PRELIMINARY DATA IN COMPARATIVE SERODIAGNOSTIC OF *NEOSPORA CANINUM* IN DOGS

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Abstract

For serological investigation of N. caninum infection in dogs more tests are available, including indirect fluorescent antibody test (IFAT), often considered as the reference test, and enzyme-linked immunosorbent assay (ELISA).
A total of 28 dogs were screened with a commercially multi-species indirect ELISA, including a subset of 9 samples previously tested by IFAT 1:50. A partial correlation was attempted between the two tests at the cutoff recommended by the manufacturer.
Seroprevalence on ELISA was 10.7% (3/28, $CI_{95\%}$=2.26-28.23), and all positive samples were also positive on IFAT. From previously tested samples by IFAT (8 positive and one negative), only 4 samples had the same result by ELISA (3 positive and one negative sample) and one sample was doubtful. Regarding the double tested samples, a poor agreement was found between the two tests (k=0.135) and difference between the prevalence obtained by the two techniques was statistically significant (p=0.05). Sensitivity and specificity were not determined because of the low number of samples tested so far, but is already planned in an outgoing experiment, as well as testing Neospora IFAT positive samples for Toxoplasma, to exclude false positive results.
It seems that IFAT is more appropriate than indirect ELISA for seroprevalence studies, and use of this indirect ELISA may require some techniques for adjustment of misclassifications.

Key words: *dogs, ELISA, IFAT, Neospora caninum.*

INTRODUCTION

Neosporosis was first described in puppies in Norway in 1984 (Bjerkas et al., 1984), but the causative organism, a protozoan parasite closely related with *Toxoplasma gondii*, was named *Neospora caninum* in 1988 (Dubey et al., 1988).
Although often *Neospora caninum* does not produce clinical signs of disease in adult dogs, this infection is epidemiologically important because the dog is the definitive host of the parasite (McAllister et al., 1998). Neosporosis is an important cause of abortion in cattle (Anderson et al., 1991), the most common intermediate host.

Diagnosis of *N. caninum* infection in dogs is based on serological assays such as the indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assays (ELISA)(Silva et al., 2007), but IFAT is considered as a reference test in dogs naturally infected with *N. caninum* (Bjorkman and Uggla, 1999). Fewer data are reported for the use ELISA in detection of *N. caninum* infection in dogs.
The aim of this study was to compare two serological tests (IFAT and ELISA), frequently used for the diagnosis of *N. caninum* infection in dogs.

MATERIALS AND METHODS

A total of 28 dog sera, collected in 2011 and 2012, as a part of a larger seroepidemiological investigation, were included in this preliminary study. Dogs came from Bucharest and surroundings.
All sera were tested by indirect ELISA, but a subset of 9 samples was also tested by IFAT in a previous study (Mitrea et al., 2012). Some of tested dogs (n=3) showed neurological disorders (paresis, ataxia, myoclonus).
In order to detect the anti-Neospora caninum antibodies of the IgG class, two commercially available tests were used: IFAT (FluoNEOSPORA c., Agrolabo S.p.A., Italy) and a multi-species indirect ELISA (ID Screen Neospora caninum Indirect Multi-Species, ID-VET Lab., Montpellier, France).
The two tests were performed following exactly the manufacturer's instructions. The optical density values of indirect ELISA were read at 450 nm, using a spectrophotometer. The cutoff of the tests were S/P>50% (obtained by an equation provided by the manufacturer) for indirect ELISA and 1:50 dilution for IFAT. Only samples that demonstrated an apple-green fluorescence of the whole membrane of *Neospora* tachyzoites, using a fluorescence microscope, were considered positive for anti- *N. caninum* antibodies. For indirect ELISA, samples with S/P between 0.4 and 0.5 were considered doubtful.
Analysis of the data was performed using Fisher's exact test or Chi-square ($\chi 2$) test (Quantitative Parasitology 3.0 software. Statistical significance was assumed at $P \leq 0.05$. The test agreement was quantified by the Kappa (K) statistic, The K value can be interpreted as follows: < 0.20 poor; 0.21 - 0.40 fair; 0.41 - 0.60 moderate; 0.61 - 0.80 good; 0.81 - 1.00 very good (Altman, 1991).

RESULTS AND DISCUSSIONS

From a total of 28 dog serum samples tested by indirect ELISA, three were clearly positive (10.7%, $CI_{95\%}$=2.26-28.23) for *N. caninum* infection, and one was doubtful (3.6%).

From the 9 samples tested previously by IFAT, 8 were positive (88.9%). In the same set of samples, prevalence by ELISA was 33.3% (3/9). Difference between the prevalence obtained by the two techniques was statistically significant (*P*=0.05). All ELISA positive samples and the doubtful one were positive on IFAT for *N. caninum* infection.

In Europe, the seroprevalence rates of *N. caninum* infection varied between 0% and 51% in different countries and in different dog categories (Dubey and Schares, 2011).

In a preveously study conducted in south of Romania, specific antibodies were detected in 20.2% of dog sera by IFAT, with higher prevalence in cattle farm dogs (38.1%) (Mitrea et al., 2012).

No dog presenting neurological disorders was positive for *N. caninum.* This fact may sustain the asymptomatic evolution of *N. caninum* infection in dogs.

Performance of the indirect ELISA compared to IFAT was not as expected, especially in terms of positive samples (Table 1). The agreement between the two techniques at a confidence level of 95% was *k*=0.135 ($CI_{se(0)}$= -0.158, 0.428; $CI_{se(1)}$= -0.440, 0.709), which corresponds to a poor agreement (Altman, 1991).

Table 1. Correlation of results obtained by IFI and indirect ELISA

		ELISA			
		-	d	+	Total
IFAT	-	1	0	0	1
	d	0	0	0	0
	+	4	1	3	8
	Total	5	1	3	9

Test agreement:	
Kappa coefficient	0.135
Confidence interval for Kappa se(0)	-0.158, 0.428
Confidence interval for Kappa se(1)	-0.440, 0.709
Proportion of observed agreement	44.4%
Proportion of expected agreement	35.8%
Proportion of expected agreement minus hazard	8.6%
Maximum agreement not due to hazard	64.2%

For *N. caninum* infection, serological tests detects antibodies against surface antigens, more specific than intracellular antigens in *Apicomplexa* (Bjorkman and Uggla, 1999).
Dubey et al. (1988b) was the first to report the successfully use of an IFAT for detecting *N. caninum* infection in dogs, with a very little cross-reactivity with related protozoa and 100% sensitivity. This IFAT was based on whole in vitro grown tachyzoites as antigenic source. Almost the same succes was reported in a number of ulterior papers and this lead IFAT to be considered an almost perfectly specific diagnostic test for *N. caninum* infection.
On the other hand, for the screening of large numbers of sera, enzyme-linked immunoassays (ELISA) are usually cost effective and less time consuming (Lasri et al., 2004).
In the present study the ELISA test was evaluated for detection of *N. caninum* infection in dogs, with the IFAT considered as ”gold standard” at a confidence level of 95%. Table 2 presents the results from test evaluation.

Table 2. Evaluation of indirect ELISA test according to the gold standard

		Gold standard (IFAT)	
		Infected	Noninfected
Evaluated test (indirect ELISA)	Positives	3	1
	Negatives	5	0
Test evaluation:			
Sensitivity		37.5% (4.0%, 71.0%)	
Specificity		0.00% (0.00%, 0.00%)	
Positive Predictive Value		75% (32.6%, 117.4%)	
Negative Predictive Value		0.00% (0.00%, 0.00%)	
True Prevalence		88.9% (68.4%, 109.4%)	
Apparent Prevalence		44.4% (12.0%, 76.9%)	
Youden's J		-62.5% (-95.048%, -28.952%)	
Fiability		33.3% (2.5%, 64.1%)	

IFAT positive samples were classified according to the intensity of the fluorescence in positive samples (+) and intense positive samples (++). IFAT titers were not determined.
The results of ELISA and IFAT on individual samples are summarized in Table 3 and Figure 1.

Table 3. Intensity of response by IFAT and indirect ELISA

No of sample	Indirect ELISA		IFAT Cut-off 1/50
	S/P of sample	Clasification	
1.	68.48	Positive	+
2.	15.41	Negative	+
3.	74.22%	Positive	++
4.	3.47%	Negative	+
5.	39.98	Negative	++
6.	5.11	Negative	+
7.	43.58	Doubtful	+
8.	79.59	Positive	++
9.	0.94	Negative	-

+/++ Intensity of response

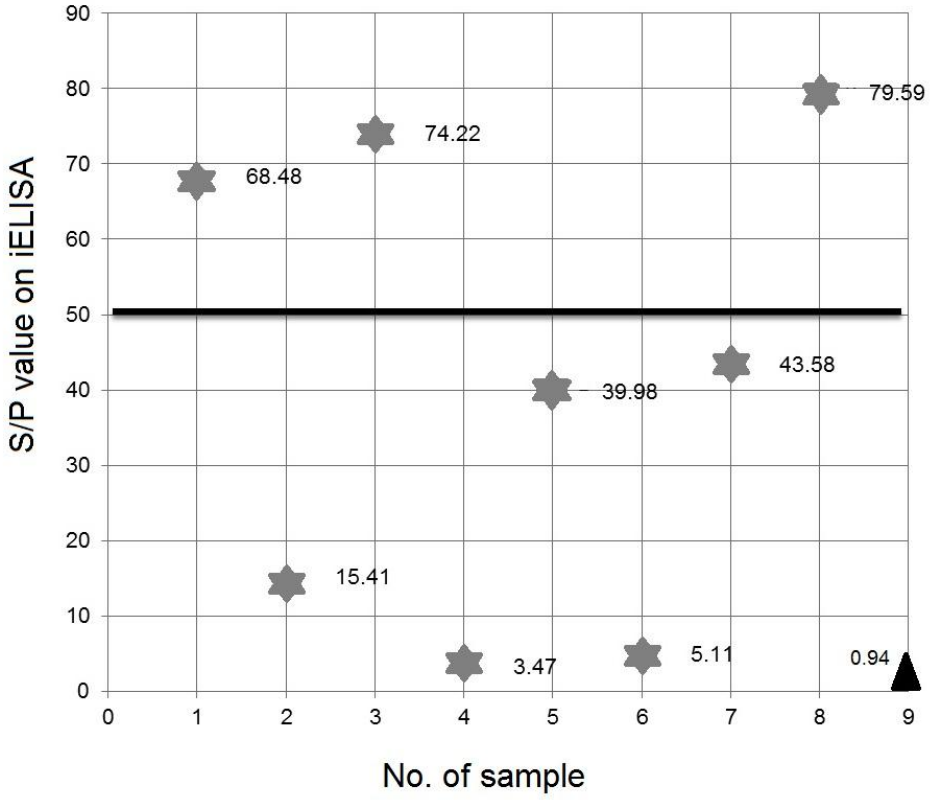

Figure 1. Procentual S/P ratio obtained in indirect ELISA: * - IFAT positive samples; Δ - IFAT negative sample.

Positive ELISA S/P values ranged from 68.48% to 79.59%. No intense positive reaction was observed by ELISA (S/P <100%). Negative and doubtful ELISA S/P values of IFAT positive samples ranged from 4.47% to 43.58% (Figure 1).

The negative sample on IFAT had the lowest S/P value on ELISA, and the 2 intense positive samples on IFAT had the highest S/P value on ELISA. From the intense positive samples (n=3) two were positive on ELISA and one was negative, but close to the doubtful zone (Table 3 and Figure 1).

Results from the present study were similar to those reported by others. Capelli et al. (2006) compared a competition ELISA and IFAT and concluded that cELISA is recommended for confirmation of clinical suspicion of neosporosis when high level of antibodies are expected. Lasri et al. (2004) found a poor positive but good negative agreement between IFAT and ELISA for the serodiagnosis of *N. caninum* infection in dogs. Silva et al. (2007) obtained a low kappa coefficient (k = 0.30), indicating a poor concordance between IFAT and an indirect ELISA results for *N. caninum* serology. In the same study, a good association was found regarding the negative agreement index (Pneg = 0.83) in contrast to the positive agreement (Ppos = 0.42) index.

According to the manufacturer, for the indirect ELISA used in the present study, correlation was found to be 100% between IFAT and ELISA on 17 dog serum samples (7 positive by IFAT), exept one serum which was negative by ELISA and positive by IFAT (the lowest titer from all IFAT tested samples, 1:80). As this serum was also ELISA-positive for *Toxoplasma*, it could be an IFAT false-positive, given that these parasites have epitopes in common. For use of this indirect ELISA in canine sera, analytical sensitivity is tested using an internal standard (pool of positive sera of different origins). Antigen used is purified *N. caninum* extract and the conjugate is an anti-multi-species IgG-HRP (Horseradish peroxidase conjugated).

Posible reasons for necorelation obtained between IFAT and ELISA in the present study are: low titer of antibodies in serum samples (not determined by IFAT), IFAT false-positive samples due to *T. gondii* cross-reactions or inappropriate cutoff value of the multi-species ELISA for dog sera.

According to Capelli et al. (2006), IFAT sensitivity of 100% assessed by Dubey et al. (1988b) was probably overestimated in the firs study about this method aplied in canine *N. caninum* infection, because was apreciated on clinical neonatal *Neospora* infections and in experimental infected animals.

Low titers of antibodies to *N. caninum* can be the expression of cross-reactivity to related parasites, particularly *T. gondii*, due to common tachyzoite and bradizoyte antigens (Bjerkas et al., 1994).

CONCLUSIONS

A poor agreement was found between indirect ELISA and IFAT (k=0.135) for detection of *N. caninum* infection in dogs.
In the same set of samples, IFAT classified more samples as positive (88.9%) than indirect ELISA (33.3%) and seems more appropriate for seroprevalence studies in asymptomatic dogs.
The discordance between the two tests was marked specially at less intense IFAT positive samples.
Further studies are planned in order to asses SE and SP of this indirect ELISA and a ROC analysis in order to verify if the cut-off recommended by the manufacturer corresponds to the highest sensitivity combined with a good specificity of indirect ELISA.
In addition, the identification of possible cross-reactions with *T. gondii* should be investigated.

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REFERENCES

Altman D.G., 1991. Practical statistics for medical research. Chapman and Hall, London.
Anderson, M.L., Blanchard, P.C., Barr, B.C., Dubey, J.P., Hoffman, R.L., Conrad, P.A., 1991. *Neospora*-like protozoan infection as a major cause of abortion in California dairy cattle. Journal of the American Veterinary Medical Association, 198, 241–244.
Bjerkas I., Mohn S. F., Presthus J., 1984. Unidentified cyst-forming sporozoon causing encephalomyelitis and myositis in dogs. Zeischrift für Parasitenkunde, 70, 271–274.
Björkman C., Uggla A., 1999. Serological diagnosis of *Neospora caninum* infection. International Journal for Parasitology, 29(10), 1497-1507.
Capelli G., Natale A., Nardelli S., Frangipane di Regalbono A., Pietrobelli M., 2006. Validation of a commercially available cELISA test for canine neosporosis against an indirect fluorescent antibody test (IFAT). Preventive Veterinary Medicine, 73, 315-320.

Dubey, J.P., Carpenter, J.L., Speer, C.A., Topper, M.J. and Uggla, A., 1988a. Newly recognized fatal protozoan disease of dogs. Journal of the American Veterinary Medical Association, 192, 1269–1285.
Dubey, J.P., Hattel, A.L., Lindsay, D.S. and Topper, M.J., 1988b. Neonatal *Neospora caninum* infection in dogs: isolation of the causative agent and experimental transmission. Journal of the American Veterinary Medical Association, 193, 1259–1263.
Dubey J.P., Schares G., 2011. Neosporosis in animals – The last five years. Veterinary Parasitology, 180, 90-109.
Lasri S., De Meerschman F., Rettigner C., Focant C., Losson B., 2004. Comparison of three techniques for the serological diagnosis of *Neospora caninum* in the dog and their use for epidemiological studies. Veterinary Parasitology, 123(1-2), 25-32.
McAllister M.M., Dubey J.P., Lindsay D.S., Jolley W.R.,Wills R.A., McGuire A.M., 1998. Dogs are definitive hosts of *Neospora caninum*. International Journal for Parasitology, 28, 1473–1478.
Mitrea I.L., Enachescu V., Ionita M., 2012. *Neospora caninum* Infection in Dogs From Southern Romania: Coproparasitological Study and Serological Follow-Up. The Journal of Parasitology, In-Press.
Silva D.A., Lobato J., Mineo T.W., Mineo J.R., 2007. Evaluation of serological tests for the diagnosis of *Neospora caninum* infection in dogs: optimization of cut off titers and inhibition studies of cross-reactivity with *Toxoplasma gondii*. Veterinary Parasitology, 143(3-4), 234-44.