DETECTION OF NEOSPORA CANINUM ANTIBODIES IN MILK ON DAIRY CATTLE

Violeta ENĂCHESCU, Mariana IONIȚĂ, Ioan Liviu MITREA

Faculty of Veterinary Medicine of Bucharest, 105 Splaiul Independentei, District 5, Zip code 050097, Bucharest, Romania

Corresponding author email: violeta.enachescu@gmail.com

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 Scharas, 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 Scharas, 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 (Scharas 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; Scharas 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 (κ), 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<sub>95%</sub>=89.4 – 100.0) and 70.34% sensitivity (CI<sub>95%</sub>=49.8-86.2), AUC of 0.873 (*P* < 0.0001) and Youden index of 0.7037 (Figure 1).
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

<table>
<thead>
<tr>
<th>Criterion</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>Likelihood ratio (%)</th>
<th>Predictive values (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Percentage (%)</td>
<td>CI95% (%)</td>
<td>Percentage (%)</td>
<td>CI95% (%)</td>
</tr>
<tr>
<td>= -0.004</td>
<td>100</td>
<td>87.2-100.0</td>
<td>0</td>
<td>0.0-10.6</td>
</tr>
<tr>
<td>&gt; 0.079</td>
<td>100</td>
<td>87.2-100.0</td>
<td>27.27</td>
<td>13.3-45.5</td>
</tr>
<tr>
<td>&gt; 0.08</td>
<td>96.3</td>
<td>81.0-99.9</td>
<td>27.27</td>
<td>13.3-45.5</td>
</tr>
<tr>
<td>&gt; 0.088</td>
<td>96.3</td>
<td>81.0-99.9</td>
<td>33.33</td>
<td>18.0-51.8</td>
</tr>
<tr>
<td>&gt; 0.09</td>
<td>92.59</td>
<td>75.7-99.1</td>
<td>39.39</td>
<td>22.9-57.9</td>
</tr>
<tr>
<td>&gt; 0.092</td>
<td>88.89</td>
<td>70.8-97.6</td>
<td>42.42</td>
<td>25.5-60.8</td>
</tr>
<tr>
<td>&gt; 0.118</td>
<td>88.89</td>
<td>70.8-97.6</td>
<td>51.52</td>
<td>33.5-69.2</td>
</tr>
<tr>
<td>&gt; 0.122</td>
<td>81.48</td>
<td>61.9-93.7</td>
<td>51.52</td>
<td>33.5-69.2</td>
</tr>
<tr>
<td>&gt; 0.232</td>
<td>81.48</td>
<td>61.9-93.7</td>
<td>75.76</td>
<td>57.7-88.9</td>
</tr>
<tr>
<td>&gt; 0.267</td>
<td>77.78</td>
<td>57.7-91.4</td>
<td>75.76</td>
<td>57.7-88.9</td>
</tr>
<tr>
<td>&gt; 0.293</td>
<td>77.78</td>
<td>57.7-91.4</td>
<td>81.82</td>
<td>64.5-93.0</td>
</tr>
<tr>
<td>&gt; 0.301</td>
<td>74.07</td>
<td>53.7-88.9</td>
<td>81.82</td>
<td>64.5-93.0</td>
</tr>
<tr>
<td>&gt; 0.398</td>
<td>74.07</td>
<td>53.7-88.9</td>
<td>90.91</td>
<td>75.7-98.1</td>
</tr>
<tr>
<td>&gt; 0.439</td>
<td>70.37</td>
<td>49.8-86.2</td>
<td>90.91</td>
<td>75.7-98.1</td>
</tr>
<tr>
<td>&gt; 0.704</td>
<td>70.37</td>
<td>49.8-86.2</td>
<td>100</td>
<td>89.4-100.0</td>
</tr>
<tr>
<td>&gt; 2.935</td>
<td>0</td>
<td>0.0-12.8</td>
<td>100</td>
<td>89.4-100.0</td>
</tr>
</tbody>
</table>

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%, CI95% = 19.55 – 43.78) at 0.704 cut-off value while serum ELISA classified 27 of 60 samples as positive (45%, CI95% = 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

<table>
<thead>
<tr>
<th>Serum prevalence (% (+/n))</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>TOTAL</th>
</tr>
</thead>
<tbody>
<tr>
<td>(16/20)</td>
<td>80%</td>
<td>40%</td>
<td>15%</td>
<td>45%</td>
</tr>
<tr>
<td>(8/20)</td>
<td></td>
<td></td>
<td></td>
<td>(27/60)</td>
</tr>
<tr>
<td>Milk prevalence (% (+/n))</td>
<td>70%</td>
<td>50%</td>
<td>10%</td>
<td>31.7%</td>
</tr>
<tr>
<td>(14/20)</td>
<td></td>
<td></td>
<td></td>
<td>(19/60)</td>
</tr>
<tr>
<td>K-value</td>
<td>0.737</td>
<td>0.419</td>
<td>0.773</td>
<td>0.723</td>
</tr>
<tr>
<td>Intense serum results*</td>
<td>87.5%</td>
<td>62.5%</td>
<td>66.7%</td>
<td>77.8%</td>
</tr>
<tr>
<td>(14/16)</td>
<td></td>
<td>(5/8)</td>
<td>(2/3)</td>
<td>(21/27)</td>
</tr>
<tr>
<td>K-value**</td>
<td>1.00</td>
<td>0.679</td>
<td>1.00</td>
<td>0.921</td>
</tr>
</tbody>
</table>

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 $\leq$ S/P $< 1$) and high positive (S/P $\geq 1$). 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).
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.

**ACKNOWLEDGEMENTS**

This study was funded by the Sectorial Operational Program Human Resources Development 2007 – 2013 through the Financial Agreement POSDRU/88/1.5/S/52614.

**REFERENCES**
