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VERSITA

Toxoplasma gondii, Neospora caninum and tick-transmitted bacterium *Anaplasma phagocytophilum* infections in one selected goat farm in Slovakia

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Abstract

Parasitic diseases of livestock together with poor welfare conditions can negatively affect the health status and production of small ruminants. Protozoan parasites and tick-borne infectious agents are common threat of livestock including small ruminants mostly during the pasture season. Therefore the priority of the study was to analyse the circulation and presence of two protozoan parasites *Toxoplasma gondii* and *Neospora caninum* as well as tick-transmitted bacterium *Anaplasma phagocytophilum* in one selected goat farm in Eastern Slovakia. Throughout a three-year study period we have repeatedly screened the sera and blood of goats and dogs from monitored farm. In total, 343 blood serum samples from 116 goats were examined by ELISA. The mean seropositivity for *T. gondii* was 56.9% (66/116, CI (95%) = 48–66.0) and 15.5% (18/116, CI (95%) = 9.3–22.7) for *N. caninum.* The permanent occurrence of anti-*Toxoplasma* and anti-*Neospora* antibodies was detected in repeatedly examined goats during the whole monitored period. The presence of both parasites in the flock was analysed by PCR. DNA of *T. gondii* was confirmed in 12 out of 25 *Toxoplasma*-seropositive goats and *N. caninum* in 14 samples out of 18 *Neospora*-seropositive animals; four goats were co-infected with both pathogens. The risk of endogenous transmission of both parasites was pursued by examination of 41 kid's sera, where seropositivity for toxoplasmosis was 31.7% and for neosporosis 14.6%. In dogs 61.1% seropositivity for *T. gondii* and 38.9% for *N. caninum* was found, however, their faeces were negative for coccidian oocysts. Eight out of 108 tested animals were infected with *A. phagocytophilum,* the causative agent of tick-borne fever. Seven of them were simultaneously infected with *T. gondii* and *A. phagocytophilum*, out of which four goats were concurrently infected with all three pathogens.

Keywords

Toxoplasma gondii, *Neospora caninum*, *Anaplasma phagocytophilum*, ELISA, PCR, goats, dogs

Introduction

In Europe, some protozoan and tick-borne diseases circulate in natural foci on farms and pastures of small ruminants. *Toxoplasma gondii* and *Neospora caninum* (the phylum Apicomplexa, class Coccidia), are morphologically close intracellular protozoan parasites with similar clinical outcome and may cause repeated abortions (Dubey *et al*. 1988). *Toxoplasma gondii* can infect many animal species and has an indirect life cycle with a cat and feline carnivores as definitive hosts (Hutchison, 1969). Sporulated oocysts in the environment contaminated by cat faeces are an important source of infection mainly to other animals. Simultaneously, with insufficient hygiene and technological measures in the processing of milk and meat products from goats, toxoplasmosis may occur also in humans (Dubey *et al*. 2011a; Spišák *et al*. 2010).

Neospora caninum was classified as *Toxoplasma gondii* until 1988. It is widespread throughout the world, causing abortions in cattle (Dubey and Lindsay 1996). Definitive hosts are dogs, coyotes, wolves. Dogs can also act as an intermediate host. The most common intermediate hosts of *N. caninum*

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include cattle, less frequently sheep, goats, horses and deer (Dubey *et al*. 2011b). Pregnant goats have been susceptible to experimental infection, at which subsequently aborted infected progeny (Lindsay *et al*. 1995). Miscarriages and stillborn kids with confirmed presence of the parasite in their brain have been reported in goats (Dubey *et al*. 1992; Eleni *et al*. 2004). Although antibodies *to N. caninum* have been observed in human sera (Petersen *et al*. 1999) the parasite has not been detected in human tissues, therefore, its transmission to humans is still debatable.

Anaplasmoses are common tick-borne zoonotic bacterial diseases of domestic animals that often become infested by ticks, such as goats and sheep on pastures. The causative agents are intracellular gram-negative bacteria that belong to the family Anaplasmataceae (Dumler *et al*. 2001). In Europe, anaplasmae are transmitted by ticks, especially *Ixodes ricinus. Anaplasma phagocytophilum* invades granulocytes and in ruminants causes tick-borne fever (Gordon *et al*. 1932, rev. Woldehivet 2010). Clinical manifestation depends on the immune status as well as welfare of the animals. Moreover, infected animals are more sensitive to the infection with other pathogens. The clinical symptoms are represented by sudden high fever, inappetence, lower production yield, weight loss and abortions in pregnant ewes (Scott *et al*. 1991; Stuen 2007). In Slovakia, the presence of *A. phagocytophilum* was detected previously in ticks and wild living animals (Štefančíková *et al*. 2008; Štefanidesová *et al*. 2008; Víchová *et al*. 2010). Recent study also confirmed its circulation in sheep flocks (Derdáková *et al*. 2011).

The aims of the work were as follows: 1) to detect specific antibodies to *Toxoplasma gondii* and *Neospora caninum* in goats, their selected kids and farm dogs; 2) to confirm the presence of the *T. gondii* and *N. caninum* DNA in the blood of animals using molecular methods. Sheep were, moreover, screened for the presence of *A. phagocytophilum.*

Materials and Methods

Goat breeding and characterisation of the farm

Goat husbandry in Slovakia is determined by natural conditions mainly in sub-mountain regions. It is a traditional branch of livestock production. In selected farm White Shothaired goats of dairy breed are kept at a two-hectare land in Eastern Slovakia that is not continuously fenced therefore it is accessible for wild animals. The goats are kept on pastures from spring to autumn and are stabled during the winter months. Animals from the monitored farm have a history of frequent abortions.

Sample collection

During 2008–2011 in seasonal intervals a total of 343 blood samples from goats (341 females and 2 males, their age varied from 1 to 4 years), 41 from kids (40 females and 1 male; younger than 6 months of age) and 18 from farm dogs (13 males, 1 female and 4 pets) were collected. Clotted blood was centrifuged for 10 min at 3000 rpm and serum samples were stored at –20°C until the serological examination for the presence of anti-*Toxoplasma* and anti-*Neospora* antibodies. For DNA isolation non-coagulated blood samples of seropositive animals were collected and maintained at +4°C until the DNA extraction.

Blood samples from goats were collected from jugular vein, samples from farm dogs were collected from the *vena cephalica antebrachii.* At the same time feacal samples of dogs were also taken.

Coprological examination

Dogs' faecal samples were examined by the flotation method according to Faust (Manual of veterinary laboratory methods, 1989).

Serological examination

Anti-*Toxoplasma* IgG antibodies were detected using indirect ELISA (ID-Vet kit, Montpellier, France) according to the manufacturer's instruction. S/P percentage was calculated for each sample (S/P% = OD sample/OD positive control × 100). Samples with S/P% ≤ 40% were considered negative, between 40% and 50% were considered doubtful and ≥ 50% were classified as positive. Samples with S/P% ≥ 200% were considered highly positive and indicated an acute stage of disease with possible clinical signs.

Anti-*Neospora* IgG antibodies were detected using competitive ELISA (cELISA, VMRD Inc., USA). The results were expressed as percentage of inhibition (% I) according to the formula: % I = 100 – [(OD sample × 100) / (Ø OD negative control)]. Samples with % I < 30% were negative, samples with % I ≥ 30% were considered positive.

DNA extraction and PCR

The DNA from the blood samples was isolated by NucleoSpin ® Blood kit (Macherey-Nagel, Germany) according to manufacturer's instructions.

The presence of *T. gondii* was confirmed by gene TGR1E that repeats in the genome from 30 to 35 times. Primers TGR1E-1 and TGR1E-2 were used according to the method by Lamoril *et al*. (1996).

The standard PCR was used for confirmation 328 bp sequence of the gene NC5 *N. caninum* (Yamage *et al*. 1996) by specific primers Np21 and Np6.

A. phagocytophilum was detectected by real time PCR amplification protocol of the msp2 gene as described by Courtney *et al*. (2004). In selected positive samples msp4 gene was amplified and sequenced using pair of MAP4AP5 and MSP4AP3 (De la Fuente *et al*. 2005).

Table I. Anti-*Toxoplasma* and anti-*Neospora* antibodies in dairy goats detected with indirect and competitive ELISA and PCR methods

Sampling point	Number of animals examined	*Toxoplasma gondii*			*Neospora caninum*		
		iELISA Positive (%)	CI	PCR Positive (%)	cELISA Positive (%)	CI	PCR Positive (%)
Spring 2008	90	43 (47.8)	37.7–58.3	–	11 (12.2)	5.3–18.7	–
Summer 2009	79	45 (57.0)	46.1–67.9	–	17 (21.5)	12.9–31.1	–
Autumn 2009	31	26 (83.9)	71.1–96.9	–	12 (38.7)	21.8–56.2	–
Winter 2010	15	12 (80.0)	59.77–100.2	–	10 (66.7)	43.2–90.8	–
Spring 2010	35	34 (97.1)	91.4–102.7	–	15 (42.9)	26.6–59.4	–
Summer 2010	34	34 (100.0)	100–100	–	10 (29.4)	13.8–44.3	–
Autumn 2010	25	25 (100.0)	100–100	12 (48.0)	18 (72.0)	54.4–89.6	14*(77.8)
Spring 2011	34	32 (94.1)	86.0–102	–	16 (47.1)	30.2–63.8	–
Total	343	253 (73.8)	69.4–78.6	–	109 (31.8)	27.1–36.9	–

% – seropositivity; *PCR positive samples from 18 examined; CI – confidence interval at 95% confidence level

Statistical analysis

The results were statistically evaluated using Mc-Callum Layton Confidence Interval Calculation Proportions at 95% confidence level. http://www.mccallum-layton.co.uk/stats/ConfidenceIntervalCalcProportions.aspx.

Results

The overall mean seropositivity for *T. gondii* was 56.9% (66/116, CI (95%) = 48% – 66.0) and for *N. caninum* 15.5% (18/116, CI (95%) = 9.3, 22.7). Together 30 animals had antibodies against both parasites (Table 1).

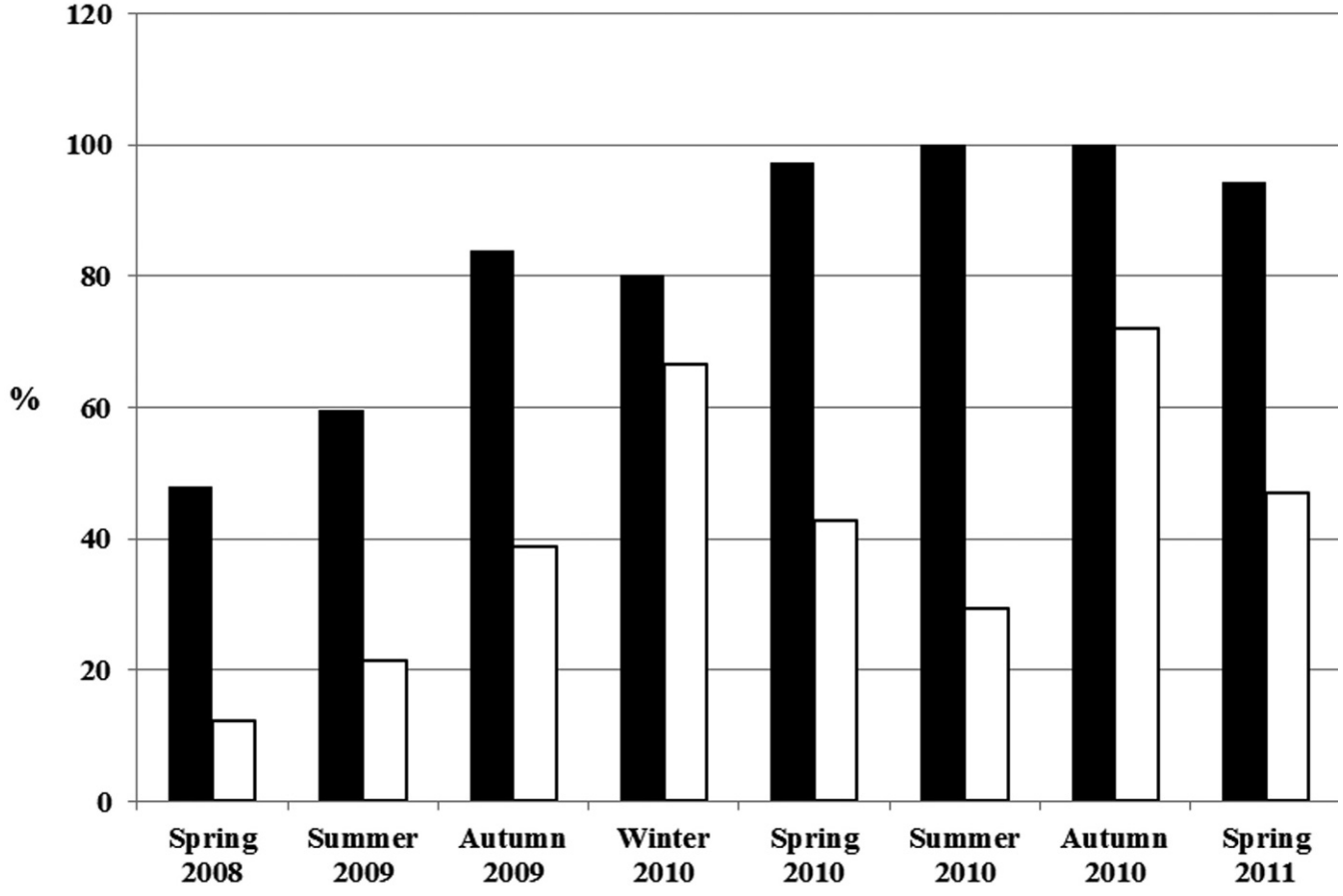

Fig 1. Seasonal occurrence of specific IgG antibodies in goat farm from 2008 to 2011 □ seropositivity of goats against *Neospora caninum* (%); ■ seropositivity of goats against *Toxoplasma gondii* (%)

Table II. Anti-*Toxoplasma* and anti-*Neospora* antibodies in kids detected with indirect and competitive ELISA techniques

Sampling point	Number of examined	*Toxoplasma gondii* positive	*Neospora caninum* positive
Spring 2008	18	ND	negative
Spring 2010	12	11	1
Summer 2010	5	1	4
Spring 2011	6	1	1
Total	41	13 (31.7%)	6 (14.6%)

ND – not done

Table III. Anti-*Toxoplasma* and anti-*Neospora* antibodies detected with indirect and competitive ELISA in dog blood sera

Sampling point	Number of examined dogs	*Toxoplasma* positive iELISA	*Neospora* positive cELISA
Spring 2008	4	ND	1
Summer 2009	6	3	3
Autumn 2009	4	4	2
Spring 2011	4	4	2
Total	18	11 (61.1%)	7 (38.9%)

ND – not done

The IgG antibody production against *Toxoplasma gondii* gradually increased during the monitored period and all animals examined in summer and autumn 2010 showed the presence of specific antibodies. Seropositivity against *N. caninum* was the highest in winter 2009 and autumn 2010 (Fig. 1).

Out of 41 examined kids, 13 (31.7%) were serologically positive for *T. gondii* and six (14.6%) animals were positive for *N. caninum* (Table II).

The presence of *T. gondii* was confirmed by PCR in 12 out of 25 *Toxoplasma*-seropositive samples. DNA of *N. caninum* out of 18 *Neospora*-seropositive animals was detected in 14 examined samples. Four animals were co-infected with both parasites.

In addition, eight (7.4%) out of 108 tested blood samples were positive for *A. phagocytophilum*. A single animal out of 18 goats tested was positive in autumn 2009, five out of 29 in autumn 2010 and two out of 39 in spring 2011. In autumn 2010 and in spring 2011, up to seven animals were at some time PCR positive for *T. gondii* and *A. phagocytophilum*; four goats were concurrently infected with *T. gondii*, *N. caninum* and *A. phagocytophilum.*

Up to 61.1% seropositivity against *T. gondii* and 38.9% against *N. caninum* was found in dogs (Table III). Faeces were negative for coccidian oocysts.

Discussion

Toxoplasmosis and neosporosis can cause great economic losses due to reproductive disorders in small ruminants by the incidence of abortion and neonatal mortality. During the grazing season, goats are exposed to further risk – anaplasmosis transmitted by infected ticks (*Ixodes ricinus*), which can also cause abortions in pregnant ewes. *T. gondii* and *A. phagocytophilum* are both zoonotic pathogens.

Our study confirms that toxoplasmosis and neosporosis persistently affect the small ruminants in Slovakia and cause losses in production due to the abortions in flock especially in conjunction with other infectious agents such as *A. phagocytophilum*. Average seroprevalence against *T. gondii* was 56.9%. Similar data were obtained in neighbouring countries. In the Czech Republic, Bártová and Sedlák (2012) recorded 66% seroprevalence in goats by latex-agglutination test against *T. gondii*, and in Austria it was more than 69% using the IFAT method (Edelhofer and Prossinger, unpublished data). The occurrence of toxoplasmosis in Europe ranges from sporadic outbreaks to the massive infection with clinical outcome in the herd (Antonis *et al*. 1998; Masala *et al*. 2003). Even 56.9% seroprevalence in our study indicates a high contamination of environment by oocysts, what may represent a risk of infection for humans due to the production of unpasteurized goat milk, cheese and other goat products from this farm. The current status is probably associated with lack of animal hygiene and unlimited contact with stray cats and wild animals. Stray cats are an important risk factor in transmission of toxoplasmosis in farm (Neto *et al*. 2008).

In Slovakia, the presence of anti-*Neospora* antibodies in cattle and sheep post abortion was confirmed by Reiterová *et al*. (2009) and Špilovská *et al*. (2009). The literature offers several epidemiological studies on the incidence of antibodies against *N. caninum* in goats detected by different serological methods. In a study conducted on farms in four regions of Romania, 2.3% of goats were seropositive using the ELISA (Iovu *et al*. 2011). In another survey from Poland only 0.47% of animals were *Neospora*-seropositive using an indirect ELISA method (Czopowicz *et al*. 2011). In the neighboring Czech Republic, Bártová and Sedlák (2012) determined 25.0% seroprevalence using competitive ELISA.

The differences in occurrence of toxoplasmosis and neosporosis in goats are there mainly due to various climatic factors and different wellfare conditions on farms. Differences in seroprevalences can be also biased by used diagnostic methods that have different sensitivity and specificity. Thus the direct molecular detection of parasites is essential. Air and land humidity is also favorable for maturation and survival of oocysts of *T. gondii* and *N. caninum* (Lindsay *et al*. 1999) and thus indirectly affects the overall prevalence of both parasitoses. Specific antibodies were detected repeatedly in 14.6% of the kids, which shows the vertical transfer of the parasite.

Totally 14 dogs were monitored on the goat farm over a period of three years. Five of them were chained, having their movement restricted and nine were allowed to move freely around the farm and in the stall. The oocysts of *N. caninum* were not found in the dog faeces; however 38.9% seroposi-

tivity of dogs points out that they might be important part in the enzootic circulation of the parasite in this area (exogenous transmission). The permanent contamination of the farm by oocysts of *T. gondii* demonstrates high seropositivity for toxoplasmosis in dogs. The presence of anti-*Toxoplasma* and anti-*Neospora* antibodies in goats indicates the contact of the parasites and animals on the farm, but it does not reflect the clinical stage of the disease. In order to confirm the parasite *T. gondii* and *N. caninum* in the blood of goats, molecular methods were used. The presence of the DNA of both parasites in the blood of goats at the time of collection demonstrates the acute stage of the disease. The definite hosts for both protozoa were constantly present on the farm. In addition, the kids were serologically positive for both parasites, reffering not only to the horizontal but also vertical transmission resulting in the permanent persistence of disease on the farm.

In Europe, the geographical distribution of *A. phagocytophilum* is practically in all areas where the vector ticks (*Ixodes ricinus*) are present. It was reported in the domestic as well as wild ruminants from many different European countries including Slovakia (Derdáková *et al*. 2011). The detected seasonal occurrence of the disease monitored in goat's farm varied from 5.5% to 6.8% in autumn, with no occurrence in winter. The presence of anaplasmosis in the herd might contribute to the abortions and immunosuppression, so the animals are more susceptible to other infectious agents.

In conclusion, we reported significantly higher seropositivity for *T. gondii* as compared with *N. caninum* in goats on the farm over the observed period. Nearly 32% of kids were infected by toxoplasmosis and 15% by neosporosis. Seasonal occurrence of specific IgG antibodies in goat farm of *T. gondii* gradually achieved 100% incidence. The results indicate that goats are more susceptible to infection with *T. gondii* than *N. caninum*. The evidence of *Toxoplasma* versus *Neospora* infections in 48 versus 77.8% of seropositive animals indicates probably a higher incidence of acute phase of the diseases. The incidence of anaplasmosis in goats was seasonal and we do not exclude that it indirectly affects the occurrence of a high susceptibility of animals to infection by protozoa. It is likely that the geographic location of the farm and its surroundings creates favorable conditions for survival of promotional stages of both parasites and allows repeated oral infections in animals. Moreover, the potential transmission of parasites from sylvatic to domestic cycle is therefore possible. Based on the obtained data we recommend that animals in the farms with reproductive disorders undergo an examination for the presence of anti-*Neospora* and anti-*Toxoplasma* antibodies, thus avoiding spread of those diseases. By eliminating a direct contact with definitive hosts, pursuing proper treatment of dogs as well as improving welfare conditions and animal hygiene the circulation of these parasites can be eradicated.

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