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Co-infection of water buffaloes in Punjab, Pakistan, with *Neospora caninum* and *Brucella abortus*

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Abstract: Neosporosis and brucellosis are 2 well-known diseases causing bovine abortion. This study determined co-infection of *Neospora caninum* and *Brucella abortus* in buffaloes for the first time in Pakistan. A monoclonal antibody based competitive ELISA was used to test buffalo sera (n = 312) for *N. caninum* specific antibodies, while rose bengal plate test assessed *B. abortus* antibodies. *N. caninum* and *B. abortus* prevalence was 42.8% (±6.7, 95% C.I.) and 12.2% (±3.6, 95% C.I.), respectively. Buffaloes >3 years old exhibited higher prevalence than younger ones. Co-infection was particularly significant as 13.2% of *Neospora* infected buffaloes were also *Brucella* infected with an overall co-existence of 12.2%, suggesting higher abortion risk in such animals than in those infected with either one of the pathogens. Proper disposal of aborted fetuses and placentae, and testing and culling infected animals are suggested to impede transmission of pathogens.

Key words: Co-infection, ELISA, *N. caninum*, *B. abortus*, rose bengal plate test, buffaloes

Neosporosis and brucellosis represent the 2 leading and well-recognized causes of bovine abortion (1). *Neospora caninum*, the etiological agent of bovine neosporosis, is an abortifacient protozoan parasite, implicated in causing infection and subsequent reproductive losses in cattle (2), water buffaloes (3) and other domestic (4) as well as wild animal species (5). Neosporosis in buffalo has been documented from several regions of the world, such as from Brazil (6), Egypt (7), and Thailand (8), and also from Pakistan (9). Economic losses instigated by *N. caninum* worldwide can be demonstrated as direct costs mediated by abortion (10), together with culling infected animals (11). Globally, *N. caninum*-induced bovine abortion median losses have been estimated as >$1298.3 million (12). Bovine neosporosis is difficult to control mainly due to inaccessibility to cost-effective vaccines (13) as well as by economic consideration devising research into efficient vaccines (14).

Brucellosis is an infectious, contagious, zoonotic, and globally important disease of animals caused by bacteria of the genus *Brucella*, comprising a group of closely related bacteria where *Brucella abortus* primarily affects cattle. It occurs almost worldwide (15) and is endemic in Pakistan (16). The significance of this incredibly contagious disease is attributed to its economic impact on the livestock industry and human health. Serious risk to human health is either by direct contact with affected animals or by utilizing contaminated milk and milk products. It inflicts heavy economic losses in the dairy sector owing to abortions, sterility, animals' replacement costs, reduced milk production, and impediment to free animal movement and export (17). The livestock sector in Pakistan is exposed to various constraints; still significant economic losses are primarily ascribed to abortion (1). As such co-infection in cattle has been reported from Pakistan, the main objective of this study was to determine co-infection status of *N. caninum* and *B. abortus* along with some associated risk factors in buffaloes for the first time.

This study was performed on 312 sera randomly collected from the Nili Ravi breed of buffaloes between August 2009 and July 2010 in the laboratory (Clinical Medicine Department) at the University of Veterinary & Animal Sciences, Lahore. None of the sampled animals (except from Dera Chahal farm) had been vaccinated against brucellosis. Sampled animals included neonatal

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calves to buffaloes >7 years old reared at public and private farms in Lahore and Narowal districts of Punjab Province.

A monoclonal antibody based cELISA (VMRD, Inc., Pullman, WA, USA) was used to evaluate the sera for *N. caninum* specific antibodies. The assay has been validated (18) for cattle and applied extensively for diagnosing neosporosis in several animal species (19). The assay was conducted as per the manufacturer's instructions using duplicate positive and negative controls on 96-well plates. Antigen-coated plates were incubated at 27 ± 3 °C for 1 h with test sera and controls and then rinsed thoroughly with wash solution. Subsequently, the plates were incubated with peroxidase-anti-mouse monoclonal antibody conjugate and then substrate solution for 20 min along with washing between steps. Finally, after adding stop solution, optical density of the wells was quantified (Multiskan EX, Thermo Scientific, USA) at 620 nm wavelength. The results were represented as

PI (Percent Inhibition) =
100 – {Sample OD × 100/Mean Negative Control}

(OD = optical density)

Samples exhibiting a PI value of ≥30 were regarded as positive and <30 as negative.

In the present study, cELISA was validated for buffaloes by comparing its performance efficacy with an Indirect Fluorescent antibody test (IFAT), a 'reference standard' test using a cut-off dilution of 1:200 (20). The relative sensitivity (Se) and specificity (Sp) of cELISA for buffalo sera were determined by comparing the cELISA kit results with IFAT determinations.

All the sera were also assessed for *B. abortus* antibodies using the rose bengal plate agglutination test. The antigen was procured from the Veterinary Research Institute, Lahore, prepared according to standard OIE protocol requirements (21). The following test protocol was employed.

Briefly, a 25-µL volume of test serum was placed on a clean, marked glass slide at 27 ± 3 °C. Then an equivalent antigen volume was dispensed in the vicinity of the serum spot and both mixed immediately using a clean wooden stick to form an oval zone 2 cm in diameter. This mixture was agitated for 4 min using a 3-directional agitator and finally the slide was read. Any observable agglutination reaction was regarded as positive. A control serum provided a positive reaction to corroborate the sensitivity of testing.

Prevalence was estimated as described by Thrusfield (22).

The simultaneous existence of *N. caninum* and *B. abortus* antibodies in buffaloes showed their co-infection status.

True prevalence was calculated by measuring % seropositivity of *N. caninum* and *B. abortus* using a chi square test with 95% C.I., through Survey Toolbox software, considering Se and Sp of cELISA (23). Differences in co-infection prevalence among various ages were evaluated by Fisher's exact test while a 2-way chi square test determined the association between age and co-infection status using SPSS 13.

Overall, of 41 IFAT positive sera (Figure) cELISA detected 38 as positive (Figure), while of 11 IFAT seronegative sera cELISA detected 8 as seronegative. Thus, the adjusted 'Se' and 'Sp' of cELISA for buffalo were 92.7% and 72.7%, respectively.

A total of 312 sera from 10 different buffalo farms were tested for determining *N. caninum* prevalence. A true prevalence of *N. caninum* antibodies at 43.3% (39.9%–46.7%, 95% C.I.) for buffalo was recorded in this study. Amongst different ages, the highest prevalence of 54.1% (45.3%–62.9%, 95% C.I.) was found in adult (>3 years) buffaloes, whilst the lowest prevalence 35.2% (24.9%-45.5%, 95% C.I.) was observed in young (2–3 years) buffaloes (Table 1). Prevalence at buffalo farms ranged from 27.6% (15.5%–39.7%, 95% C.I.) to 66.8% (56.5%–77%, 95% C.I.), with the highest farm prevalence (66.8%) recorded for Riasat dairy while the lowest (27.6%) was recorded for Bengali farm (Table 2). Sex of the animals appeared to have no association with the prevalence of *N. caninum* antibodies (Table 3).

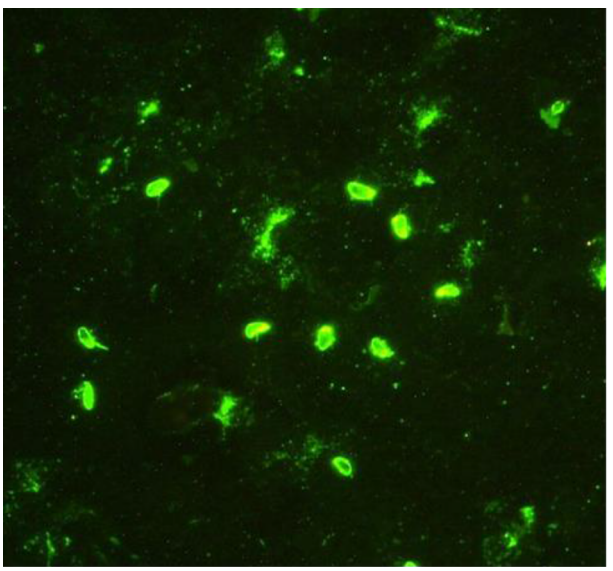

Figure. Fluorescent slide of a positive serum sample showing complete peripheral fluorescence of *Neospora caninum* tachyzoites at 400× magnification (Olympus, BX40-Japan).

Table 1. Age-specific prevalence and co-infection of *B. abortus* and *N. caninum* in various age groups of buffalo.

Buffalo age	Tested (n)	*B. abortus*		*N. caninum*		Prevalence of co-infection	
		N	%	N	%	N	%
Calves <1 year	76	3	3.9[a] (±4.4)	42	42.8[a] (±6.7)	2	4.8[a] (±6.5)
1–2 years	45	4	8.9[a] (±8.3)	22	33.0[a] (±8.8)	3	13.6[a] (±14.3)
2–3 years	35	5	14.3[a](±11.6)	17	32.5[a](±10.0)	2	11.8[a] (±15.3)
3–5 years	45	9	20[a] (±11.7)	27	50.0[a] (±8.7)	5	18.5[a] (±14.7)
5–7 years	47	7	14.9[a](±10.2)	26	42.8[a] (±8.6)	4	15.4[a] (±13.9)
>7 years	64	10	15.6[a] (±8.9)	33	37.1[a] (±7.4)	6	18.2[a] (±13.2)
Total	312	38	12.2 (±3.6)	167	40.1 (±3.3)	22	13.2 (±5.1)

Mean ± S.D. Values in the same column bearing different superscript letters (a, b, c) are statistically significantly different ($P < 0.05$, chi square), while those sharing the same superscripts are nonsignificant ($P > 0.05$).

Table 2. Herd-specific prevalence of *B. abortus* and its co-infection with *N. caninum* in dairy buffaloes.

Dairy properties	Tested (n)	*B. abortus*		*N. caninum*		Buffaloes with co-infection	
		N	(%)	N	(%)	N	(%)
Rakh Dera Chahal	46	0	0[a]	24	(38.0[a] ± 8.7)	0	0[a]
Saggian Farm	21	5	(23.8[a] ± 18.2)	13	(52.9[b] ± 12.6)	4	(30.8[a] ± 25.1)
Bengali Farm	25	2	(8[a] ± 10.6)	11	(25.5[c] ± 11.8)	0	0[a]
Riaz dairy	26	5	(19.2[a] ± 15.1)	16	(52.3[d] ± 11.3)	3	(18.8[a] ± 19.1)
Riasat dairy	31	2	(6.5[a] ± 8.6)	21	(61.8[e] ± 9.9)	2	(9.5[a] ± 12.6)
M.D Farm	23	5	(21.7[a] ± 16.9)	13	(44.7[f] ± 12.3)	3	(23.1[a] ± 22.9)
Nawabzada dairy	27	3	(11.1[a] ± 11.9)	12	(26.2[g] ± 11.3)	1	(8.3[a] ± 15.6)
Abdul Ghani	43	4	(9.3[a] ± 8.7)	23	(40.0[h] ± 9.0)	3	(13[a] ± 13.8)
Sarwar dairy	34	6	(17.6[a] ± 12.8)	18	(39.2[i] ± 10.1)	2	(11.1[a] ± 14.5)
Mehaar Sharif	36	6	(16.7[a] ± 12.2)	16	(26.2[j] ± 9.8)	4	(25[a] ± 21.2)
Total	312	38	38 (12.2 ± 3.6)	167	(40.1 ± 3.3)	22	(13.2 ± 25.1)

Mean ± S.D. Values in the same column bearing different superscript letters (a, b, c) are statistically significantly different ($P < 0.05$)

Table 3. Sex-specific prevalence of *N. caninum* and *B. abortus* and co-infection in dairy buffaloes.

Buffalo sex	Tested (n)	*N. caninum* positive (%)	*B. abortus* positive (%)	Co-infection (%)
Female	252	138 (54.76%)[a]	32 (12.69%)[b]	19 (7.53%)[c]
Male	60	29 (48.33%)[a]	6 (10.0%)[b]	3 (5.0%)[c]
Total	312	167 (53.52%)	38 (12.17%)	22 (7.05%)

Values in the same column bearing the same superscript letters (a, b, c) are statistically nonsignificant ($P > 0.05$)

Overall, 38 of 312 sera were detected positive for *B. abortus* antibodies, showing an overall prevalence of 12.2% (8.6%–15.8%, 95% C.I.) for brucellosis. Generally, higher prevalence was discernible in buffaloes >2 years of age, with the highest prevalence of 20% (9.3%–31.7%, 95% C.I.) in 3–5 year olds while buffaloes >7 years old demonstrated 15.6% (6.7%–24.5%, 95% C.I.) prevalence. Prevalence in 2–3-year-old and 5–7-year-old buffaloes was 14.3% (2.7%–25.9%, 95% C.I.) and 14.9% (4.7%–25.1%, 95% C.I.), respectively. Amongst young stock (1–2 years old) prevalence was lower at 8.9% (0.6%–17.2%, 95% C.I.), while calves up to 1 year of age were the least infected (3.9%; 0.5%–8.3%, 95% C.I.). Prevalence of *B. abortus* antibodies between the 2 sexes and amongst various age groups did not vary significantly ($P > 0.05$, chi square test) (Tables 1 and 3); however, the difference was significant ($P < 0.05$) between young (calves <2 years old) and adult (2–7 years old) buffaloes.

Prevalence of *B. abortus* antibodies at different farms ranged from 0% to 23.8% (±18.2) with 13.2% (8.1%–18.3%, 95% C.I.) of 43.3% *N. caninum* seropositive buffaloes also harboring *B. abortus* infection. Co-infection at different farms ranged from 0% to 30.8%. The highest co-infection (30.8%) was observed at a farm located near the River Ravi where buffaloes were often taken for outdoor foddering and watering. The dairy farm (Riasat dairy) with the highest *N. caninum* prevalence (66.8%) was experiencing repeated abortions. Co-infection prevalence among various age groups was nonsignificant as there was no association between age and co-infection state ($P > 0.05$). Similarly, the sex of the animals appeared to have no association with the prevalence of *N. caninum* antibodies (Table 3).

The prevalence status of *N. caninum* determined in the current study is in agreement with a previous study conducted in dairy cattle reporting 43.8% prevalence for bovine neosporosis in Pakistan (1). Adult buffaloes exhibited a higher prevalence than young ones in this study, which is comparable with the results described by other researchers (20). The close association of dogs with dairy farms in urban areas has been validated to augment *N. caninum* transmission to intermediate hosts (9). Differences in the prevalence of *B. abortus* antibodies amongst various farms was nonsignificant ($P > 0.05$), showing a general prevalence pattern trend of brucellosis in this species as reported earlier (24). These results intensify the findings of previous studies (1,24). The substantial prevalence of brucellosis in buffaloes at present could be attributable to the lack of a vaccination program. Owing to potential zoonotic risk, brucellosis should be regarded as a priority disease demanding strict control measures. Execution of valid vaccination campaigns, and proper disposal of aborted fetuses and placentae are requisite to avoid its perpetuation. Milk should only be consumed after being pasteurized.

Co-infection of *B. abortus* within 13.2% of all *N. caninum* seropositive buffaloes is quite considerable, indicating higher probability of abortion in adult buffalo having co-infection than in those infected with a single pathogen. A recent study in this country has reported a 16.3% share of *B. abortus* abortions within an overall abortion rate of 3.53% in Kundi buffalo in Hyderabad District (25), Sindh, Pakistan. This suggests the likelihood of even higher abortion rates in buffalo attributed to co-existence of pathogens. Our findings on co-infection are in compliance with a previous study (26) on dairy cattle in Turkey demonstrating 13.8% co-existence of *N. caninum* and *B. abortus*. The widespread occurrence of *N. caninum* co-infection in cattle with abortifacient pathogens has been recently described in Punjab (Pakistan) (27). Cattle are generally an integral part of the communal dairy farming system in this country with plausibly high risk of mutual transmission of the pathogens between bubaline herd mates. These abortifacient pathogens may cause high abortion ramifications in adult buffalo (>3 years old) particularly in farms with higher (≥18%) co-infections. No sex association was found with co-infection, suggesting no predilection of the pathogens for sex. At present such studies are lacking and further studies focusing on co-infection and bovine abortions are needed. It is anticipated that by curtailing the transmission of *N. caninum* and *B. abortus* using efficient hygienic measures, immunoprophylaxis, and testing and culling of infected farm animals could help to reduce bubaline abortions.

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