Prevalence of Neospora caninum using Milk and Serum ELISA and its Hematological Effect in Dairy Buffaloes

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INTRODUCTION

Neospora (N.) caninum, an intracellular apicomplexan protozoon, is widely implicated as an abortifacient in bovine, which imposes serious economic impact on dairy and beef industry worldwide (Reichel, 2013). Besides abortion, bovine neosporosis is associated with stillbirths or occasionally premature birth or neurologically impaired calves (Micheloud et al., 2015). N. caninum infection in buffaloes have been reported from many countries around the globe, including Argentina (Konard et al., 2013), Italy (Auriemma et al., 2014), Australia (Neverauskas et al., 2015), Thailand (Kengradomkij et al., 2015) and Pakistan (Nasir et al., 2011). Several studies demonstrate both congenital (Chryssafidis et al., 2011) as well as horizontal transmission to buffalo from definitive hosts (canine) as the major portal of exposure (Kengradomkij et al., 2015). Recent literature reveals growing exposure of buffalo to N. caninum infection with age (Moore et al., 2014). In Thailand, N. caninum was determined as one of the important pathogens causing low milk productivity and slow growth rate of livestock animals (Inpankaew et al., 2014). Globally, the estimated median losses due to N. caninum induced abortions were in excess of US $1.298.3 million (Reichel, 2013).
*Neospora caninum* infection in cattle is serologically diagnosed chiefly by assessing protozoan specific antibodies in serum, plasma or milk (Garcia et al., 2013). These assays include indirect fluorescent antibody (IFA) test, agglutination test and enzyme-linked immunosorbent assays (ELISA). Now a days, antibodies detection has been the most applicable technique for research studies on *N. caninum* prevalence in cattle. Schares et al. (2004) firstly demonstrated the use of a commercial available bovine *N. caninum* specific serum antibody ELISA for the comparison of antibody levels in paired serum and milk samples.

But in healthy dairy animals, physiological stressful conditions (Lacetera et al., 2005) such as, pregnancy and milk yield (Detilleux et al., 1995) have been documented to affect the peripheral white blood cells profile. Peripheral immune responses have been used to assess the health status of an animal. As in other species, the populations of peripheral immune cells in bovines undergo alteration during pregnancy on account of conceptus-maternal conjoint relationship developed through placentomes (Oliveira et al., 2008). This physiological change may affect the results of immunological diagnostic tests. Thus, it was hypothesized that milk ELISA could be an efficient alternate of serum ELISA in determining the prevalence of *N. caninum* in dairy buffaloes and the protozoan may also affect the hematological profile of the host.

**MATERIALS AND METHODS**

The study was got approved by the Ethical Committee of the Institution (University of Veterinary & Animal Sciences, Lahore). For this study, milk samples were collected from individual lactating buffaloes (composite milk) as well as from the bulk milk just before blood sampling.

**Milk sampling and *Neospora caninum* antibodies testing using immune stimulating complex (iscom) ELISA:** About 15 ml of milk was sampled out into clean, sterile plastic tube and preserved for transportation with 0.1 g of sodium azide. A total of 64 milk samples (n=57 individual buffalo milk samples and n=7 bulk milk samples) were collected from the dairy buffaloes located at different farms of the districts of Lahore and Narowal. All the milk samples were centrifuged at 1000 × g for 15 min and the skimmed milk samples thus obtained were stored at -20 °C until assayed.

The presence of *N. caninum* specific antibodies in buffalo milk samples was detected by iscom ELISA (SANOVIR® Svanova Biotech AB, Uppsala, Sweden) as described by Bjorkman et al. (1997) with subsequent determinations worked out following manufacturer’s instructions. Thus, milk samples showing a percent positive (PP) of ≥20 were determined positive while the test samples with a PP value of <20 were declared negative.

**Serological and hematological analysis for *Neospora caninum:*** Monoclonal antibodies based cELISA (VMRD, Inc., Pullman, Washington) was performed to appraise *N. caninum* specific antibodies in the sera. This assay was validated (Jakubeck and Uggala, 2005) for cattle and also being used in other species. In the current study, cELISA was validated for buffaloes by comparing its performance efficiency with that of Indirect Fluorescent antibody test (IFAT), a ‘reference standard’ test keeping a cut-off dilution of 1: 200 (Guarino et al., 2000). IFAT is valued for very little cross reactivity to other coccidian parasites (Dubey et al., 1996). Resultantly, relative sensitivity (Se) and specificity (Sp) of cELISA for buffalo’s sera was calculated and determined by comparing cELISA results with IFAT outcomes. The results of ELISA were interpreted in accordance with the manufacturer’s recommendations wherein, serum samples exhibiting percent inhibition (PI) value of ≥30 were declared positive while of <30 were evaluated negative. Both the milk and serum ELISA assays were then compared to determine the agreement level in their performance efficiencies.

Hematological analyses of 50 blood samples from the seropositive buffalo and 15 samples from seronegative buffaloes were accomplished to determine the total leukocyte count (TLC) and differential leukocyte count (lymphocytes, monococytes and granulocytes).

Blood samples containing anticoagulant were used for determination of hematological variables in Nili-Ravi buffaloes. Red blood cells (RBCs) count, packed cell volume (PCV), hemoglobin (Hb) concentration and platelets count were also performed using an automated Hematology analyzer (Diatron Abacus Junior Vet; Diamond Diagnostics-USA). Blood glucose level in the seropositive and seronegative buffalo was also evaluated using Glucose Assay kit (Abcam, USA).

The data regarding the prevalence were analyzed by using Pearson Chi Square Test while General Linear Model (GLM) with all the two-way interactions was performed for hematological parameters using SPSS (SPSS, 18; USA).

**RESULTS**

Analysis of serum *N. caninum* antibodies using cELISA and its validation by IFAT: Overall 52 serum samples collected from lactating buffaloes were tested using IFAT (VMRD, Pullman-USA), which showed 41 seropositive and 11 seronegative samples. A cut-off dilution of 1: 200 was used to ascertain the positivity of the serum samples as described by Guarino et al. (2000) in water buffaloes. All the IFAT positive samples exhibited complete peripheral tachyzoite fluorescence (Fig. 1) under 400X magnification of Fluorescent microscope (Olympus Fluorescence Microscope, Japan). Different dilutions producing fluorescence in sera were 1: 200 (12 samples), 1: 600 (12), 1: 1000 (11 samples) and 1: 1200 (6 samples), while 11 samples did not show fluorescence and were thus declared negative. The relative diagnostic sensitivity and specificity of the cELISA for buffalo sera was calculated through comparing cELISA outcomes with that of IFAT. Subsequently, out of 41 IFAT positive sera, cELISA detected 38 sera as positives. While, out of 11 IFAT negative sera, cELISA detected 8 samples as negative. Consequently, the adjusted sensitivity and specificity of cELISA for buffaloes was obtained as 92.7% and 72.7%, respectively which was then used for indirect comparison with reference standard IFAT test.
Analysis of milk anti-*N. caninum* antibodies using iscom ELISA: In total, 64 milk samples were evaluated using iscom ELISA to determine the presence and possibility of the transmission of *N. caninum* through milk. These individual buffalo milk samples (n=57) originated from the 42 *N. caninum* seropositive buffaloes and 15 seronegative buffaloes (Table 1). According to manufacturer, the sensitivity (Se) and specificity (Sp) of iscom ELISA was 91 and 98% and for cELISA it was adjusted for buffalo sera at 92.7 and 72.7%, respectively as described earlier.

Overall, out of 57 individual milk samples, 35 (61.4% ±12.6, 95% CI) samples were determined positive by milk ELISA while the same animals evaluated serologically by cELISA represented 42 buffaloes (76.6% ±7.1) positive by serum cELISA. In bulk milk samples, four out of seven samples were detected positive for *N. caninum* antibodies. Though, seven cELISA seropositively evaluated buffalo milk samples were negative in iscom ELISA, the means of sera tested by cELISA showed a relatively better linear association with IFAT tested sera means (R²=0.857) compared to the level of agreement for individual samples (Fig. 2).

**Fig. 1:** Sample (1:200) showing complete peripheral fluorescence of the tachyzoites of *N. caninum* at 400X magnification.

**Percent inhibition means cELISA**

\[ y = -2E-05x^2 + 0.052x + 37.519 \]

\[ R^2 = 0.8575 \]

**IFAT titre means**

**Percent positivity iscom ELISA**

\[ y = 2.2271x + 5.5272 \]

\[ R^2 = 0.7692 \]

**Hematological impact of *Neospora caninum***: The mean values of different hematological determinants along with standard error estimates are shown in Table 2. The General linear model (GLM) analyses of the hematological parameters were performed taking into account all the possible two way interactions. The analysis showed a significant (P<0.05) effect of the interaction of season × *N. caninum* seropositivity on the monocyte count of buffaloes between seropositive and seronegative buffaloes. The monocyte count was relatively lower in seropositive buffaloes of different ages tested among various seasons of the year except in winter, compared to the counts in seronegative animals (Fig. 4).

Likewise, the interaction of *N. caninum* seropositivity with season depicted a significant effect (P<0.05) on the blood glucose level amongst the seropositive and seronegative buffaloes. Blood glucose values were higher in seropositive buffaloes than their seronegative counterparts particularly during the warm months of the year (Fig. 5).

**Table 1:** Prevalence and comparison of individual milk and serum *N. caninum* antibodies by two assays in buffaloes.

<table>
<thead>
<tr>
<th>Name of dairy farms</th>
<th>Number of buffaloes in the farms (n)</th>
<th>Number of bulk milk samples (n)</th>
<th>cELISA positive (n=50)</th>
<th>cELISA positive serum samples (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nawabzada</td>
<td>45</td>
<td>12</td>
<td>6 (53.9±15.0)</td>
<td>8 (65.0±16.6)</td>
</tr>
<tr>
<td>Riaz</td>
<td>50</td>
<td>15</td>
<td>10 (72.6±12.7)</td>
<td>11 (76.0±14.0)</td>
</tr>
<tr>
<td>Riaz</td>
<td>42</td>
<td>13</td>
<td>9 (75.5±13.3)</td>
<td>10 (82.0±14.3)</td>
</tr>
<tr>
<td>Mehaar</td>
<td>48</td>
<td>17</td>
<td>10 (58.8±23.4)</td>
<td>13 (81.2±12.6)</td>
</tr>
<tr>
<td>Total</td>
<td>185</td>
<td>57</td>
<td>35 (61.4±12.6)</td>
<td>42 (76.6±7.1)</td>
</tr>
</tbody>
</table>

**Table 2:** Haematological values of *Neatspora caninum* seropositive and seronegative groups of buffalo.

<table>
<thead>
<tr>
<th>Haematology Parameters</th>
<th>Reference Values</th>
<th>Seropositive (n=50)</th>
<th>Seronegative (n=15)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WBCs (4-12) x 10^9/l</td>
<td></td>
<td>13.2±0.7</td>
<td>11.5±0.9</td>
</tr>
<tr>
<td>Lymphocytes (45-75)%</td>
<td></td>
<td>49.1±3.3</td>
<td>46.6±6.2</td>
</tr>
<tr>
<td>Monocytes (2-7)%</td>
<td></td>
<td>3.8±0.4</td>
<td>5.7±1.0</td>
</tr>
<tr>
<td>Granulocytes (15-45)%</td>
<td></td>
<td>47.4±3.3</td>
<td>44.0±6.1</td>
</tr>
<tr>
<td>RBCs (5-10) x 10^12/l</td>
<td></td>
<td>6.9±0.2</td>
<td>7.6±0.4</td>
</tr>
<tr>
<td>Platelets (150-400) x 10^9/l</td>
<td></td>
<td>296.2±19.2</td>
<td>292.0±33.2</td>
</tr>
<tr>
<td>Packed cell Volume (24-48) %</td>
<td></td>
<td>32.4±0.7</td>
<td>34.2±1.4</td>
</tr>
<tr>
<td>Haemoglobin (8-14) mg/dl</td>
<td></td>
<td>10.3±0.2</td>
<td>10.9±0.5</td>
</tr>
<tr>
<td>Blood plasma glucose (mg/dl)</td>
<td></td>
<td>48.6±2.8</td>
<td>44.2±4.7</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>56.4±3.4</td>
<td>55.2±6.0</td>
</tr>
</tbody>
</table>

Values are Mean±SE.
Some of the hematological parameters of the buffaloes were influenced by lactation and season, regardless of their seropositivity, such as buffaloes in lactation demonstrated a significant effect (P<0.05) on the lymphocytic count. Lymphocytes were counted higher in dry animals compared to lactating buffaloes. Season alone was found to have a significant effect (P<0.05) in terms of high hematocrit (packed cell volume) values in the cooler months of the year compared to hot summer months of the year. Likewise, hemoglobin concentration of buffaloes was found to have a significant effect (P<0.05) in terms of high hematocrit (packed cell volume) values in the cooler months of the year compared to hot summer months of the year. Anyhow, some of the negative samples were actually low positive. But when the cut-off threshold is lowered to 0.15 instead of 0.20, agreement between the two ELISAs becomes quite substantial.

Relatively lower detection limit of the iscom ELISA may be attributable to the role of lactation stage of the animal resulting in fluctuation of antibodies level in the milk of tested buffaloes at different milking stages and parities (Scharres et al., 2004). Also, antibody level (IgG) had been reported to decline within few weeks after calving (Madsen et al., 2004) which might have contributed in the overall lower detection efficacy of milk ELISA compared to serological evaluations as most of the sampled animals were in late lactation. Furthermore, since the milk samples in the bulk milk are further diluted owing to the number and stage of lactating animals contributing in the pooled tank milk which may also dilute the antibody level to an undetectable limit, though the significant number of individual animals may still be at serologically detectable level. The findings in the current study are also augmented by the demonstrated low performance of the bulk milk ELISA compared to individual serum or milk analysis (Bjorkman and Lunden, 1998) as bulk milk ELISA has been reported to assess as low as 10-15% prevalence with in herd which reflects its inability to detect the lower prevalence than this percentage.

In iscom ELISA, two milk samples from the buffaloes tested seronegative were found positive which may be due to the false positive test detection of the milk ELISA as has been considered by Bjorkman et al. (1997) due to the possibility of antibodies being raised to other antigenic proteins. This may also be explained on the basis of separate pool of antibodies in udder and serum as reported by the earlier findings, demonstrating only 30% share of serum IgG in milk while the remaining are produced locally (Tizard, 2012). The overall and differential leukocyte count within the reference values were reported for healthy cattle (Roland et al., 2014). The following interactions were found to have significant effect on different hematological aspects. The interaction of N. caninum seropositivity × season demonstrated significant (P<0.05) effects on monocyte count of buffaloes. The reason for the lower monocyte counts in the current study may be the presence of chronic N. caninum infection as high monocyte count is usually present in severe acute type of infections or sepsis.

The reason for higher blood glucose level of infected buffaloes may be the stress induced by the combined factors of N. caninum positivity and main the hot season resulting in persistent and slightly elevated level of plasma glucocorticoids thus increasing the blood glucose level. Blood glucose level in the seropositive buffaloes was higher in autumn and lower in winter. The overall and differential leukocyte count within the reference values were reported for healthy cattle (Roland et al., 2014). The following interactions were found to have significant effect on different hematological aspects. The interaction of N. caninum seropositivity × season demonstrated significant (P<0.05) effects on monocyte count of buffaloes. The reason for the lower monocyte counts in the current study may be the presence of chronic N. caninum infection as high monocyte count is usually present in severe acute type of infections or sepsis.

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**DISCUSSION**

The individual milk sample analysis showed that the performance of iscom ELISA for milk was in moderate to good agreement (kappa value=0.567) with that of serological analysis by cELISA (VMRD, Washington, USA) keeping cELISA as the reference test. These findings were supported by the fact that the milking buffaloes originated from dairy properties having >15% seroprevalence of N. caninum antibodies (Nasir et al., 2014). These findings are in line with previously reported comparisons of different ELISA studies. The correlation coefficient between milk ELISA and serum ELISA was R² = 0.76, in the current study, which is comparable to the finding by Wapenaar et al. (2007) describing a correlation coefficient of 0.87 between serology and bulk milk analysis reflecting a good reliability of this diagnostic alternative for lactating herds. The reason for slightly lower agreement may be the higher percent positivity threshold set for detecting milk samples in the current study which probably is not well suited for analyzing buffalo milk samples as some of the negative samples were actually low positive. But when the cut-off threshold is lowered to 0.15 instead of 0.20, agreement between the two ELISAs becomes quite substantial.

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The lymphocytic count in the current study regardless of seropositivity was significantly (P<0.05) higher in dry buffaloes compared to lactating buffaloes which is in agreement with previous study describing the relationship of higher milk production with decrease in peripheral white blood cells owing to recruitment of leukocyte populations into the mammary tissues of high milk yielding cattle without distressing immune functions (Detilleux et al., 1995). A non-significant difference in the total leukocytic count between the seropositive and seronegative buffaloes is in accordance with the previous stance that the effects of N. caninum were chiefly limited to reproductive parameters (Hall et al., 2006) in cattle. The hematocrit values recorded were significantly (P<0.05) higher in cooler seasons of the year regardless of seropositivity compared to hot summer and autumn seasons which may be due to high blood plasma ratio of the animals during hot months resulting in dilution of the hematocrit and consequently lower cell to plasma ratio and vice versa in cooler months (winter and spring) in study areas.

The effect of N. caninum seropositivity and season interaction indicated significant (P<0.05) effect on blood hemoglobin concentration of the tested buffaloes which differs from the previous findings demonstrating a decline in PCV% in barn with increase in the temperature and relative humidity (Coban and Nilufer, 2005). Since, most of the sampled animals in the current study were adult buffaloes, thus overall high leukocytic and granulocytic count, in seropositive animals were recorded which are in level with the findings of Serrano et al. (2011) describing elevated counts in higher parities in cattle.

Conclusions: In conclusion, the utility of milk ELISA was limited in determining the prevalence of N. caninum in lactating herds only while serum ELISA is superior by virtue of determining the prevalence in general added by higher sensitivity. Reduced monocyte count and higher blood glucose level in N. caninum positive buffaloes may be due to oblivious stress of infection on the animal.

Authors contribution: AN conceived the idea and designed the study. AS and TR helped in the conduct of milk and serum testing. AN and IK performed the statistical analyses. AS, SE, SM, HA and MAZ contributed in the intellectual & scholarly write up of the manuscript. All the authors approved the final version for submission.

REFERENCES


