

Detection of antibodies against *Brucella abortus*, *Leptospira* spp., and Apicomplexa protozoa in water buffaloes in the Northeast of Argentina

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Abstract Water buffalo industry has become a profitable activity worldwide, including the Northeast of Argentina (NEA). However, research on diseases affecting this species is scarce. The aim of the present study was to detect antibodies against *Brucella abortus*, *Leptospira* spp., *Neospora caninum*, *Toxoplasma gondii*, and *Sarcocystis* spp. in 500 water buffalo cows from five ranches (100 animals each) in the NEA. Serum samples were tested for *B. abortus* by fluorescence polarization assay, *Leptospira* spp. by microagglutination test, and *N. caninum*, *T. gondii*, and *Sarcocystis* spp. by indirect fluorescent antibody tests. Overall, the proportion of seropositive animals was 6.4, 22.2, 42.2, 25.4, and 50.8 % for brucellosis, leptospirosis, neosporosis, toxoplasmosis, and sarcocystosis, respectively. The proportion of seropositive animals for all diseases was statistically different among herds ($p < 0.05$). Statistical differences were also detected among age groups for brucellosis and neosporosis ($p < 0.05$). The detection of specific antibodies to *B. abortus*, *Leptospira* spp., and several Apicomplexa protozoans in water buffaloes in the NEA is reported in this study.

Keywords *Bubalus bubalis* · *Brucella abortus* · *Leptospira* spp · Apicomplexa protozoa

Introduction

Water buffalo (*Bubalus bubalis*) is one of the best adapted species of livestock to hot and humid tropical areas, where the predominant environment has rivers and swamp soils with poor-quality forage. Buffaloes are reared on poor pasture extensive systems (Food and Agriculture Organization and FAO 2005). Buffalo industry is an important economic resource, based on production of milk, hide, and beef (Food and Agriculture Organization and FAO 2005). Over the past few years, buffalo-breeding has become an important economic activity worldwide, including in the Northeast of Argentina (NEA). Under extensive conditions in the subtropical environment and wet areas of the NEA, water buffaloes are better adapted than cattle being a more profitable industry and having a relevant impact in the regional economy (Crudeli and Patiño 2011). Water buffalo population in the NEA consists of approximately 100,000 heads and most of the breeds, which were imported from Brazil, are Mediterranean, Murrah and Jafarabadi (Crudeli and Patiño 2011).

The impact of infectious and parasitic diseases on the water buffalo industry has not been thoroughly investigated (Borriello et al. 2006; Adesiyun et al. 2009). Although brucellosis does not appear to affect severely the water buffalo production, caution should be taken due to its zoonosis character and the fact that water buffaloes may act as carriers of *Brucella abortus* (Borriello et al. 2006). A few authors have reported that water buffaloes are genetically resistant to brucellosis (Borriello et al. 2006; Capparelli et

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al. 2007); although *Brucella*-related abortions have been recorded in this species (Galiero et al. 2006).

Leptospirosis is also a zoonotic bacterial disease that may affect livestock (Adesiyun et al. 2009). *Leptospira* spp. infections have been reported in water buffaloes in several countries (Adesiyun et al. 2009; Felt et al. 2011; Suwancharoen et al. 2013). Many serovars of *Leptospira* spp. detected in water buffaloes without clinical signs could infect humans thus showing its relevance in public health (Adesiyun et al. 2009).

Diseases caused by Apicomplexa protozoa have already been reported on water buffaloes (Dubey et al. 1998; Fujii et al. 2001; Huong 1999; Xiang et al. 2011); but the role and epidemiology of parasites like *Neospora caninum*, *Toxoplasma gondii*, and *Sarcocystis* spp. are partially understood in the NEA. Serological evidence of *Neospora* exposure has been reported in water buffaloes in Argentina (Campero et al. 2007). The pathogenicity to fetuses of *N. caninum* in water buffalo has been demonstrated (Konrad et al. 2012), but spontaneous abortions have not been recorded so far. Toxoplasmosis and sarcocystosis are zoonoses with a worldwide distribution (Dubey et al. 1989; Dubey et al. 2005). Antibodies against *T. gondii* and *Sarcocystis* spp. have been shown in water buffalo in various countries (Dubey et al. 1998; Huong 1999; Jehle et al. 2009; Xiang et al. 2011), but there are no data regarding toxoplasmosis and sarcocystosis in this species in Argentina.

The aim of the present study was to detect antibodies against *B. abortus*, *Leptospira* spp., *N. caninum*, *T. gondii*, and *Sarcocystis* spp. in water buffaloes in the NEA.

Materials and methods

Area, animals, and sampling

The NEA (Fig. 1) is characterized by very warm, humid summers with mild drier winters. The main rivers in this region are Paraná and Uruguay. The studied ranches were located in Corrientes (A, B, C), Chaco (D) and Formosa (E) provinces in the NEA at 27.3° South latitude and 58.1° West longitude, approximately. Herds were selected on the basis of a previous history of good management, acceptable reproductive and productive performances, accessibility, and the willingness of the producer to be included in the study. Ranches A, B, C, and D had beef herds; meanwhile, ranch E had a double-purpose beef and dairy herd. The animals were maintained under extensive conditions in marshy areas.

Following the National Plan for the Control and Eradication of the Bovine Brucellosis and Foot and Mouth Disease (FMD) carried out by National Service of Animal Health, all female water buffaloes were vaccinated between 3 and 8 months of age using the live strain S19 of *B. abortus*



Fig. 1 The NEA embraces the Formosa, Chaco, Misiones, and Corrientes provinces and North of Santa Fe and Entre Ríos provinces. Location of each ranch is referenced as A, B, C, D, and E, respectively

and annually against FMD. The vaccine against *B. abortus* only interferes with any serological tests if the period for immunization is not well-thought-out. No other vaccine to prevent any disease was used.

Female water buffaloes of Mediterranean, Murrah, and Jafarabadi breeds and their crossbreed were randomly sampled. Blood samples from 500 animals (100 representing over 20 % of the total stock in each of the five herds) were obtained. With ages from 2 to 18 years old, the animals were arbitrarily grouped into four categories: 1 (2 to 4 years), 2 (5 to 7 years), 3 (8 to 10 years), and 4 (≥ 11 years) (Table 1). Blood samples were taken by jugular venipuncture, were centrifuged, and the sera obtained was aliquoted and stored at -20°C until use.

Serology

The presence of specific antibodies against *B. abortus*, *Leptospira* spp., *N. caninum*, *T. gondii*, and *Sarcocystis* spp. were evaluated as described below.

Brucellosis

Serum samples were tested for *B. abortus* antibodies by buffered plate antigen test (BPA) as previously described (OIE 2009) as screening test. Results were expressed as positive or negative. Positive BPA samples were then subject to analysis by fluorescence polarization assay (FPA) (OIE 2009). FPA antibody test kit was conducted using 20 μl of antigen *Brucella* O-polysaccharide conjugated with isothiocyanate fluorescein (Lab. Biológico Tandil, Argentina). Positive and

Table 1 Twenty-four leptospiral serovars used by MAT to test the serum samples

Australis Ballico
Autumnalis Akiyami
Ballum Castellon Castellon 3
Bataviae Swart
Canicola Hond Utrecht IV
Celledoni Celledoni
Cynopteri Cynopteri
Djasiman Djasiman
Grippotyphosa Moskva V
Hebdomadis Hebdomadis
Icterohaemorrhagiae Copenhageni M 20
Javanica Veldrat 45
Louisiana LSU 1945
Manhao Manhao
Mini Sari
Panama CZ 214
Pyrogenes Salinem
Ranarum ICF
Sarmin Sarmin
Sejroe Wolffi 3705
Hardjo Hardjoprajitno
Shermani 1342
Tarassovi Perepelicin

negative sera were used as controls. After incubation, samples were read with a polarization reader (Sentry 100, Diachemix Corporation, MI, USA). Data were converted to millipolarization units (mP) (Nicoletti 1992), where values ≥ 105 mP were considered positive, and values below 105 mP were considered negative.

Leptospirosis

Serum samples were assessed by microscopic agglutination test (MAT) (OIE 2008) for the following 24 leptospiral serovars (Table 2). *Leptospira* spp. were maintained in Ellinghausen-McCullough-Johnson-Harris medium (Difco, MI, USA). A volume of 0.1 ml of each serum dilutions was added to equal volume of live reference strain antigen of each serogroup (Levett 2008). Samples were incubated at 29 ± 1 °C for 2 h, and observed under dark-field microscope. Samples with agglutination ≥ 50 % were considered as positive, and samples with agglutination < 50 % were considered as negative. All samples were tested at dilution of 1:100 as cutoff (Adesiyun et al. 2009). All positive samples were tested at twofold ranging from 1:100 to 1:6,400. Positive and negative control sera were run in each test as control.

Neosporosis

Detection of specific *N. caninum* antibodies in serum samples was carried out by indirect fluorescent antibody test (IFAT), as previously described (Dubey et al. 1998). A fluorescein isothiocyanate (FITC)-labeled affinity-purified rabbit anti-bovine IgG antibody (Sigma, St. Louis, USA) (Fujii et al. 2001; Campero et al. 2007) was used. *N. caninum* specific antibodies were measured using dilutions of sera from 1:100 (Fujii et al. 2001; Campero et al. 2007). Positive and negative control sera were obtained from inoculated and non-inoculated experimental water buffaloes (Konrad et al. 2012). Slides were examined by epifluorescence microscopy at $\times 400$ (Olympus Bx 51, Olympus Inc., Tokyo, Japan), and serum samples were considered positive when the tachyzoites showed a complete peripheral fluorescence. Positive reactions in serum dilutions of 1:100 were regarded as indicative for the presence of *N. caninum* antibodies (Fujii et al. 2001; Campero et al. 2007).

Toxoplasmosis

Serum antibody response to *T. gondii* was screened by IFAT. Cell culture-derived tachyzoites from RH strain was used as antigen. Sera were diluted twofold in phosphate buffered saline (PBS) starting at 1:25. A FITC-labeled affinity-purified rabbit anti-bovine IgG antibody (Sigma, St. Louis, USA) was used (Moré et al. 2008). Positive and negative control sera were used. Positive reactions in serum dilutions $\geq 1:100$ were considered as indicative for the presence of *T. gondii* antibodies.

Sarcocystosis

Sera were tested by IFAT for the detection of antibodies to *Sarcocystis* spp. as previously described (Moré et al. 2008). Bradyzoites from naturally infected bovine hearts were used as antigen. One hundred grams of myocardium were mixed with 400 ml of digestion solution (2.5 g pepsin [0.7 U-FIP/mg] and 10 ml HCl in 1 l of water solution) and put

Table 2 Distribution of water buffalo females according to age

Ranch	Category (n)			
	2 to 4 years	5 to 7 years	8 to 10 years	≥ 11 years
A	54	11	17	18
B	31	28	41	-
C	27	29	30	14
D	70	18	12	-
E	31	37	32	-

in a magnetic stirrer for 20 min at 37 °C. The suspension was filtered through 300-, 150-, and 53- μ m sieves into 50 ml centrifuge tubes and centrifuged at 500 \times g for 5 min. Supernatants and the upper layer of the sediment were discarded. The pellet was washed three times with PBS and diluted in PBS to a final concentration of 4 million bradyzoites/ml. Bradyzoites were fixed onto 12-well IFAT slides and stored at -20 °C. Sera were diluted twofold in PBS starting at 1:25 dilution. Positive and negative control sera were used as control (Moré et al. 2008). Positive reactions in sera dilutions \geq 1:100 were considered as indicative for the presence of *Sarcocystis* spp. antibodies.

Statistical analysis

Multivariate statistical analysis was performed using discriminated analysis technique. Biplot technique was carried out in order to observe grades of association among the studied diseases with age or ranch of origin. By canonical correlations, partial correlations among diseases were assessed. Data classified in categories were analyzed by contingency tables, so as to establish association among diseases, age, and herds (Di Rienzo et al. 2011). ANOVA and Duncan tests were performed to evaluate such associations. *P* values \leq 0.05 were considered statistically significant.

Results

The studied ranches had animals with specific antibodies for all the tested diseases, except for ranch C where all animals were negative to brucellosis. Serum antibody results for all diseases are summarized in Table 3. Brucellosis was the disease with the lowest proportion of seropositive animals (6.4 %) (Table 3). The highest proportion of seropositive animals to brucellosis observed in ranch E showed statistically significant differences when compared to proportions of seropositive animals in the other ranches ($p < 0.05$). In addition, categories 1 and 4 showed the lowest proportion of seropositive animals. For brucellosis, the eldest animals were negative

for brucellosis tests and statistically significant differences in comparison with younger animals from categories 2 and 3 ($p < 0.05$) were found. The proportion of seropositive animals to leptospirosis was 22.2 %. There were no statistically significant differences among age groups ($p > 0.05$), but there were statistically significant differences among ranches being higher in ranches A, D, and E ($p < 0.05$) (Table 3). Serum antibody responses to *L. Pomona*, *Canicola*, *Grippytyphosa*, *Pyrogenes*, *Wolffi* and *Hardjo* with titers from 1:100 to 1:3,200 (data not shown) were detected. The highest titers 1:3,200 were detected to *Leptospira interrogans* serovar *Pomona* serogroup *Pomona* on ranches B ($n=1$), C ($n=2$), and E ($n=2$) (data not shown), while the other serovars showed lower titers.

The overall proportion of seropositive animals to neosporosis, toxoplasmosis, and sarcocystosis was 42.2, 25.4, and 50.8 %, respectively. Statistical significant differences among ranches were observed for the three parasitic diseases ($p < 0.05$) (Table 3). According to age, the lowest proportion of seropositive animals for neosporosis was recorded in young animals ($p < 0.05$) (Table 4). In contrast, there were no statistically significant differences considering the age factor in toxoplasmosis and sarcocystosis between different age groups ($p > 0.05$).

Discussion

In the last few years, a remarkable growth in the buffalo industry has resulted in a clear necessity to study the diseases affecting this species and to evaluate its potential role as reservoir of zoonotic pathogens. In an effort to identify and develop management tools to control these diseases, the present study was conducted to detect seropositive animals to *B. abortus*, *Leptospira* spp., and some Apicomplexa protozoans. It is worth pointing out that the presence of seropositive females to different diseases does not necessarily correlate with a low reproductive performance. Information submitted by the owners of the sampled herds showed a weaning of 69 to 82 % (data not shown), which is a very good productive performance considering the Argentinean

Table 3 Distribution of 500 water buffalo females according to their serostatus to several diseases

Ranch	Brucellosis (%)	Leptospirosis (%)	Neosporosis (%)	Toxoplasmosis (%)	Sarcocystosis (%)
A	1	28	26	35	49
B	1	13	25	6	25
C	0	7	65	33	59
D	5	35	39	37	82
E	25	28	56	16	39
Total	6.4	22.2	42.2	25.4	50.8

Hundred animals were tested per ranch

Table 4 Distribution of positive water buffalo females to several diseases according to age

Age groups	Brucellosis (%)	Leptospirosis (%)	Neosporosis (%)	Toxoplasmosis (%)	Sarcocystosis (%)
2 to 4 years ($n=213$)	4.7	24.4	35.7	29.1	54.9
5 to 7 years ($n=123$)	9.7	20.3	50.4	25.2	52
7 to 10 years ($n=132$)	7.5	19.7	42.4	20.4	44.7
≥ 11 years ($n=32$)	0	25	53.1	21.9	43.8

marginal extensive management conditions. Association between serostatus and presence of any clinical signs was not evaluated in this work. In addition, further research is needed to evaluate the impact of these infectious agents on the water buffalo industry in Argentina.

Brucellosis was the disease with the lowest proportion of seropositive animals. Noteworthy, the high seroprevalence of brucellosis observed in the beef and dairy ranch (25 %) emphasizes the potential risk of human infection by the intake of milk or derived products without pasteurization. On the other hand, water buffaloes may act as carriers of *B. abortus* (Borriello et al. 2006) being a potential source of bacteria for cattle cohabitating the same ranch.

According to our results, the exposure to *Leptospira* spp. seems to be frequent. However, it is important to highlight that the proportion of seropositive animals can be affected by the number of serovars and the type of diagnostic test selected. In Argentina, *L. Pomona* is the most common serovar found in bovine outbreaks of abortion, stillbirth, and weak calf syndrome (Draghi et al. 2011). Suwancharoen et al. (2013) recorded a seroprevalence of 30.5 % in buffaloes; this value was slightly lower than our findings (22.2 %). The mentioned authors also found that the most commonly detected antibodies against *L. interrogans* serovars Mini, Sejroe, and Bratislava which is not in agreement with our finding of antibodies against *L. Pomona*, *Canicola*, *Grippotyphosa*, *Pyrogenes*, *Wolffi*, and *Hardjo*. The finding of titers as 1:3,200 to *L. Pomona* suggests an active stage of infection; but no attempt to *Leptospira* isolation was carried out in the present work.

The role of *N. caninum* on the reproductive performance of water buffalo is still uncertain; although natural or experimental congenital transmission in female water buffalo was recently reported (Chryssafidis et al. 2011; Konrad et al. 2012). The serological prevalence of neosporosis in water buffaloes is in agreement with previous work from Argentina (Campero et al. 2007) and Brazil (Fujii et al. 2001; Gennari et al. 2005). In contrast, works carried out in Asia found lower proportion of seropositive animals (Yu et al. 2007).

The proportion of seropositive animals for *T. gondii* in the studied ranches ranged from 6 to 37 %. Due to the role of the water buffalo as a reservoir for this protozoa and the high proportion of seropositive animals found in this work,

it may be advisable to continue with research work in this matter. Our results show higher values than those reported in Iran (14.3 %) (Hamidinejat et al. 2010) and in Trinidad (7.8 %) by Persad et al. (2011) using MAT and latex agglutination tests, respectively, as a diagnostic tool.

This is the first report of water buffalo exposure to *Sarcocystis* spp. in Argentina. The high proportion of seropositive animals found in this study (50.8 % and >25 % in all ranches) suggests that water buffalo could represent an important reservoir of this pathogen. Moré et al. (2011) found that 99.7 % of cattle were positives at IFAT titers of $\geq 1:25$ in Argentina. Studies were conducted to determine the prevalence of *Sarcocystis* species by macroscopic and histological examination in slaughtered water buffaloes in Iran and Egypt and found a value of 83 and 78.9 %, respectively (Oryan et al. 2010; El-Dakhly et al. 2011). By analyzing striated muscle from natural infected water buffaloes, a variety of species were identified, like *Sarcocystis fusiforme*, *Sarcocystis cruzi*, *Sarcocystis hominis*, *Sarcocystis hirsuta* and *Sarcocystis sinensis* (Jehle et al. 2009; Chen et al. 2011; Xiang et al. 2011). In our work, only serology was used as a diagnostic tool.

Water buffaloes in the NEA are exposed to all the diseases tested including four zoonoses. Since the water buffalo industry has become an important economic activity, authorities from National Animal Institutes should consider water buffaloes as carriers of many diseases affecting any Eradication National Programs. The origin of infections associated with the water buffaloes from the NEA is hard to establish since the first introduction of water buffaloes imported from Brazil and Italy date to 1970 and there is also the possibility that imported animals free of pathogens could become infected at local ranches. International regulations on animal trading must be improved in order to control diseases.

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Conflict of Interest None of the authors of this paper has a financial or personal relationship with other people or organizations that could inappropriately influence or bias the content of this article.

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