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Seroepidemiological study of *Neospora caninum* in equids using c-ELISA in Erbil Province, Iraq

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Abstract: Equine neosporosis is an intracellular protozoan disease with a global distribution, affecting a diverse range of warm-blooded animals. *Neospora caninum* Dubey, Carpenter, Speer, Topper et Uggla, 1988 is associated with foetal loss, neurological disease and abortion in equids. No information was available regarding equine *N. caninum* infection among equids in Iraq. Thus, the aim of this study was to determine the prevalence rate of *N. caninum* in equines by using a competitive enzyme-linked immunosorbent assay (c-ELISA). A total of 329 blood samples randomly selected from equines, comprising 268 horses and 61 donkeys were examined. The seroprevalence rate of *N. caninum* was determined as 46% (28/61) for donkeys and 24% (64/268) for horses. The prevalence of *N. caninum* indicated a significantly higher risk of infection in donkeys compared to horses ($P < 0.001$). However, the odds of *N. caninum* infection in draught equids were 8.2 times greater than other equids with a significant difference ($P < 0.001$). The current study revealed no significant differences in the prevalence of *N. caninum* across various genders, breeds, clinical statuses, disease histories and among equids that had contact with dogs. While outdoor feeding and mixed (grazing), showed a significant difference ($P = 0.003$) and ($P = 0.75$), respectively, in the presence of antibodies against *N. caninum* compared to indoor feeding (stable). Moreover, the odds of infection in equids with a history of late abortion were 4.8 times higher than those without such a history of abortion (2.20–10.56) with statistical significance ($P < 0.001$).

Keywords: Neosporosis, seroprevalence, risk factors, equines.

Equine neosporosis is an obligate intracellular protozoan disease with global distribution, caused by a cyst-forming coccidian parasite *Neospora caninum* Dubey, Carpenter, Speer, Topper et Uggla,1988 (Dubey et al. 2003, 2007). In horses and donkeys, *N. caninum* associated with preterm deliveries, stillbirths, foetal malformations, neonatal mortality and abortion (Gharekhani et al. 2013, Leszkowicz Mazuz et al. 2020). This parasite is prevalent among equids worldwide and can cause neurological disorders and diseases of reproductive system (Vardeleon et al. 2001, Javanmardi et al. 2020, Leszkowicz Mazuz et al. 2020).

Neospora caninum has a multi-host life cycle, where the sexual stage of the parasite takes place in the intestines of dogs and wild canids, and asexual reproduction results in formation of cysts in the tissues of domestic livestock (Marsh et al. 1999, Pitel et al. 2001, Veronesi et al. 2008). However, this parasite is kept in the existence by horizontal and vertical transmission. While congenital transmission plays a significant role in maintaining the presence of *Neospora* in their descendants, neosporosis-induced abortions might span across multiple generations (Dubey and Lindsay 1996, Dubey 2003). The prevalence of *Neospora* spp. exposure has been extensively documented in asymptomatic horses (Dubey et al. 2017).

Several studies reported on *N. caninum* infection in equine population in many parts of the world such as in South America with positivity (2.5–15 %) (Hoane et al. 2006), 2% in New Zealand (Vardeleon et al. 2001), 1–28 % in Europe (Pitel et al. 2001, Ciaramella et al. 2004, Jakubek et al. 2006, Bártová et al. 2010, Bártová et al. 2015, Cruz et al. 2019), 70% in Israel (Tirosh-Levy et al. 2020), 3% in Jordan (Talafha et al. 2015) and 40.8% in Iran (Gharekhani and Heidari 2014).

Several laboratory methods have been employed for diagnosing *N. caninum*. Serological assays including immunoblotting (IB), direct agglutination tests, indirect fluorescent antibody test (IFAT) and an array of ELISAs are used to identify specific antibodies in the blood sera of affected animals (de Waal 2012). For epidemiological studies, a c-ELISA test with high sensitivity and specificity has been developed for the detection of *N. caninum* infection (Hiasa et al. 2012, Zhou et al. 2017, Pagmadulam et al. 2018).

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Table 1. Relative risk factors associated with seropositivity to infection with *Neospora caninum.*

Factor	No. of equine tested	Positive (%)	*N. caninum* OR (95%CI)	*P-Value*	X^2
Type of equine					
Donkey	61	28 (46%)	1		< 0.001
Horse	268	64 (24%)	0.36 (0.20–0.65)	< 0.001	
Gender					
Female	211	62 (29%)	1		0.441
Male	118	30 (25%)	0.82 (0.49–1.36)	0.443	
Age group					
< 3 Years	52	9 (17%)	0.35 (0.16–0.77)	0.01	
3–10 Years	153	57 (37%)	1		0.002
> 10 Years	124	26 (20%)	0.44 (0.25–0.76)	0.004	
Breed					
Thoroughbred	79	22 (28%)	1.0 (0.57–1.90)	0.88	
Crossbreed	163	44 (27%)	1		0.88
Other breeds	87	26 (30%)	1.1 (0.64–2.04)	0.62	

OR – odds ratio; CI – confidence interval; X^2 – Chi square; Thoroughbred – originating in England; Crossbreed – breeding between two different breeds or types of horses; Other breeds – includes Arabians; quarter horses and local horses.

Although there is lack of data regarding the prevalence of *N. caninum* infection in equids in Iraq. Therefore, this study was carried out to identify the prevalence of such an infection in naturally exposed equids with respect to various determinants using c-ELISA.

MATERIALS AND METHODS

Sample collection and processing

This study was carried out under the supervision and regulations of the Ethic Committee at College of Veterinary Medicine, Salahaddin University, Iraq. This research was conducted between 2022 and 2023 in different geographical regions within Erbil governorate. A total of 329 blood samples randomly selected from equines, comprising 268 horses and 61 donkeys. Jugular vein blood samples were collected using anticoagulant free sterile vacutainers® tubes, then placed on ice and transported to the laboratory. After centrifugation at 3,000 rpm for 15 minutes, serum was separated from the coagulated blood and stored at (-20 °C) for subsequent analysis. Animal data, including type of equine, gender, age category, breeds, contact with dogs, management, purpose of keeping, clinical status, history with previous diseased and previous history of abortion, were also recorded during this study.

All serum sample obtained from horses was examined for the presence of *Neospora caninum* antibodies using a commercially available cELISA test kit (ID. Vet, Grabels, France) methods following the manufacturer's instructions. For each specimen, the S/P (%) ratio was computed by dividing the optical density of the tested serum by the average OD of the positive control, as indicated by the formula: S/P (%) = (OD sample/OD positive control) × 100. Samples exhibiting an S/P (%) value of ≥ 50% were categorised as positive.

Statistical analysis

The seropositive analysis of *N. caninum* infection was analysed using X^2 and Fisher's exact test to distinguish the occurrence rates among different groups. Binomial logistic regression in GenStat 12th Edition (https://genstat.kb.vsni.co.uk/knowledge-base/hnewr12/) was employed to compute the odds ratio and determine the 95% confidence intervals for prevalence values, assessing the impact of risk factors such as type of equine, gender, age category, purpose of keeping, management and history of abortion. Variables were considered statistically significant when ($P \leq 0.05$).

Table 2. Management and purpose of keeping factors associated with seropositivity to infection with *Neospora caninum*

Factors	No. of equine tested	Positive (%)	*N. caninum* OR (95% CI)	*P-Value*	X^2
Contact with dogs					
No	41	8 (20%)	1		0.18
Yes	288	84 (29%)	1.7 (0.75–3.83)	0.2	
Management					
In grazing	93	29 (31%)	1		
In stable	79	11 (14%)	0.35 (0.16–0.77)	0.009	0.003
Mixed	157	52 (33%)	1.0 (0.63–1.89)	0.75	
Purpose of keeping					
Breeding	37	8 (22%)	1.18 (0.47–2.92)	0.723	
Recreation	116	22 (19%)	1		< 0.001
Racing	87	18 (21%)	1.11 (0.56–3.27)	0.76	
Draught	89	44 (49%)	4.18 (2.24–7.79)	< 0.001	

OR – odds ratio; CI – confidence interval; X^2 – Chi square; Draught horses are commonly used in harness for heavy work.

RESULTS

This seroprevalence study investigated four variables: type of equine, gender, age group and breeds (Table 1). The analysis of our collected data revealed that 28 (46%) of donkey and 64 (24%) of horses were seropositive for neosporosis. *Neospora* antibodies were found in 62 (29%) female and 30 (25%) males. The following seroprevalences were found in age groups: 9 (17%) for those < 3 years, 57 (37%) for those 3–10 years, and 26 (20%) for those > 10 years. Table 1 displays the results of the multivariable logistic regression analysis. Donkeys exhibited a significantly higher risk of *Neospora* spp. infection compared to horses ($P < 0.001$). The seroprevalence of *Neospora* antibodies in equines varied significantly across age groups: 37% in those aged 3–10 years, which was significantly higher ($P < 0.01$) than the 20% in those over 10 years and 17% in those under 3 years. However, the prevalence of *N. caninum* did not significantly differ between genders ($P = 0.443$) or breeds ($P = 0.88$).

Table 2 shows that the odds of *N. caninum* infection in equids in contact with dogs were 1.7 times greater than in those not in contact with dogs. However, this difference was not statistically significant ($P = 0.2$). Outdoor feeding (grazing) and mixed grazing exhibited a significant difference ($P = 0.003$) and ($P = 0.75$) in the presence of antibodies against *N. caninum* compared to indoor feeding (stable). It should also be noted that the odds infection of *N. caninum* in draught equids are 4.2 times greater than in other equids, with CI (2.24–7.79), and this difference is statistically significant ($P < 0.001$).

The results summarised in Table 3 showed that the odds of infection in equids with a history of late abortion were 4.82 times higher than in equids without a history of abortion, with CI (2.20–10.56) and a significant differ-

Table 3. Clinical status and history of abortion factors associated with seropositivity to infection with *Neospora caninum*

Factors	No. of equine tested	Positive (%)	*N. caninum* OR (95% CI)	*P-Value*	X^2
Clinical status					
Clinically healthy	298	85 (28.5%)	1		
Clinically ill	31	7 (22.5%)	0.7 (0.30–1.70)	0.48	0.48
History of previous neosporosis infection					
No	111	29 (26.1%)	1		
Yes	218	63 (28.9%)	1.14 (0.68–1.92)	0.59	0.59
History of previous abortion					
No abortion	149	31 (20.8%)	1		
Early abortion	28	12 (42.8%)	2.85 (1.22–6.65)	0.015	<0.001
Late abortion	34	19 (55.9%)	4.82 (2.20–10.56)	<0.001	

OR – odds ratio; CI – confidence interval; X^2 – Chi square; Clinically ill – equines exhibit general clinical signs of disease.

ence (P < 0.001). Additionally, the seropositivity rate was found to be 2.9 times higher in equids with a history of early abortion (42.8%) compared to those without a history of abortion (20.8%) (P = 0.015). According to the present study, statistical analyses revealed no significant differences in seropositivity to *Neospora* spp. among categories based on clinical status (P = 0.48) and history of previous disease (P = 0.59).

DISCUSSION

This study represents Iraq's inaugural serological survey on antibodies against infection with *Neospora caninum* and investigates the risk factors for equine neosporosis. It found a higher seroprevalence rate in donkeys (46%) compared to horses (24%). This disparity is likely due to behavioural differences, such as donkeys grazing closer to the ground and increased potential exposure to contaminated grass or soil (Dubey and Schares 2011, Gennari et al. 2016). Additionally, variations in water sources, habitat preferences and innate immune responses may contribute, with donkeys possibly having distinct immune profiles or genetic predispositions (Saqib et al. 2015). Environmental factors, including management practices and hygiene standards, further influence exposure risks, as donkeys often inhabit less controlled environments (Javanmardi et al. 2020). Species-specific differences and regional variations in seroprevalence highlight the complex interplay of factors affecting *N. caninum* infection rates (Santolaria et al. 2011).

Regarding sex differences, the data revealed a higher prevalence of antibodies against *N. caninum* in female equines (29%) compared to males (25%). This suggests that females may be more susceptible, potentially due to hormonal variations during the estrous cycle influencing their immune responses. (Dubey et al. 2003). Behavioural differences, such as grazing habits during estrus, may also increase females' exposure to oocysts of *N. caninum* (Talafha et al. 2015, Selim et al. 2020a). Additionally, environmental management and exposure levels could contribute to these gender-specific differences (Selim et al. 2020b). However, the precise reasons for these disparities are not fully understood, underscoring the need for more comprehensive studies to investigate gender-specific factors influencing *N. caninum* susceptibility in equines.

Furthermore, the seroprevalence of antibodies against *N. caninum* varies significantly across different age groups of equines: 17% in those < 3 years, 37% in those 3–10 years, and 20% in those > 10 years. These variations suggest age-related differences in susceptibility. Younger equines may have lower exposure due to shorter lifespans and potential maternal immunity, while horses aged 3–10 years, more active and exposed to outdoor environments, show the highest seroprevalence. Older equines (> 10 years) exhibit lower seroprevalence, possibly due to acquired immunity or reduced outdoor activity (Kligler et al. 2007, Karatepe and Karatepe 2012). Environmental factors and management practices also influence these patterns. Further research is needed to understand the mechanisms driving these age-related differences and to inform targeted control measures.

The higher prevalence of *N. caninum* infection in working equids compared to breeding, recreation and racing equids, although not statistically significant (4.2 times higher), suggests potential differences in environmental exposures and management practices. Working equids may face increased exposure to contaminated environments and less controlled grazing conditions, contributing to higher infection rates (Gharekhani and Heidari 2014, Bártová et al. 2015). Variations in nutritional and health management practices between these groups could also influence susceptibility. Further research with larger sample sizes and detailed epidemiological studies is needed to better understand these trends and identify specific risk factors contributing to *N. caninum* infection in different equid populations. Moreover, no significant differences in the prevalence rates of neosporosis were found between equids that had contact with dogs and those without such contact. *Neospora caninum*, the causative agent, is transmitted through ingestion of oocysts shed in dog faeces, contaminating shared environments. This environmental exposure appears to play a significant role in infection dynamics, regardless of direct contact between equids and dogs (Vanleeuwen et al. 2010, Dangoudoubiyam et al. 2011).

Additionally, outdoor feeding and mixed grazing showed significant differences in the presence of antibodies against *N. caninum* compared to indoor feeding, with a P-value of 0.75 and 0.09, respectively. The odds of infection in equids with a history of abortion were approximately 4.8 times higher than in equids without such a history, with significant differences (P < 0.001). This finding aligns with other research (Alshammari et al. 2003, Leszkowicz Mazuz et al. 2020), which reported significantly higher seroprevalence of *N. caninum* in grazing equines and aborting mares compared to those kept indoors and healthy equines. The preferential outdoor feeding of horses might expose them to contamination with *N. caninum* oocysts, increasing the likelihood of infection and the consequent risk of abortion (Jakubek et al. 2006, Kligler et al. 2007). Identifying different risk factors and understanding their contribution to disease transmission and epidemiology are essential for formulating and executing effective measures to manage equine neosporosis.

In conclusion, this study provides data on the seroprevalence of antibodies against *N. caninum* in equines in Iraq, indicating significant differences based on species, sex, age and environmental factors. Understanding these dynamics is vital for developing targeted strategies to mitigate the risk of infection with *N. caninum* and its impact on equine health.

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