J. Vet. Sci. (2008), **9**(4), 381–385

JOURNAL OF
Veterinary
Science

Serosurveillance for Japanese encephalitis, Akabane, and Aino viruses for Thoroughbred horses in Korea

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Recent global warming trends may have a significant impact on vector-borne viral diseases, possibly affecting vector population dynamics and disease transmission. This study measured levels of hemagglutination-inhibition (HI) antibodies against Japanese encephalitis virus (JEV) and neutralizing antibodies against Akabane virus (AKAV) and Aino virus (AINV) for Thoroughbred horses in Korea. Blood samples were collected from 989 racehorses in several provinces, between October 2005 and March 2007. Sera were tested using either an HI assay or a virus neutralization test. Approximately half (49.7%; 492/989) of the horses tested were antibody-positive for JEV. The HI titer against JEV was significantly correlated with racehorse age ($p < 0.05$). Horses with an HI antibody titer of 1 : 160 or higher accounted for 3.9% of the animals tested, indicating that vectors transmitting arthropod- borne viruses bit relatively few horses. In contrast, 3.8% (19/497) and 19.5% (97/497) of horse sera collected in March 2007 were positive against AKAV and AINV, respectively. The presence of antibodies against AKAV and AINV may indicate the multiplication of AKAV and AINV in these horses.

Keywords: arbovirus, racehorse, serosurveillance

Introduction

Populations of Korean horses, including native ponies, have been gradually increasing with the growth of the racing industry. Approximately 23,000 horses including 8,000 Thoroughbred horses and 15,000 other breeds (Jeju horses and Jeju racehorses) are raised on 1,142 premises in Korea [12]. Racehorses are important industrial animals in Korea, and therefore great care has been taken to prevent arbovirus infections in these animals. However, there is growing concern that global warming may affect the prevalence of vector-borne diseases and, consequently, the racehorse industry.

Japanese encephalitis virus (JEV) is a Flavivirus belonging to the family *Flaviviridae*. Japanese encephalitis (JE) is a typical zoonosis caused by JEV and is transmitted by several species of mosquito, principally *Culex tritaeniorhynchus*, which breed in small pools or paddy fields [16]. JEV can infect both domestic and wild animals, including swine, horses, chickens, reptiles, and grey herons. Although adult animals do not develop clinical signs, they may serve as viral reservoirs or amplifying hosts. After JEV was first identified in Koreans in 1946, a number of JE cases were reported in domestic animals as well as in humans through the 1950s and 1960s [9]. When an attenuated live vaccine was developed and administered to both swine and horses in 1980, the number of outbreaks in animals was significantly reduced [8]. Recently, JEV has been considered an emerging virus, with new JE cases being reported in regions of Australia and Africa [15]. JEV has been identified from a diseased horse in Japan in 2006 [19] and JEV antibodies were detected in 52% of Indonesian horses [18]. A horse that is naturally infected with JEV displays several symptoms, such as anorexia, lethargy, and fever, and is considered to be a dead-end host [19]. There have been no reports on horses that were clinically infected with JEV in Korea until recently; however, there are reports of other infected animals in Korea [20,21]

Akabane virus (AKAV) and Aino virus (AINV) are members of the Simbu serogroup, genus *Orthobunyavirus*, family *Bunyaviridae*, and consist of three segments of single-stranded negative RNA. These two viruses are responsible for many reproductive disorders, including abortion, stillbirth, and congenital malformation, in ruminants [13]. AKAV and AINV are transmitted via biting midges such as *Culicoides* (*C.*) *brevitarisis, C. oxystoma*, and *C. nebulous* [7]. Although AKAV and AINV are not known to cause illness in horses, there have

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been several reports on the presence of antibodies against arboviruses in these animals [2,3].

The risk of exposure of domestic animals to arbovirus-infected mosquito bites depends on several environmental factors, including climate, host abundance, and vector populations. Among the arboviruses, JEV, AKAV, and AINV are the most significant vector-borne viral agents in South Korea. Sero-epidemiological studies are critical for predicting potential outbreaks of vector-borne viral diseases among horses. These studies also provide data for establishing a system to prevent these diseases. In our study, we conducted a serological survey to determine the prevalence of antibodies against JEV, AKAV, and AINV in Thoroughbred horses in Korea.

Materials and Methods

Viruses and cells

The strain of JEV used as an antigen for the hemagglutination inhibition test was KV1899. This particular strain was isolated from Korean pig blood in 1999 [20]. The strains of Akabane and Aino viruses used for the virus neutralization test were K-9 and KSA9910, respectively [11]. The latter two viruses were propagated using Vero cells cultured in α-minimum essential medium (MEM; Gibco BRL, USA) supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), an antimycotic (0.25 μg/ml amphotericin B), and 5% fetal bovine serum (FBS; Gibco BRL, USA). Uninfected cell cultures were used as negative controls.

Collection of sera

For the seroprevalence study, blood samples were collected from thoroughbred racehorses in several provinces of Korea, between October 2005 and March 2007. The species of racehorse tested in this study was Thoroughbred, and about 70% of the racehorses were produced in Korea; the others were imported from several countries such as the United State of America, Australia, Japan, New Zealand, India and Ireland. Most of the racehorses are vaccinated once a year against *Streptococcus equi*, JEV and equine influenza virus. None of the horses used in this study had been vaccinated against AKAV or AINV. Clotted blood samples were separated by centrifugation, and the sera were stored at -20°C until use.

Hemagglutination inhibition (HI) test

To estimate JEV antibody prevalence in horse sera samples, an HI test was performed in 96-well microplates, using slightly modified standard methods. Using a sucrose-acetone extraction method, viral antigens were prepared from the brains of suckling mice infected with the Korean isolate of strain KV1899 [1,20]. Briefly, the sera were treated in round bottom microplates (96-well). To remove non-specific inhibitors, 10 μl of serum and 50 μl of 4% bovine albumin were mixed with 40 μl of 25% kaolin (Sigma, USA) and incubated for 30 min. After pipetting, the kaolin was removed by centrifugation at 3,600 rpm for 15 min in a microfuge. The resultant clear supernatant was mixed with 5 μl of packed goose erythrocytes to remove any natural agglutinins. After incubation for 1 h at 37°C, the treated serum was separated from the goose erythrocytes by centrifugation. For the HI test, four to eight HA units of JEV (in 25 μl) were added to 25 μl of treated serum. After incubation for 1 h at 37°C, 50 μl of 0.33% goose erythrocytes were added, and the microplates were incubated at 37°C for 30 min. The HI titer was expressed as the reciprocal of the highest dilution of serum showing complete inhibition of hemagglutination. An HI titer of 1 : 20 or higher was considered positive.

Virus neutralization (VN) test

The VN tests for AKAV and AINV were carried out in 96-well microplates using Vero cells [11]. A 50 μl aliquot of a two-fold serial dilution of heat-inactivated serum was mixed with an equal volume of 200 $TCID_{50}$ of each virus and incubated at 37°C for 1 h. A total of 100 μl of Vero cells in α-MEM containing 10% FBS were then added to each well at a concentration of 200,000 cells per ml. The microplates were incubated for 5 days at 37°C under 5% CO_2, after which time virus-induced cytopathic effects were evaluated visually. The VN titer was expressed as the reciprocal of the highest serum dilution that completely inhibited cytopathic effects in the wells. The serum dilution ranged from 1 : 2 to 1 : 64, and an antibody titer higher than 1 : 2 was considered positive.

Statistical analysis

Chi-squared tests were used to analyze differences in seroprevalence between the sexes, ages, and provinces, respectively. A p-value less than 0.05 was considered to be statistically significant.

Results

Seroprevalence of JEV

Approximately half (49.7%; 492/989) of all horses tested were positive for JEV; there were no significant differences in JEV seroprevalence by year ($p > 0.05$). Horses with an HI titer of 1 : 160 or higher accounted for only 3.9% of the animals tested (Table 1). The HI titer against JEV increased with increasing racehorse age ($p < 0.05$; Fig. 1). This age-dependent trend was consistent with sera obtained in two subsequent years, but the positive relationship tended to be higher in 2006 than in 2007 ($p < 0.05$; Fig. 1).

No differences were found among females, males, and geldings with respect to JEV antibody prevalence ($p > 0.05$; Fig. 2). JEV antibody rates were numerically higher in Gyeongnam province as compared to Jeju and Gyeonggi

Table 1. Seroprevalence and distribution of hemagglutination inhibition titer against Japanese encephalitis virus in serum samples collected from Thoroughbred horses between 2005 and 2007 in Korea

Year	No. of samples	No. of positive samples(%)	Hemagglutination inhibition titer							
			< 1 : 20	1 : 20	1 : 40	1 : 80	1 : 160	1 : 320	1 : 640	1 : 1,280
2005	230	122 (53.0)	108	45	47	25	2	2	1	0
2006	262	132 (50.4)	130	44	32	40	12	4	0	0
2007	497	238 (47.9)	259	86	82	52	15	2	1	0
Total	989	492 (49.7)	497	175	161	117	29	8	2	0

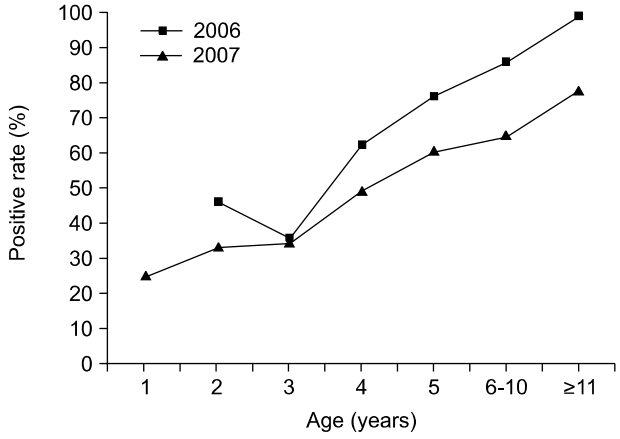

Fig. 1. Distribution of hemagglutination-inhibition titer by age among Thoroughbred horses that were positive against Japanese encephalitis virus in 2006 and 2007.

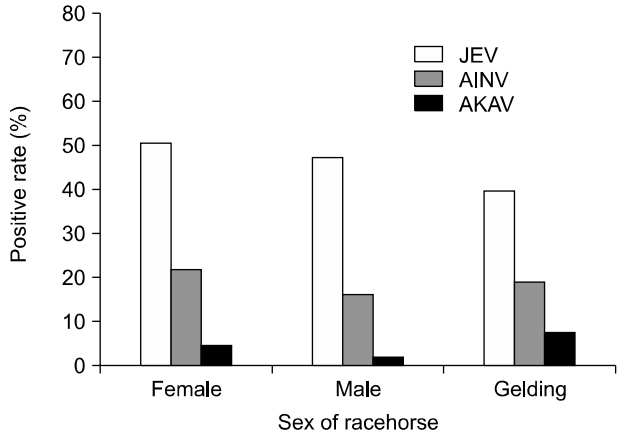

Fig. 2. Seropositive rates for Japanese encephalitis virus (JEV), Aino virus (AINV) and Akabane virus (AKAV) grouped by gender among 497 Thoroughbred horses whose blood samples were collected in 2007.

provinces, but statistical differences between regions were not observed: Gyeongnam, 58.1% (97/167); Jeju, 51.5% (34/66); and Gyeonggi provinces, 40.5% (107/264) (χ^2 = 4.54, df = 2, p = 0.103; Fig. 3).

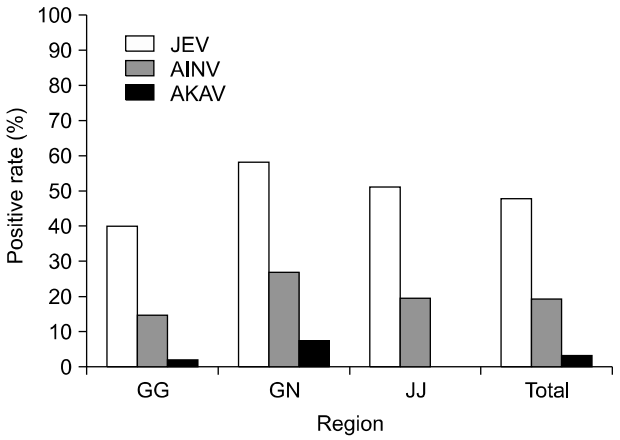

Fig. 3. Regional distribution of Japanese encephalitis virus (JEV), Aino virus (AINV) and Akabane virus (AKAV) antibodies from Thoroughbred horses in 2007. GG:Gyeonggi province, GN: Gyeungnam province, JJ: Jeju province.

Seroprevalence of Akabane and Aino viruses

Seroprevalence against AKAV and AIV was examined in sera obtained in March 2007 from 497 racehorses. The seropositive rates for AKAV and AINV individually were 3.8% (19/497) and 19.5% (97/497), respectively (Table 2). AINV antibody rates were relatively low compared with those of JEV: Gyeongnam, 26.9% (45/167); Jeju, 19.7% (13/66); and Gyeonggi provinces, 14.8% (39/264) (χ^2 = 6.37, df = 2, p = 0.042; Fig. 3). AKAV antibody rates were lowest among those of the viruses: Gyeongnam, 7.8% (13/167); Gyeonggi, 2.3% (6/264): and Jeju provinces, 0% (0/66); (χ^2 = 10.5, df = 2, p = 0.005; Fig. 3).

Discussion

Most of the racehorses raised in Korea are vaccinated with a live attenuated JE vaccine in May of each year. The seropositive rates found in this study ranged from 53.5 to 47.9% over the course of 3 years and were similar to rates obtained in a 1985 survey [14]. Sugiura and Shimada [17] reported that horses showing a titer of 1 : 1,280 or higher were classified as infected with field JEV. JE vaccine

Table 2. Distribution of serum neutralizing titers against Akabane and Aino viruses in Thoroughbred horses

Pathogen	No. of samples	No. of positive samples (%)	Serum neutralizing titer						
			< 1 : 2	1 : 2	1 : 4	1 : 8	1 : 16	1 : 32	1 : 64
Akabane virus	497	19 (3.8)	478	16	3	0	0	0	0
Aino virus	497	97 (19.5)	400	50	30	11	5	1	0

produced in Japan is killed vaccine and is known to induce an HI titer of less than 1 : 640. Therefore, the Japanese scientists considered that a titer of 1 : 1,280 or higher was induced by JEV infection. In contrast to Japanese JE vaccine, Korean JE vaccine is live vaccine and most of the antibody titers induced by Korean JE vaccine are less than 1 : 160 [8]. In addition, Yamanaka *et al*. [19] reported that horses infected with JEV induced SN antibody titers from 1 : 160 to 1 : 640. Moreover, most of the Korean racehorses have been vaccinated against JEV and the antibody induced by JEV infection can not be differentiated from the one induced by JEV vaccination. After full consideration of the previous reports, we concluded that an HI titer greater than 1 : 160 could be a meaningful titer, one that could be induced by JEV infection. In our study, racehorses showing antibody titers equal to or higher than 1 : 160 accounted for only 3.9% of horses tested. There were no horses with HI antibody titers higher than 1 : 1,280 and clinical illness due to JEV infection, indicating that JEV infection is not widespread among South Korean racehorses at this time. The presence of antibodies to JEV non-structural 1 (NS1) protein also has been considered an indicator of natural JEV infection among populations vaccinated with inactivated JE vaccine [5,6]. However, because a live attenuated JE vaccine, named as Anyang 300 strain, has been inoculated into horses at every spring season since 1980, it is not able to be applied the detection methods of the NS1 antibodies for differentiating vaccinated horses from naturally infected horses in Korea. In this study, the seroprevalence of JEV was significantly correlated with racehorse age, indicating either an immune response against repeated vaccinations or JEV infection. Continuous serosurveillance of JEV has been reported for goats and wildlife species in Korea [10,21]. Therefore, we predict that clinical JEV infection could appear in horses at any time.

Neutralizing antibodies against AKAV have been observed in many countries in buffalo, sheep, camels, and cattle. Relatively high incidence rates of antibodies against AKAV, ranging from 50 to 85%, have also been reported for horses [2,3]. In the present study, we observed a low prevalence (3.8%) and low antibody titers ($\leq$1 : 4) in the racehorses we examined. These data suggest that either the virus replicates at a low level in horses or only a small number of horses are exposed to mosquitoes infected with AKAV.

AINV is a member of the Simbu serogroup and causes congenital defects in calves. Thirty three percent of dairy cattle in Japan had a positive reaction in the serosurveillance for AINV [4]. According to a study by Cybinski *et al*. [2], specific antibodies against AINV have been detected in cattle, sheep, and goats, but not in Australian horses. In 2006, seropositive rates against AINV were 17.8% and 24.3% in Korean goats and cattle, respectively (data not shown). Our results demonstrate the presence of AINV antibodies in horses (19.5%). Regional prevalence rates ranged from 14.8 to 26.9%, indicating that horses seropositive for AINV are widely distributed throughout the country. We can also infer that vectors transmitting AINV are active in the provinces surveyed.

In conclusion, the present results suggest that the incidence rate of antibody against AINV infection is much higher (about 20%) than those of antibodies to AKAV and JEV infection (about 4%). Further studies are necessary to model and predict the transmission of vector-borne viral diseases between horses and mosquitoes. The effects of climate change on the distribution and disease transmission of vectors in the Korean Peninsular region should also be surveyed in the near future.

Acknowledgments

This work was supported financially by a grant from the National Veterinary Research and Quarantine Service, Ministry for Food, Agriculture, Forestry and Fisheries, Korea. The authors would like to thank Ms. S.S. Choi for technical assistance.

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