Veterinary Parasitology 178 (2011) 64–69

Contents lists available at ScienceDirect

Veterinary Parasitology

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

Toxoplasma gondii and *Neospora caninum* in wildlife: Common parasites in Belgian foxes and Cervidae?

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ARTICLE INFO

Article history:
Received 23 September 2010
Received in revised form 1 December 2010
Accepted 13 December 2010

Keywords:
Toxoplasma gondii
Neospora caninum
Red fox
Deer
PCR
Antibodies

ABSTRACT

Sera from Cervidae were tested for the presence of antibodies against *Neospora caninum* using ELISA; and against *Toxoplasma gondii* using SAG1-ELISA and a commercially available agglutination test. The *T. gondii* seroprevalence was 52% (38/73) in roe deer (*Capreolus capreolus*), 0% in bred fallow deer (0/4) (*Dama dama*) and red deer (0/7) (*Cervus elaphus*). We found 2.7% of the roe deer samples and none of the bred deer samples positive for *N. caninum*. Brain samples from wild roe deer, red deer and red foxes (*Vulpes vulpes*) were tested for the presence of *T. gondii* and *N. caninum* DNA using multiplex real-time PCR. We detected *T. gondii* in 18.8% (57/304) of the red foxes and in 1 of the 33 deer samples. *N. caninum* was found in 6.6% of the red foxes and in 2 roe deer samples. Twenty-six of the *T. gondii* positive DNA extracts from the red fox samples were genotyped. Twenty-five were type II and only one was found to be type III.

1. Introduction

Toxoplasma gondii and *Neospora caninum* are closely related obligate intracellular protozoan parasites with worldwide distributions. Their lifecycles involve carnivore definitive hosts, felids for *Toxoplasma*, and dogs and coyotes for *Neospora*, and a wide range of warm-blooded intermediate hosts. Both domestic and wild animals can be infected (Frenkel, 1970; McAllister et al., 1998; Tenter et al., 2000; Gondim et al., 2004). Carnivorous animals get infected by feeding on chronically infected meat, prey and carrion. For herbivores infection occurs by ingestion of oocysts on vegetation or in feed. While *T. gondii* is considered an important zoonotic disease and can cause life-threatening infections in immunocompromised hosts and foetuses (Montoya and Liesenfeld, 2004), *N. caninum* does not infect humans, although there is some evidence that it might occur (Tranas et al., 1999). Humans get toxoplasmosis by horizontal and vertical transmission; horizontal transmission occurs by ingestion of sporulated oocysts or by consumption of meat containing tissue cysts, the latter way being considered the most common in Europe (Cook et al., 2000). In veterinary

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doi:10.1016/j.vetpar.2010.12.016

Table 1
Primers and probe sequences used in the triplex PCR to detect *T. gondii*, *N caninum* and cellular r18S DNA.

	Target	Name	Sequence 5′ → 3′	5′ Modification[a]	3′ Modification[b]
Primers	*T. gondii*	T2	CGGAGAGGGAGAAGATGTT		
		T3	GCCATCACCACGAGGAAA		
	N. caninum	NF1	GAGAATGAGAGCGATTTCCAG		
		NR1	CTCCTGAAGTCCCAGCGA		
	Cellular r18S	VF1	GATTAAGTCCCTGCCCTTT		
		VR1	CACACCGCCCGTCGCTACTACC		
Probes	*T. gondii*	TP	CTTGGCTGCTTTTCCTGGAGGG	FAM 488	BHQ1
	N. caninum	NP	CCTTCTGAGTCGGGTTGTGTTTGGC	Atto 647	BHQ3
	Cellular r18S	VP	CACACCGCCCGTCGCTACTACC	Texas Red 547	BHQ2

[a] Fluorophore used as reporter.
[b] Quencher molecule.

medicine, *T. gondii* is an important cause of abortion in goats and sheep (Tenter et al., 2000). Neosporosis is considered a major cause of abortion in cattle (Dubey, 2003; Thilsted and Dubey, 1989). Because of their structural and genetic similarities and common hosts misdiagnosis between both parasites is not uncommon.

Toxoplasma can evolve both in domestic and sylvatic lifecycles. *Toxoplasma* isolates in the domestic cycle seem to be mainly clonal in Europe and designated as universal types I, II and III clonal lineages. In contrast, recombination of genetic material seems to occur more frequently in the sylvatic cycle (Ajzenberg et al., 2004), and atypical strains are more often associated with clinical outbreak types of infections (Grigg and Sundar, 2009). These observations have been made mainly in the Americas and there is a need to study occurrence and characterisation of *T. gondii* in wildlife in other continents.

The objectives of the present study were to assess the prevalence of *T. gondii* and *N. caninum* in a sample of the Belgian red fox and deer population, to compare different molecular and serological detection techniques and to genetically characterize the *T. gondii* isolates. Fox and deer have different feeding habits; while foxes can be considered sentinels of *Toxoplasma* infections occurring in wildlife, venison is a potential source of human infection. Few data are available on the sylvatic cycle of *N. caninum* (Panadero et al., 2010), and the role of the fox as final host is still debated (Wapenaar et al., 2006; Marco et al., 2008). Toxoplasmosis is a very common infection in humans in Belgium and seroconversion occurs in 9/10,000 pregnancies (Breugelmans et al., 2004), which is among the highest rates in Europe.

2. Materials and methods

2.1. Samples

Brain (from both Cervidae and *Vulpes vulpes*) and serum samples (from Cervidae only) were obtained from various sources. Three hundred and four brain samples from Belgian red foxes (*V. vulpes*) and 33 brain samples from deer were obtained from the National Reference Laboratory for Rabies at the Belgian Institute of Public Health. These samples (fox and deer) involve dead-found animals, which died from an unknown cause, and were collected for rabies diagnosis to verify the continued absence of rabies in Belgian wildlife populations. The 20 *Capreolus capreolus* (roe deer) and 13 *Cervus elaphus* (red deer) samples obtained from this laboratory originated from Wallonia. Eighty-four blood samples from wild and bred deer were obtained from hunters and taken just after the kill. The samples originated from Flanders and belonged to three deer species: 73 samples from wild roe deer; 4 and 7 samples from bred fallow deer (*Dama dama*) and red deer, respectively. The samples were collected between 2004 and 2009 and the serum, separated from the blood clot by centrifuging 10 min at 3200 × *g* as soon as possible after reception of the sample, was stored at −20 °C. For each sample, the hunters recorded the postal codes of the geographical location where the animal was shot.

2.2. Molecular methods

2.2.1. DNA extraction

After several freezing and thawing cycles, between 5 and 10 g of each brain sample (depending on the quantity available) was homogenized in a Potter Homogenizer and stored at −20 °C. DNA extraction was performed with the Qiagen DNA Mini Kit (Qiagen, Venlo, The Netherlands), using a slightly modified protocol: to 400 μl of homogenized brain sample, 40 μl of proteinase K and 400 μl AL lysis buffer were added and incubated at 56 °C until complete lysis. Then 400 μl of a 24/1 mixture of chloroform and isoamyl-alcohol was added. This was mixed and centrifuged at 22,000 × *g* (4 °C) for 20 min. The supernatant was transferred to a new 1.5 ml micro tube and mixed with 200 μl of 95+% ethanol to precipitate the DNA. From hereon the manufacturer's instructions were followed: the lysate/ethanol mixture was transferred to a spin column, washed once with 500 μl AW1 buffer and once with 500 μl AW2 buffer. The DNA was eluted in 200 μl AE buffer and stored at −20 °C till further use.

2.2.2. Real-time PCR

DNA was tested by triplex quantitative real-time PCR with dual labeled probes on a BioRad iCycler (Bio-Rad, Hercules, CA). To detect *T. gondii*, *AF146527* was used as target (Homan et al., 2000) and for *N. caninum X84238* (Yamage et al., 1996). To check for PCR inhibition and DNA quality, cellular r18S was used as target (see Table 1 for primers and probes). A *T. gondii* positive control was made by extracting DNA from cultured tachyzoites from the RH strain. *N. caninum* DNA was obtained from Moredun

Research Institute, Pentland Science Park, Edinburgh, UK. Each PCR reaction contained 10 μl of extracted DNA, mixed with 15 μl of a PCR master mix containing 12.5 μl of PowerMix (BioRad, Nazareth, Belgium), 0.5 μl of each primer (IDT, Belgium) at a concentration of 20 μM and 0.5 μl dual labeled probe at a concentration of 2 μM. Cycling protocol: Initial denaturation and activation of the Taq polymerase at 95 °C for 3 min; followed by 45 cycles at 95 °C for 20 s and 60 °C for 20 s. Results were analysed with the iCycler software.

2.2.3. Genotyping analysis

T. gondii DNA samples with a Ct value ≤ 32 by real-time PCR were submitted to a genotyping analysis with 15 microsatellite markers in a Multiplex PCR assay described elsewhere (Ajzenberg et al., 2010). Briefly, in each pair of primer, the forward one was 5′-end labeled with fluorescein to allow sizing of PCR products electrophoresed in an automatic sequencer. PCR was carried out in a 25-μl reaction mixture consisting of 12.5 μl of 2× QIAGEN Multiplex PCR Master Mix (Qiagen, France), 5 pmol each primer and 5 μl of DNA. Cycling conditions were 15 min at 95 °C; 30 s at 94 °C, 3 min at 61 °C, and 30 s at 72 °C (35 cycles); and 30 min at 60 °C. PCR products were diluted 1:2 in deionised formamide. One μl of each diluted PCR product was mixed with 0.5 μl of a dye-labeled size standard (ROX 500, Applied Biosystems) and 23.5 μl of deionised formamide. This mixture was denatured at 95 °C for 5 min and then electrophoresed using an automatic sequencer (ABI PRISM 3130xl, Applied Biosystems). The sizes of the alleles in base pairs (bp) were estimated using GeneMapper analysis software (version 4.0, Applied Biosystems).

2.3. Serological methods

2.3.1. T. gondii SAG1-ELISA

The cervid sera were tested for the presence of anti-*T. gondii* IgG antibodies as described earlier (De Craeye et al., 2008). Briefly, sera were diluted 1/100 in PBS with 10% foetal calf serum (FCS). SAG1 coated 96 well plates (Meddens Diagnostics BV, The Netherlands) were incubated at 37 °C with 100 μl of serum dilution per well. Detection of SAG1-specific IgG's was done with horseradish peroxidase (HRP) labeled rabbit anti-deer IgG (KPL, Maryland, USA) conjugate, diluted 1/10,000 in PBS with 10% FCS. The chromogen used was 3-3′, 5,5′-tetramethylbenzidine (TMB, Sigma, St. Louis, MO) and the reaction was stopped with 0.1 N H_2SO_4. The plate was read with the iMark microplate plate reader (Bio-Rad, Hercules, CA) at 450 nm with background correction at 620 nm. The cut-off was determined as the mean corrected OD_{450} +3X standard deviation OD_{450} of two negative reference sera. The reference sera, two negative and one positive control, were tested by the Sabin Feldman lysis test.

2.3.2. Modified agglutination test (MAT)

The deer were also tested for the presence of *T. gondii* IgG with the Toxoscreen DA kit (BioMerieux, Craponne, France). Sera were diluted 1/40, 1/100 and 1/4000 and tested according to manufacturer's instructions.

2.3.3. N. caninum ELISA

All deer sera were tested for the presence of anti-*Neospora* IgG using ID Screen *N. caninum* Indirect ELISA (IDVet, Montpellier, France). Samples were tested according to the manufacturer's instruction.

2.4. Statistical analysis

The PCR prevalence and exact confidence intervals were computed by Region for Belgium. The agreement between the results of the diagnostic tests used was assessed by calculating positive and negative agreement indices with credibility intervals according to the method described by Graham and Bull (1998). These specific agreement indices were used in several applications (Erhart et al., 2002; Bhattarai et al., 2009) and do not have the limitations of the kappa statistic (K), like the influence of trait prevalence. Following comparisons were conducted on the deer sera: the agreement between *T. gondii* and *N. caninum* on serology outcomes, the agreement between *T. gondii* and *N. caninum* on PCR outcomes, and for *T. gondii* specifically: the agreement between the MAT test and the SAG1-ELISA test at a 1/100 dilution. For the red fox brain samples, the agreement between *T. gondii* and *N. caninum* on PCR outcomes was computed as well.

Considering the outcome of 2 tests in general, the values a, b, c and d denote the observed frequencies for each possible combination of ratings by tests 1 and 2: a, being the number of samples positive with both tests; b, the number of samples negative with test 1 and positive with test 2, c, the number of samples positive with test 1 and negative with test 2 and d, the number of samples negatives with both tests. The proportion of specific agreement for the positive ratings (pr+) (the positive agreement index), and for the negative ratings (pr−) (the negative agreement index) were calculated as follows: $\mathrm{pr}+ = 2a/(2a+b+c)$ and $\mathrm{pr}- = 2d/(2d+b+c)$. pr+, for example, estimates the conditional probability, given that one of the test results, randomly selected, is positive, the other will also be positive. Credibility intervals (95%) were calculated using the Bayesian method proposed by Graham and Bull (1998). In this analysis a non-informative prior distribution was used because no specific prior knowledge about the long-run cell probabilities was available.

3. Results

3.1. Serological tests

In total, 84 serum samples from deer were tested by SAG1-ELISA for the presence of anti-*T. gondii* IgG. No positives were found in the bred species, whereas 52% of the wild animals were positive.

Three dilutions were tested in the MAT test: 1/40, 1/100 and 1/4000. The 1/40 dilution as described in the manufacturer's manual resulted in too much background and made the reading too difficult. This could be due to the fact that many serum samples were strongly haemolysed. Although the samples where taken immediately after shooting the animal, it sometimes took a long time before they reached the laboratory and could be centrifuged. When comparing

Table 2
Results of the serological tests on the deer sera. (A) Comparison of *T. gondii* versus *N. caninum*. (B) Comparison between the commercial MAT test and the SAG1-ELISA test at a 1/100 dilution.

(A) Serology Cervidae		
	T. gondii	
	+	–
N. caninum		
+	1	1
–	38	44
(B) *T. gondii* Serology		
	MAT 1/100	
	+	–
SAG1-ELISA 1/100		
+	38	0
–	0	46

the results with the quality of the serum, it was clear that all samples testing positive at a 1/40 dilution in MAT and negative at a 1/100 dilution in the MAT and the SAG1-ELISA, were very strongly haemolysed. With a 1/100 dilution the test was much clearer to interpret and identical results were obtained as in the ELISA test (Table 2). In the wild animal group, 41% of the sera were still positive at a 1/4000 dilution. Only 2 of the 84 deer serum samples tested positive in the ID Screen *N. caninum* Indirect ELISA. The samples came from wild adult roe deer and one was also positive for *T. gondii* in MAT (1/100) and SAG1-ELISA (Table 2).

Positive and negative agreement indices with their 95% credibility intervals (indicated between brackets) were used to assess the agreement between *T. gondii* and *N. caninum* serology in cervids. A positive agreement of 0.0488 (0.003–0.193) and a negative agreement of 0.693 (0.59–0.77) indicated that the agreement is better in the negative rather than the positive direction. Furthermore for *T. gondii* the agreement assessment between the commercial MAT test and the SAG1-ELISA test indicated a positive agreement of 1.0 (0.97–1.00) and a negative agreement of 1.0 (0.98–1.00). Here the agreement is nearly similar in the positive rather than the negative direction.

3.2. Molecular identification

3.2.1. PCR on red fox samples

In total, 57 (18.8%) of the 304 collected brain samples tested positive for *T. gondii* and 20 samples tested positive (6.6%) for *N. caninum* (Table 3). Most samples were collected in the Brussels Capital region and Wallonia. Only 48 samples originated from Flanders. In Brussels we found 15.4% *T. gondii* positive samples, in Wallonia 21.7% and in Flanders 20.8%. Fewer *N. caninum* positives were found: 5.9% in Brussels, 6.7% in Wallonia and 8.3% in Flanders. Only one sample tested positive for both parasites (Table 4).

3.2.2. PCR on cervids

On a total of 33 brain samples, 1/20 roe deer was positive for *T. gondii* and 2 were positive for *N. caninum*. All red deer (13) were negative for both parasites (Tables 3 and 4).

Positive and negative agreement indices with their 95% credibility intervals (indicated between brackets) were used to assess the PCR results agreement between *T. gondii* and *N. caninum* in deer and in red foxes. For the deer a positive agreement of 0.000 (0.000–0.610) and a negative agreement of 0.952 (0.88–0.99) were obtained. For the foxes a positive agreement 0.0260 (0.0027–0.0968) and a negative agreement 0.858 (0.82–0.89) were obtained. In both cases the agreement was better in the negative rather than the positive direction.

3.2.3. Genotyping

Out of the 57 *T. gondii* PCR positive fox samples, 26 had a Ct lower than 32 and were sent to the National Reference Center for toxoplasmosis, Limoges, France, for genotyping analyses. All 26 samples were *T. gondii* genotype II, except for one sample, which was of genotype III.

4. Discussion

While the seroprevalence of human toxoplasmosis and seroconversion rates during pregnancy in Belgium is among the highest in Europe, little is known about the occurrence of *T. gondii* in domestic animals and wildlife and on genotypes circulating. In this study, we provided data on the molecular evidence and on seroprevalence of toxoplasmosis and neosporosis in Belgian wildlife. In addition, we have genotyped *T. gondii* isolates from this study.

In red foxes, using PCR on brain samples, we found prevalences of 18.8% for *T. gondii* and 6.6% for *N. caninum*. In brain samples of deer, *T. gondii* and *N. caninum* were found in one and two samples, respectively. In 52% of the serum samples from wild roe deer we detected antibodies against *T. gondii*, while only two animals had antibodies against *N. caninum*. These figures demonstrate that both parasites are circulating in wildlife. Both animal species have a different diet and probably a different susceptibility to *T. gondii* and *N. caninum*. While foxes are defined as carnivores, they are also very opportunistic feeders, especially the urban fox.

Table 3
Summary of samples from red fox and deer, collected per region, and corresponding prevalence of *T. gondii* and *N. caninum*.

PCR	Region	*T. gondii*			*N. caninum*			Sample size
		# positives	%	CI 95%[a]	# positives	%	CI 95%[a]	
Red fox	Flanders	10	20.83%	10.47–34.99%	4	8.33%	2.31–19.98%	48
	Wallonia	26	21.67%	14.66–30.11%	8	6.67%	2.92–12.71%	120
	Brussels	21	15.44%	9.82–22.63%	8	5.88%	2.57–11.26%	136
Deer	Wallonia	1	3.03%	0.07–15.75%	2	6.06%	0.74–20.23%	33

[a] CI 95%: confidence interval at 95%.

Table 4
Comparison of *T. gondii* versus *N. caninum* PCR results on the brain samples of (A) red fox and (B) deer.

(A) Red fox		
	T. gondii	
	+	–
N. caninum		
+	1	19
–	56	228
(B) Deer		
	T. gondii	
	+	–
N. caninum		
+	0	2
–	1	30

Because of their feeding habits, they are likely to be more exposed to both parasites' tissue cysts and oocysts compared to deer, which are feeding mainly on grass, leaves, young shoots and berries.

It is difficult to compare results from screenings of wildlife samples, because different methods, both serological and molecular, each with their own sensitivity and specificity, are used. However, geographical differences in prevalence may reflect disparities in transmission of the parasites due to varying epidemiological factors, such as, type of habitat, presence of domestic or wild felidae, climate and soil. When compared to prevalences in fox and deer from other European countries, the figures from this study indicate a rather high prevalence of both parasites in Belgian wildlife. The high demographic pressure in the country, with patchy distribution of forests and pasture, and a high level of urbanization, may allow frequent contacts at the interface between wildlife and domestic animals, increasing opportunities for parasites to pass from the domestic to the sylvatic lifecycle or vice versa. Interestingly, negligible geographical differences were found in the occurrence of both parasites within the country, although the northern part of the country is more urbanized than the south.

Previous studies indicate that *T. gondii* seroprevalence in European foxes ranges from 20% in the UK (Dubey, 2009), to more than 60% in Spain and Hungary (Jakubek et al., 2007). The highest seroprevalence (98%) of *T. gondii* in European red foxes was found in a previous study on 123 Belgian foxes (Buxton et al., 1997). Only one study in the Czech Republic used molecular diagnosis on fox brain samples for prevalence estimation of *T. gondii* and could identify only 1.32% positives (Hurkova and Modry, 2006). Seroprevalence of *N. caninum* in red foxes in Europe is much lower, from 0% in Austria and Sweden (Jakubek et al., 2001; Wanha et al., 2005) to 3.2% in Spain (Sobrino et al., 2008). In contrast, molecular surveys on *N. caninum* in red foxes found higher prevalences: 4.61% and 10.7% in the Czech Republic and Spain, respectively (Almeria et al., 2002; Hurkova and Modry, 2006). No previous studies on *T. gondii* and *N. caninum* prevalence in Belgian deer were reported. The 52% seroprevalence in roe deer of *T. gondii* in this study is similar to recent findings in France (60%) (Aubert et al., 2010), but higher than most previous surveys in other European countries (Dubey, 2009). Only two roe deer sera were positive for *N. Caninum* antibodies (2.7%). Although the sample size was too low to accurately estimate the prevalence, higher numbers were found in other European countries: from 6.8% in Spain to 14% in the Czech Republic, on 160 and 79 animals respectively (Bartova et al., 2007; Panadero et al., 2010).

The ELISA's used in this study did only detect one samples that was positive for both *T. gondii* and *N. caninum*, indicating as described elsewhere that cross reactivity and co-infection between both related parasites are rare (Panadero et al., 2010). In this study we show that real-time multiplex PCR is a useful method to demonstrate the presence of different parasites in brain samples of wild animals. The same extracted DNA, if enough concentrated, can then be used for genotyping the detected strains. One of the main advantages is that there is no need to first perform bio-assay to isolate the parasite. Samples can be frozen before analysis, which is an advantage because it is not easy to get a large number of samples from wild animals due to regulations and ethical restrictions. By using multiplexing, both parasite species can be detected in the same PCR reaction; this makes the screening more time and cost efficient. The method used here shows a high specificity as only one sample was detected positive for both pathogens. The amplification fragments were sequenced and blasted to ensure that the correct species were detected (data not shown). From the 26 samples of *T. gondii* DNA isolated from red foxes that could be genotyped, 25 were characterized as genotype II, while one sample was identified as genotype III. All *T. gondii* strains recently isolated from wildlife in France were genotype II (Aubert et al., 2010). Interestingly, genotype II is the predominant genotype among patients who acquired toxoplasmic infection in Europe (Ajzenberg et al., 2009) and in congenital toxoplasmosis in France (Ajzenberg et al., 2002). It also appears to be the main genotype in sheep from Denmark and France (Jungersen et al., 2002; Halos et al., 2010). Similar results were recently obtained for strains isolated from red foxes (Aubert et al., 2010) and wild boar (*Sus scrofa*) (Richomme et al., 2009) in France.

The high prevalences found in this study and the finding that the same genotypes of *T. gondii* are circulating in wildlife, domestic animals and humans, indicates that wild animals may play a role as reservoir. Meat from game can thus be considered as a potential risk factor for the transmission of *T. gondii* to humans. More investigations should be performed to establish the route of transmission of both, *T. gondii* and *N. caninum* from wildlife to domestic animals. The ELISA's and the multiplex PCR evaluated in this study are valuable tools for such investigations.

Acknowledgements

This work was supported by the Belgian Federal Public Service for Health, Food Chain Safety and Environment. We also wish to acknowledge Dr. Frank Katzer from Moredun Research Institute for providing a *N. caninum* DNA sample; A. Laeremans and M. Abady, from the Laboratory for

Toxoplasmosis, and F. Mosselmans and A. Francart from the Laboratory for Rabies for their technical assistance.

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