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Original Article

Age, gender and climate associations with the seroprevalence of *Neospora* species infection in horses in Jordan

Idade, sexo e associações climáticas com a soroprevalência da infecção por *Neospora* em cavalos da Jordânia

Marwan Abu-Halaweh[1]; Mahmoud N. Abo-Shehada[2]*; Raida Khalil[1]

[1]Department of Biotechnology and Genetic Engineering, Faculty of Scienctable, Philadelphia University, Jerash, Jordan
[2]Faculty of Epidemiology and Population Health, London School of Hygiene and Tropical Medicine, University of London, London, UK

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Abstract

A cross-sectional study was carried out on a sample of 379 horses to determine the seroprevalence of *Neospora* spp. in Jordan using the indirect fluorescent antibody test. Five variables, namely locality (n=10), climatic zone (n=4), age group (n=3), gender, and breed were tested as risk factors for *Neospora*–immunoglobulin (Ig)G seropositivity at four cutoff titers (1:50, 1:200, 1:400, and 1:800) using univariate and multivariate logistic regression analyses. A total of 122 (32%; 95% CI: 28, 37) sera samples had anti-*Neospora*–IgG at a cutoff titer of 1:50. Increased *Neospora*–IgG seropositivity was found in horses in three localities (Madaba, Zarka, and Petra) and was associated with the following variables: cool temperate climate; age >14 years; and female gender. Seropositivity was found among horses from Madaba at all cutoff titers, Zarka at titers >1:200, and Petra at titers <1:200. Cool temperate climate was associated with titers <1:400. Horses aged >14 years were found to be associated with seropositivity at titers ≥1:200. Female gender was associated with high seropositivity at >1:800.

Keywords: *Neospora* spp., horse, seroprevalence, risk factors, climate, Jordan.

Resumo

Um estudo transversal foi realizado, na Jordânia, em uma amostra de 379 cavalos, para determinar a soroprevalência de *Neospora* spp., usando-se o teste de anticorpos fluorescentes indiretos. Cinco variáveis: localidade (n=10), zona climática (n=4), grupo etário (n=3), sexo e raça, foram testadas como fatores de risco para soropositividade para *Neospora*-imunoglobulina (Ig)G, considerando-se quatro pontos de corte (1:50, 1:200, 1:400 e 1:800) por meio de análises de regressão logística univariada e multivariada. Um total de 122 (32%; 95% CI: 28, 37) amostras de soros apresentaram anti-*Neospora*-IgG, utilizando-se como ponto de corte o título de 1:50. Cavalos de três localidades apresentaram aumento da soropositividade para *Neospora*-IgG (Madaba, Zarka e Petra) o que foi associado às seguintes variáveis: clima temperado fresco; idade >14 anos; e sexo feminino. Os cavalos de Madaba apresentaram soropositividade em todos os títulos utilizados como ponto de corte; os cavalos de Zarka em títulos >1:200; e os cavalos de Petra em títulos <1:200. O clima temperado fresco foi associado aos títulos <1:400. Cavalos com idade >14 anos estiveram associados à soropositividade nos títulos ≥1:200. O sexo feminino esteve associado à alta soropositividade nos títulos >1:800.

Palavras-chave: *Neospora* spp., cavalo, soroprevalência, fatores de risco, clima, Jordânia.

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*Corresponding author: Mahmoud N. Abo-Shehada. E-mail: mahmoud.abo-shehada@lshtm.ac.uk

Introduction

Neospora is a coccidian parasite of animals. The genus *Neospora* has two species: *N. caninum* and *N. hughesi*. *N. caninum* is a well-known parasite of domestic and wild animals. It was first isolated from canines that suffered from inflammatory lesions of the skeletal muscles and central nervous system (Anderson et al., 1991; Barr et al., 1994; Dubey & Lindsay, 1996; Dubey et al., 2007; Donahoe et al., 2015; Dubey et al., 1988). *N. caninum* is an obligate intracellular pathogen that is capable of utilizing a wide range of animals as intermediate hosts (Dubey et al., 2007; Silva & Machado, 2016). Cattle, sheep, horses, goats, foxes, deer, buffaloes, and camels are its natural intermediate hosts, with cats, mice, rats, gerbils, and monkeys serving as experimental intermediate hosts (Dubey et al., 2007). Dogs have been identified as both intermediate and definitive hosts of this parasite (Dubey et al., 2007). Dogs are likely to become infected through the ingestion of contaminated tissues, including aborted fetuses, dead calves, or placentas of infected animals. In addition to domestic and wild dogs (Mcallister et al., 1998), other Canids including grey wolves (Dubey et al., 2011), coyotes (Gondim et al., 2004), British red foxes (Bartley et al., 2013), and Australian dingoes (King et al., 2010) are hosts that shed oocysts in the feces, which can serve as a major source of infection for other species. Horizontal and vertical transmissions of *N. caninum* have been reported in cattle, goats, and sheep (Dubey et al., 2007; González-Warleta et al., 2018). A direct association between *N. caninum* with spontaneous abortion was reported in naturally infected cattle, sheep, goats, (González-Warleta et al., 2018; Dubey & Schares, 2011; Ghalmi et al., 2011) and horses (Anderson et al., 2019; Leon et al., 2012; Portella et al., 2017; Veronesi et al., 2008).

Conversely, *N. hughesi* is a poorly known parasite with an unknown life cycle, host range, and infection prevalence, and a definitive host has not yet been determined (Hoane et al., 2005). Recently, serological cross-reactivity was found between *N. caninum* and *N. hughesi* (Gondim et al., 2009). Many serologic surveys of *N. caninum* have been performed without considering potential cross-reactions with *N. hughesi* (Gondim et al., 2009).

In Jordan, the seroprevalence for *N. caninum* was found at high rates in sheep, goats (Abo-Shehada & Abu-Halaweh, 2010), and cattle (Talafha & Al-Majali, 2013). Seroprevalence of *N. caninum* in horses in other countries of the Middle East were 0.3% Turkey (Zhou et al., 2017), 10% Saudi Arabia (Alanazi et al., 2014), to 32% Iran (Moraveji et al., 2011). This is a cross-sectional study investigates the seroprevalence of *Neospora* spp. infection among horses in Jordan and the possible association between locality, climatic zone, age, gender, and breed with *Neospora* spp. seropositivity at four cutoff titers.

Materials and Methods

Animals and study area

Horses in Jordan are bred for different purposes, including for agricultural activities, transport, racing, showing, breeding, and tourism, with estimated numbers of 5,250 horses in this region (Anon, 2007). The examined horses originated from the following 10 localities (Governorates): Irbid, Jerash, Zarqa, Mafraq, Ajlon, Jordan River Valley, Balqa, Amman, Madaba, and Petra, within four climatic zones (cool steppe, cool temperate rainy, warm temperate rainy and cool desert) Figure 1. Most horses live in individual pens (holdings) and small herds in stables.

Sample size determination

The prevalence of *Neospora* spp. infection among horses in Jordan has not been previously reported. However, a prevalence rate of 11.9% was reported from neighboring country (Kligler et al., 2007). According to Thrusfield (Thrusfield, 1995), based on an expected prevalence of 12%, the appropriate number of horses to be examined as part of this investigation is 267, as a 99% level of confidence and 5% absolute precision were required. Representative samples were selected according to the estimated density in each area. A total of 379 samples were collected. The study had at least 80% power at a 5% significance level to detect an odds ratio (OR) ≥2 for risk factors present in 50% of controls, and an OR ≥3 for those present in 20% of controls.

Horse sampling and data collection

During the period spanning April–September 2008, the main areas where horses are raised were visited, and at least 10%of the herd /holding were sampled systematically (i.e., the first one or two horses), and then every tenth animal (90% of holdings) was also sampled. On some occasions (5%), permission to sample was denied and the next

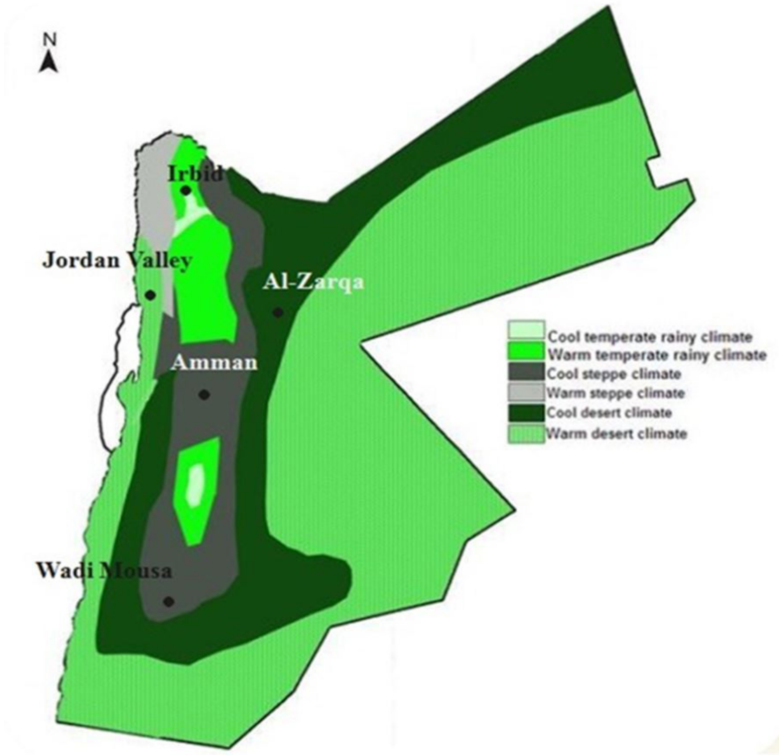

Figure 1. Map of Jordan colors show the different climate regions (Anon, 1984) and the locations were samples have been collected for this study. 1. Warm temperate rainy (Irbid and Jarash (%21)), 2. Cool steppe (Amman (%39)), 3. Cool desert (Zarqa (%38)), 4. Warm desert (Jordan valley), 5. Cool temperate rainy (Wadi Mousa, Petra (%9)), and 6. Warm steppe (not sampled).

holding was used. Herds and holdings (n= 268, ranged from 45 horses to one horse) were sampled once during the study period. Thus, 379 local horses were surveyed for *N. caninum*. Both male (46%) and female (45%) horses were sampled. The majority were of the local breed (93%) and the rest were thoroughbreds that were imported more than 5 years earlier. The horses ranged from 6 months to 22 years of age (quartiles: Q1, 5; Q2, 8; and Q3, 11.75 years) and were divided into three age groups (<8 years, 45%; 8–14 years, 42%; and >14 years; 13%).

Blood samples (n=379) were collected aseptically from the jugular vein in vacuum tubes. At the time of sampling, information was collected regarding the date of collection, locality, climatic zone, age, gender, and breed.

Serology

Sera were tested for anti *Neospora* spp IgG. Slides spotted with whole *N. caninum* (NC-1 strain) tachyzoites were purchased from VMRD, Inc. (Pullman, WA, USA). Cattle-positive sera and anti-cattle sera labeled with fluorescein were used until positive horse sera were found; then, positive horse sera were used. IgG polyclonal anti-serum anti-horse were produced in goats and conjugated with fluorescein isothiocyanate (VMRD). Tests were carried out as described by the manufacturer and the samples were tested on diluted concentrations of 1:50, 1:100, 1:200, 1:400, and 1:800 in 12-well plates filled with tachyzoites grown in VERO cells and fixed on a Teflon surface. After the reactions ended, glycerol was added, the samples were covered with coverslips, and the reading was carried out immediately using an ultraviolet emission microscope (Olympus CBA, Tokyo, Japan) with 250X magnification and confirmed at 400X magnification.

Statistical methods

The data were stored in a database. Statistical analyses were performed using SPSS software version 11 (SPSS Inc., Armonk, NY, USA) and Epi-Info (CDC, Atlanta, GA, USA). A *P*-value <0.05 was considered statistically significant. The 95% confidence intervals (CIs) were calculated for seroprevalences at four cutoff titers.

A total of five risk factors were tested, including locality [Governorate (n=10)], climatic zone (n=4), age (n=3 groups), gender, and breed. The dependent variable was *Neospora* spp. seropositivity status, coded as 0 (negative horse) or 1 (positive horse). This was repeated for positivity at four cutoff titers (1:50, 1:200, 1:400, and 1:800).

Data were analyzed according to a case–control design, where *Neospora* spp.-positive and -negative horses were compared in relation to their exposure to potential risk factors (Thrusfield, 1995). Initially, when screening for a significant association between variables in order to identify candidate variables ($P<0.05$) for the multiple logistic regression model, one risk factor at a time was tested to assess its association with *Neospora* spp. seropositivity using the chi-squared test and simple logistic regression. For ordered categorical variables, the chi-squared test for trends was employed and Fishers exact test was used when the frequency observed was less than five. ORs with 95% CIs were calculated. A multivariate analysis was then conducted with all factors that had a $P\leq0.05$ or an OR≤0.3 or an OR≥3.0 in the univariate analysis. Only those factors that were included in the final models are presented. A *P*-value of <0.05 was considered statistically significant.

Results

The seroprevalence of *Neospora* spp. among horses was 32%. Table 1 summarizes the seropositivity of *Neospora* spp. among horses in Jordan at four cutoff titers.

Table 1. Seropositivity of *Neospora* spp. infections in horses in Jordan at four cutoff titers (n=379).

Titer	Positive (N)	Seroprevalence (%)	95% CI
1:50	122	32	28, 37
1:200	74	20	16, 24
1:400	37	10	7, 13
1:800	30	8	5, 11

In the univariate analysis, localities, climatic zones, and breed were associated with *Neospora* spp. seropositivity at the four cutoff titers of 1:50, 1:200 1:400, and 1:800 (Tables 2 and 3), in addition to age at cutoff titers of 1:200 and 1:800, and gender at a cutoff titer of 1:800.

Table 2. Univariate association between risk factors and *Neospora* spp. seropositivity at 1:50 and 1:200 cutoff titers among 379 horses in Jordan.

Variable	Category	Coding	Total N	*+ve 1: 50* (n = 122)	*P*	+ve1:200 (n = 74)	*P*
Locality	Irbid	0	17	0 (0)	0.01	0 (0)	0.01
	Jerash and Mafraq	1	16	10 (63)		6 (38)	
	Zarqa	2	70	25 (36)		19 (27)	
	Balqa	3	34	10 (29)		4 (12)	
	Amman	4	157	33(21)		23 (15)	
	Madaba	5	35	23(66)		11 (31)	
	Petra	6	50	21 (42)		11 (22)	
Climate	Cool steppe	0	157	33 (21)	0.01	23 (15)	0.01
	Cool temperate rainy	1	9	8 (89)		5 (56)	
	Warm temperate rainy	2	91	35 (39)		16 (18)	
	Cool desert	3	122	46 (38)		30 (25)	
Age (Years)	<8	0	172	158 (92)	0.20	33 (19)	0.09
	8–14	1	158	44 (28)		26 (17)	
	>14	2	49	20 (41)		15 (31)	

Table 2. Continued...

Variable	Category	Coding	Total N	*+ve 1: 50* (n = 122)	*P*	+ve1:200 (n = 74)	*P*
Gender	Male	0	176	54 (31)	0.32	31 (18)	0.23
	Female	1	203	68 (34)		43 (21)	
Breed	Thoroughbred	0	28	1 (4)	0.01	1 (4)	0.02
	Local	1	351	121 (35)		73 (21)	

Table 3. Univariate association between the risk factors and *Neospora* spp. seropositivity at 1:400 and 1:800 cutoff titers among 379 horses in Jordan.

Variable	Category	Coding	Total N	+ve 1:400 (n = 37)	*P*	+ve 1:800 (n = 30)	*P*
Locality	Irbid	0	17	0 (0)		0 (0)	0.02
	Jerash and Mafraq	1	16	0 (0)	0.01	0 (0)	
	Zarqa	2	70	13 (19)		12 (17)	
	Balqa	3	34	0 (0)		0 (0)	
	Amman	4	157	12 (8)		8 (5)	
	Madaba	5	35	6(17)		5(14)	
	Petra	6	50	6 (12)		5 (10)	
Climate	Cool steppe	0	157	12 (8)	0.01	8 (5)	0.04
	Cool temperate rainy	1	9	0 (0)		0 (0)	
	Warm temperate rainy	2	91	6 (7)		5 (6)	
	Cool desert	3	122	19 (48)		17 (14)	
Age (Years)	<8	0	172	20 (11)	0.20	16 (9)	0.05
	8–14	1	158	9 (6)		7 (4)	
	>14	2	49	8 (16)		7 (12)	
Gender	Male	0	176	13 (7)	0.32	9 (5)	0.04
	Female	1	203	24 (12)		21(10)	
Breed	Thoroughbred	0	28	0 (0)	0.01	0 (0)	0.09
	Local	1	351	37 (11)		30 (9)	

Following forward selection, three localities (Madaba, Zarqa, and Petra), cool temperate climate, age >14 years, and female gender were included in the final models of *Neospora* spp. seropositivity at cutoff titers of 1:50, 1:200, 1:400, and 1:800 (Tables 4 and 5).

Table 4. Multivariate logistic regression models of the factors associated with *Neospora* spp. seropositivity at four cutoff titers among 379 horses in Jordan.

Cutoff titer	Variable	Category	*P*	OR	95% CI
1: 50 [a]	Locality	Petra	0.009	2.3	1.2, 4.3
		Madaba	0.001	6.1	2.9, 12.8
	Climate	Cool temperate	0.001	3.1	4.0, 251

[a]Likelihood ratio of chi-squared (LR $_x2$)=44.4 on 3 degrees of freedom (d.f.), [b]LR $_x2$=20.3 on 4 d.f., [c]LR $_x2$=10.4 on 2 d.f., [d]LR $_x2$=20.8 on 4 d.f.

Table 4. Continued...

Cutoff titer	Variable	Category	*P*	OR	95% CI
1: 200 [b]	Locality	Madaba	0.006	3.1	1.4, 6.8
		Zarqa	0.014	2.2	1.2, 4.2
	Climate	Cool temperate	0.004	6.2	1.8, 21.8
	Age	>14 years	0.043	2.1	1.1, 4.2
1:400 [c]	Locality	Madaba	0.035	2.9	1.1, 8.0
		Zarqa	0.003	3.2	1.5, 7.0
1: 800 [d]	Locality	Madaba	0.012	4.3	1.4, 13.5
		Zarqa	0.001	4.5	1.9, 10.7
	Gender	Female	0.036	2.4	1.1, 5.6
	Age	>14 years	0.035	2.9	1.1, 7.6

[a]Likelihood ratio of chi-squared (LR χ^2)=44.4 on 3 degrees of freedom (d.f.), [b]LR χ^2=20.3 on 4 d.f., [c]LR χ^2=10.4 on 2 d.f., [d]LR χ^2=20.8 on 4 d.f.

Table 5. Summary of risk factors for *Neospora* spp. seropositivity at four cutoff titers in Jordan.

Variable	Category	***Neospora*–IgG titer**			
		1:50	**1:200**	**1:400**	**1:800**
Locality					
	Madaba	*	*	*	*
	Zarqa		*	*	*
	Petra	*			
Climate	Cool temperate	*	*		
Age	>14 years		*		*
Gender	Female				*

* = increasing risk factor

Discussion

As far as we know, this is the first study to describe the presence of antibodies to *Neospora* spp. in asymptomatic horses in Jordan. The results demonstrated that horses in Jordan were exposed to *Neospora* spp. with a high seroprevalence of 32%, indicating a wide distribution of the infection in Jordan across four climatic zones and all age groups. Seroprevalences of *Neospora* spp. in horses were reported worldwide (Table 6) and ranged from 0% in several regions of Brazil and Argentina (Dubey et al., 1999a, b) to 47% in Parana State, southern Brazil (Locatelli-Dittrich et al., 2006). However, the seroprevalence in horses in Jordan was similar to that of central Wyoming (Dubey et al., 2003) and lower than those reported southern Brazil (47%) (Locatelli-Dittrich et al., 2006).

Serological cross-reactivity between *N. caninum* and *N. hughesi* antigens has been reported when the indirect fluorescent antibody test (IFAT) and *Neospora* agglutination test (*NAT*) were used (Dubey et al., 2001; Figliuolo et al., 2004; Marsh et al., 1996, 1998). Thus, it is difficult to distinguish between these two species using serological tests; as such, molecular techniques are used to identify the *Neospora* species responsible for the infection (Spencer et al., 2000).

Many factors were reportedly associated with an increased risk for *N. caninum* seropositivity in cattle, including age and gender (Dyer et al., 2000; Jensen et al., 1999; Rinaldi et al., 2005; Sanderson et al., 2000). The current results showed that seropositivity to *Neospora* spp. with high titers was associated with horses aged >14 years (OR=2.9). This result is in accordance with previous reports (Kligler et al., 2007; Ciaramella et al., 2004) and may support the notion that vertical transmission in horses might be less efficient than in cattle, which is due to differences in placentation,

Table 6. Seroprevalences of *Neospora* spp. in horses in some countries.

Region prevalence (%)	Test	Antigen		No. positive/ No. examined	Seroprevalence % (95% CI)	Reference
		N. hughesi	***N. caninum***			
USA						
CA, FL, MO, MT	IFAT	NH		23/208	11 (7, 15)	(Vardeleon et al., 2001)
Alabama	IFAT	NH		59/536	11 (8, 17)	(Cheadle et al., 1999)
Central Wyoming	NAT		NC	86/276	31.1 (26, 37)	(Dubey et al., 2003)
South America						
Brazil, several states	ELISA	NH*		24/961	2.5 (1.7, 3.7)	(Hoane et al., 2006)
Brazil, Sao Paulo	IFAT		NC	107/1106	10.3 (8.1, 11.6)	(Villalobos et al., 2006)
Brazil, Parana State	IFAT		NC	17/36	47 (32.0, 63.0)	(Locatelli-Dittrich et al., 2006)
Brazil	IFAT	NC	NC	84/174	(40.68.50–55.93)	(Cazarotto et al., 2016)
Mexico	ELISA	NH*		15/495		(Yeargan et al., 2013)
Argentina	NAT		NC	76	0 (0.0, 4.1)	(DUBEY et al., 1999b)
Europe						
Sweden	ELISA	NH**		39/414	9 (7, 12)	(Jakubek et al., 2006)
France	NAT		NC	100/434	23 (19, 27)	(Pitel et al., 2001)
Italy	IFAT		NC	42/150	28 (21, 35)	(Ciaramella et al., 2004)
Middle East						
Turkey	ELISA		NC	7/75	9 (3, 16)	(Vural et al., 2006)
Iran (northeast) Iran (south)	N-MAT		NC-1	51/150 64/200	34 (26.9, 41.9) 32 (25.9, 38.8)	(Hosseini et al., 2011) (Moraveji et al., 2011)
Saudi Arabia	IFAT		NC	29/200	10	(Alanazi et al., 2014; Kligler et al., 2007)
Jordan	IFAT		NC	122/379	32 (28, 37)	Current study
Far East						
South Korea	IFAT		NH	4/191	2 (0.1, 4)	(Gupta et al., 2002)
Africa Nigeria, West Africa	IFAT		NC	10/120		(Bartova et al., 2017)

*Recombinant NhSAG1; **IH-ISCOM.

as was previously suggested (Pitel et al., 2003). Also, it may indicate horizontal transmission by sporulated oocyst ingestion (Locatelli-Dittrich et al., 2006) and can be taken as evidence of frequent acquired infection.

Likewise, female horses had higher odds (OR=2.9) of seropositivity with high titers. Infections in general are known to be associated with stress factors, including parturition and other diseases. Females are at a disadvantage when faced with the stresses of pregnancy and parturition. Such stress is aggravated with local malnutrition practices and the prevalence of other concomitant diseases. Furthermore, higher seroprevalence at high titers in females, as reported here, may be similar to that seen in cattle, where the intensity and duration of the specific antibodies during gestation could reflect the intensity of recrudescence of an existing latent infection (Vural et al., 2006) and could be related to the risk of fetal infection (Guy et al., 2001; Stenlund et al., 1999).

Locality was a determining factor in the distribution of *Neospora* spp. seropositivity. *N. caninum* infections were evident across all climate ranges available in Jordan (Abo-Shehada & Abu-Halaweh, 2010), though with different seroprevalences, likely following the presence and relative abundance of the definitive host (i.e., dogs; Table 2 and 3). Furthermore, climate was reported to influence the seroprevalence of *N. caninum* in farm animals, including cattle (Rinaldi et al., 2005; Schares et al., 2004), sheep, and goats (Abo-Shehada & Abu-Halaweh, 2010). In this study, the cool temperate climatic zone was found to be an enhancing factor for lower titers <1:400 (cutoff titers of 1: 50 and 1: 200), with ORs of 3.1 and 6.2, respectively. However, such an association was not evident with higher cutoff titers (1:400 and 1:800). In a previous study of sheep and goats in northern Jordan, there was evidence of an association between seropositivity to *Neospora* spp. and cool temperate climate, with an OR of 0.01 (Abo-Shehada & Abu-Halaweh, 2010). *N. caninum* oocysts sporulate in the environment (Vural et al., 2006) and the present results suggest an adverse effect on sporulation in cool, temperate, rainy climates that translate into horses and other animals being exposed to lower numbers of sporulated oocysts.

Overall, this study provided serologic evidence for naturally occurring *Neospora* spp. infection in Jordanian horses. *Neospora* spp. infections are widespread in Jordan and pose risks for the development of health problems among horses and other animals in the Middle East. Increased seroprevalences of low and high titers of *Neospora* spp. were associated with two localities (Madaba and Zarqa), and ages >14 years old, while the increased seroprevalence of low titers was associated with the Petra locality and a cool temperate climate. The increased seroprevalence of *Neospora* spp. was associated with high titers and female gender in horses.

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