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DETECTION OF *NEOSPORA CANINUM* ANTIBODIES IN MILK ON DAIRY CATTLE

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Abstract

Serodiagnostic of Neospora caninum infection in cattle is generally based on using the enzyme-linked immunosorbent assay (ELISA) method for detection of specific antibodies in serum samples, but the use of milk is also possible. The present study was undertaken to assess the potential of an ELISA kit for testing individual and pooled milk samples in dairy farms. Pairs of milk and blood samples (n = 60) were collected from 3 dairy farms (A, B, and C) in southern Romania. Additionally, four pooled milk samples were obtained (one pooled milk sample for each farm and a total sample as a pooled sample from all three farms). Skimmed milk was obtained and tested by using a commercially available ELISA kit (HerdChek N. caninum Antibody Test Kit, IDEXX Lab.). The optimized cut-off value of S/P> 0.704 was determined by receiver operating characteristic (ROC) analysis, using serum results as 'gold standard'. The sensitivity and specificity of the assay at this cut-off were 70.4% and 100.0%, respectively and the agreement with classic serology, expressed as kappa values, was good (K=0.723). When samples with low positive response on sera were excluded, the correlation obtained was even better (K= 0.921). For pooled milk samples a lower cut-off was necessary in order to identify as positive all dairy farms with a 15% or higher within-herd seroprevalence.
The results of this study demonstrate that the prevalence of N. caninum in dairy farms can be estimated by using this indirect ELISA kit on individual and pooled milk samples.

Key words: *ELISA, milk, Neospora caninum, Romania.*

INTRODUCTION

N. caninum is one of the main primary etiologic agents of abortion in cattle (Dubey, 2003) causing significant economic losses around the world. Congenitally infected bovine fetuses may die in uterus, may be born dead or alive with clinical signs or apparently healthy, but with persistent chronic infection that can be later transmitted by females to their progeny (Dubey and Schares, 2011). *N. caninum* is one of the most effective vertically transmitted pathogens in cattle (Bjorkman et al., 1996).
Many serosurveys have been reviewed recently showing worldwide distribution of *N. caninum* infection (Dubey and Schares, 2011).
N. caninum infection can cause repeated abortions in some cows (Anderson et al., 1995) and seropositive cows are more susceptible to abortion than seronegative ones (Dubey et al., 2007). Studies from U.S., but also from Europe, calculated significant economic losses due to reproductive problems associated with *N. caninum* infection in cattle (Dubey et al., 2007).
The routine diagnosis for *N. caninum* infection in cattle is based on detection of specific antibodies in serum samples, but also milk samples can be used for lactating cows (Conraths and Gottstein, 2007).
Testing of milk samples presents some advantages over testing of blood samples, like easily and lowered costs of collecting samples, noninvasiveness of the method with reduction of some disease transmission by needle and reduction of productions losses caused by stress (Schares et al., 2004).
In previous studies conducted in different regions of the world some ELISAs for detection of *Neospora caninum* antibodies in cattle were adapted for use in individual or bulk milk samples (Bjorkman et al., 1996; Schares et al., 2004; Bartels et al.2005; Frossling et al., 2006; Hall et al., 2006; Wapenaar et al., 2007 González-Warleta et al., 2011), but different test characteristics were obtained. In a recent study whole and skim milk samples were analyzed with a commercial serum ELISA test and both were equally suited as a screening tool (Byrem et al., 2012). However, no studies have

been performed in South Romania to evaluate characteristics of testing milk samples for *N. caninum* antibodies.

In a serological survey performed in 13 dairy farms from south of Romania seroprevalence rates of *N. caninum* infection ranged between 6.3% and 80%, with a medium of 40.3% (Mitrea et al., 2012; Enachescu et al., 2012). *N. caninum* infection in cattle has been also reported in west with 27.7% prevalence (Imre et al., 2012), north-west and center with 34.6% prevalence (Gavrea et al., 2011) of Romania.

In the context of an already existing milk quality testing, testing milk rather than sera would be a cost-effective approach for evaluating *N. caninum* exposure in dairy farms.

This study was undertaken to assess the potential of an ELISA kit for testing individual and pooled milk samples in order to determine the *N. caninum* infection status in dairy farms from southern Romania. Therefore, the aim of this study was to evaluate use of skim milk samples for estimation of *N. caninum* prevalence by two commercially available indirect ELISAs in dairy farms from the southern Romania, compared with serum samples. This study also investigated the potential use of pooled milk samples with an indirect ELISA to determine the *N. caninum* infection status of the cattle herds.

MATERIALS AND METHODS

Serum and milk samples

The study was conducted in south area of Romania comprising three counties (Ilfov, Olt and Dambovita). A total of individual 60 pairs of milk and blood samples were collected in 2010 from 3 dairy farms (A, B, and C) as a part of a larger serological survey (Mitrea et al., 2012; Enachescu et al., 2012). Animals were randomly selected. Additionally, pooled milk samples were obtained by homogenization of all milk samples for each farm and a total sample as a pooled sample from all three farms. About 5 ml of blood and 5 ml of milk were collected in plain vacutainer tubes and rapidly transported to the laboratory in cold conditions. Blood and milk samples were centrifuged at 2,500 rpm and 8000 rpm respectively for 10 min in order to separate serum and skimmed milk. Serum and skimmed milk samples were aliquoted and stored at-20°C until used.

Antibody analyses

Skimmed milk and serum samples were analysed by using a commercially available indirect ELISA: HerdChek *Neosporacaninum* Antibody Test Kit, IDEXX Lab. Manufacturer's instructions were strictly followed, with an exception: skimmed milk was diluted 1:2 in the dilution buffer delivered with the ELISA kit, as per recommendation of Schares et al. (2004). Plates were read at 620 nm and the test results were expressed as an S/P ratio obtained by an equation provided by the manufacturer. Serum samples with an S/P ratio equal or higher than 0.5 were considered positive.

Optimized cut-off values were calculated for skimmed milk samples with serum results considered as "gold standard" (see *Results*).

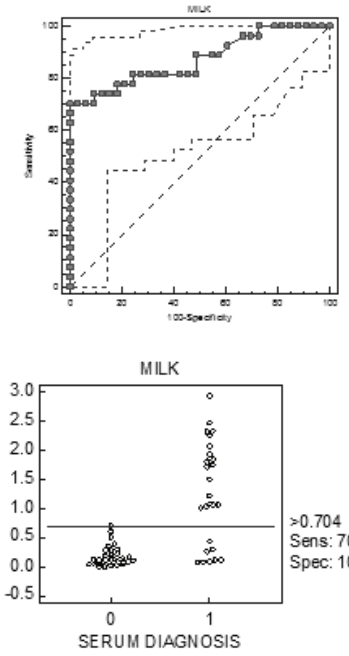
Data analysis

Receiver operating characteristic (ROC) curve analysis, test agreement, sensitivity, specificity, 95% confidence intervals, positive and negative likelihood ratio, positive and negative predictive value, Youden index, and significance levels were calculated using a statistical software program (MedCalc for Windows, version 12.4.0.0, MedCalc Software, Mariakerke, Belgium). Statistical significance was assumed at $P< 0.05$.

The agreement between classic serology and milk ELISA (Inter-rater agreement) was quantified by Weighted Kappa (*K*), 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

When the serum results were considered as the gold standard, ROC curve analysis revealed the associated criterion of S/P? 0.704, with 100% specificity ($CI_{95\%}$=89.4 – 100.0) and 70.34% sensitivity ($CI_{95\%}$=49.8-86.2), AUC of 0.873 ($P < 0.0001$) and Youden index of 0.7037 (Figure 1).



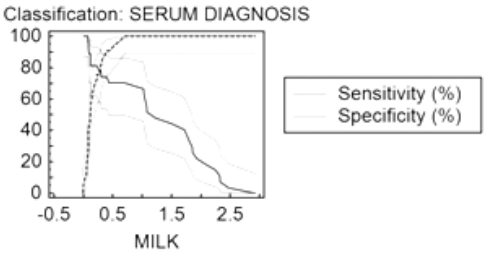


Figure 1. Results of analyzing milk by IDEXX ELISA, with serum results as „gold standard": (a) ROC curve for milk IDEXX ELISA with results shown as dots representing sensitivity/specificity pairs; (b) Interactive dot diagram for milk IDEXX ELISA results classified after serum diagnosis (0=negative, 1=positive); (c) Plotting of milk IDEXX ELISA sensitivity and specificity with 95% confidence intervals.

The associated criterion is the value with the highest specificity and sensitivity and was chosen as cut-off for using IDEXX ELISA on skimmed milk samples.

Other important criterion values revealed by ROC curve analysis on milk samples are presented in Table 1.

Table 1. Criterion values revealed by ROC analysis for milk samples. with serum sample as the true status

Criterion	Sensitivity		Specificity		Likelihood ratio (%)		Predictive values (%)	
	Percentage (%)	$CI_{95\%}$ (%)	Percentage (%)	$CI_{95\%}$ (%)	Positive	Negative	Positive	Negative
=-0.004	100	87.2-100.0	0	0.0-10.6	1		45	
> 0.079	100	87.2-100.0	27.27	13.3-45.5	1.37	0	52.9	100
> 0.08	96.3	81.0-99.9	27.27	13.3-45.5	1.32	0.14	52	90
> 0.088	96.3	81.0-99.9	33.33	18.0-51.8	1.44	0.11	54.2	91.7
> 0.09	92.59	75.7-99.1	39.39	22.9-57.9	1.53	0.19	55.6	86.7
> 0.092	88.89	70.8-97.6	42.42	25.5-60.8	1.54	0.26	55.8	82.4
> 0.118	88.89	70.8-97.6	51.52	33.5-69.2	1.83	0.22	60	85
> 0.122	81.48	61.9-93.7	51.52	33.5-69.2	1.68	0.36	57.9	77.3
> 0.232	81.48	61.9-93.7	75.76	57.7-88.9	3.36	0.24	73.3	83.3
> 0.267	77.78	57.7-91.4	75.76	57.7-88.9	3.21	0.29	72.4	80.6
> 0.293	77.78	57.7-91.4	81.82	64.5-93.0	4.28	0.27	77.8	81.8
> 0.301	74.07	53.7-88.9	81.82	64.5-93.0	4.07	0.32	76.9	79.4
> 0.398	74.07	53.7-88.9	90.91	75.7-98.1	8.15	0.29	87	81.1
> 0.439	70.37	49.8-86.2	90.91	75.7-98.1	7.74	0.33	86.4	78.9
> 0.704	**70.37**	**49.8-86.2**	**100**	**89.4-100.0**		**0.3**	**100**	**80.5**
> 2.935	0	0.0-12.8	100	89.4-100.0		1		55

Because area under the ROC curve (AUC) is significantly different from 0.5, milk IDEXX ELISA has the ability to distinguish between positive and negative bovines, regarding *N. caninum* infection. When the variable under study cannot distinguish between the two groups the AUC will be equal to 0.5 and the ROC curve will coincide with the diagonal, but when there is a perfect separation of the values of the two groups the AUC equals 1 and the ROC curve will reach the upper left corner of the plot (Zweig & Campbell, 1993).

Milk ELISA classified 19 of 60 samples as positive (31.7%, $CI_{95\%}$ = 19.55 – 43.78) at ?0.704 cut-off value while serum ELISA classified 27 of 60 samples as positive (45%, $CI_{95\%}$ = 32.04 – 57.96). The agreement between

serum and milk was *K*=0.723, corresponding to a good agreement.

The within-herd prevalence of dairy farms with serum and milk ELISA is presented in Table 2.

Table 1. The within-herd prevalence of dairy farms with serum and milk ELISA

	A	B	C	TOTAL
Serum prevalence (% (+/n))	80% (16/20)	40% (8/20)	15% (3/20)	45% (27/60)
Milk prevalence (% (+/n))	70% (14/20)	15% (3/20)	10% (2/20)	31.7% (19/60)
K-value	0.737	0.419	0.773	0.723
Intense serum results*	87.5% (14/16)	62.5% (5/8)	66.7% (2/3)	77.8% (21/27)
K-value**	1.00	0,679	1.00	0.921

Previous studies have used IDEXX ELISA to test milk samples for *N. caninum* infection (Schares et al., 2004; Bartels et al., 2005; Byrem et al., 2012) and revealed also a relative high sensitivity and a good agreement with serum results. Schares et al. (2004) reported a TG-ROC determined cut-off value of 0.261, with 90% sensitivity (Se) and specificity (Sp) and *K* = 0.80, for individual skim milk samples compared with serum samples. Bartels et al. (2005) determined a cut-off value of 0.6, Sp of 92% and Se of 61% for testing bulk milk samples. Byrem et al. (2012) calculated a cut-off value of 0.3 for skim milk with Sp of 95%, Se of 77.0% and *K*=0.77.

The sensitivity of 70.34% obtained for skimmed milk samples by IDEXX ELISA was lower than that of 100% reported for using this kit on serum samples (Wu et al., 2002). Others also previously reported a lower sensitivity on milk than on serum of different ELISAs for detecting *N. caninum* antibodies (Bartels et al., 2005; Schares et al., 2004; Byrem et al., 2012).

Differences in cut-off values reported for milk ELISAs may be caused by different commercial or in-house ELISAs that were evaluated or different laboratory techniques, such the use of manual or automated washing steps. Moreover, the stage of lactation in which the paired samples were taken may have played a role. IgG concentration in milk can vary depending on the stage of lactation, so that in late lactation milk quantity decreases but milk protein concentration increases, including IgG (Caffin et al., 1993). Lactation stage was identified as a factor associated with increasing agreement between milk and serum result in individual paired samples in animals (Schares et al., 2004).

The association between seroprevalence level and risk for reproductive losses may be different in distinct dairy industry situations (Wapenaar et al., 2007), involving unknown factors with influence in choosing the appropriate cut-off value and explaining the variance in parameters of milk ELISA.

Based on the value of the S/P ratio, the positive serum samples were divided into 2 categories: low positive (0.5< S/P< 1) and high positive (S/P?1). Milk ELISA performed better when samples with low positive result on sera were excluded (Table 2). A higher intensity of positive reaction (higher S/P values) can be correlated with a higher titer of antibodies indicating increasing performance of milk ELISAs with increasing antibody titer in analyzed samples.

In farm A, reproductive history of animals was known and permitted a correlation of diagnostic performance with abortions. All seropositive bovines with history of abortions were also positive on milk samples and presented a high positive reaction both on serum and milk. Thereby, high positive reaction both in serum and milk from animals that have aborted may be an additional clue for neosporosis as a cause of abortion, but require confirmation by other diagnostic techniques.

Schares et al. (2004) found that using IDEXX ELISA on milk samples, more aborting animals were identified positive than using the same kit on serum samples, but in that study tested cattle farms presented recent history of epidemic or endemic abortions, statistically associated with *N. caninum* infection.

Subsequently, we also analyzed four pooled milk samples of three herds with known seroprevalence of *N. caninum* infection.

When the optimized cut-off value for individual skimmed samples was used also for pooled milk samples only the two batches of samples with higher seroprevalence – farm A and total pooled sample with seroprevalence of 80 and 45%, respectively – were correctly classified, but the test failed to classify as positive the other two batches – farm B and C with seroprevalence of 40 and 15%, respectively (Table 3).

Table 2. Classification of pooled milk samples according to different cut-off values

Pooled samples	Cut-off values			
	> 0.704[a]	**> 0.61**[b]	**> 0.51**[c]	**> 0.398**[d]
A S/P=1.754	+	+	+	+
B S/P=0.687	-	+	+	+
C S/P=0.562	-	-	+	+
Total S/P=**1.573**	+	+	+	+

[a]Se =70.37%, Sp = 100%
[b]Se =70.37%, Sp = 96.97%
[c]Se =70.37%, Sp = 93.94%
[d]Se =74.07%, Sp = 90.91%

The relatively low sensitivity for pooled milk samples in the present study may be the consequence of the cut-off values chosen for the test. According to Bartels et al. (2005) the IDEXX ELISA performed satisfactorily in bulk milk samples at a calculated cut-off value of 0.6 – with 61% sensitivity and 92% specificity – to detect a within-herd seroprevalence of *N. caninum* in lactating cows of at least 15%. When calculated this cut-off value, Bartels et al. (2005) were based on previous studies in the Netherlands which suggested that a within-herd *N. caninum* seroprevalence of 15% can be associated with increased risk for reproductive losses.

When the interpretation by Bartels et al. (2005) of bulk milk IDEXX ELISA results with respect to seroprevalence levels was used, 3 of the 4 herds were classified correctly, at a similar cut-off value of 0.61, but with higher sensitivity and specificity (Table 3). The herd classified as negative had a seroprevalence of 15%, making the interpretation challenging. The cut-off value that classified correctly all pooled milk samples in the present study was > 0.51, with 93.94% specificity and 70.37% sensitivity. A lower cut-off value can be chosen, with increasing sensitivity but decreasing specificity. When a test is used either for the purpose of screening or to exclude a diagnostic possibility, a cut-off value with a high sensitivity may be selected, but when the test is used to confirm a disease, a higher specificity may be required (Zweig & Campbell, 1993).

Testing pooled milk samples may represent an alternative to testing individual milk samples, especially when a high prevalence is suspected.

CONCLUSIONS

When evaluating a diagnostic test it is essential to consider its future utility. The diagnostic performance of the IDEXX ELISA for individual milk samples regarding *N. caninum* infection creates opportunities for implementing an economical and reliable testing scheme for dairy farms, while the diagnostic performance for pooled milk samples justify further consideration.

When considering these aspects, from the present study following conclusions can be drawn:

The *Kappa* value of 0.723 suggests that the use of optimized cut-off value (S/P> 0.704) is adequate for testing bovine milk for *N. caninum* infection in southern Romania.

The specificity for milk IDEXX ELISA was 100% and the sensitivity was 70.4%.

Milk ELISA performed better when low positive sera were excluded (K=0.921) indicating increasing performance with increasing antibody titer in analyzed samples.

Testing pooled milk samples with the IDEXX ELISA may represent an alternative to testing individual milk samples for *N. caninum* infection in laboratory conditions, identifying dairy farms with a 15% or higher within-herd seroprevalence at the cut-off value of S/P> 0.51.

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REFERENCES

1. Altman D.G., 1991. Practical statistics for medical research. Chapman and Hall, London.
2. Anderson M.L., Palmer C.W., Thurmond M.C., Picanso J.P., Blanchard P.C., Breitmeyer R.E., Layton A.W., McAllister M., Daft B., Kinde H., Read D.H., Dubey J.P., Conrad P.A., Barr B.C., 1995. Evaluation of abortions in cattle attributable to

neosporosis in selected dairy herds in California. Journal of the American Veterinary Medical Association, 207, 1206–1210.

3. Bartels C.J., van Maanen C., van der Meulen A.M., Dijkstra T., Wouda W., 2005. Evaluation of three enzyme-linked immunosorbent assays for detection of antibodies to *Neospora caninum* in bulk milk. Veterinary Parasitology, 131, 235–246.
4. Björkman C., Johannsson O., Stenlund S., Holmdahl O.J.M., Uggla, A., 1996. *Neospora* species infection in a herd of dairy cattle. Journal of the American Veterinary Medical Association, 208, 1441–1444.
5. Byrem T.M., Bartlett P.C., Donohue H., Voisinet B.D., Houseman J.T., 2012. Performance of a commercial serum ELISA for the detection of antibodies to *Neospora caninum* in whole and skim milk samples. Veterinary Parasitology, 190 (1-2), 249-253.
6. Caffin J.P., Poutrel B., Rainard P., 1993. Physiological and pathological factors influencing bovine immunoglobulin G1 concentration in milk. Journal of Dairy Science, 66, 2161–2166.
7. Conraths J., Gottstein B., 2007. Neosporosis: General considerations. In: Ortega-Mora L. M., Gottstein B., Conraths F. J., Buxton D. (Eds.), Protozoal abortion in farm ruminants. CAB International, Wallingford, 42–46.
8. Dubey J.P., 2003. Review of *Neospora caninum* and neosporosis in animals. Korean Journal of Parasitology, 41, 1–16.
9. Dubey J.P., Schares G., 2011. Neosporosis in animals – The last five years. Veterinary Parasitology, 180, 90-109.
10. Dubey J.P., Schares G., Ortega-Mora L.M., 2007. Epidemiology and Control of Neosporosis and *Neospora caninum*. Clinical Microbiology Reviews, 20 (2), 323–367.
11. Enachescu V., Ioni?a M., Mitrea I.L., 2012. Serosurveillance of *Neospora caninum* in farm and courtyard cattle. Scientific Works C series, Veterinary Medicine, LVIII (3), *in press*.
12. Frossling J., Lindberg A., Bjorkman C., 2006. Evaluation of an iscom ELISA used for detection of antibodies to *Neospora caninum* in bulk milk. Preventive Veterinary Medicine, 74, 120–129.
13. Gavrea R.R., Iovu A., Losson B., Cozma V., 2011. Seroprevalence of *Neospora caninum* in dairy cattle from north-west and centre of Romania. Parasite, 18 (4), 349-351.
14. González-Warleta M., Castro-Hermida J.A., Carro-Corral C., Mezo M., 2011. Anti-*Neospora caninum* antibodies in milk in relation to production losses in dairy cattle. Preventive Veterinary Medicine, 101 (1-2), 58-64.
15. Hall C.A., Reichel M.P., Ellis J.T., 2006. Prevalence of *Neospora caninum* infection in Australian (NSW) dairy cattle estimated by a newly validated ELISA for milk. Veterianry Parasitology, 142 (1-2), 173-178.
16. Imre K., Morariu S., Ilie M.S., Imre M., Ferrari N., Genchi C., Darabus G., 2012. Serological survey of *Neospora caninum* infection in cattle herds from Western Romania. The Journal of Parasitology, 98 (3), 683-685.
17. Mitrea I.L., Enachescu V., Radulescu R., Ionita M., 2012. Seroprevalence of *Neospora caninum* infection on dairy cattle in farms from southern Romania. The Journal of Parasitology, 98 (1), 69-72.
18. Schares G., Barwald A., Staubach C., Wurm R., Rauser M., Conraths F.J., Schroeder C., 2004. Adaptation of a commercial ELISA for the detection of antibodies against *Neospora caninum* in bovine milk. Veterinary Parasitology, 120, 55–63.
19. Wapenaar W., Barkema H.W., O'Handley R.M., Bartels C.J., 2007. Use of an enzyme-linked immunosorbent assay in bulk milk to estimate the prevalence of *Neospora caninum* on dairy farms in Prince Edward Island, Canada. The Canadian Veterinary Journal, 48 (5), 493-499.
20. Wu J.T.Y., Dreger S., Chow E.Y.W., Bowlby E.E., 2002. Validation of 2 commercial *Neospora caninum* enzyme linked immunosorbent assays. Canadian Journal for Veterinary Research, 66, 264–271.
21. Zweig M.H., Campbell G., 1993. Receiver-operating characteristic (ROC) plots: a fundamental evaluation tool in clinical medicine. Clinical Chemistry, 39, 561-577.