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PROTOZOOLOGY - SHORT COMMUNICATION

Evidence of *Neospora caninum* infection in buffaloes (*Bubalus bubalis*) from Northwestern Romania

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Abstract

Neospora caninum is a heteroxenous parasitic Apicomplexan protozoan, able to infect a variety of domestic and wild animals, mainly associated with reproductive disorders and abortions in susceptible species. Considering the importance of Romania in the European buffalo industry and the severe economic losses caused by *N. caninum* infection in livestock, the aim of the present study was to assess the occurrence of neosporosis in buffaloes raised in household and farming systems in northwestern Romania. Overall, 197 serum samples were tested for the presence of *N. caninum*-specific antibodies by ELISA. Additionally, from 74 slaughtered buffaloes, diaphragm digest, heart, and lymph node samples were tested for the presence of *N. caninum* DNA. The overall seroprevalence was 68.5%. The seroprevalence was significantly higher in adult animals compared to calves and young ones and in the household system compared to farm. By nPCR, six samples were found to be positive for *N. caninum* DNA, of which three were serologically negative. The present study reveals a high prevalence of *N. caninum*-specific antibodies in buffaloes in northwestern Romania and to our best knowledge, it is the first one performed in Romania.

Keywords *Neospora caninum* · Buffaloes · Seroprevalcence · PCR

Introduction

Neosporosis represents a major cause of reproductive disorders and abortions in susceptible animal populations worldwide, causing major economic losses, particularly among bovines (Dubey and Schares 2011). It is caused by *Neospora caninum* a heteroxenous parasitic Apicomplexan protozoan, able to infect a variety of domestic and wild animals, but which infects mainly bovines and domestic dogs (Dubey and Schares 2011). Since the first identification of the parasite in Norvegian dogs (Bjerkås et al. 1984) and following the original description of the parasite by Dubey et al. (1988), neosporosis has been identified and studied worldwide. The definitive hosts are represented by canids (domestic dogs, coyotes, dingoes and wolves) (Dubey and Schares 2011). Although exposure to *N. caninum* has been demonstrated in a large variety of wild and domestic animal species, viable parasites have been isolated from a limited number species, including cattle, bison, water buffaloes, white-tailed deer, sheep, and horses (Dubey and Schares 2011).

Due to similarity with *Toxoplasma gondii*, it is assumed that the environmental resistance of *N. caninum* oocysts is likewise similar, the survival and sporulation of the oocysts being favored by humidity and a moderate temperature (Dubey et al. 2007). The infection of intermediate hosts occurs by ingestion of sporulated oocysts along with contaminated water or feed, or, more importantly, by transplacentar, vertical transmission (Dubey and Schares 2011). Postnatal transmission by maternal milk has also been demonstrated (Dubey and Schares 2011).

Diana Bărburaş and Adriana Györke contributed equally.

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In buffaloes, the role of *N. caninum* infection in abortions has also been demonstrated (Guarino et al. 2000; Gennari et al. 2005), with parasitic cysts having been found in the tissues of aborted fetuses presenting encephalitis and myocarditis (Guarino et al. 2000). In Romania, raising buffaloes is a traditional activity, particularly in the Transylvania region. The buffalo population was estimated at around 300,000 animals in 1985 and decreased gradually since, to around 20,000 animals in 2016 being the 2nd largest population in Europe, after Italy (National Institute of Statistics 2017). The buffaloes are raised for milk and meat production, and to a lesser extent, for traction work.

Considering the importance of Romania in the European buffalo industry and the severe economic losses caused by *N. caninum* infection in livestock, the aim of the present study was to assess the prevalence of neosporosis in buffaloes raised in households and farms in northwestern Romania.

Materials and methods

Animals and samples

The estimated sample size for a population of 20,000 animals with 5% margin of error, 95% confidence level, and a 10% response distribution was 139. Buffaloes were sampled for blood in the field and for blood and tissue samples in a slaughterhouse. All sampled animals were identified by a unique code and for each animal, the sex, age, and origin were recorded.

Blood samples were collected from the jugular vein, either in the field, or at the slaughterhouse. The attained serum was separated, labeled, and stored at − 20 °C until further processing. From the slaughtered buffaloes, individual tissue samples (diaphragm, heart, and mesenteric lymph nodes) were also collected. Heart and mesenteric lymph node samples were labeled and stored at − 20 °C until further processing. Diaphragm tissue samples were the subject of artificially digestion for *T. gondii* isolation by bioassay and the obtained digest was used for DNA extraction.

Enzyme-linked immunosorbent assay (ELISA)

The serum samples were processed by ELISA technique, using the Monoscreen AbELISA *Neospora caninum* (rSRS2) (Bio-X Diagnostics S.A., Belgium) commercial kit, according to the manufacturer's instructions. The results were calculated according to the following formula: sample inhibition % = [(negative control OD−sample OD)/negative control OD]*100, where OD represents the optical density. Samples having an inhibition percentage of ≥ 33% were considered positive. The sensitivity and specificity of the test according to manufacturer's information is 89.0% and 96.0%, respectively.

Molecular assays

The genomic DNA was extracted from 25 mg heart and mesenteric lymph node tissues and 100-μl diaphragm digests, using a commercially available kit (Isolate II Genomic DNA Kit, Bioline, UK), according to the manufacturer's instructions. *N. caninum* DNA was detected by amplification of fragments of the *Nc*5 gene, using previously published primers and under previously described conditions: a 337-bp fragment by conventional PCR (Müller et al. 1996) and a 299-bp fragment by nested PCR (Fish et al. 2007).

All positive samples were sequenced using an external service (performed by Macrogen Europe, Amsterdam, The Netherlands) and the attained sequences were compared to those available in GenBank by Basic Local Alignment Tool (BLAST) analysis.

The sequences were aligned using MEGA X software (Kumar et al. 2018) and the phylogenetic relationships were inferred by Maximum Parsimony method. The analysis involved 22 *N. caninum* nucleotide sequences and one sequence of *Cytospora*, as outgroup. The MP tree was obtained using the Subtree-Pruning-Regrafting (SPR) algorithm (Nei and Kumar 2000) with search level 1 in which the initial trees were obtained by the random addition of sequences (10 replicates). There were a total of 213 positions in the final dataset.

Statistical analysis

All statistical analysis was performed using Epi Info ™ 7 software (CDC, USA). The frequency, prevalence, and the 95% confidence interval for anti-*N. caninum* antibodies and *N. caninum* DNA were calculated. These were calculated both in total, as well as by age, sex (females, males), and rearing system (household, farm). According to age, the animals were divided in three categories: calves (0–6 months), youth (6–30 months) and adults (over 30 months). The differences among categories were assessed by means of chi-square testing. A p value of < 0.05 was considered statistically significant. Logistic regression analysis was performed to quantify the association between the presence of *N. caninum* antibodies and risk factors (age, gender, and rearing system). The risk factors remained in the logistic regression analysis if the goodness of fit of the model was significant ($p \leq 0.05$).

Results

For the present study, there were a total of 197 blood samples collected from buffaloes (*Bubalus bubalis*, Mediterranean Carpathian breed) originating from five counties of northwestern Romania (Table 1). Tissue samples were collected from 76

Table 1 Distribution of sampled animals

County	Age category			Gender		Breeding system		Place of sampling		Total
	Buffalo calves	Youth	Adults	Females	Males	Household	Farm	Field	Slaughterhouse	
Cluj	14	15	23	37	15	21	31	23	29	52
Sălaj	11	36	89	128	8	136	0	100	36	136
Bihor	0	0	3	2	1	3	0	0	3	3
Bistrița-Năsăud	0	0	1	0	1	1	0	0	1	1
Maramureș	0	1	4	5	0	5	0	0	5	5
Total	25	52	120	172	25	166	31	123	74	197
Age (months)	5.0 (± 1.6)	17.6 (± 8.3)	129.9 (± 67.2)	93.5 (± 78.4)	21.9 (± 27.1)	95.1 (± 77.9)	27.5 (± 44.7)	69.0 (± 71.9)	110.1 (± 80.3)	84.4 (± 77.6)

slaughterhouse buffaloes. The age ranged between 2 weeks and 25 years (300 months) with an average age of 84.4 (± 77.6) months. By sex, most of the samples were collected from females (172/197) and according to origin, the majority (166/179) came from the household system (Table 1).

The overall seroprevalence of anti-*N. caninum* antibodies assessed by ELISA in the sampled buffaloes was of 68.5% (135/197). According to the age group, the seroprevalence was significantly higher ($p = 0.002$) in adults compared to calves and youth. According to sex, the prevalence was significantly higher ($p = 0.009$) in females, while according to origin, the prevalence was significantly higher ($p = 0.00004$) in households compared to farming system (Table 2). Buffaloes from household system were 3.87 (OR 95% CI 1.58–9.47) more likely to present antibodies against *N. caninum*, comparing with farming system ($p = 0.003$).

Diaphragm digest, heart, and mesenteric lymph node samples were negative by conventional PCR. By means of nPCR, 6 (8.1%) of diaphragm sample digests were positive. Among these, three animals were serologically negative. The positive buffaloes by nPCR originated from all three age groups: calves ($n = 3$), youth ($n = 1$), and adults ($n = 2$) (Table 2).

The BLAST analysis revealed five unique sequences, which were 97–100% identical to the Liverpool strain of *N. caninum* (Accession umber LN714488). The sequences were placed into the Genbank database, under the accession numbers MK012391–MK012395. Our sequences are clustered in a single clade, together with three other *N. caninum* isolates, originating from Europe and Asia (Fig. 1).

Discussion

In the present study, we aimed to find out the prevalence of neosporosis by indirect (ELISA) and direct (PCR) detection techniques in buffaloes from Northwestern Romania. Several studies report the seroprevalence of *N. caninum* infection in buffaloes worldwide (Supplementary file), but only one presents data about parasite direct detection in naturally infected buffaloes (Rodrigues et al. 2004).

The seroprevalence (68.5%) of anti-*N. caninum* antibodies in buffaloes from Romania is similar to other values reported worldwide (Supplementary file). Regardless of the utilized method (IFAT, ELISA, DAT), most of the studies (21/29) reported a seroprevalence of over 30%, with the highest values being reported in Australia (88.3%) (Neverauskas et al. 2015), Brazil (up to 88%) (Chryssafidis et al. 2015), and Egypt (68%) (Dubey et al. 1998). Comparing with other studies from Europe done in Italy (34.6–51.0%) (Guarino et al. 2000;

Table 2 Frequency and prevalence of *N. caninum* antibodies in sera and *N. caninum* DNA in diaphragm digest, stratified by age category, gender and breeding system

	ELISA			nPCR (diaphragm digest)		
	Frequency	Prevalence (95% CI)	*p*	Frequency	Prevalence (95% CI)	*p*
Age category						
Calves ($n = 25/9$)	12	48.0 (27.8–68.7)	0.002	3	33.3 (7.5–70.1)	0.009
Youth ($n = 52/9$)	30	57.7 (43.2–71.3)		1	11.1 (0.3–48.2)	
Adults ($n = 120/56$)	93	77.5 (69.0–84.6)		2	3.6 (0.4–12.3)	
Gender						
Males ($n = 25/14$)	11	44.0 (24.4–65.07)	0.009	3	21.4 (4.7–50.8)	0.14
Females ($n = 172/60$)	124	72.1 (64.76–78.65)		3	5.0 (1.0–13.9)	
Breeding system						
Household ($n = 166/66$)	124	74.7 (67.38–81.12)	0.00004	6	9.1 (3.4–18.7)	0.84
Farming ($n = 31/8$)	11	35.4 (19.23–54.63)		0	0 (0.0–36.9)	
Total	135	68.5 (61.55–77.94)		6	8.1 (3.0–16.8)	

Auriemma et al. 2014) and Czech Republic (9.1%) (Sedlák and Bártová 2006), the seroprevalence in our study was higher.

According to age category, in our study, the prevalence was significantly higher in adult animals compared to young ones. Similarly, some other authors noted a significant correlation between the age and number of positive animals (Guarino et al. 2000; Moore et al. 2014; Supplementary file). Indeed, older age would imply a potentially longer contact with contaminated water and/or feed, which would account for the higher prevalence. Interestingly, the seroprevalence in calves was high (48.0%). This may be due to trans-placental transmission (Guarino et al. 2000) or to persistence of maternal antibodies. *N. caninum* maternal antibodies can persist until up 21 weeks of age (Cardoso et al. 2008).

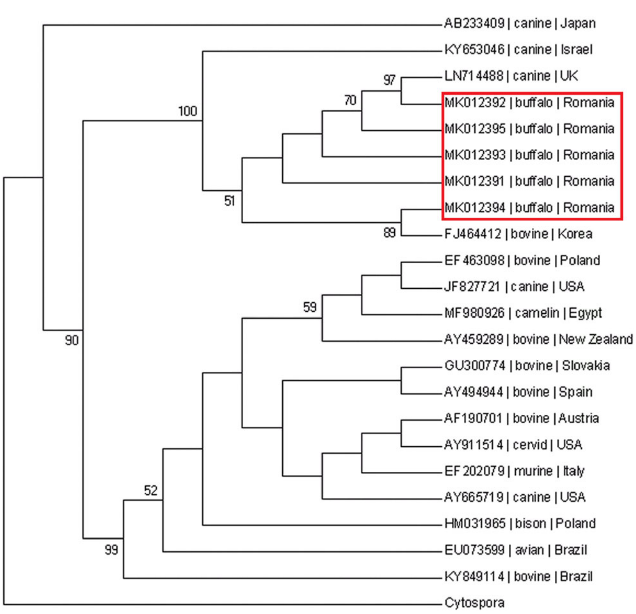

Fig. 1 Maximum parsimony bootstrap consensus tree inferred from 1000 replicates. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. For each *N. caninum* sequence, the Accession number, isolation source, and country are provided

The risk factor (OR 3.87) identified in the present study is the rearing system, with a significantly higher seroprevalence in household animals (74.7%) compared to those originating from farm (35.4%). This can be explained by the permanent contact between the home-raised buffaloes and definitive hosts as contamination source.

The direct detection of *N. caninum* DNA in naturally or experimentally infected buffaloes has been reported by a limited number of studies based on low sample sizes (Rodrigues et al. 2004; Chryssafidis et al. 2014, 2015). In our study, six diaphragm samples were identified as being positive by nested PCR. Among them, three animals were serologically negative. These results show a discrepancy between the presence of *N. caninum*-specific antibodies and the actual detection of the parasite. Also, the probability of direct detection of *N. caninum* in seropositive animals compared to seronegative ones seems to be similar. The present study reveals a high seroprevalence of *N. caninum*-specific antibodies in buffaloes in Northwestern Romania and indicates no correlation between the presence of antibodies and the occurrence of parasites in this species. To our best knowledge, this is the first study in Romania regarding the prevalence of *N. caninum* in buffaloes.

Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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