235.

Capelli, G., Natale, A., Nardelli, S., Frangipane di Regalbono, A. and Pietrobelli, M. (2006). Validation of a commercially available cELISA test for canine neosporosis against an indirect fluorescent antibody test (IFAT). *Preventive Veterinary Medicine* **73**, 315–320.

Collantes-Fernández, E., Gómez-Bautista, M., Miró, G., Alvarez-García, G., Pereira-Bueno, J., Frisuelos, C. and Ortega-Mora, L. M. (2008). Seroprevalence and risk factors associated with *Neospora caninum* infection in different dog populations in Spain. *Veterinary Parasitology* **152**, 148–151.

Conrad, P. A., Sverlow, K., Anderson, M., Rowe, J., BonDurant, R., Tuter, G., Breitmeyer, R., Palmer, C., Thurmond, M., Ardans, A., Dubey, J. P., Duhamel, G. and Barr, B. (1993). Detection of serum antibody responses in cattle with natural or experimental *Neospora* infections. *Journal of Veterinary Diagnostic Investigation* **5**, 572–578.

Dempster, R. P. (1984). *Toxoplasma gondii*: purification of zoites from peritoneal exudates by eight methods. *Experimental Parasitology* **57**, 195–207.

Díaz-Ruiz, F., Delibes-Mateos, M., García-Moreno, J. L., López-Martín, J. M., Ferreira, C. and Ferreras, P. (2013). Biogeographical patterns in the diet of an opportunistic predator: the red fox *Vulpes vulpes* in the Iberian Peninsula. *Mammal Review* **43**, 59–70.

Donahoe, S. L., Lindsay, S. A., Krockenberger, M., Phalen, D. and Šlapeta, J. (2015). A review of neosporosis and pathologic findings of *Neospora caninum* infection in wildlife. *International Journal for Parasitology: Parasites and Wildlife*. **4**, 216–238.

Duarte, A., Fernandes, M., Santos, N. and Tavares, L. (2012). Virological Survey in free-ranging wildcats (*Felis silvestris*) and feral domestic cats in Portugal. *Veterinary Microbiology* **158**, 400–404.

Dubey, J. P. (1999). Neosporosis in cattle: biology and economic impact. *Journal of the American Veterinary Medical Association* **214**, 1160–1162.

Dubey, J. P. (2003). Review of *Neospora caninum* and neosporosis in animals. *Korean Journal of Parasitology* **41**, 1–16.

Dubey, J. P. and Lindsay, D. S. (1993). Neosporosis. *Parasitology Today* **9**, 452–458.

Dubey, J. P., Lindsay, D. S., Adams, D. S., Gay, J. M., Baszler, T. V., Blagburn, B. L. and Thulliez, P. (1996). Serologic responses of cattle and other animals infected with *Neospora caninum*. *American Journal of Veterinary Research* **57**, 329–336.

Dubey, J. P., Schares, G. and Ortega-Mora, L. M. (2007). Epidemiology and control of neosporosis and *Neospora caninum*. *Clinical Microbiology Reviews* **20**, 323–367.

Dubey, J. P., Jenkins, M. C., Ferreira, L. R., Choudhary, S., Verma, S. K. and Kwok, O. C. (2014). Isolation of viable *Neospora caninum* from brains of wild gray wolves (*Canis lupus*). *Veterinary Parasitology* **201**, 150–153.

Dubey, J. P., Hemphill, A., Calero-Bernal, R. and Schares, G. (2017). Neosporosis in Wild Canids and Other Carnivores. In Neosporosis in animals (ed. Dubey, J. P., Hemphill, A., Calero-Bernal, R. and Schares, G.) pp. 379–389. CRC Press, Boca Raton, Florida, USA.

Ferroglio, E., Rossi, L. and Gennero, S. (2000). Lung-tissue extract as an alternative to serum for surveillance for brucellosis in chamois. *Preventive Veterinary Medicine* **43**, 117–122.

Galvão, C. M., Rezende-Gondim, M. M., Chaves, A. C., Schares, G., Ribas, J. R. and Gondim, L. F. (2015). Brazilian donkeys (*Equus asinus*) have a low exposure to *Neospora* spp. *Brazilian Journal of Veterinary Parasitology* **24**, 340–344.

Gavrea, R., Mircean, V., Pastiu, A. and Cozma, V. (2012). Epidemiological survey of *Neospora caninum* infection in dogs from Romania. *Veterinary Parasitology* **188**, 382–385.

Gondim, L. F., McAllister, M. M., Mateus-Pinilla, N. E., Pitt, W. C., Mech, L. D. and Nelson, M. E. (2004*a*). Transmission of *Neospora caninum* between wild and domestic animals. *Journal of Parasitology* **90**, 1361–1365.

Gondim, L. F., McAllister, M. M., Pitt, W. C. and Zemlicka, D. E. (2004*b*). Coyotes (*Canis latrans*) are definitive hosts of *Neospora caninum*. *International Journal for Parasitology* **34**, 159–161.

Gondim, L. F., Meyer, J., Peters, M., Rezende-Gondim, M. M., Vrhovec, M. G., Pantchev, N., Bauer, C., Conraths, F. J. and Schares, G. (2015). *In vitro* cultivation of *Hammondia heydorni*: generation of tachyzoites, stage conversion into bradyzoites, and evaluation of serologic cross-reaction with *Neospora caninum*. *Veterinary Parasitology* **210**, 131–140.

Goździk, K., Wrzesień, R., Wielgosz-Ostolska, A., Bień, J., Kozak-Ljunggren, M. and Cabaj, W. (2011). Prevalence of antibodies against *Neospora caninum* in dogs from urban areas in Central Poland. *Parasitolgy Research* **108**, 991–996.

Hamilton, C. M., Gray, R., Wright, S. E., Gangadharan, B., Laurenson, K. and Innes, E. A. (2005). Prevalence of antibodies to *Toxoplasma gondii* and *Neospora caninum* in red foxes (*Vulpes vulpes*) from around the UK. *Veterinary Parasitology* **130**, 169–173.

Hernandez, J., Risco, C. and Donovan, A. (2001). Association between exposure to *Neospora caninum* and milk production in dairy cows. *Journal of the American Veterinary Medical Association* **219**, 632–635.

Hughes, J. M., Thomasson, D., Craig, P. S., Georgin, S., Pickles, A. and Hide, G. (2008). *Neospora caninum*: detection in wild rabbits and investigation of co-infection with *Toxoplasma gondii* by PCR analysis. *Experimental Parasitology* **120**, 255–260.

Ibrahim, H. M., Huang, P., Salem, T. A., Talaat, R. M., Nasr, M. I., Xuan, X. and Nishikawa, Y. (2009). Short report: prevalence of *Neospora caninum* and *Toxoplasma gondii* antibodies in northern Egypt. *American Journal of Tropical Medicine and Hygiene* **80**, 263–267.

Jakubek, E. B., Bröjer, C., Regnersen, C., Uggla, A., Schares, G. and Björkman, C. (2001). Seroprevalences of *Toxoplasma gondii* and *Neospora caninum* in Swedish red foxes (*Vulpes vulpes*). *Veterinary Parasitology* **102**, 167–172.

Jakubek, E. B., Farkas, R., Pálfi, V. and Mattsson, J. G. (2007). Prevalence of antibodies against *Toxoplasma gondii* and *Neospora caninum* in Hungarian red foxes (*Vulpes vulpes*). *Veterinary Parasitology* **144**, 39–44.

Jakubek, E. B., Mattsson, R., Mörner, T., Mattsson, J. G. and Gavier-Widén, D. (2012). Potential application of serological tests on fluids from carcasses: detection of antibodies against *Toxoplasma gondii* and *Sarcoptes scabiei* in red foxes (*Vulpes vulpes*). *Acta Veterinaria Scandinavica* **54**, 1–5.

King, J. S., Slapeta, J., Jenkins, D. J., Al-Qassab, S. E., Ellis, J. T. and Windsor, P. A. (2010). Australian dingoes are definitive hosts of *Neospora caninum*. *International Journal for Parasitology* **40**, 945–950.

Kottek, M., Grieser, J., Beck, C., Rudolf, B. and Rubel, F. (2006). World Map of the Köppen–Geiger climate classification updated. *Meteorologische Zeitschrift* **15**, 259–263.

Kuruca, L., Spasojevic-Kosic, L., Simin, S., Savovic, M., Laus, S. and Lalosevic, V. (2013) *Neospora caninum* antibodies in dairy cows and domestic dogs from Vojvodina, Serbia. *Parasite* **20**, 40.

Machacova, T., Bártová, E., Sedlak, K., Budikova, M. and Piccirillo, A. (2015) Risk factors involved in transmission of *Toxoplasma gondii* and *Neospora caninum* infection in rabbit farms in Northern Italy. *Annals of Agricultural and Environmental Medicine* **22**, 677–679.

Maia, C., Cortes, H., Brancal, H., Lopes, A. P., Pimenta, P., Campino, L. and Cardoso, L. (2014). Prevalence and correlates of antibodies to *Neospora caninum* in dogs in Portugal. *Parasite* **21**, 29.

Marco, I., Ferroglio, E., López-Olvera, J. R., Montané, J. and Lavín, S. (2008). High seroprevalence of *Neospora caninum* in the red fox (*Vulpes vulpes*) in the Pyrenees (NE Spain). *Veterinary Parasitology* **152**, 321–324.

Mörner, T., Sandström, G. and Mattsson, R. (1988). Comparison of serum and lung extracts for surveys of wild animals for antibodies to *Francisella tularensis* biovar palaearctica. *Journal of Wildlife Diseases* **24**, 10–14.

McAllister, M. M., Dubey, J. P., Lindsay, D. S., Jolley, W. R., Wills, R. A. and McGuire, A. M. (1998). Dogs are definitive hosts of *Neospora caninum*. *International Journal for Parasitology* **28**, 1473–1478.

McInnes, L. M., Irwin, P., Palmer, D. G. and Ryan, U. M. (2006). *In vitro* isolation and characterization of the first canine *Neospora caninum* isolate in Australia. *Veterinary Parasitology* **137**, 355–363.

Mineo, T. W., Silva, D. A., Costa, G. H., von Ancken, A. C., Kasper, L. H., Souza, M. A., Cabral, D. D., Costa, A. J. and Mineo, J. R. (2001). Detection of IgG antibodies to *Neospora caninum* and *Toxoplasma gondii* in dogs examined in a veterinary hospital from Brazil. *Veterinary Parasitology* **98**, 239–245.

Murphy, T. M., Walochnik, J., Hassl, A., Moriarty, J., Mooney, J., Toolan, D., Sanchez-Miguel, C., O'Loughlin, A. and McAuliffe, A. (2007). Study on the prevalence of *Toxoplasma gondii* and *Neospora caninum* and molecular evidence of *Encephalitozoon cuniculi* and *Encephalitozoon* (Septata) *intestinalis* infections in red foxes (*Vulpes vulpes*) in rural Ireland. *Veterinary Parasitology* **146**, 227–234.

Packham, A. E., Sverlow, K. W., Conrad, P. A., Loomis, E. F., Rowe, J. D., Anderson, M. L., Marsh, A. E., Cray, C. and Barr, B. C. (1998). A modified agglutination test for *Neospora caninum*: development, optimization, and comparison to the indirect fluorescent-antibody test and enzyme-linked immunosorbent assay. *Clinical and Diagnostic Laboratory Immunology* **5**, 467–473.

Paré, J., Hietala, S. K. and Thurmond, M. C. (1995). Interpretation of an indirect fluorescent antibody test for diagnosis of *Neospora* sp. infection in cattle. *Journal of Veterinary Diagnostic Investigation* **7**, 273–275.

Płoneczka, K. and Mazurkiewicz, M. (2008). Seroprevalence of *Neospora caninum* in dogs in south-western Poland. *Veterinary Parasitology* **153**, 168–171.

Regidor-Cerrillo, J., Pedraza-Diaz, S., Rojo-Montejo, S., Vazquez-Moreno, E., Arnaiz, I., Gomez-Bautista, M., Jimenez-Palacios, S., Ortega-Mora, L. M. and Collantes-Fernandez, E. (2010). *Neospora caninum* infection in stray and farm dogs: seroepidemiological study and oocyst shedding. *Veterinary Parasitology* **174**, 332–335.

Robbe, D., Passarelli, A., Gloria, A., Di Cesare, A., Capelli, G., Iorio, R. and Traversa, D. (2016). *Neospora caninum* seropositivity and reproductive risk factors in dogs. *Experimental Parasitology* **164**, 31–35.

Schaefer, J. J., White, H. A., Schaaf, S. L., Mohammed, H. O. and Wade, S. E. (2011). Modification of a commercial *Toxoplasma gondii* immunoglobulin G enzyme-linked immunosorbent assay for use in multiple animal species. *Journal of Veterinary Diagnostic Investigation* **23**, 297–301.

Sergeant, E. S. G. (2016). Epitools epidemiological calculators. Ausvet Pty Ltd. http://epitools.ausvet.com.au.

Shkap, V., Reske, A., Pipano, E., Fish, L. and Baszler, T. (2002). Immunological relationship between *Neospora caninum* and *Besnoitia besnoiti*. *Veterinary Parasitology* **106**, 35–43.

Silva, D. A., Lobato, J., Mineo, T. W. and Mineo, J. R. (2007). Evaluation of serological tests for the diagnosis of *Neospora caninum* infection in dogs: optimization of cut off titers and inhibition studies of cross-reactivity with *Toxoplasma gondii*. *Veterinary Parasitology* **143**, 234–244.

Sobrino, R., Dubey, J. P., Pabón, M., Linarez, N., Kwok, O. C., Millán, J., Arnal, M. C., Luco, D. F., López-Gatius, F., Thulliez, P., Gortázar, C. and Almería, S. (2008). *Neospora caninum* antibodies in wild carnivores from Spain. *Veterinary Parasitology* **155**, 190–197.

Thompson, G., Canada, N., do Carmo Topa, M., Silva, E., Vaz, F. and Rocha, A. (2001). First confirmed case of *Neospora caninum*-associated abortion outbreak in Portugal. *Reproduction in Domestic Animals* **36**, 309–312.

Thurmond, M. C. and Hietala, S. K. (1996). Culling associated with *Neospora caninum* infection in dairy cows. *American Journal of Veterinary Research* **57**, 1559–1562.

Thurmond, M. C. and Hietala, S. K. (1997). Effect of congenitally acquired *Neospora caninum* infection on risk of abortion and subsequent abortions in dairy cattle. *American Journal of Veterinary Research* **58**, 1381–1385.

Trees, A. J., Guy, F., Tennant, B. J., Balfour, A. H. and Dubey, J. P. (1993). Prevalence of antibodies to *Neospora caninum* in a population of urban dogs in England. *Veterinary Record* **132**, 125–126.

Uzeda, R. S., Costa, K. de S., Santos, S. L., Pinheiro, A. M., De Almeida, M. A., McAllister, M. M. and Gondim, L. F. (2007). Loss of infectivity of *Neospora caninum* oocysts maintained for a prolonged time. *Korean Journal of Parasitology* **45**, 295–299.

Václavek, P., Sedlák, K., Hůrková, L., Vodrázka, P., Sebesta, R. and Koudela, B. (2007). Serological survey of *Neospora caninum* in dogs in the Czech Republic and a long-term study of dynamics of antibodies. *Veterinary Parasitology* **143**, 35–41.

Waap, H., Cardoso, R., Marcelino, E., Malta, J., Cortes, H. and Leitão, A. (2011). A modified agglutination test for the diagnosis of *Besnoitia besnoiti* infection. *Veterinary Parasitology* **178**, 217–222.

Waap, H., Nunes, T., Vaz, Y. and Leitão, A. (2016). Serological survey of *Toxoplasma gondii* and *Besnoitia besnoiti* in a wildlife conservation area in southern Portugal. *Veterinary Parasitology Regional Studies and Reports* **3–4**, 7–12.

Waller, T., Lyngset, A., Elvander, M. and Morein, B. (1980). Immunological diagnosis of encephalitozoonosis from *post-mortem* specimens. *Veterinary Immunology and Immunopathology* **1**, 353–360.

Wang, S., Yao, Z., Zhang, N., Wang, D., Ma, J., Liu, S., Zheng, B., Zhang, B., Liu, K. and Zhang, H. (2016). Serological study of *Neospora caninum* infection in dogs in central China. *Parasite* **23**, 25.

Wanha, K., Edelhofer, R., Gabler-Eduardo, C. and Prosl, H. (2005). Prevalence of antibodies against *Neospora caninum* and *Toxoplasma gondii* in dogs and foxes in Austria. *Veterinary Parasitology* **128**, 189–193.

Yakhchali, M., Javadi, S. and Morshedi, A. (2010). Prevalence of antibodies to *Neospora caninum* in stray dogs of Urmia, Iran. *Parasitology Research* **106**, 1455–1458.

Zhang, D., Wang, Z., Fang, R., Nie, H., Feng, H., Zhou, Y. and Zhao, J. (2010). Use of protein AG in an enzyme-linked immunosorbent assay for serodiagnosis of *Toxoplasma gondii* infection in four species of animals. *Clinical and Vaccine Immunology* **17**, 485–486.