



## SEROLOGICAL AND MOLECULAR SURVEY OF SERIOUS INTRACELLULAR COCCIDIA IN RED FOXES IN EASTERN SLOVAKIA

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### ABSTRACT

We tested the sera or meat juices of 215 red foxes (*Vulpes vulpes*), collected during 2009—2014 at different localities of Eastern Slovakia for the presence of anti-Toxoplasma and anti-Neospora antibodies. We also examined the brains or muscle tissues and uncoagulated blood samples for the presence of both parasite's DNA. The mean seropositivity to *T. gondii* was 72.6 % (95 % Confidence Interval CI 66.1—78.4) and to *N. caninum* 33.9 % (95 % CI 27.7—40.7). We observed higher Toxoplasma-seropositivity in adults (71.1 %) than in juveniles (60.0 %). Neospora-seropositivity was almost the same in both age groups about of 30 %. In general, we observed less frequently the DNA of parasites, *T. gondii* (14.5 %) and *N. caninum* (20.3 %) in the tissue samples and uncoagulated blood samples. Coccidiosis are considerably common in red foxes and circulate in locations of Eastern Slovakia. The high infection rate in foxes is probably due to their infected prey. On the other hand, the contamination of the environment with oocysts and their subsequent transfer to other farm and wild animals is also possible.

**Key words:** antibodies, DNA; ELISA; *Neospora caninum*; PCR; red foxes; *Toxoplasma gondii*; Slovakia

### INTRODUCTION

The population density of red foxes (*Vulpes vulpes*) after successful oral anti-rabies vaccination has significantly increased throughout Europe. Their expansion to nearby human settlements (the phenomenon of urbanization) has also been going on [6]. Foxes participate in the spread of several parasitic diseases, such as toxoplasmosis and neosporosis.

*Toxoplasma gondii* and *Neospora caninum* are two closely related protozoan parasites with worldwide occurrence, having an indirect life cycle with cats and other wild feline carnivores as definitive hosts for *T. gondii*; and dogs and other canine carnivores for *N. caninum*. Oocysts are excreted in the faeces of the definitive host to the environment where they mature and can cause infection in some host species, including both domestic and wild animals [4], [7].

Toxoplasmosis is a widespread zoonotic disease, causing serious medical problems especially in pregnant women and immunosuppressed patients [16]. Humans become infected due to the consumption of inadequately cooked meat containing tissue cysts or by ingestion of sporulated oocysts, and also by transplacental transmission [4].

Neosporosis causes repeated abortions in cattle and other farm ruminants and has a negative economic impact on their breeding [3]. Both parasitoses circulate in both sylvatic and domestic cycles. The infections can be transmitted to stables due to poor zoohygiene by rodents or by contact with free living animals.

The aim of this study was to evaluate the occurrence of anti-Toxoplasma and -Neospora antibodies in red foxes from different locations of Eastern Slovakia and to associate the seroprevalence with their DNA evidence.

## MATERIALS AND METHODS

Within the period of 2009 to 2014, we collected blood, brain and muscle tissue samples of red foxes from different localities of Eastern Slovakia; in particular from localities of agricultural lands and farms. The blood and tissue samples were obtained from the Department of Pathology of the State Veterinary and Food Institute in Košice from foxes which had been trapped or shot for rabies control and also from individual hunting. Sera were prepared from coagulated blood by centrifugation at 600 g for 10 min. In case when blood samples were absent, meat juices were prepared by freeze-thawing from muscle samples for serological testing. Tissue and uncoagulated blood samples were removed for molecular analysis. All samples were kept at  $-20^{\circ}\text{C}$  up to further analyses.

The circulating anti-Toxoplasma antibodies were tested by the multi-species indirect ELISA (ID-Vet, Montpellier, France) using the manufacturer's protocol. We examined a total of 150 serum samples and 65 meat juices. The sera were diluted at 1:10 and meat juices 1:2. Protein G peroxidase labelled conjugate, which recognizes mammalian IgG antibodies and a TMB (3,3',5,5'-Tetramethylbenzidine) substrate, were used. The absorbance (OD) was measured at 450 nm by a spectrophotometer (Thermolabsystem, Optsys MR, U.S.A.). The results were calculated as S/P % index

according to the formula:  $S/P\% = (OD_{\text{sample}} - OD_{\text{NC}} / OD_{\text{PC}} - OD_{\text{NC}}) \times 100$ . The samples with  $S/P\% \leq 40\%$  were negative; between 40% and 50% doubtful and with  $S/P\% \geq 50\%$ , were categorized as positive.

For the detection of the anti-Neospora antibodies, a competitive ELISA (VMRD, Inc., Pullman, USA) test was used according to the manufacturer's instructions. Non-diluted serum samples or meat juices were added to the wells of the antigen-coated plates. The absorbance was measured at 630 nm. A test was considered valid, if the mean of the Positive Controls formed  $\geq 30\%$  inhibition of the reaction,  $\%I = 100 - (OD_{\text{sample}} / OD_{\text{NC}} \times 100)$ . The results were negative when the tested sample produced  $< 30\%$  inhibition and positive if they produced  $\geq 30\%$  inhibition.

We isolated DNA from the brain and skeletal muscle tissues by NucleoSpin®Tissue kit (Macherey-Nagel, Germany) with a previous overnight digestion with proteinase K (Promega, Madison, WI) at  $56^{\circ}\text{C}$ . Isolation from non-coagulated blood samples was performed by NucleoSpin®Blood kit (Macherey-Nagel, Germany), according to the manufacturer's instructions. The isolates were stored at  $-20^{\circ}\text{C}$  until the analysis.

The amplification of the TGR1E gene of 191 bp in size repeated itself in the *T. gondii* genome 30–35 times was made using two primers: TGR1E-1 and TIGRIE-25 [13]. DNA-free molecular water was used as the negative and *T. gondii* RH-strain (NRL for Toxoplasmosis, Prague, Czech Republic) as the positive control. The PCR products were analysed in 1.5% agarose gel with GelRed dye and evaluated under UV light.

The detection of the Nc5 gene of *N. caninum* was performed with specific primer pairs: Np6 and Np21 [19]. The amplifications were approved in a gradient thermocycler (Bioer, China). The first Slovak *N. caninum* isolate [14] was used as positive and DNA-free water as negative control. The PCR products were analysed in 1% agarose gel and evaluated under UV lamp using a digital camera (Panasonic AC 40).

The results of the serological investigations were evaluated statistically by the Fisher exact test (GraphPad Software). The prevalence and exact confidence intervals at 95% level were computed. The significance of the differences were evaluated using STATISTICA 6 Base (StatSoft, Inc., 2001).

## RESULTS

In 215 foxes from the localities of eight eastern Slovak districts the mean level of anti-Toxoplasma and anti-Neospora antibodies were detected in 72.6 % (95 % Confidence Interval CI 66.1—78.4) and 33.5 % (95 % CI 27.2—40.2) of the samples, respectively. The higher Toxoplasma-seropositivity was detected in adults (71.1 %; 95 % CI 62.4—78.8) compared to the juveniles (60.0 %; 95 % CI 36.1—80.9). The Neospora-seropositivity was almost the same in the adults and the juvenile categories, or only those of unknown age.

Toxoplasma-infection was present on a similar level in both genders (72.3 % in males and 69.7 % in females). However, males were more frequently infected with *N. caninum* (39.8 %) compared to the females (23.7 %). Toxoplasmosis

was detected in all districts at a high level, varying between 50.0 % and 78.4 %. The level of specific antibodies was high and values of S/P % indexes fluctuated from 96.5 % to 260.3 %. Neosporosis was present in seven districts with fluctuation from 25.9 % to 46.3 %. The percentage of inhibition of cELISA varied between 33.0 % and 105.5 %. The serological follow-up of toxoplasmosis revealed a slight decrease in individual years from 80.6—54.5 %. On the other hand, the yearly occurrence of neosporosis was most variable with the significantly lowest seropositivity in 2014 (4.5 %;  $P=0.0009$ ) (Table 1). The occurrence and the linear trend of Toxoplasma- and Neospora-seropositivity in red foxes of both examined coccidiosis is shown in Figure 1.

The molecular detection revealed a significantly lower rate of *T. gondii* (14.5 %) and *N. caninum* (20.3 %) DNA evi-

**Table 1. The occurrence of specific antibodies in red foxes according to the year, age, gender and the hunting locality**

Years	2009		2010		2012		2014		Total		Total	
	N/n		N/n		N/n		N/n		N/n/%	95% CI	N/n/%	95% CI
	<i>T. g.</i>	<i>N. c.</i>	<i>T. g.</i>	<i>N. c.</i>	<i>T. g.</i>	<i>N. c.</i>	<i>T. g.</i>	<i>N. c.</i>				
									<i>Toxoplasma gondii</i>		<i>Neospora caninum</i>	
Age category												
Juvenile	2/1	2/0	–	–	17/11	17/6	1/0	1/0	20/12/60.0	36.1—80.9	20/6/30.0	11.9—54.3
Adult	28/23	28/13	–	–	58/44	58/24	42/24	42/2	128/91/71.1	62.4—78.8	128/39/30.5	22.7—39.2
Unknown	6/5	6/0	58/46	58/26	2/2	2/2	1/0	1/0	67/53/79.1	67.4—88.1	67/28/41.8	29.8—54.5
Gender												
Male	17/15	17/6	3/3	3/1	40/32	40/21	23/10	23/1	83/60/72.3	61.4—81.6	83/29/39.8	29.2—51.5
Female	19/14	19/7	1/1	1/0	36/24	36/10	20/14	20/1	76/53/69.7	58.1—79.6	76/18/23.7	14.7—34.8
Unknown	–	–	54/42	54/25	1/1	1/1	1/0	1/0	56/43/76.8	63.6—87.0	56/26/46.4	32.9—60.2
Districts												
Gelnica	–	–	–	–	9/7	9/4	4/2	4/0	13/9/69.2	38.6—90.9	13/4/30.8	9.1—61.4
Košice	31/25	31/11	19/17	19/8	9/8	9/5	15/8	15/1	74/58/78.4	67.3—87.1	74/24/32.4	22.0—44.3
Michalovce	–	–	8/7	8/3	24/18	24/10	9/6	9/1	41/31/75.6	59.7—87.6	41/14/34.1	20.1—50.6
Rožňava	–	–	2/1	2/1	4/2	4/1	–	–	6/3/50.0	11.8—88.2	6/2/33.3	4.3—77.7
S.N. Ves	–	–	13/11	13/5	7/5	7/2	7/4	7/0	27/20/74.1	53.7—88.9	27/7/25.9	11.1—46.3
Sobrance	1/1	1/0	–	–	3/3	3/0	3/1	3/0	7/5/71.4	29.0—96.3	7/0/0	0.0—34.8
Trebišov	–	–	16/10	16/9	21/14	21/10	4/2	4/0	41/26/63.4	46.9—77.9	41/19/46.3	30.7—62.6
Vranov	4/3	4/2	–	–	–	–	2/1	2/0	6/4/66.7	22.3—95.7	6/2/33.3	4.3—77.7
Total N/n	36/29	36/13	58/46	58/26	77/57	77/32	44/24	44/2	215/156/72.6	66.1—78.4	215/73/33.9	27.7—40.7
[%]	80.6	36.1	79.3	44.8	74.0	41.6	54.5	4.5				

*T. g.* — *Toxoplasma gondii*; *N. c.* — *Neospora caninum*; N — number of animals examined  
n — number of positive animals; % — seropositivity

Table 2. Molecular evidence of *Toxoplasma gondii* and *Neospora caninum* in red foxes by the years and examined sample types

Years/ <i>T.g.</i> / <i>N.c.</i>	2009			2010			2012		2014		Total N/n/%
	A	B	C	A	B	C	A	B	B	C	
Examined [N]	–	20	20	4	38	–	–	47	40	4	
Positive [n]	–	9	1	0	0	–	–	7	6	2	
Prevalence [%]	–	4.5	0.5	0	0	–	–	14.9	15.0	50.0	
<b>Total <i>T.g.</i> N/n/%</b>	<b>40/10/25.0</b>			<b>42/0/0.0</b>			<b>47/7/14.9</b>		<b>44/8/18.2</b>		<b>173/25/14.5</b>
Examined [N]	1	24	20	9	30	33	13	23	40	4	
Positive [n]	1	11	0	5	12	3	3	4	1	0	
Prevalence [%]	100	45.8	0	55.6	40.0	9.1	23.1	17.4	2.5	0	
<b>Total <i>N.c.</i> N/n/%</b>	<b>45/12/26.7</b>			<b>72/20/27.8</b>			<b>36/7/19.4</b>		<b>44/1/2.3</b>		<b>197/40/20.3</b>

*T. g.* — *T. gondii*; *N. c.* — *N. caninum*; A — DNA isolated from blood; B — DNA isolated from brain; C — DNA isolated from muscle tissue

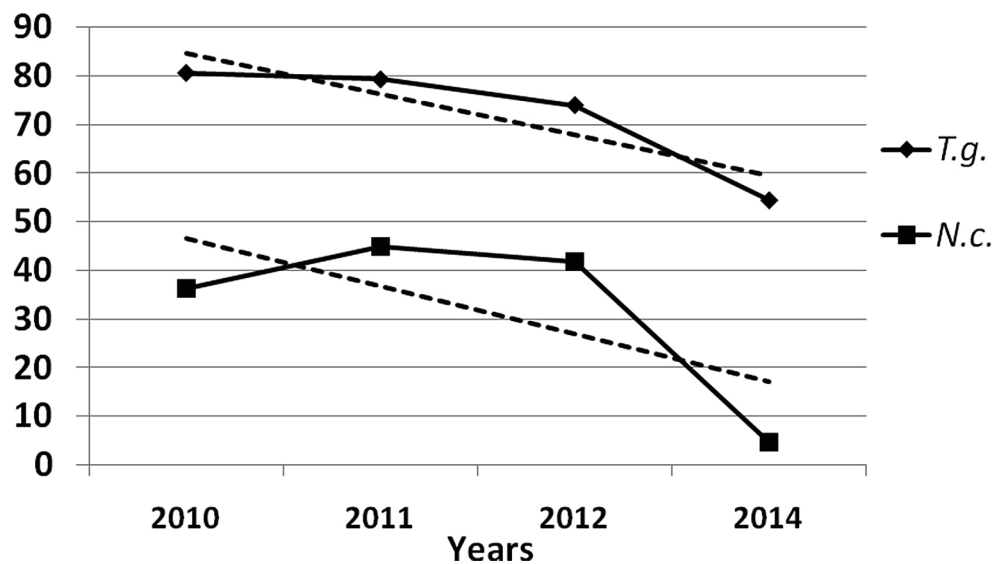


Fig. 1. The occurrence and linear trend of *Toxoplasma*- and *Neospora*-seropositivity in red foxes from different locations of Eastern Slovakia

dence in the tissues and blood samples (Table 2) when compared with the serological evidence. The presence of *T. gondii* was confirmed in 15.2% of the brain tissue samples and 12.5% of the muscle samples. The genomic DNA of *N. caninum* was confirmed in 23.9% of the brain samples, 39.1% in the uncoagulated blood and only 5.3% of the muscle samples (Table 2). Coinfection was detected in three samples, which were simultaneously PCR positive for both parasites.

## DISCUSSION

During the past three decades, the rapidly increasing population density of red foxes has had a significant effect on the prevalence of infections, including coccidiosis. This fact has resulted in an increased risk of contact with oocysts due to the greater amount of animals in a small area [10]. The high incidence of toxoplasmosis, and likewise neosporosis, in wildlife is explained by the high abundance of the causative agents in the diets of the foxes. An important prey for foxes represents rodents. On the other hand, the carcasses of the infected foxes can serve as the source of infection for the definitive hosts by contaminating the environment with oocysts. This phenomenon corresponds to our overall detection rate up to 72.6% *Toxoplasma*-seroprevalence in foxes. The occurrences of toxoplasmosis in red foxes from localities of Eastern Slovakia are higher in comparison to other Western European countries where seropositivity vary from 20% to 47% [8, [11], [17], [18]. In accordance with our high seropositivity rate, also in neighbouring Hungary, high (68%) seroprevalence has been detected in red foxes [12]. Similarly, in Central and Eastern Germany, 74.5% and 84.7% of the foxes had antibodies to *T. gondii* [9]. The age and gender of foxes did not influence the seropositivity significantly; however, older animals were infected more frequently than juveniles. We could deduce that the infection is transmitted mainly via infected prey or sporulated oocysts and only sporadically by transplacental transmission.

Despite the high seropositivity rate, the molecular evidence of *T. gondii* was one fifth lower. Similarly, in foxes from Germany 13.4% were PCR-positive [9]. By multiplex real-time PCR, *T. gondii* was detected in 18.8% of the brain samples in Belgian red foxes [2]. In a recent study 6% of *T. gondii* DNA was confirmed in the fox brain tissues from Romania [15].

Concerning the neosporosis in wild carnivores, their natural prey are frequently the source of infections [5]. The presence and distribution of neosporosis in wildlife may represent a high infection risk for livestock, especially during the grazing season. Our results revealed 24.6% mean *Neospora*-seropositivity, which is higher than the prevalence in other countries of Western Europe, where the seropositivity varied between 11% and 17% [1], [18]. In Hungary, only 1.5% *Neospora*-seroprevalence of red foxes was estimated [12]. The absence of significant age-related differences of *Neospora*-seropositivity may suggest that infections could be transmitted mainly via the transplacental way, as in cattle.

## CONCLUSIONS

The high occurrence of anti-*Toxoplasma gondii* (72.6%) and anti-*Neospora caninum* (33.9%) antibodies in red foxes from different locations of Eastern Slovakia suggests their important role in the maintenance of sylvatic circulation of both coccidian parasites as a reservoir host. The serological follow-up revealed a reasonable decreasing trend during the examined period. Despite the high serological evidence, DNA of both parasites was significantly less frequently confirmed from tissue and blood samples. A simultaneously positive molecular finding for both coccidia was detected only in three cases. In order to disclose the actual role of red foxes in the epidemiology of toxoplasmosis and neosporosis, more extensive study is required in widespread ranges of natural ecosystems.

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