DOI: 10.2478/s11686-014-0202-x

Acta Parasitologica, 2014, 59(1), 5–10; ISSN 1230-2821

VERSITA

Comparative study for the detection of antibodies to *Neospora caninum* in milk and sera in dairy cattle in southern Romania

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Abstract

The study aimed to assess the within-herd *Neospora caninum* exposure in dairy cattle in southern Romania, based on the detection of specific antibodies in milk and serum. A total of 104 paired samples of milk and serum were collected from four dairy farms. Individual samples were analyzed for *N. caninum* antibodies by ELISA: IDEXX Neospora Ab (Idx) (three farms: A, B, C; n = 60) and ID-VET Lab (Idv) (farm D; n = 44). Additionally, four pooled milk samples, one per each farm (A, B, C) and a composed one (A+B+C), were analyzed with Idx ELISA. Optimized cut-off values for milk samples were determined by receiver operating characteristic (ROC) analysis, with serum results considered as true status. The agreement was expressed by *K values*. The overall seroprevalence of *N. caninum* infection was 45% in the farms tested by Idx ELISA and 56.8% in the farm tested by Idv ELISA. A good agreement between serum and milk was obtained for both ELISA kits ($K = 0.72$ and 0.77, respectively). The specificity and sensitivity at optimized cut-off of S/P>0.704 for Idx and S/P%>7.966% for Idv were 100% and 70.37% for Idx and 89.47% and 88% for Idv. Testing pooled milk samples, there were identified as *N. caninum* positive the dairy farms with a 15% or higher within-herd seroprevalence at the cut-off value of S/P>0.51. This is the first study in Romania in which milk samples were tested to determine the *N. caninum* infection status in dairy farms, providing a base for further researches.

Keywords

Cattle, *Neospora caninum*, IgG antibodies, serum, milk, Romania

Introduction

Neosporosis is a major cause of abortion in cattle worldwide (Dubey and Schares 2011) and has a great economic impact in dairy farming (Hemphill and Gottstein 2006). The causative agent, *Neospora caninum*, is a heteroxenous apicomplexan protozoan capable of infecting various species of domestic and wild animals with dogs, coyotes, Australian dingoes and gray wolves serving as definitive hosts (McAllister *et al*. 1998, Gondim *et al*. 2004, King *et al*. 2010, Dubey *et al*. 2011).

Conventional serological techniques, such as indirect fluorescent antibody test and enzyme-linked immunosorbent assay (ELISA), are routinely used in adult animals and aborted fetuses for the detection of anti–*N. caninum* antibodies (Dubey and Schares 2006, Conraths and Gottstein 2007). Although *N. caninum* antibodies in cows indicate only exposure to this parasite (Dubey and Schares 2006), seropositive dams identified by ELISA have higher risk for abortion (2- to 4- fold comparing to the seronegative cows) (Pare *et al*. 1997, Moore *et al*. 2009).

Antibodies to *N. caninum* can also be measured in milk (Björkman *et al*. 1996; Conraths and Gottstein 2007). The antibodies found in milk are selectively transported from the serum into the mammary gland and the IgG is the primary immunoglobulin class for bovine milk (Hurley and Theil 2011). ELISA values for milk are linearly correlated to serum ELISA values (Schares *et al.* 2004, González-Warleta *et al*. 2011).

Testing of milk samples presents some advantages over testing of blood, like easily and lower costs of collecting samples, noninvasiveness of the method and reduction of stress-related production loss (Schares *et al*. 2004). When bulk tank milk samples are used, collecting samples is even easier and

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is an economical way of estimating *N. caninum* prevalence in cattle herds (Frossling *et al*. 2006; Wapenaar *et al*. 2007).

In previous studies conducted in different regions of the world several ELISAs were adapted for detection of *Neospora caninum* antibodies in cattle using individual milk samples (Björkman *et al*. 1996, Schares *et al*. 2004, Hall *et al*. 2006), bulk milk samples (Bartels *et al*. 2005, Frossling *et al*. 2006, Wapenaar *et al*. 2007) or both (González-Warleta *et al*. 2011).

In Romania, *Neospora caninum* infection in dairy cattle has only been recently serologically diagnosed in different areas of the country, with prevalences up to 56% (Gavrea and Cozma 2010, Gavrea *et al*. 2011, Imre *et al*. 2011, Mitrea *et al*. 2012). Despite of the high seroprevalence reported, no studies have been performed in Romania to evaluate characteristics of testing milk for *N. caninum* antibodies. Testing milk samples already collected for infectious disease testing or quality control will provide a competitive cost advantage over other tests for evaluating *N. caninum* exposure in dairy farms. Therefore, due to an increasing interest in screening Romanian dairy farms for the presence of *N. caninum*, the aim of this study was to assess the potential of two ELISA kits for testing individual and pooled milk samples in order to determine the *N. caninum* infection status in dairy farms from southern Romania.

Materials and Methods

Serum and milk samples

The study was conducted in four dairy farms in a southern area of Romania. Animals were randomly selected for individual sampling. A total of 104 paired samples of milk and serum were collected. Of them, 60 paired samples were collected in 2010 from three farms (A, B, C) and 44 paired samples were collected in 2012 from one farm (D). Additionally, four pooled milk samples, one per each farm (A, B and C) and a composed one (A+B+C), were obtained. For each polled sample, 1 ml of milk was drawn from every individual sample from each farm and mixed together. The fourth pooled sample was obtained by mixing 1 ml of each pooled samples from the three farms, mentioned above. No pooled milk samples were analysed for farm D because of the restricted number of samples that could be tested.

About 5 ml of blood and 5 ml of milk were individually collected in plain vacutainer tubes and transported to the laboratory in cold conditions. Blood and milk samples were centrifuged at 500 × g and 1000 × g, respectively for 10 min. Obtained sera and skim milk samples were aliquoted and stored at −20°C until used.

Antibody analyses

Aliquots of skim milk and sera samples were analysed for the presence of IgG antibodies specific for *N. caninum* infection using two commercially available indirect ELISA tests: Herd-Chek *Neospora caninum* Antibody Test Kit, IDEXX Lab (Idx ELISA) and ID Screen *Neospora caninum* Indirect Multi-Species, ID-VET Lab (Idv ELISA).

Manufacturer's instructions were followed for serum and for milk, but with an exception for the Idx ELISA: milk was diluted 1:2 in the dilution buffer delivered with the ELISA kit, as per recommendation of Schares *et al*. (2004). For Idv ELISA, milk was diluted 1:2 as per manufacturer's recommendations.

Plates were read at 620 nm (Idx ELISA) and 450 nm (Idv ELISA), and the test results were expressed as an S/P ratio and S/P%, respectively, obtained by an equation provided by the manufacturer.

Serum samples with an S/P ratio equal or higher than 0.5 (Idx ELISA) or S/P% equal or higher than 50% (Idv ELISA) were considered positive and those 40%<S/P%<50% were considered doubtful (only for Idv ELISA).

For detection of *N. caninum* antibodies in skim milk samples using Idx ELISA, calculation of an optimized cut-off value was necessary because of the lack of specific instructions. For Idv ELISA, the manufacturer's cut-off values were followed (S/P%≥30% were considered positive and those with 25%<S/P%<30% were considered doubtful), but also revised cut-off values were calculated similar to those for Idx ELISA.

Data analysis

Optimized cut-off values for milk samples were determined by ROC (Receiver Operating Characteristic) curve analysis, with serum results considered as the true status (Schares *et al*. 2004, Byrem *et al*. 2012).

ROC curve analysis, test agreement, sensitivity, specificity, 95% confidence intervals, positive and negative likelihood ratio, positive and negative predictive values, 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 serum 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

ROC curves

Results on all milk–serum pairs were examined by ROC curve analysis in order to find the appropriate cut-off values for milk that should provide similar results as the use of serum with the same test.

When the serum results were considered as the true status, ROC analysis revealed different criterion values, but the as-

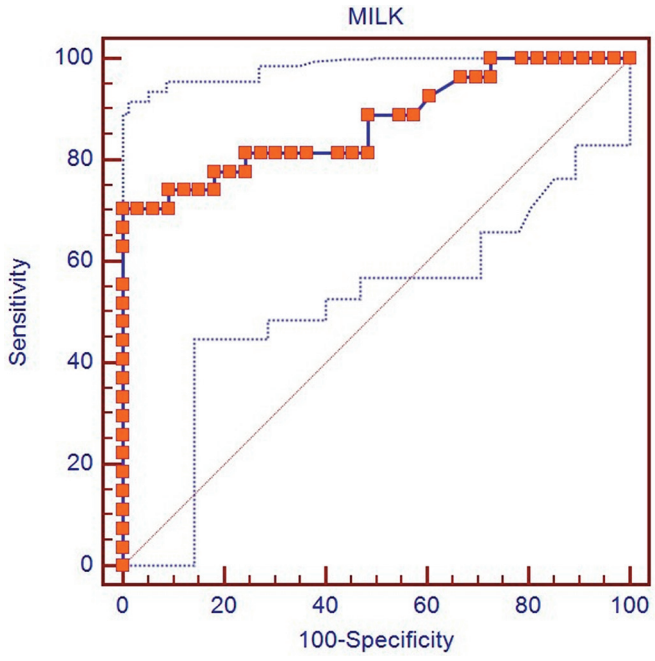

Fig. 1. ROC curve for milk Idx ELISA with serum results considered as the true status. Each point on the ROC curve represents a sensitivity/specificity pair

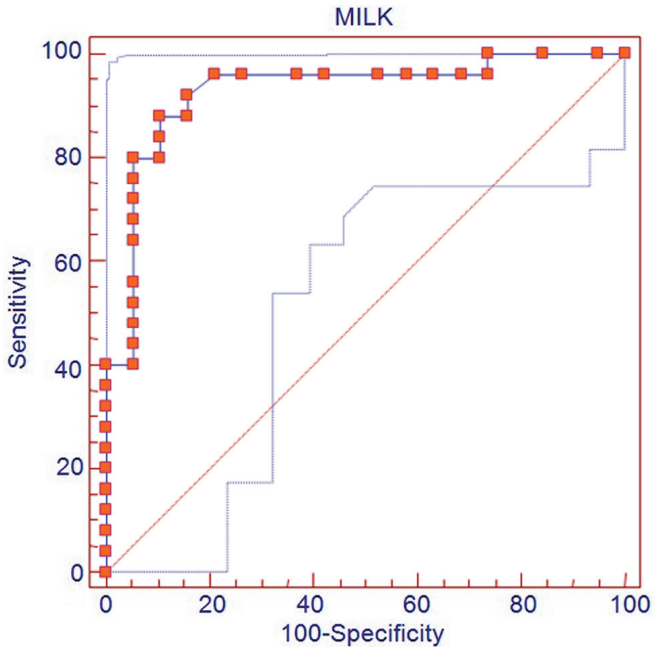

Fig. 2. ROC curve for milk Idv ELISA with serum results considered as the true status. Results are shown as dots representing sensitivity/specificity pairs

sociated criterion revealed by MedCalc software was chosen as a threshold value. The associated criterion is the value with the highest specificity and sensitivity. ROC curves for the two ELISAs used in the present study are shown in Figure 1 (Idx ELISA) and Figure 2 (Idv ELISA).

For Idx ELISA, ROC curve analysis revealed an associated criterion of S/P>0.704, with 100% specificity ($CI_{95\%}$ = 89.4–100.0) and 70.4% sensitivity ($CI_{95\%}$ = 49.8–86.2), AUC of 0.873 (P<0.0001) and Youden index of 0.7037. The positive and negative predictive values were 100% ($CI_{95\%}$ = 82.4–100.0) and 80.5% ($CI_{95\%}$ = 65.1–91.2), respectively and the negative likelihood ratio was 0.3% ($CI_{95\%}$ = 0.2–0.5). The revealed associated criterion for Idv ELISA was S/P%>7.966, with 89.5% specificity ($CI_{95\%}$ = 66.9–98.7) and 88% sensitivity ($CI_{95\%}$ = 68.8 –97.5), AUC of 0.927 (P <0.0001) and Youden index of 0.7747. The positive and negative predictive values were 91.7% ($CCI_{95\%}$ = 72.4–99.0) and 85% ($CI_{95\%}$ = 61.4–97.0) and the positive and negative likelihood ratio were 8.36% ($CI_{95\%}$ = 2.2–31.3) and 0.13% ($CI_{95\%}$ = 0.05–0.4), respectively.

Prevalence of antibodies and tests agreement between milk and serum

Serum Idx ELISA classified 27 of 60 samples as positive (45%, $CI_{95\%}$ = 32.04–57.96), while milk Idx ELISA classified

Table I. Comparative results of within herd prevalence obtained by serum and milk ELISAs

		Serum prevalence % (+/n)	Milk prevalence % (+/n)		*K*-value	Agreement
Idx ELISA	Farm A	80% (16/20)	70% (14/20)		0.737	Good
	Farm B	40% (8/20)	15% (3/20)		0.419	Moderate
	Farm C	15% (3/20)	10% (2/20)		0.773	Good
	Average	45% (27/60)	31.7% (19/60)		0.723	Good
Idv ELISA	Farm D	56.8% (25/44)	Manufacturer's cut-off	22.7% (10/44)	0.365	Fair
			Revised cut-off	54.5% (24/44)	0.77	Good

Table II. Classification of pooled milk samples according to different cut-off values and compared to serum prevalence and S/P value

Farms	Serum prevalence % (+/*n*)	Pooled milk samples				
		S/P	Cut-off values			
			>0.704[a]	>0.61[b]	>0.51[c]	>0.398[d]
A	80% (16/20)	1.754	+	+	+	+
B	40% (8/20)	0.687	–	+	+	+
C	15% (3/20)	0.562	–	–	+	+
A+B+C	**45% (27/60)**	**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%.

19 of 60 samples as positive (31.7%, $CI_{95\%}$ = 19.55–43.78) at S/P>0.704 as cut-off value. The agreement between serum and milk Idx ELISA was K = 0.723 ($CI_{95\%}$ = 0.552–0.895, standard error = 0.088), corresponding to a good agreement. The within-herd prevalence of dairy farms with serum and milk ELISAs is shown in Table I.

In the farm D serum Idv ELISA was used and it classified 25 of 44 samples as positive (56.8%, $CI_{95\%}$ = 41.58–72.05). When Idv ELISA was used on milk at the manufacturer's cut-off, 10 out of 44 samples were positive (22.7%, $CI_{95\%}$ = 9.83 -35.62) and 3 out of 44 were doubtful (6.8%), with K = 0.365 ($CI_{95\%}$ = 0.164–0.567, standard error = 0.103), corresponding to a fair agreement. With the same test at the revised cut-off, 24 of 44 samples were classified as positive (54.5%, $CI_{95\%}$ = 39.23–69.86) and K = 0.77 ($CI_{95\%}$ = 0.580–0.960, standard error = 0.097), corresponding to a good agreement.

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 for Idx ELISA and 50%<S/P%<100% for Idv ELISA) and high positive (S/P>1 for Idx ELISA and S/P%>100% for Idv ELISA). Both milk ELISA performed better when low positive sera were excluded (K = 0.921 for Idx ELISA and K = 0.509 for Idv ELISA at the manufacturer's cut-off, or K = 0.886 at the revised cut-off).

Subsequently, we also analyzed four pooled milk samples from three farms with known seroprevalence of *N. caninum* infection using Idx ELISA. The test results at different cut-off values are shown in Table II. The cut-off value for pooled milk samples that identified as positive all farms tested in this study (with seroprevalence values ranging from 15% to 80%) was S/P>0.51 with 93.94% specificity and 70.37% sensitivity.

Discussion

As ROC curves can be used to evaluate the accuracy of a test to discriminate diseased cases from normal cases (Zweig and Campbell 1993), this statistical analysis was frequently used to evaluate performance of serum ELISAs on milk samples. Because area under the ROC curve (AUC) was significantly different from 0.5, there is evidence that the both milk ELISA kits were able to distinguish between positive and negative cattle, regarding *N. caninum* infection. The closer the ROC curve is to the upper left corner, the higher is the overall accuracy of the test (Zweig and Campbell 1993).

High relative sensitivities for use of Idx ELISA on milk samples and good agreement with serum results for diagnosis of *N. caninum* bovine infection have also been described before, but the cut-off values determined for use of this kit on milk samples vary greatly. Therefore, the ROC determined cut-off values varied from 0.261 to 0.600, with sensitivity (Se) and specificity (Sp) ranging between 61–90% and 90–95%, respectively and K value from 0.77 to 0.80 (Schares *et al*. 2004, Bartels *et al*. 2005, Byrem *et al*. 2012). In all the studies referred above the milk dilution was 1:2, as well as in our study. Using a 1:2 dilution for skim milk samples analyzed by Idx ELISA showed a higher correlation with serum analysis than using a higher dilution, and a low background noise than using undiluted milk samples (Byrem *et al*. 2012).

Regarding Idv ELISA, more milk samples were classified as positive and a better agreement was achieved with the revised cut-off revealed by ROC curve analysis than by using the cut-off recommended by the manufacturer. Hall *et al*. (2006) validated this Idv ELISA for detection of *N. caninum* antibodies in bovine milk, optimizing cut-off values by TG-ROC analysis and obtained a 97% sensitivity and specificity, respectively. In the present study serum and milk samples were tested by the same method, Idv ELISA, but in the study of Hall *et al*. (2006) cut- off determination for milk Idv ELISA was made in relation to serum samples tested by an Idx ELISA. Difference in method used for testing reference serum samples might explain the different optimized cut-off values reported for Idv ELISA.

The sensitivities of the Idx ELISA and the Idv ELISA were 70.4 and 88%, respectively for use on skim milk samples. Both values are lower than those reported for serum samples: 100% for Idx ELISA (Wu *et al*. 2002) and 100% for Idv ELISA. These results confirm previous assessments on the use different ELISAs for detecting *N. caninum* antibodies in milk samples that have established their lower sensitivity compared

to serum samples (Schares *et al*. 2004, Bartels *et al*. 2005, Byrem *et al*. 2012).

Differences in parameters of milk ELISA were registered in different regions of the world. The association between seroprevalence level and risk for reproductive losses may be different in distinct dairy industry situations (Wapenaar *et al*. 2007).

Concerning the better performance of both ELISAs when low positive sera were excluded, 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.

Cows with a high serum antibody titer are capable to transmit *N. caninum* to the fetus by transplacental route (Jenkins *et al*. 1997) and cows with abortions due to neosporosis frequently present specific antibody titers higher than infected but non-aborting cows (Dubey and Schares 2006), so that using milk ELISAs can be particularly important in detecting these animals.

Using the Idx ELISA at cut-off value of S/P >0.704 as defined by the ROC curve analysis for milk samples, testing of pooled milk samples classified as positive the two batches of samples with higher seroprevalence – from farm A and the composed sample (A+B+C) with seroprevalence of 80 and 45% – but failed to classify as positive the other two batches – from farm B and C, for which the seroprevalence was 40% and 15%, respectively (Table II).

The relatively low sensitivities of the Idx ELISA for pooled milk samples in the present study may be the consequence of the cut-off values chosen for the tests. According to Bartels *et al*. (2005) the Idx 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 a similar cut-off as the one indicated by Bartels *et al*. (2005) for bulk milk Idx ELISA (S/P>0.61) was applied in our study on pooled milk samples (Table II), only one batch of samples did not classified correctly, but it had a seroprevalence of 15%, at the limit of the test, making the interpretation challenging. These results are consistent with the study mentioned above (Bartels *et al*. 2005).

When a test is used either for the purpose of screening or to exclude the infection a cut-off value with a high sensitivity may be selected. The cut-off value that classified correctly all pooled milk samples in the present study was S/P>0.51, with 93.94% specificity and 70.37% sensitivity.

In conclusion, milk ELISAs can represent valuable tools in screening and monitoring *N. caninum* prevalence in dairy farms. Additionally, testing pooled milk samples may represent an alternative to testing individual milk samples, especially when a high prevalence is suspected.

Finally, serum ELISAs must to be used in seroprevalence studies because of their higher sensitivity and specificity and milk positive herds should benefit of a complete serological screening and a subsequent protocol with adequate control measures regarding *N. caninum* infection.

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. We are grateful to Dr. Radulescu R. from the Institute for Diagnosis and Animal Health for technical assistance.

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Received: May 13, 2013
Revised: August 2, 2013
Accepted for publication: December 4, 2013