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Article

Detection of anti-*Neospora* spp. antibodies associated with different risk factors in horses from Mexico

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Abstract:

Neospora spp. is a protozoan parasite that causes abortions and diseases in the Central Nervous System (CNS) of several domestic and wild animal species. In horses, this parasite causes abortions, neonatal mortality, and CNS diseases. The *Neospora* species identified in horses is different from *Neospora caninum* and is called *Neospora hughesi*. This study aimed to detect the presence of anti-*Neospora* spp. antibodies associated with different risk factors in horses from Mexico. Risk factors were identified by surveying each stable and individual animal from four

different regions (Center, North, West, and South). A total of 684 serum samples were obtained from horses in the different regions, 52.3 % (358) males and 47.7 % (326) females. Samples were subjected to an indirect immunofluorescence (IIF) assay; results were analyzed to estimate the association between seropositivity and risk factors. The seroprevalence of *Neospora* spp. was 2.34 %. The positive cases were mainly found in three of the four regions included in this study and were significantly associated with anti-*Neospora* spp. antibodies. The coexistence of the horses with other animals obtained an OR value of 2.34 (95% CI : 0.28 - 19.0; $P<0.04$). This study concludes that *Neospora* spp. is present in horses from Mexico.

Key words: *Neospora hughesi*, *Neospora* spp., Horses, Risk factors.

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Introduction

The first case of neosporosis reported in horses was that of a late-term fetus and placenta infected with *Neospora caninum*[1]. The second case corresponded to a one-month-old foal with congenital blindness and neurological disorders[2]. Molecular, antigenic, and structural studies have shown that the species found in horses with neurological problems does not correspond to the one reported, identifying it as *Neospora hughesi*[3]. *N. caninum* and *N. hughesi* are obligate intracellular protozoa. These species belong to the phylum Apicomplexa, class Sporozoea, order Eucoccidiida, and family Sarcocystidae[4]. In horses, *N. caninum* has been associated with abortions and reproductive problems[5-8], and *N. hughesi* with neurological diseases[9-11] and *Sarcosystis neuronae*, the causal agent of the equine protozoal myeloencephalitis (EPM). EPM is a severe neurological disease that produces significant losses in equine production; this has been mainly reported in the United States[12].

The definitive hosts of *N. caninum* are domestic dogs[13], coyotes[14], Australian dingoes[15], and gray wolves[16]. Additionally, several domestic and wild animal species have been identified as intermediate hosts[17]. However, the definitive hosts of *N. hughesi* are still unknown, and horses are considered the only potential intermediate host[18]. Infection can occur after ingesting sporulated oocysts in contaminated feed or water. Vertical transmission is currently considered only as an alternative route[19,20].

The serological techniques used to detect anti-*Neospora* spp. antibodies are indirect immunofluorescence (IIF), enzyme-linked immunoassay (ELISA), *Neospora* agglutination test (NAT), and Western blot (WB). Additionally, *N. caninum* tachyzoite antigens are used to evaluate seropositivity for *Neospora* spp. due to the cross-reaction of this parasite[21]; DNA-based techniques are used to differentiate between *N. hughesi* and *N. caninum*[22]. This study aimed to detect the presence of anti-*Neospora* spp. antibodies associated with different risk factors in horses from Mexico.

Material and methods

Area of study

This study was carried out in the Central, North, West, and South regions of Mexico (Figure 1). Regions were selected according to their geographic and climatic characteristics.

Figure 1: Geographic division of Mexico by study region

Study design

A transversal epidemiological study was conducted from October 2016 to October 2017. This study consisted of two stages: field and laboratory. During the field stage, 5 mL of venous blood were collected in Vacutainer® tubes without anticoagulant (BD Vacutainer®) by puncturing the jugular vein of apparently healthy horses. A total of 684 samples were collected; 75 corresponded to the Central region (10.96 %), 54 to the North region (7.89 %), 298 to the West region (43.57 %), and 257 to the South region (37.57 %).

All horses were subjected to a physical exam to determine their health status. Additionally, a survey was applied to the owners to collect information about the general characteristics of the animals (breed, age, sex, and reproductive status) and the specific management of the stable (use of horses, feed, housing, water source, and contact with other animals). Blood samples were transported under refrigeration at 4 °C. In the laboratory, samples were centrifuged at 3,500 rpm for 10 min to separate the serum, which was then transferred to 1.5-mL microtubes and stored at -20 °C until further analysis.

Serologic test

The IIF assay was performed using a commercial kit to detect the circulating IgG antibodies against *Neospora* spp. This kit (SLD-IFA-NC) employs slides antigenated with *N. caninum* tachyzoites (strain NC-1) and an anti-equine IgG-fluorescein isothiocyanate conjugate (FITC-Conjugate VMRD). Serum samples were diluted 1:25 in phosphate-buffered saline (PBS). Positive and negative controls were used as standards. The assay was performed following the manufacturer's instructions. All sera that fluoresced at the initial dilution were considered positive and were further diluted to the titer endpoint. The highest serum dilution showing fluorescence was considered the endpoint titer.

Data analysis

The data obtained through the surveys (independent variables) and the presence of anti-*Neospora* spp. antibodies (dependent variable) were analyzed using the statistical software STATA, version 10. The seroprevalence distribution was obtained with the independent variables, and the association between the seroprevalence of *N. caninum* in horses and other animals was obtained using a logistic regression model (Chi-square), considering $P<0.05$. Finally, the odds ratios (OR)

between the positive results, the negative results, and the relationship with the confidence interval were calculated.

Results

The presence of anti-*Neospora* spp. antibodies was detected in serum samples obtained from horses using the IIF technique (*N. caninum* NC-1). Serum samples with titers at a 1:50 dilution were considered positive. The overall seroprevalence was 2.34 % (16/684).

The reproductive status variable was analyzed based on the total number of animals seropositive for *Neospora* spp. A total of 50 % (8/16) of the animals had anti-*Neospora* spp. antibodies at different dilutions, greater at the cut-off point (1:50). Table 1 shows that in entire males (4/16) the maximum dilution was 1:200; however, pregnant females (3/16) reached dilutions of 1:200, two of them reached 1:400.

Table 1: Percentage of anti-*Neospora* spp. antibody titers according to serial dilutions using IIF technique

Animals	**Titers**			
	1:50	**1:100**	**1:200**	**1:400**
Castrated	18.75 (3/16)	-	-	-
Entire	25.00 (4/16)	18.75 (3/16)	6.25 (1/16)	-
Filly	6.25 (1/16)	-	-	-
Mare	12.50 (2/16)	-	-	-
Pregnant	18.75 (3/16)	18.75 (3/16)	18.75 (3/16)	12.50 (2/16)
Empty	6.25 (1/16)	-	-	-
Recent foaling mare	12.50 (2/16)	12.50 (2/16)	-	-
Total	100.0 (16/16)	50.00 (8/16)	25.00 (4/16)	12.50 (2/16)

The survey variables included in the study were analyzed to identify possible risk factors associated with the presence of anti-*Neospora* spp. antibodies. Table 2 shows the results obtained from this analysis; the highest number of positive cases (62.5 %) was observed in the horses from the West region, 6.25 % corresponded to the Central region, and 31.25 % to the South. There were no positive cases in the North region. This variable had no statistical significance ($P<0.05$). Of the positive animals, 43.75 % were male and 56.25 % female. The variable of coexistence of horses with other animals obtained an OR of 2.34 (95% CI: 0.28 - 19.0; $P=0.04$). The use of horses and housing variables had a *P*-value <0.20. After analysis with logistic regression, the housing variable had an OR of 3.12. The age, breed, reproductive status, and water source variables were not statistically significant ($P>0.05$).

Table 2: Distribution of *Neospora* spp. in positive and negative sera and associated factors of horses from Mexico

Variable	Samples		*Neospora* spp.		*P*-value	OR (95% CI)
	N	%	+	-		
Region:						
Central	75	10.96	1 (6.25)	74		
North	54	7.89	0 (0.00)	54	0.36	79 (0.49-1.29)
West	298	43.57	10 (62.50)	288		
South	257	37.57	5 (31.25)	252		
Sex:						
Male	358	52.34	7 (43.75)	351	0.49	0.45 (0.03-6.18)
Female	326	47.66	9 (56.25)	317		
Age:						
Young (7-24 m)	68	9.94	1 (6.25)	67	0.21	1.09 (0.47-2.51)
Adult (25-48 m)	375	54.82	10 (62.50)	365		
Old (>48 m)	241	35.23	5 (31.25)	236		
Breed:						
Creole	262	38.30	6 (37.50)	256	0.68	1.09 (0.22-5.40)
Pure	422	61.70	10 (62.50)	412		
Reproductive status:						
Entire	293	42.84	4 (25.00)	289		
Castrated	65	9.50	3 (18.75)	62		
Filly	26	3.80	1 (6.25)	25		
Mare	121	17.69	2 (6.25)	119	0.13	0.69 (0.32-1.49)
Pregnant	53	7.75	3 (18.75)	50		
Empty	115	16.81	1 (6.25)	114		
Recent foaling mare	11	1.61	2 (12.50)	9		
Use of horses:						
Racing	329	48.10	6 (37.50)	323	0.10	0.25 (0.04-1.35)
Not racing	355	51.90	10(62.50)	345		
Feed:						
Forage	325	47.51	9 (56.25)	316	0.06	1.14 (0.27-4.76)
Grazing	269	39.33	4 (25.00)	265		
Mixed	90	13.16	3 (18.75)	87		
Housing:						
Stable	340	49.71	8 (50.00)	332	0.20	3.12 (0.49-21)
Paddock	344	50.29	8 (50.00)	336		
Coexistence with other animals:						
Yes	598	87.43	15 (93.75)	583	0.04	2.34 (0.28-19)
No	86	12.57	1 (6.25)	85		
Water sources:						
Open pit	341	49.85	8 (50.00)	333	0.78	0.86 (0.31-2.41)
Water bank	343	50.15	8 (50.00)	335		
Total	684	100.00	16 (100.00)	668	-	-

Discussion

The seroprevalence reports of neosporosis in horses vary worldwide[23-26]. This study, reported a seroprevalence for anti-*Neospora* spp. antibodies of 2.34 %. Other studies have showed similar results. In Brazil, a study reported a seroprevalence of 2.5 % and 4.1 % of *Neospora* spp.[6,18]; 3.5 % in Costa Rica; 3.7 % in Nineveh, Iraq; and 3% in Durango, Mexico, for the presence of anti-*N. hughesi* antibodies[27-29]. However, some studies have reported higher values for anti-*Neospora caninum* antibodies; 34 % in the United States, 20 % in Iran, 48.27 % in Brazil, and 12 % in Peru[23-26]. The detection of antibodies against *Neospora* spp. contributes to the epidemiological information of this disease in Mexico and horses, representing an important production system in livestock farms. The seroprevalence of *Neospora* spp. in females and males was 2.7 % and 2.0 %, respectively. Studies in Brazil reported a seroprevalence of 4.3 % in females and 3.7 % in males[6], while in Israel, a study reported 10.9 % in females and 13.0% in males[8]. These results coincide with those reported in Brazil, which despite not being statistically significant, it should be noted that both females and males have a similar risk of infection.

The horses in this study were from different regions in Mexico. The West and South regions had the highest percentage of horses with anti-*Neospora* spp. antibodies compared to the Central and North regions. Similar studies in Brazil reported differences in the coastal (5.6 %) and mountain (2.6 %) regions[6]. In Jordan, the presence of *Neospora* spp. in horses varies across regions[30]. This suggests that the geographic localization and the climate of each region influence the presence of *Neospora* spp. in horses.

The OR in this study was 1.1 for the variables of age, breed, and feed. There were no statistical differences. These results are similar to those reported in Israel[8], Brazil[25], Jordan[30], and Italy[31] and suggest that the increase of these variables, although not statistically different, augments the probability of infection by *Neospora* spp. As for the housing variable, the OR obtained in this study was 3.12, similar to that reported in Israel[8], which means that housing is an important risk factor that increases the probability of infection with *Neospora* spp. The variable of coexistence with other animals was associated with the presence of *Neospora* spp., the OR= 2.34 (P<0.05), which coincides with several studies in Brazil that analyzed the same variable with an OR= 1.35 (P<0.05)[5,6,25]. These results indicate that coexistence with other animals increases the risk of infection with *Neospora* spp.

Conclusions and implications

In this study, was detected anti-*Neospora* spp. antibodies in horses from different regions in Mexico; this demonstrates the presence of Neosporosis in equine farms. None of the positive cases showed characteristic clinical signs of the disease. The coexistence with other animals was the risk factor with the greatest association to seropositivity. However, it is necessary to perform an analysis considering the different categories of each variable. Further studies are required to identify the definitive host of *Neospora* spp. and its transmission mechanisms to horses. Additionally, it is essential to identify the *Neospora* species that affects horses.

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