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RESEARCH ARTICLE

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

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ABSTRACT

The current study compared the performance of serum and milk ELISA in determining *Neospora caninum* prevalence in lactating Nili-Ravi buffaloes. Overall, 64 milk samples (n=57 individual milk samples; n=7 bulk milk samples) collected from buffaloes of districts Lahore and Narowal were tested for anti-*N. caninum* antibodies using milk iscom ELISA. The same animals were evaluated serologically using serum cELISA for this purpose. Furthermore, hematological profiling of 50 seropositive and 15 seronegative buffaloes was performed to appraise any effect of *N. caninum* seropositivity on the hematological parameters of the host. Resultantly, milk ELISA detected lesser milk samples positive i.e., 61.64% (±12.6, 95% C.I. 35/57) for *N. caninum* against higher seropositivity (76.6% ±7.16, 42/57) by serum cELISA. The agreement level (Kappa ratio=0.568 & R^2 =0.769) between the performance of two assays was found good. General Linear Model analysis of hematological parameters reflected significantly ($P<0.05$) lower monocyte count but higher blood glucose levels in seropositive buffaloes associated with different seasons. It was concluded that, owing to the limitations of the milk ELISA (also lower sensitivity than cELISA) it is best suited for finding prevalence in lactating dairy herds having substantial number of infected animals but in general, serum ELISA is best for prevalence studies. While, decreased monocyte count and higher blood glucose levels in the affected animals was observed, which may be attributed to persistent inherent stress of infection on the host.

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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).

N. 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 commercially 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, monocytes 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-of 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 (R^2=0.857) compared to the level of agreement for individual samples (Fig. 2).

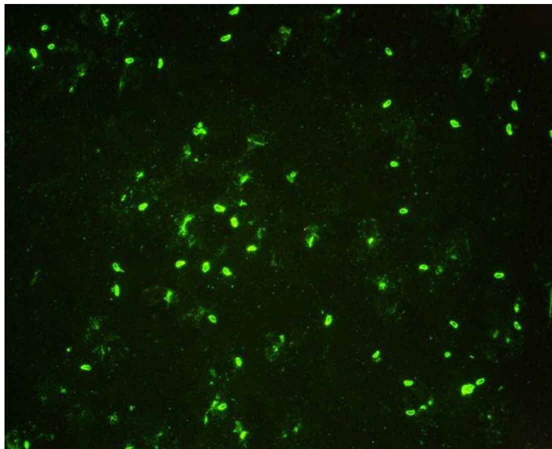

Fig 1: Sample (1:200) showing complete peripheral fluorescence of the tachyzoites of *N. caninum* at 400X magnification.

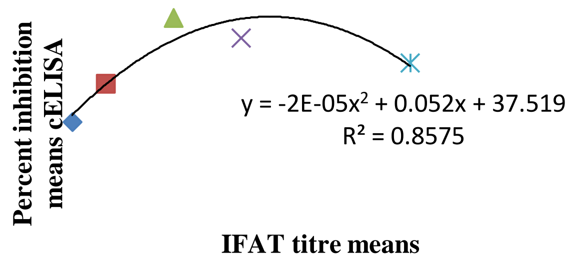

Fig. 2: Comparison of cELISA means against IFAT for *N. caninum* antibodies detection.

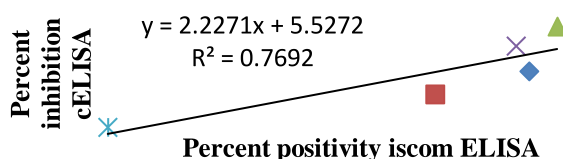

Fig. 3: Comparison of iscom ELISA (milk) and cELISA (serum) for milk and serum antibody analysis in sampled dairy buffaloes.

The Kappa value was calculated using software Win episcope, 2.0 (Thrusfield *et al.*, 2001) for both the tests (cELISA and iscom ELISA) to determine the level of agreement between the two assays. The Kappa ratio determined between the performances of two tests was 0.568 which can be regarded as moderate to good agreement (Pfeiffer, 2010). The linear association for comparison between two tests was, R^2=0.769 indicating a good agreement level between the two ELISAs (Fig. 3). The reason for slight difference in conformity between the two tests may be the cut-off threshold for milk iscom ELISA, which appeared to be slightly higher as some of the low negative milk samples ranged in PP between 15-20%. On lowering the cut-off value to 0.15 or 15%, the level of compliance between the two tests increased from 0.567 to Kappa ratio of 0.69, which is a good agreement between the two different format tests (Altman, 1991).

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 sero-negative 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

Name of dairy farms	Number of buffaloes in the farms (n)	Individual buffalo milk samples (n)	iscom ELISA positive (%±95% C.I)	cELISA positive serum samples (%±95% C.I)
Nawabzada	45	12	6 (53.9±15.0)	8 (65.0±16.6)
Riasat	50	15	10 (72.6±12.7)	11 (76.0±14.0)
Riaz	42	13	9 (75.5±13.3)	10 (82.0±14.3)
Mehaar	48	17	10 (58.8±23.4)	13 (81.2±12.6)
Total	185	57	35 (61.4±12.6)	42 (76.6±7.1)

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

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

†Values are Mean±SE.

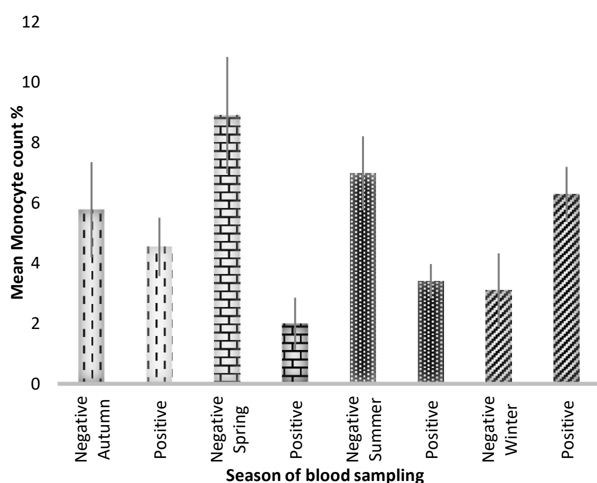

Fig. 4: Effect of the interaction of *N. caninum* seropositivity × season on monocyte counts of buffaloes.

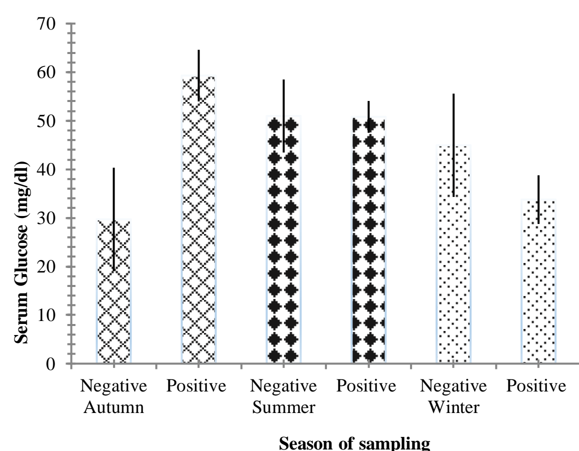

Fig. 5: Effect of the interaction of *N. caninum* seropositivity × season on blood glucose level of buffaloes.

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 lower ($P<0.05$) in summer months than in the other months of the year in buffaloes.

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 $R^2 = 0.76$, 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.

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 (Schares *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 mainly 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. Anyhow, nearly resembling blood glucose level in summer season in both the seropositive and seronegative buffaloes suggest the effect of a more dominant summer stress rather than due to being seropositive.

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

Altman DG, 1991. Practical Statistics for Medical Research. Chapman and Hall, London.

Alvarez-Garcia G, Garcia-Culebras A, Gutierrez-Exposito D, *et al*., 2013. Serological diagnosis of bovine neosporosis: a comparative study of commercially available ELISA tests. Vet Parasitol 198:85-95.

Auriemma C, Lucibelli MG, Borriello G, *et al*., 2014. PCR detection of *Neospora caninum* in water buffalo foetal tissues. Acta Parasitol 59:1-4.

Bjorkman C and Lunden A, 1998. Application of iscom antigen preparations in ELISAs for diagnosis of Neospora and Toxoplasma infections. Int J Parasitol 28:187-93.

Bjorkman C, Holmdahl O and Uggla A, 1997. An indirect enzyme-linked Immunosorbent assay (ELISA) for demonstration of antibodies to *Neospora caninum* in serum and milk of cattle. Vet Parasitol 68:251-60.

Chryssafidis AL, Soares RM, Rodrigues AA, *et al*., 2011. Evidence of congenital transmission of *Neospora caninum* in naturally infected water buffalo (*Bubalus bubalis*) fetus from Brazil. Parasitol Res 108:741-3.

Coban O and Nilufer S, 2005. Blood characteristics of dairy calves as affected by age, breed and types of barn. J Anim Vet Adv 4:459-61.

Detilleux JC, Kehrli Jr ME, Stabel JR *et al*., 1995. Study of immunological dysfunction in periparturient Holstein Friesian cattle selected for high and average milk production. Vet Immunol Immunopathol 44:251-67.

Dubey JP, Lindsay DS, Adama DS, *et al*., 1996. Serologic responses of cattle and other animals infected with *Neospora caninum*. Am J Vet Res 57:329-36.

Guarino A, Fusco G, Savini G, *et al*., 2000. Neosporosis in water buffalo (*Bubalus bubalis*) in Southern Italy. Vet Parasitol 91:15-21.

Hall CA, Reichel MP and Ellis JT, 2006. Prevalence of *Neospora caninum* infection in Australian (NSW) dairy cattle estimated by a newly validated ELISA for milk. Vet Parasitol 142:173-8.

Inpankaew T, Jittapalapong S, Mitchell TJ, *et al*., 2014. Seroprevalence of *Neospora caninum* infection in dairy cows in Northern provinces, Thailand. Acta Parasitol 59:305-9.

Jakubeck EB and Uggala A, 2005. Persistence of *Neospora caninum* specific immuno-globulin G antibodies in bovine blood and lung tissue stored at room temperature. J Vet Diagn Invest 17:458-60.

Kengradomkij C, Inpankaew T, Kamyingkird K, *et al*., 2015. Seroprevalence and risk factors associated with exposure of water buffalo (*Bubalus bubalis*) to *Neospora caninum* in northeast Thailand. Vet Parasitol 207:156-60.

Konrad JL, Campero LM, Caspe GS, *et al*., 2013. Detection of antibodies against *Brucella abortus*, *Leptospira* spp. and Apicomplexa protozoa in water buffaloes in the Northeast of Argentina. Trop Anim Health Prod 45:1751-6.

Lacetera N, Bernabucci U, Scalia D, *et al*., 2005. Lymphocyte functions in dairy cows in hot environment. Int J Biometeorol 50:105-10.

Madsen BD, Rasmussen MD, Nielsen MO, *et al*., 2004. Physical properties of mammary secretions in relation to chemical changes during transition from colostrum to milk. J Dairy Res 71:263-72.

Micheloud JF, Moore DP, Canal AM, *et al*., 2015. First report of congenital *Neospora caninum* encephalomyelitis in two newborn calves in the Argentinean Pampas. J Vet Sci Technol 6:251.

Moore DP, Konrad JL, Martino SS, *et al*., 2014. *Neospora caninum* serostatus is affected by age and species variables in cohabiting water buffaloes and beef cattle. Vet Parasitol 203:259-63.

Nasir, A, Ashraf M, Shakoor A, *et al*., 2014. Co-infection of water buffaloes in Punjab, Pakistan, with *Neospora caninum* and *Brucella abortus*. Turk J Vet Anim Sci 38: 572-76.

Nasir A, Ashraf M, Khan MS, *et al*., 2011. Seroprevalence of *Neospora caninum* in Dairy Buffalo in Lahore District, Pakistan. J Parasitol 97:541-3.

Neverauskas CE, Nasir A and Reichel MP, 2015. Prevalence and distribution of *Neospora caninum* in water buffalo (*Bubalus bubalis*) and cattle in the Northern Territory of Australia. Parasitol Int 64:392-6.

Oliveira LJ and Hansen PJ, 2008. Deviations in populations of peripheral blood mononuclear cells and endometrial macrophages in the cow during pregnancy. Reproduction 136:481-90.

Pfeiffer DU, 2009. Interpreting Diagnostic Tests. In: Veterinary Epidemiology: An introduction. Epidemiology Division, Department of Veterinary clinical Sciences, Willey-Blackwell Publishing, The Royal Veterinary College, University of London, UK. pp:110-41.

Reichel MP, Alejandra Ayanegui-Alcerreca M and Gondim LFP, 2013. "What is the global economic impact of *Neospora caninum* in cattle - the billion dollar question. Int J Parasitol 43:133-42.

Schares G, Barwald A, Staubach C, *et al*., 2004. Adaptation of a commercial ELISA for the detection of antibodies against *Neospora caninum* in bovine milk. Vet Parasitol 120:55-63.

Serrano B, Almeria S, Garcia-Ispierto I, *et al*., 2011. Peripheral white blood cell counts throughout pregnancy in non-aborting *Neospora caninum*-seronegative and seropositive high-producing dairy cows in a Holstein Friesian herd. Res Vet Sci 90:452-62.

Roland L, Drillich M and Michael I, 2014. Hematology as diagnostic tool in bovine medicine. J Vet Diag Invest 26:592-8.

Thrusfield M, Ortega C, de Blas ID, *et al*., 2001. Win Episcope 2.0: Improved epidemiological software for Veterinary Medicine. Vet Rec 48:567-72.

Tizard IR, 2012. Veterinary Immunology: An Introduction. 9th edition, vol. xxiv. Saunders, Philadelphia, PA USA, pp:568.

Wapenaar W, Barkema HW, O'Handley RM, *et al*., 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. Can Vet J 48:493-9.