

# Seroprevalence of *Toxoplasma gondii* and *Neospora caninum* infection in sheep, goats, and fallow deer farmed on the same area<sup>1</sup>

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**ABSTRACT:** *Toxoplasma gondii* and *Neospora caninum* are coccidian parasites with a global distribution that cause reproductive failure and production losses in livestock. The seroprevalence of both parasite species in ruminants and *Cervidae* has been investigated worldwide and found to vary greatly. Studies carried out on mixed flocks with 3 ruminant species (sheep, goats, and fallow deer) living under the same conditions are excellent models for identifying any differences in the rate of infection with the 2 parasites between the animal species. Additionally, the species used in the present study differ in their feeding categories: grazers, browsers, and intermediate feeders. The aim of the study is to identify any variation in the prevalence of the 2 parasites in mixed flocks and to identify any possible relationships with food choice. The seroprevalence against *T. gondii* and *N. caninum* in 167 captive fallow deer, 64 sheep, and 39 goats were detected using commercially available ELISA. The seroprevalence for *T. gondii* achieved 10% in fallow deer, 21% in goats, and 47% in sheep. The seroprevalence for *N. caninum* achieved 13% in

sheep and fallow deer and 21% in goats. Overall, 53% of the sheep, 33% of the goats, and 22% of the fallow deer were seropositive for both infections. Coinfection of *T. gondii* and *N. caninum* was detected in 6% of sheep, 8% of goats, and 2% of fallow deer. Statistical analyses of the seroprevalence levels observed between 2 parasites for each animal species revealed that only the results obtained for sheep were significant ( $P < 0.01$ ). Additionally, the differences in the seroprevalence levels for *T. gondii* between sheep and goats and between sheep and fallow deer were statistically significant ( $P < 0.01$ ). The results of the *N. caninum* seroprevalence levels observed among animal species were not significant. Although the variations in susceptibility to *T. gondii* and *N. caninum* infections demonstrated by the examined animals may affect the differences in seropositivity, these appear to be related to the feeding habits of the animal species. Therefore, the risk of infection by agents found close to the ground, such as coccidian oocysts, varies. Sheep as grazers are at a greater risk of infection by *T. gondii* than goats and fallow deer.

**Key words:** Apicomplexan parasites, mixed flock, ruminants, seropositivity

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they have no competing interests. All animals belong to the Witold Stefański Institute of Parasitology, Polish Academy of Sciences. The Breeding Station is located in Kosewo Górne. Material collection was performed by a veterinary officer during routine examinations.

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## INTRODUCTION

*Toxoplasma gondii* and *Neospora caninum* are closely related Coccidian parasites of medical and veterinary importance. Their life cycles involve different definitive carnivorous hosts: Felidae for *Toxoplasma* and Canidae for *Neospora*. Both parasites can use a wide range of ungulates as intermediate hosts (Dubey, 2010; Almería, 2013; Donahoe et al., 2015).

Both parasite species are involved in reproductive failure and production losses in livestock.

The seroprevalence of *T. gondii* and *N. caninum* in sheep and goats has been investigated worldwide and found to vary greatly (Chikweto et al., 2011; Bartova and Sedlak, 2012; Čobádiová et al., 2013; Guimarães et al., 2015). In *Cervidae*, the prevalence of *T. gondii* varies from 6.6% to 53.5% and *N. caninum* from 0% to 88% (Dubey et al., 2009; Goździk et al., 2010; Halová et al., 2013).

Ruminants hosts are infected by Apicomplexan parasites by ingesting food or water contaminated with oocysts excreted with the feces of definitive hosts. Several studies have implied that vertical transmission, as occurs in cattle, is also the route of transmission (reviewed by Dubey, 2010; Almería, 2013; Donahoe et al., 2015).

Ruminant species are classified into 3 main feeding categories: those that feed mainly on browse material; those that feed mainly on grass; and those that switch their diet from grasses to browse (Hofmann, 1989). Ruminants are assumed to have morphological and physiological adaptations that allow them to develop more specialized food intake, which are thought to influence all aspects of their life history (Gordon, 2003).

The aim of the study was to determine the seroprevalence of *T. gondii* and *N. caninum* infection in sheep, goats, and fallow deer (*Dama dama*), in a mixed flock farmed on the same area and under the same conditions, and to find possible relationships with food choice.

## MATERIALS AND METHODS

### Animals

The studies were conducted from 2014 to 2015 in the breeding station in Kosewo Górne, in the Mazurian lake district, north-east Poland (latitude 53°41' North, longitude 21°25' East). This area is located on 15 hectares and consists of meadows with several small lakes partially bordered with mixed forest. The animals are kept on pastures

from early spring to late autumn and are stabled during the winter months with the possibility to freely range outdoors.

Blood samples were taken from the jugular vein by a veterinary officer during routine examination of 270 living animals breeding together in the same area: 167 captive fallow deer, 64 sheep (Merino), and 39 goats (Polish Fawn Improved). All examined animals were female and aged from 1 to 2 yr old. The samples were left to clot overnight at +4 °C and centrifuged at 1,000 × g for 15 min. The collected sera were stored at –20 °C until assayed by ELISA.

### Serology

The presence of antibodies to *T. gondii* in sheep, goats, and fallow deer was detected using indirect ELISA (ID Screen Toxoplasmosis Indirect Multi-species kit, ID VET, Montpellier, France). Cutoff was calculated based on sample-to-positive (S/P) percentage according to the formula  $S/P = [\text{optical density (OD) sample} - \text{OD negative control (NC)}] / [\text{OD positive control (PC)} - \text{OD NC}] \times 100$ ; samples with  $S/P \geq 50\%$  were considered positive.

The presence of antibodies to *N. caninum* in sheep and goats was detected by competitive ELISA (*Neospora caninum* Antibody Test Kit, VMRD, Pullman, WA). Percentage inhibition was calculated using the formula  $\%I = 100 [1 - (\text{sample OD}/\text{NC OD})]$ ; the samples with  $\%I > 30\%$  were considered as positive.

To detect the presence of antibodies to *N. caninum* in fallow deer, IDEXX ELISA (IDEXX Laboratories Inc., Westbrook, ME) was performed according to the manufacturer's instructions, with some modifications according to Bieñ et al. (2012).

### Immunoblot Technique

Antigens were prepared from tachyzoites of *T. gondii* (RH strain) and *N. caninum* (NC1 isolate) that were maintained in Vero cells in RPMI 1640 medium supplemented with 1% horse serum, 50 U/mL of penicillin, and 50 µg/mL of streptomycin and incubated at 37 °C in 5% CO<sub>2</sub> in a humidified incubator (Bieñ et al., 2012). The parasites were purified by centrifugation through 30% isotonic Percoll (Pharmacia, Uppsala, Sweden) and washed 3 times in PBS pH 7.2. Next, the tachyzoites were suspended in distilled water ( $1 \times 10^9$  parasites/mL), freeze-thawed 3 times, and sonicated for 3 min. The resulting solution was centrifuged ( $2,000 \times g$ , for 20 min at 4 °C), the pellet was discarded, and the

supernatant was used as antigen. Antigen was prepared according to Cabaj et al. (2005).

The positive results obtained by ELISA examination of the serum samples were verified using an immunoblot technique previously performed for fallow deer serum samples (Bień et al., 2012), with some modifications. Anti-sheep IgG (Bethyl Laboratories, Inc., Montgomery, TX), anti-goat IgG (Sigma–Aldrich, St. Louis, MO), and anti-deer IgG (KPL, Gaithersburg, MD) conjugated to horse-radish peroxidase were used as a second antibody; they were diluted 1:3,000, added to each strip, and incubated for 1 h at room temperature. The immunocomplexes were detected using SuperSignal West Pico Chemiluminescent Substrate (Thermo Science, Waltham, MA) and visualized using the ChemiDoc MP Imaging System (Bio-Rad Laboratories, Hercules, CA).

Antigens from tachyzoites of *T. gondii* and *N. caninum* for Western blot were prepared according to Cabaj et al. (2005). They were prepared from tachyzoites of *T. gondii* (RH strain) and *N. caninum* (NC1 isolate) that were maintained in Vero cells in RPMI 1640 medium supplemented with 1% horse serum, 50 U/mL of penicillin, and 50 µg/mL of streptomycin and incubated at 37 °C in 5% CO<sub>2</sub> in a humidified incubator (Bień et al., 2012).

### Statistical Analysis

*T* test and chi-square test were used for statistical analysis (<http://www.socscistatistics.com>). Mc-Callum Layton Confidence Interval Calculation Proportions were used at 95% confidence level (<https://www.allto.co.uk/tools/statistic-calculators/confidence-interval-for-proportions-calculator/>). A  $P < 0.05$  was considered significant.

The occurrence of antibodies to *T. gondii* and *N. caninum* within a single animal species, that is sheep, goats, or fallow deer, was analyzed using binominal analysis: This was performed by calculating the proportion of positive ( $P_A$ ), negative ( $N_A$ ), and overall ( $p_o$ ) agreements. The kappa coefficient was calculated to indicate the degree of reliability between rates for each animal species and for the

mixed flock (Cicchetti and Feinstein, 1990; Graham and Bull, 1998).

## RESULTS

Antibodies to *T. gondii* were confirmed in 47% of serum samples from the examined sheep, 21% of goats, and 10% of fallow deer (Table 1). Anti-*N. caninum* antibodies were present in 13% of sheep and fallow deer and 21% of goat serum samples. Coinfection of *T. gondii* and *N. caninum* was detected in 6% of sheep, 8% of goats, and 2% of fallow deer.

ELISA-positive results revealed seroreactivity to *T. gondii* and *N. caninum* antigens in immunoblot; selected results are presented in Fig. 1. Immunoblot analysis of the *T. gondii* antigen revealed a specific pattern for sera taken from all 3 animal species. The sheep and goat sera gave high reactivity and more bands between 25 and 250 kDa (Fig. 1A, lanes 1 and 2), whereas the fallow deer sera displayed more bands between 35 and 250 kDa (Fig. 1A, line 3).

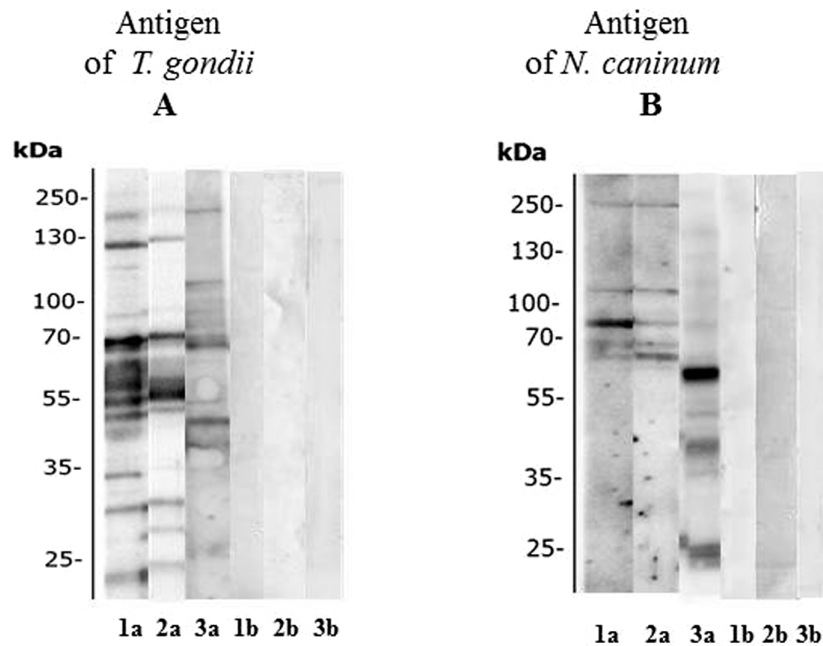
Immunoblot analysis using the *N. caninum* antigen revealed similar protein recognition patterns between the sera taken from sheep and goats; 5 immunodominant protein bands were located in the area between 55 and 250 kDa (Fig. 1B, lanes 1 and 2). However, the serum from fallow deer revealed seroreactivity to immunodominant *N. caninum* antigens at 25 and 130 kDa (Fig. 1B, lane 3).

Statistical analysis of the difference in total seroprevalences (20% for *T. gondii* and 14% for *N. caninum*) revealed that the result was significant ( $P < 0.01$ ). However, the differences in the seroprevalence levels observed between 2 parasites for each animal species revealed that only the results obtained for sheep were significant ( $P < 0.01$ ). Statistical analysis of the seroprevalence levels observed among species for *T. gondii* revealed statistical significance between the results obtained for the seroprevalence detected in sheep and goats and in sheep and fallow deer ( $P < 0.01$ ). The results of the *N. caninum* seroprevalence levels observed among animal species were not significant.

**Table 1.** *Toxoplasma gondii* and *Neospora caninum* seropositivity in sera of sheep, goats, and fallow deer

Animal species	Total number	<i>Toxoplasma gondii</i> positive (%)	<i>Neospora caninum</i> positive (%)	Mixed infection (%)	Overall seroprevalence (%)	$P_A$	$N_A$	$p_o$
Sheep	64	30 (47)	8 (13)	4 (6)	34 (53)	0.21	0.75	0.53
Goats	39	8 (21)	8 (21)	3 (8)	13 (33)	0.54	0.83	0.74
Fallow deer	167	17 (10)	21 (13)	3 (2)	35 (22)	0.16	0.92	0.80
Total	270	50 (20)	37 (14)	10 (4)	82 (30)	—	—	—

$P_A$  = positive agreement;  $N_A$  = negative agreement;  $p_o$  = overall agreement.



**Figure 1.** Immunoblot analysis of antibody responses of sheep, goats, and fallow deer to *Toxoplasma gondii* (A) and *Neospora caninum* (B) infections. Strips were incubated with sera demonstrated as positive (a) and negative (b) by ELISA; serum samples (lines 1: sheep; lines 2: goat; lines 3: fallow deer).

The total seroprevalence of *T. gondii* for 3 animal species was 20% [55/270; 95% confidence interval (CI) = 9.43 to 30.57] and 14% for *N. caninum* (37/270; 95% CI = 2.82 to 25.18). The  $P_A$  value indicates the conditional probability of the examined animal species being infected with both diseases, whereas  $N_A$  indicates the conditional probability that if the examined animal does not have 1 disease, then it will not have the second. In the present study, low positive  $P_A = 0.21$  and higher negative  $N_A = 0.75$  indexes were found between the 2 infections in sheep, with median overall agreement  $p_o = 0.53$ . By contrast, median positive  $P_A = 0.54$ , high negative  $N_A = 0.83$ , and high overall agreement  $p_o = 0.74$  were found in goats. Finally, low positive  $P_A = 0.16$  and negative  $N_A = 0.92$  indexes were found in fallow deer, with a high value of overall agreement  $p_o = 0.80$  (Table 1). The kappa coefficients calculated for fallow deer, sheep, and goats (0.05, 0.02, and 0.1, respectively) indicate that the degree of reliability between rates is not significant.

## DISCUSSION

Earliest studies performed in European countries found that antibodies to *T. gondii* were detected even in 79% of tested goats and 24% of tested sheep (Misurova et al., 2009; Špilovská et al., 2009). They indicated that goats are associated with higher *T. gondii* seroprevalence than sheep. Additionally, it has been found that goats are less frequently

seropositive to *N. caninum* (up to 6%; Díaz et al., 2016).

Until now, only one report on the seroprevalence of *T. gondii* and *N. caninum* in different animal species kept together in mixed flocks has been available (Diakoua et al., 2013). The authors noted that goats demonstrate higher *T. gondii* seroprevalence than sheep, whereas sheep had higher *N. caninum* seroprevalence.

Our findings indicate that although the seroprevalence of both parasite species was similar in goats (21%), the sheep sera demonstrated higher seroprevalence of *T. gondii* (47%).

Our results are opposite to those published earlier, and the cause may be the breed of sheep identified as an associated factor for the occurrence of anti-*T. gondii* antibodies (Guimarães et al., 2015; Deksne et al., 2017).

The study on the seroprevalence of *T. gondii* and *N. caninum* in fallow deer in European countries are limited (Bartova et al., 2007; Bień et al., 2012; Cabaj et al., 2017).

In the present study, specific IgG to *T. gondii* and *N. caninum* were detected in 10% and 13% of examined fallow deer, respectively, and the difference was not significant. The earlier screening studies on fallow deer from this region found *N. caninum* seropositivity to be only 3.3% (Bień et al., 2012). The differences may be influenced by the age of animals and number of individuals tested (Halová et al., 2013; De Craeye et al., 2011). Antibodies to

*T. gondii* and *N. caninum* in fallow deer from the Czech Republic were detected in 17% and 1%, respectively (Bartova et al., 2007).

The study was carried out on mixed flocks, in which 3 animal species live under the same conditions. This is an excellent model for identifying any differences between the animal species with regard to the rate of infection by the 2 parasites. The seroprevalence of *T. gondii* varied considerably (10% to 47%), although the prevalence of *N. caninum* was found to be similar in sheep and fallow deer (13%), and higher in goats (21%). However, analysis of the seroprevalence levels observed among 2 parasites for each animal species revealed statistical significance only in the results obtained for sheep. Additionally, the seroprevalence levels for *T. gondii* observed among species were significant between sheep and goats and between sheep and fallow deer. In case of the *N. caninum*, seroprevalence levels among animal species were not significant. Vikoren et al. (2004) suggested that the seroprevalence level of parasite species in examined animals is attributed to differences in susceptibility to infections. Low seroprevalence of both parasite species in mixed flock does not indicate a high contamination of the environment by oocysts and high vertical transmission rates, although the animals were not tested for antibodies when they were born.

In the present study, coinfection by both Apicomplexan parasites was found to be rare (4%), being found in only 6% of sheep, 8% of goats, and 2% of fallow deer. Similar results for serum coinfection were found in studies on sheep and goats in Slovakia and the Czech Republic (Špilovská et al., 2009; Bartova and Sedlak, 2012; Čobádiová et al., 2013).

Although binomial analysis revealed that the degree of reliability between rates is not significant, the overall seroprevalence calculated for each of the examined animal species is reflected in the negative agreement  $N_A$  value. The highest  $N_A$ , calculated for fallow deer (0.92), related to the lowest overall seroprevalence (22%), whereas the lowest  $N_A$ , calculated for sheep (0.75), related to the highest overall seroprevalence (53%). In addition, the percentage of mixed infection was reflected in a positive agreement  $P_A$  value. The highest  $P_A$ , calculated for goats (0.54), related to the highest percentage of coinfection (8%), whereas the lowest  $P_A$ , calculated for fallow deer (0.16), related to the lowest percentage of coinfection (2%).

In our study, anti-*Toxoplasma* IgG antibodies were detected using indirect ELISA (ID VET, Montpellier, France) validated for many animal

species (Špilovská et al., 2009; Čobádiová et al., 2013). Due to our earlier experience (Bień et al., 2012; Cabaj et al., 2017), antibodies to *N. caninum* in fallow deer were detected by modified IDEXX ELISA. Antibodies to *N. caninum* in sheep and goats were detected by VMRD ELISA. Although it has been validated only for 1 animal species, it has been used in earlier studies to detect antibodies to *N. caninum* in goats, sheep, and moose (Čobádiová et al., 2013; Moskwa et al., 2014).

In the present study, an immunoblot technique confirmed the positive ELISA results, with a 100% accordance observed between ELISA and immunoblot. The results of earlier (Harkins et al., 1998; Vitor et al., 1999; Bień et al., 2012) and presented studies show that numerous *T. gondii* or *N. caninum* antigens have been recognized with little consistency within and between animal species. The different numbers of immunoreactive protein bands and their intensity probably reflect the intensity of the infection and time after ingestion.

In the present study, the overall seroprevalence of both coccidian parasites was 53% for sheep, 33% for goats, and 22% for deer. Although the difference in the antibodies level to 2 parasites was significant only for sheep, it appears that the seroprevalence may be related to the feeding habits of the animal species. Sheep are very selective grazers. Goats are more likely to obtain their food by browsing than by grazing. They do not graze monotonically in the pasture but explore the area looking for new plants, typically weeds and herbs (Ekesbo, 2011). Fallow deer predominantly graze on a wide variety of grasses, herbs, forbs, and sedges, but they also commonly browse trees and shrubs (Jensz and Finley, 2013). Thus, it is expected that due to the type of grazing, goats and fallow deer are at less risk of infection by agents found close to the ground, such as coccidian oocysts in general.

## CONCLUSION

Although the variations in susceptibility to *T. gondii* and *N. caninum* infections demonstrated by the examined animals affect the observed differences in seropositivity, these may also be influenced by the food intake and food choice of the examined animal species.

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