SEROSURVEILLANCE OF *NEOSPORA CANINUM* IN FARM AND COURTYARD CATTLE

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Abstract

Neospora caninum is an apicomplexan parasite related to Toxoplasma gondii, well known as an important cause of abortion in cattle. In live animals the routine diagnostic of infection is detection of anti-N. caninum antibodies in serum. This study aimed to investigate the exposure to N. caninum infection in cattle from south area of Romania, and to survey the seroprevalence in some herds. A total of 542 sera from dairy cattle (16 herds in 9 counties) were sampled at different intervals. In the first sampling only farm animals were included, but in the second sampling courtyard cattle were added (n=47). Anti-N. caninum antibodies were detected using an indirect ELISA test. Anti-N. caninum antibodies were found in all herds. A total of 189 samples (34.9%, $CI_{95\%}$=30.85-39.05) were positive, with 104/258 (40.3%, $CI_{95\%}$=43.27-46.58) in the first sampling and 85/284 (29.9%, $CI_{95\%}$=24.66-35.63) in the second sampling. The highest rate of infection was in Giurgiu County, with 18/29 infected animals (62.1%). Prevalence increased with age and abortions were more frequent in seropozitive caws (9/74, 12.2%) than in seronegative ones (5/83, 6%). Seroprevalence was significantly higher in farm (37.2%, 184/495) than in courtyard cattle (10.6%, 5/47). At the second investigation in the same farms prevalence decreased significantly, compared with the first one (35.1%, 46/131 vs. 49%, 47/96). N. caninum infection is widespread in dairy farms from the studied area and may be a cause of abortion in some herds. The prevalence decreased at the second investigation in the same farms.

Key words: *cattle, Neospora caninum, Romania, seroprevalence.*

INTRODUCTION

Neospora caninum is an apicomplexan protozoan recognized as an important cause of abortion in cattle. Mainly affected species are dogs and cattle (Dubey, 2003). The parasite was first isolated from dogs in Norway as a cyst-forming sporozoan closely related to *Toxoplasma gondii* (Bjerkas et al., 1984).

In cattle, the most frequent transmission pattern is vertical, from infected dams to their offspring with resulting lifelong infection, but postnatal infection is also possible, via ingestion of oocysts shed by infected dogs.

In cattle, neosporosis causes severe economic losses by reproductive disorders, especially abortions. Fetuses may die in utero, be stillborn, born alive with clinical signs, or born clinically normal but chronically infected (Dubey, 2003).
The routine diagnosis of *N. caninum* infection in bovines is based on detection of specific antibodies in sera (Dubey and Schares, 2006). Serological studies show a widely varied prevalence between herds, regions, and countries. Prevalence of the infection in cattle can reach 90% in some herds (Dubey and Schares, 2011).
This study aimed to investigate the exposure to *N. caninum* infection in cattle from south area of Romania, and to survey the seroprevalence in some herds.

MATERIALS AND METHODS

The serological survey was performed in 9 counties from the south of Romania: Ilfov, Giurgiu, Teleorman, Olt, Argeș, Dâmboviţa, Prahova, Ialomița and Călăraşi.
In 2010 and 2012, 542 blood samples were collected from 16 cattle herds randomly selected: 13 dairy farms (n = 495) and 3 villages (n = 47). Distribution of herds according to county is shown in Table 1.

Table 1. Distribution of herds according to provenience county

	Ilfov	Giurgiu	Teleorman	Olt	Argeș	Dâmbovița	Prahova	Ialomița	Călărași
Farms	A, B	C	D	E	F	G	H, I	J, K	L, M
Courtyard cattle	N	O	P	-	-	-	-	-	-

The herd's size varied within 23 – 840 cattle and the most common cattle breed was Holstein/Friesian. Animals were randomly sellected for individual sampling. Animals were divided in two groups: the first sampling (2010, n=258) and the second sampling (2012, n=284). In the first sampling only farm animals were included, but in the second sampling courtyard cattle were added. Three age categories were analyzed: 24 calves (12 days -15 months), 30 heifers (16 – 24 months) and 230 adult cows (25 months and over: 82 primiparous and 148 multiparous). Serological investigation was repeated in 4 farms (A, B, D and I, n=96, respectively A', B', D' and I', n=131) at 4 months – 2 years intervals between samplings. In addition to the 542 samples mentioned above, serum samples from two aborted fetuses were tested, one at 3 months of gestation and the other at almost 5 months of gestation. Mother of 5-month fetus was coming from farm M. Data on herd's size, age and reproductive history were obtained from herd owners' records, staff and local veterinarians.
Blood samples were taken from jugular, mammary, or caudal veins, using disposable needles and vacuum plane tubes. All samples were immediately

transported to the laboratory. Serum was removed after centrifugation at 2500 rpm for 10 min and stored at – 20ºC until use.
Sera were analyzed for anti-*N. caninum* antibodies using two commercially available indirect ELISA kits (HerdChek Neospora caninum Antibody Test Kit, IDEXX Lab. and ID Screen *Neospora caninum* Indirect Multi-Species, ID-VET Lab.) as per the manufacturer's instructions. Briefly, serum samples diluted 1:100 were analyzed for the presence of anti-*N. caninum* IgG antibodies. Plates were read at 620 nm (HerdChek test) or 450 nm (ID Screen test), and the test results were expressed as an S/P ratio obtained by an equation provided by the manufacturer. Samples with an S/P ratio equal or higher than 0.5, were considered positive. For ID Screen test samples with S/P between 0.4 and 0.5 were considered doubtful.
Data analysis was performed using Fisher's test and Chi-square test (Quantitative Parasitology 3.0 software). Statistical significance was assumed at $p \leq 0.05$.

RESULTS AND DISCUSSIONS

Seropositive animals were found in all examined herds, indicating a wide extending of *N. caninum* infection in cattle from analyzed area.
The average prevalence was 34.9% (189/542, $CI_{95\%}$=30.85-39.05), with 40.3% (104/258 $CI_{95\%}$=43.27-46.58) in the first sampling and 29.9% (85/284, $CI_{95\%}$=24.66-35.63) in the second sampling (p=0.012).
The highest rate of infection was in Giurgiu County, with 62.1% prevalence (18/29). The seroprevalence rates in different counties are mentioned in Table 2.

Table 2. Seroprevalence of *N. caninum* infection in cattle from the south of Romania

County	***Farms***				**Courtyard cattle**	***TOTAL***
	First investigation	***Second investigation***				
Ilfov	A	A'	B	B'	N	73/163, 44.8%
	19/27 70.4%	15/56 26.8%	13/19 68.4%	25/44 56.8%	1/17 5.9%	
Giurgiu	C	-			O	18/29, 62.1%
	16/20 80%				2/9 22.2%	
Teleorman	D	D'			P	8/67, 11.9%
	5/30 16.7%	1/16 6.3%			2/21 9.5%	
Olt	E	-			-	3/20 15%
	3/20 15%					

County	***Farms***			**Courtyard cattle**	***TOTAL***
	First investigation	***Second investigation***			
Argeș	F 7/52 13.5%	-		-	7/52 13.5%
Dâmbovița	G 8/20 40%	-		-	8/20 40%
Prahova	H 13/35 37.1%	I 10/20 50%	I' 5/15 33.3%	-	28/70, 40%
Ialomița	J 16/20 80%	K 3/23 13%		-	19/43, 44.9%
Călărași	L 17/23 73.9%	M 8/44 18.9%		-	25/67, 37.3%
TOTAL	104/258 40.3%	80/237 33.8%		5/47 10.6%	197/542 34.9%
	184/495 37.2%				

Overall, one county (Giurgiu) had a high prevalence of positive animals (over 60%), five counties (Ilfov, Dâmbovița, Prahova, Ialomița, Călărași) had a medium prevalence (between 30 – 60%), and three (Teleorman, Olt, Argeș) showed a low prevalence (less than 30%). The counties within the same group of prevalence had a grouped distribution on the map (Figure 1).

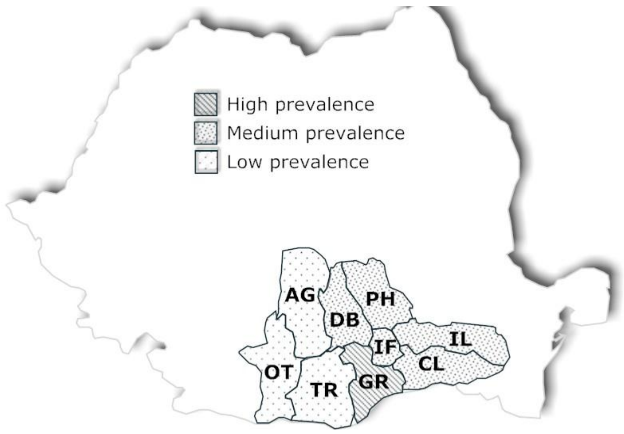

Figure 1. Grouped distribution of counties by prevalence category

In other regions of Romania, *N. caninum* infection in cattle was also reported, with 27.7% prevalence in west (Imre et al., 2012) and 34.6% prevalence in north-west and centre (Gavrea et al., 2011).

Prevalence increased with age (Figure 2), but there were no statistically significant differences between the age groups (p=0.792).

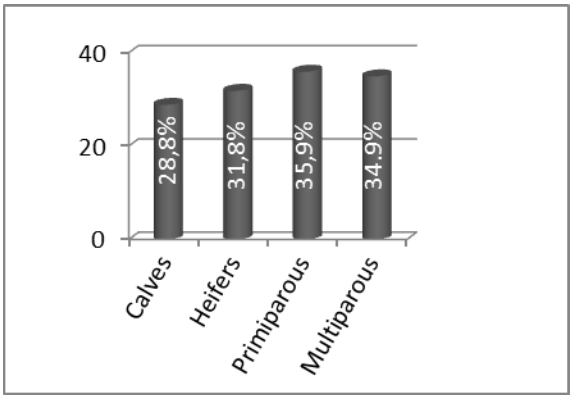

Figure 2. Prevalence of *N. caninum* infection by age groups

The presence of specific antibodies in clinically healthy calves may be caused by infection in the last period of gestation after development of their fetal immune system. The fetus begins to develop a specific immune response against the parasite in the fourth to sixth month of gestation; thus, if the fetus survives, the calf can be born clinically healthy, but congenitally infected (Innes et al., 2005). The extent of endogenous transplacental transmission is estimated to be 78.0–95.0% (Paré et al., 1996). It is also possible that calves were infected post-partum via pooled colostrum (French et al., 1999) or ingestion of oocysts, but unlikely because of their young age. We must consider the possibility that antibodies may be passively transmitted, giving false positive results for very young calves. Maternal antibodies in calves persist for 6 months (Paré et al., 1996). Hietala and Thurmond (1999) showed that, after 1 month, such passively acquired antibodies could still be demonstrated in 50% of sera from uninfected calves. However, in most of the calves, antibodies were not detected after 2 months, in the same study.

In two farms (A, B') where a reproductive history was available, abortions were twice more frequent in seropozitive caws (9/74, 12.2%) than in seronegative ones (5/83, 6%), although no statistical association was found (p=0.262).

The main symptoms of neosporosis in cows are abortion and stillbirth. Several studies have demonstrated that seropositive cows are more likely to abort than seronegative cows (Dubey et al., 2007). The risk of abortion is increased 2- to 4-fold for seropositive dams as compared with seronegative cows (Paré et al., 1997).

Seroprevalence was significantly higher (p=0.0001) in farm cattle (37.2%, 184/495) than in courtyard cattle (10.6%, 5/47). In farms, prevalence of *N. caninum* infection was 3.5 higher than in courtyard herds (Figure 3).

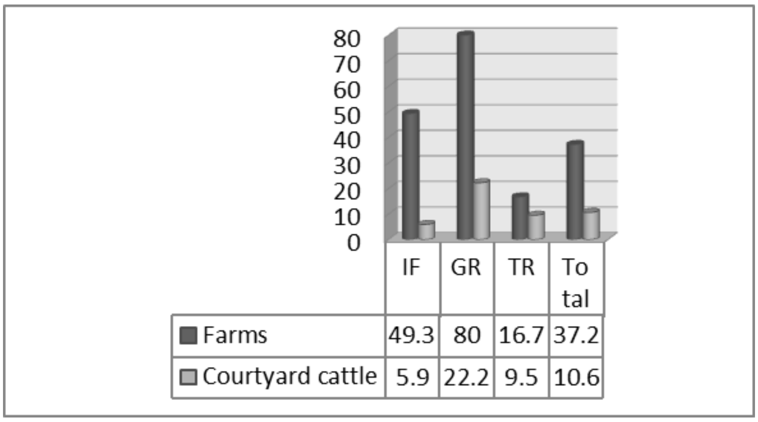

Figure 3. Prevalence of *N. caninum* infection in farm and courtyard cattle

The difference between breeding systems can be attributed to individual care for the animal. In addition, in the yards most dogs had restricted access. In pastures, oocysts contaminations caused by definitive hosts may be too low to pose a significant infection risk or oocysts may not survive during the summer months if they are very hot and dry (Dubey et al., 2007).

Major differences between breeding systems in our country have been also found in the north-west, where Gavrea et al. (2009) obtained a prevalence of 19.3% in a village from Cluj County, while in cattle from dairy farms in the northwest and center prevalence was 34.6% (Gavrea et al., 2011). In western Romania no correlation was found between seropositivity and cattle breeding system (Imre et al., 2012).

Most positive samples reacted intensely positive (Table 2). On this basis we can say that the specific antibody titer to *N. caninum* infection is high in herds in southern Romania. As the antibody titer is an indirect indicator of antigenic exposure to the immune system, an increase in antibody titer may reflect an increase in parasite activity and multiplication in the host (Innes et al., 2005). Based on this, a high antibody titer, reflected by a high S/P ratio, may reflect a recent infection or reactivation of the infection.

Table 2. Classification of positive samples according to the intensity of color reaction expressed as S/P ratio

Positive samples	Low positive (0.5<S/P≤1) n (%)	High positive (S/P>1) n (%)
First sampling	27/104 (26%)	77/104 (74%)
Second sampling	39/85 (45.9%)	46/85 (54.1%)
TOTAL	66/189 (34.9%)	123/189 (65.1%)

In farms with repeated serological investigation (A and A', B and B', D and D', I and I'), the second set of samples revealed a significantly decreased prevalence (*p*=0.036) compared with the first one (35.1%, 46/131 vs. 49%, 47/96) (Table 3).

Table 3. Seroprevalence in farms with repeated serological investigation

Farm and sampling interval	First sampling			Second sampling		
	Prevalence	Mean S/P of positive	Intense positive reactions	Prevalence	Mean S/P of positive	Intense positive reactions
D/D' 2 years	5/30 (16.7%)	1.085	1/5 (20%)	1/16 (6.3%)	0.756	0/1 (0%)
B/B' 1 year	13/19 (68.4%)	1.113	8/13 (61.5%)	25/44 (56.8%)	1.21	16/25 (64%)
A/A' 10 months	19/27 (70.4%)	2.004	16/19 (84.2)	15/56 (26.8%)	0.901	6/15 (40%)
I/I' 4 months	10/20 (50%)	0.916	4/10 (40%)	5/15 (33.3%)	0.655	0/5 (0%)
TOTAL	47/96 (49%)	1.279	29/47 (61.7%)	46/131 (35.1%)	0.880	22/46 (47.8%)

Decreasing prevalence was sustained by decreasing mean S/P ratio of positive samples (from 1.279 to 0.880) and decreasing percent of high positive samples (S/P≥1) from the total of positive samples (S/P≥0.5) (from 61.7% to 47.8%). Same tendency was observed in every farm with repeated serological investigation, except farm B (Table 3). In the second sampling of farm B (B') more animals were included, compared with the first one, including primiparous and multiparous animals. These factors can be involved in decreasing prevalence of *N. caninum* infection associated with the increase of the mean S/P and percent of intense positive samples.

Some individual animals were repeatedly tested. In farm B, two animals remained negative in both investigations, but in farm I one animal remained negative, one remained positive with decreasing of antibody level at the second sampling (S/P decreased from high positive, 1.22 to low positive, 0.851), and the last one, low positive at the first investigation (S/P ratio=0.921) become negative at the second investigation (S/P ratio=0.274).
Decreasing prevalence may indicate a slowly decreasing tendency of *N. caninum* infection in the area, and that can be explained by culling of cows with reproductive failure or decreasing of dogs' number, but, however, these facts could not been verified. Another factor that can be incriminated is fluctuation of specific antibody level. In *N. caninum* infection levels of specific antibodies may persist for life, but fluctuate, and sometimes are below the detection limits of serological tests (Dubey and Scares, 2006). A policy of annual testing and culling of all seropositive cattle in one population reduced the seroprevalence from 12% to <1% in the first year of simulation (Häslet et al., 2006).
Results from the present study are different from those of others, regarding repeated serological investigation: Woodbine et al. (2008) did not observe strong temporal changes in a four year longitudinal seroepidemiological study of *N. caninum* infection in 114 herds, but Piagentini și col. (2012) observed an increse in seroprevalence at 3 years interval, comprising a total of 615 animals. In the second study the predominance of horizontal infection was demonstrated by testing caw-offspring pairs.
In the present study, since there was no significant association between seroprevalence and age of the animals tested, associated with significant decrease of seroprevalence in the second testing, it can be said that in these herds vertical infection was preponderent. Cows may transmit the infection to their offspring in several pregnancies (Fioretti et al., 2003). Congenital infection rates are high, varying from 80% in heifers, 71% in second parity cows, 67% in third parity cows and 66% in fourth parity and older cows (Dijkstra et al., 2003).
On the other hand, free walking dogs were observed on all examined farms. Also, the higher within-herd prevalence (Table 1) could be due to a greater external exposure to oocysts (French et al., 1999). In farm K a pair caw-calf was tested. The mother, primiparous, tested negative at serological investigation, but the calf, 5 months old, tested positive, with intense reaction. The other two positive samples from the same farm also presented an intense reaction, and came from animals of 17 and respectively 18 months. Based of these results, a horizontal source of infection can be suspected in this farm, either through oocysts shed by dogs, or by pooled

colostrum, including colostrum from infected animals. Feeding of pooled colostrum is a putative risk factor for seropositivity (Corbellini et al., 2006), but, however, cross-suckling of calves born to seronegative mothers on seropositive cows has not led to an infection (Davison et al., 2001).

Serum samples from the two aborted fetuses tested negative, but the mother of the older fetus tested positive, with intense positive reaction (S/P ratio=1.140). A low sensitivity was reported in several studies when serology is performed on aborted fetuses (Dubey and Schares, 2006), and these may be due to lack of fetal immunocompetence, a short interval between infection and fetal death or autolysis with degradation of immunoglobulins (Wouda et al., 1997). Thus, a negative serological result in an aborted fetus does not rule out *N. caninum* infection (Dubey and Schares, 2006).

CONCLUSIONS

N. caninum infection is widespread in cattle from the studied area (34.9% prevalence) and may be a cause of abortion in some herds.

Cattle from dairy farms are more exposed to *N. caninum* infection than courtyard cattle (37.2% vs. 10.6%).

The prevalence decreased at the second investigation in the same farms (35.1%), compared with the first one (49%).

Both horizontal and vertical infection may be suspected, depending on the analyzed herd.

ACKNOWLEDGEMENTS

This research was supported by the Sectorial Operational Programme Human Resources Development 2007-2013 through the Financial Agreement POS-DRU/88/1.5/S/52614.

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