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Serological and molecular detection of *Anaplasma phagocytophilum* in horses reared in Korea

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ABSTRACT: The objective of this study was to investigate the nationwide prevalence of *Anaplasma phagocytophilum* in horses reared in Korea using a commercial ELISA kit and two different nested PCR (nPCR) analyses. Our analysis showed that 2.9% (true prevalence: 3.1%, 16/549) of the horses were seropositive by ELISA, and none were positive by the two nPCR analyses. Significant differences were observed in the ELISA results when the data were analysed according to breed and geographic region. In light of a recent case of human granulocytic anaplasmosis in Korea and the predicted increase in the number and distribution of ticks due to global warming, continuous monitoring and appropriate control programs for *Anaplasma* spp. and other tick-borne pathogens need to be established.

Keywords: anaplasmosis; ELISA; equine; PCR; Korea

Anaplasma phagocytophilum is a gram-negative obligate intracellular bacterium belonging to the order Rickettsiales. *A. phagocytophilum* is transmitted by ticks in the family Ixodidae (Cho et al. 2010; Mencke 2013; Veronesi et al. 2014), and the bacterium can infect a variety of animals, including ruminants, rodents, dogs, cats, horses, and humans (Rikihisa 2011). When *A. phagocytophilum* infects horses, it causes equine granulocytic anaplasmosis (EGA), and when it infects humans, it is called human granulocytic anaplasmosis (HGA). The clinical manifestations of EGA include fever, depression, anorexia, leukopenia, thrombocytopenia, limb oedema, and ataxia (Rikihisa 2011).

Several Korean studies have reported the presence of *A. phagocytophilum* in a variety of mammals, including rodents, dogs, and cattle (Chae et al. 2008b; Jung et al. 2012; Kang et al. 2013), and a case of HGA has recently been documented in Korea (Kim et al. 2014). In recent decades, there has been a gradual increase in temperatures worldwide due to global warming, and this climate change is expected to influence the abundance and distribution of ticks (Leger et al. 2013). Therefore, the recent temperature increase on the Korean peninsula might expand the distribution of ticks and consequently increase the risk of tick-borne diseases (Chae et al. 2008a). Since little is known about EGA in Korea, the objective of

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this study was to investigate the prevalence of *A. phagocytophilum* in horses reared in Korea using PCR and ELISA analyses.

MATERIAL AND METHODS

Study area. This study included horses reared throughout Korea. Korea is located between 34°20' to 37°11' northern latitude and 126°07'–129°19' eastern longitude (Jung et al. 2014). The study area has an annual precipitation of 1300 mm and an annual mean temperature of 12.9 °C (cold season mean: –2.1 °C, warm season mean: 27.3 °C).

Sample size determination and sample collection. Between 2009 and 2013, blood samples were collected from the jugular veins of 549 horses reared in Korea. The total number of horses currently in Korea is approximately 30 000. This sample size was determined by the following formula using an expected disease prevalence of 3%, an accepted error of 1.5%, and a confidence level of 95% with a simple random sampling design (Thrusfield 2005):

$$n = \frac{1.962 p_{exp} (1 - p_{exp})}{d^2}$$

where:

n = the required sample size

p_{exp} = expected prevalence

d = desired absolute precision

According to the formula, a minimum of 497 samples were needed, and the samples were collected from various regions.

For the epidemiological study, we recorded data for age, sex, breed, and the region where the samples were collected (Figure 1, Table 1). The mean age of the study animals was 8.3 years, and the standard deviation was 5.0 years. The analysis criteria and the number of samples were as follows: age of study animals: ≤ 4 years (n = 138), 5–10 years (n = 224), and ≥ 11 years (n = 187); sex: male (n = 94), female (n = 214), and gelding (n = 241); breed: thoroughbred (n = 393), warmblood (n = 61), Korean native pony (n = 15), and mixed breed (n = 80); and region: northern (n = 127), central (n = 179), and southern (n = 243). The chi-squared test was used to test for significant relationships between categories according to region. Samples were stored at –20 °C until analysis.

DNA extraction and PCR. DNA was extracted from whole blood using the DNeasy® Blood & Tissue Kit (Qiagen, Germany) according to the manufacturer's instructions. To amplify the 16S rRNA gene of *A. phagocytophilum*, two different nested PCRs (nPCR) were performed using the EE1/EE2 and EE3/EE4 primers, which amplify a 928-bp fragment (Barlough et al. 1996), and ge3a/ge10r and ge9f/ge2, which amplify a 546-bp fragment (Liz et al. 2002). For each nPCR analysis, *A. phagocytophilum* DNA isolated from dog blood was included as a positive control. The PCR products were separated on 1% agarose gels stained with ethidium bromide and viewed using a UV transilluminator.

Serological assay. A commercial ELISA kit (SNAP® 4Dx test; IDEXX Laboratories, USA) was used to detect antibodies against *A. phagocytophi-*

Table 1. Characteristics of study horses reared in Korea from which samples were collected for the detection of antibodies against *Anaplasma phagocytophilum*

Group		Number of tested	Northern (n = 127)	Central (n = 179)	Southern (n = 243)	P-value
Age	≤ 4 years	138	58	25	55	< 0.001
	5–10 years	224	21	90	113	
	≥ 11 years	187	48	64	75	
Sex	male	94	38	33	23	< 0.001
	female	214	65	62	87	
	gelding	241	24	84	133	
Breed	thoroughbred	393	94	107	192	< 0.001
	Korean native pony	15	2	0	13	
	warmblood	61	0	36	25	
	mixed	80	31	36	13	

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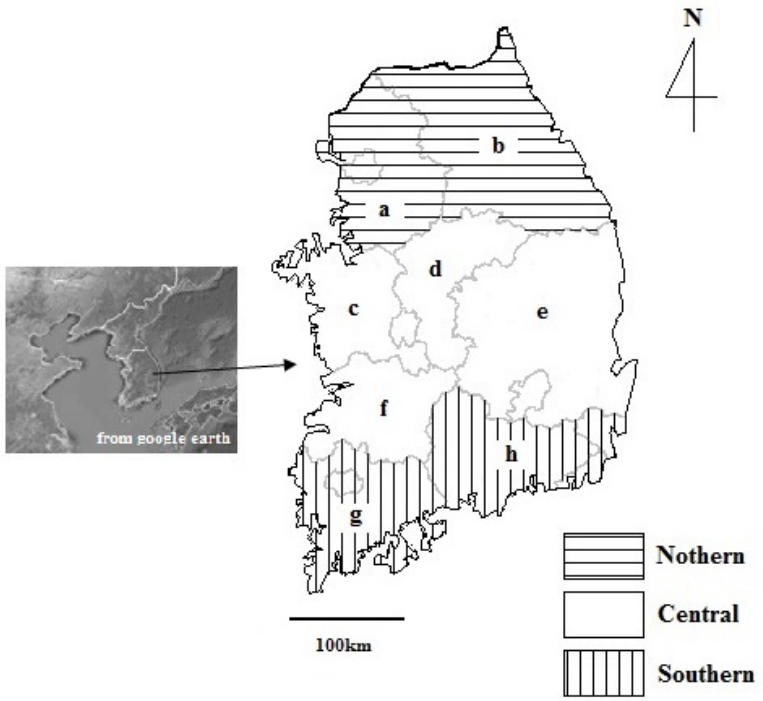

Figure 1. Regional map of Korea showing the three study regions where horse blood samples were collected to detect the presence of *Anaplasma phagocytophilum* antibodies: northern [Gyeonggi-do (**a**) and Gangwon-do (**b**)], central [Chungcheongnam-do (**c**), Chungcheongbuk-do (**d**), Gyeongsangbuk-do (**e**), and Jeollabuk-do (**f**)], and southern [Jeollanam-do (**g**) and Gyeongsangnam-do (**h**)]

lum according to the manufacturer's instructions. Although the SNAP® 4Dx test was originally developed to detect canine antibodies against *A. phagocytophilum*, it can also be used to detect antibodies against *A. phagocytophilum* in blood samples from horses (Chan et al. 2010; Vieira et al. 2013; Veronesi et al. 2014). The SNAP® 4Dx test detects antibodies from both active infection and previous exposures to the pathogen. The test has good sensitivity (86.7%) and specificity (99.8%) for blood samples from horses when compared to the indirect fluorescence antibody test (IFAT), which is considered the "gold standard" assay for detecting *A. phagocytophilum* antibodies (Veronesi et al. 2014).

Statistical analysis. The observed prevalence (OP) was calculated as the number of positive samples/total number of samples. The true prevalence (TP) was estimated using the following formula (Reiczigel et al. 2010):

$$TP = (OP + Sp - 1)/(Se + Sp - 1)$$

where:

Se = sensitivity

Sp = specificity

Seroprevalence was analysed using the chi-squared and Fisher's exact tests, and a *P*-value of less than 0.05 was considered statistically significant. The 95% confidence interval for the adjusted prevalence of each estimate was calculated using Blaker's method (Reiczigel et al. 2010).

RESULTS

Sixteen of the 549 horses (2.9%; TP: 3.1%) were seropositive for *A. phagocytophilum* using ELISA (Table 2). However, none of the samples were positive by either of the two nPCR analyses. When the ELISA results were analysed according to age, *A. phagocytophilum* seropositivity was detected in 2.2% (TP: 2.3%, 3/138) of horses ≤ 4 years of age, in 1.8% (TP: 1.8%, 4/224) of horses aged 5–10 years, and in 4.8% (TP: 5.3%, 9/187) of horses over the age of 10 years. The highest seroprevalence was detected in horses over the age of 10; however, the difference was not statistically significant ($P > 0.05$).

Analysis according to sex revealed that 0% (TP: 0%, 0/94) of males, 4.7% (TP: 5.2%, 10/214) of females, and 2.5% (TP: 2.6%, 6/241) of geldings were seropositive. The seroprevalence observed in females was significantly higher than that in males ($P = 0.035$), but not higher than that in geldings ($P = 0.308$).

Analysis according to breed showed that 2.3% (TP: 2.4%, 9/393) of thoroughbreds, 8.2% (TP: 9.2%, 5/61) of warmbloods, 6.7% (TP: 7.5%, 1/15) of Korean native ponies, and 1.3% (TP: 1.2%, 1/80) of mixed breeds were seropositive for *A. phago-*

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Table 2. Seroprevalence of *Anaplasma phagocytophilum* in 549 horses reared in Korea according to age, sex, breed, and region

Group		Number of tested	Number of positive	Observed prevalence (%)	True prevalence (%)	95% CI	*P*-value
Age	≤ 4 years	138	3	2.2	2.3	0.5–7.0	0.143
	5–10 years	224	4	1.8	1.8	0.5–5.0	
	≥ 11 years	187	9	4.8	5.3	2.6–10.0	
Sex	male	94	0	0	0	0–4.2	0.068
	female	214	10	4.7	5.2	2.5–9.3	
	gelding	241	6	2.5	2.6	1.0–5.8	
Breed	thoroughbred	393	9	2.3	2.4	1.1–4.7	0.045
	Korean native pony	15	1	6.7	7.5	0.2–34.7	
	warmblood	61	5	8.2	9.2	3.6–20.1	
	mixed	80	1	1.3	1.2	0–7.2	
Region	northern	127	0	0	0	0–3.0	0.027
	central	179	5	2.8	3.0	1.0–7.0	
	southern	243	11	4.5	5.0	2.5–8.9	
Total		549	16	2.9	3.1	1.8-5.2	

CI = confidence interval; calculated using Blaker's method

cytophilum. Significant differences in seropositivity were observed according to breed ($P = 0.045$). The highest seroprevalence was observed in warmbloods, which was significantly different from that in thoroughbreds ($P = 0.028$), but not significantly different from mixed breeds ($P = 0.085$).

In the regional analysis, 0% (TP: 0%, 0/127), 2.8% (TP: 3.0%, 5/179), and 4.5% (TP: 5.0%, 11/243) of horses tested seropositive in the northern, central, and southern regions, respectively. Significant differences in seropositivity were observed according to region ($P = 0.027$). The highest seroprevalence was observed in the southern region, which was significantly higher than in the northern region ($P = 0.019$), but not the central region ($P = 0.444$).

DISCUSSION

Approximately 235 species of Ixodidae ticks are known worldwide, and they can transmit pathogens that cause various diseases such as anaplasmosis, babesiosis, and ehrlichiosis (Mencke 2013). Climate change could affect the abundance and distribution of ticks (Leger et al. 2013). In recent years, there has been a gradual increase in temperatures worldwide due to global warming. The recent temperature elevations on the Korean peninsula are predicted to expand the distribution of ticks and consequently increase the risk of tick-borne diseases (Chae et al. 2008a).

Numerous studies conducted outside Korea have also reported seropositivity for *A. phagocytophilum* in horses: 73% (67/90) of horses in the Czech Republic tested positive by IFAT (Praskova et al. 2011); 13% (21/162) of horses in Portugal tested positive by IFAT (Ribeiro et al. 2013); 13.7% (41/300) and 6.7% (20/300) of horses in Italy tested positive by IFAT and PCR, respectively (Laus et al. 2013); 16.3% (56/343) of horses in Tunisia tested positive by IFAT (Ben Said et al. 2014); and 10.1% (45/444) and 9.0% (40/444) of horses in Italy tested positive by IFAT and ELISA, respectively (Veronesi et al. 2014). The prevalence of seropositivity in the present study was lower than that reported in these studies; however, it is consistent with another Korean study that showed 2.2% (2/92) seropositivity for *A. phagocytophilum* in horses reared on Jeju Island, as determined by IFAT (Chae et al. 2009).

In the present study, seropositivity differed significantly according to region ($P = 0.027$). The highest seropositivity was detected in the southern region. Korea is located in the northern hemisphere, and at the lower latitudes in Korea, the annual mean temperature, precipitation, and humidity are higher than in the higher latitudes. This climate provides a favour-

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able environment for ticks that transmit tick-borne pathogens. Similar epidemiological characteristics were observed in our previous study of antibodies against *Anaplasma* spp. in goats (Lee et al. 2015).

Significant differences in seropositivity were observed among the different breeds ($P = 0.046$). Ben Said et al. (2014) reported higher seroprevalence in thoroughbreds than other breeds, and M'ghirbi et al. (2012) reported higher seroprevalence in barbs than in Arabians. The authors of these previous studies suspected that the statistically significant differences between breeds were more a result of the activity of the horses rather than genetic factors. Similarly, in this study, a significantly higher seroprevalence was observed in warmbloods when compared to thoroughbreds ($P = 0.028$), but not when compared to mixed breed horses ($P = 0.085$). There are two possible explanations for this observation. The first is the relationship between seroprevalence and geographical region. There were statistically significant differences according to region ($P = 0.027$), and between region and breed ($P < 0.001$; Table 1). Therefore, the statistically significant difference according to breed could be related to regional seroprevalence. The other possibility is, as suggested in previous studies (Ben Said et al. 2014; M'ghirbi et al. 2012), related to the relationship between seroprevalence and the activity of the horse breed. However, we did not collect data on the activity of the horses in this study; therefore, such analyses were not possible.

In this study, there were no statistically significant differences between sexes ($P = 0.068$); however, the seroprevalence was significantly higher in females than in males ($P = 0.035$). This result is consistent with the findings of Praskova et al. (2011) and Ben Said et al. (2014), who reported higher seroprevalence in females than in males or geldings. However, M'ghirbi et al. (2012) reported no significant differences in seroprevalence according to sex. In addition, Praskova et al. (2011) and Ben Said et al. (2014) also attributed the significant difference according to sex to the activity of the horses, as was suggested for the differences according to breed. Therefore, we suspect that there was no actual relationship between sex and seroprevalence, and we think that seroprevalence is related to the activity of the horses, which resulted in an observed difference in seroprevalence according to sex.

Although 16 samples were seropositive, none of the samples tested positive for *A. phagocytophilum* 16s rRNA by nPCR. This may be attributed to the fact that antibodies against *A. phagocytophilum* persist in the blood longer than the antigen. Horses inoculated intravenously with *A. phagocytophilum* showed seroconversion at 12–16 days post-inoculation (Franzen et al. 2005), and serum antibodies persisted for 300 days after inoculation (Nyindo et al. 1978). Therefore, the fact that 16 samples were positive by serological assay but negative by nPCR indicates that the horses were previously exposed and were not currently infected. In addition, although antibodies against *A. phagocytophilum* can persist for a long time, in this study, horses were categorised into three groups by age, with five years between each group. Antibodies cannot persist for such a long period of time. Therefore, it is suspected that the effect of antibody persistence was limited to seroprevalence according to age.

To the best of our knowledge, this is the first large-scale, nationwide investigation into the prevalence of *A. phagocytophilum* in horses raised in Korea. A case of HGA was recently reported in Korea (Kim et al. 2014). Although there is no evidence of transmission from animals to humans, *A. phagocytophilum* is transmitted to horses and humans by the same tick species. In the future, the prevalence of tick-borne diseases, such as those caused by *A. phagocytophilum* is expected to increase due to global warming. Therefore, continuous monitoring programs for EGA, HGA, and other tick-borne diseases as well as appropriate control programs need to be established.

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