

Sero-surveillance of Getah Virus among Thoroughbred Horses in Korea

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Getah virus (GETV), which is transmitted by mosquitoes, causes lower limb edema and stiffness in horses. In this study, we investigated the sero-surveillance of GETV among Thoroughbred racehorses in Korea during 2013 and 2014. A total of 1,182 equine serum samples collected from Thoroughbred racehorses in four provinces (Gyeongnam, Gyeonggi, Jeonbuk and Jeju provinces) were analyzed using virus neutralization (VN) tests. An antibody titer of $\geq 1:2$ was considered positive. Overall, the seropositivity rate for GETV was found to be 12.4% (146/1,182) among the racehorses; the annual seropositivity rates were 12.4% and 12.2% in 2013 and 2014, respectively. The seropositivity rates in April and September in 2013 turned out to be 8.6% and 15.2%, respectively. The regional distribution of seropositivity ranged from 5.0% to 22.3% in 2013 and from 0.0% to 15.0% in 2014, respectively. Gyeongnam province had the highest seropositivity rate than other provinces. By analyzing the distribution of VN titers according to horse age, we found that the highest GETV seropositivity rate was in horses over 6 years of age (22.4% and 28.1%, 2013 and 2014, respectively), and that the incidence of GETV was higher in geldings (17.6% and 18.6%, 2013 and 2014, respectively) than in males and females. These results indicate that Thoroughbred horses raised in Korea were bitten by mosquitoes harboring GETV.

Key Words: Getah virus, Sero-surveillance, Equine, Korea

INTRODUCTION

Approximately 30,000 horses, including 14,000 Thoroughbred horses and 16,000 horses belonging to other breeds, are bred on about 1,900 horse farms in Korea. Approximately 700 horses are imported each year from the United States of America, New Zealand, and Australia (1). Recent legislation has sought to promote the development of the horse industry by establishing instructions on how to support and promote it. These laws can guide the horse

industry and the improve quality of rural economies by strengthening the competitiveness of the industry overall.

Getah virus (GETV), a mosquito-borne virus, has been categorized in the *Semliki Forest complex* with *Bebaru*, *Chikungunya*, *Mayaro*, *O'nyong nyong*, *Ross River*, *Semliki Forest*, and *Una virus* (2). GETV can be divided into several viral subtypes, including *Sagiyama*, *Ross River*, and *Bebaru* (3). GETV is an enveloped, positive-strand RNA virus that belongs to the *Alphavirus* genus of the family *Togaviridae* (4). GETV is composed of four non-structural proteins (nsP1-nsP4) and five structural proteins (C, E3, E2, 6K,

Received: May 26, 2015/ Revised: August 17, 2015/ Accepted: September 8, 2015

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**This work was supported financially by a grant (N-1543083-2014-18-01) from Animal, and Plant Quarantine Agency, Ministry of Agriculture, Food and Rural Affairs (MAFRA), Republic of Korea.

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and E1) (5). E1, a class 2 viral fusion protein that promotes the release of the viral nucleocapsid in the cytoplasm, is bound to E2 in mature GETV (6).

GETV was isolated in Malaysia in 1955 from *Culex gelidus* mosquitoes (7), and has since been isolated from a variety of mosquitoes, including *Culex*, *Aedes*, and *Armigeres* in Japan, China, Korea, and Australia (8, 9, 10, 11). GETV infections in horses have been reported in Japan and India (12, 13). Serological surveys indicate that GETV is widespread (9, 14~16). Getah viral infections can occur in a wide range of vertebrates, including humans, but pigs and horses are known to be amplifying hosts (8, 17, 18). Horses infected experimentally with GETV via the nasal route secrete high levels of virus, indicating that horse-to-horse transmission is possible (19). Although the morbidity rate of GETV infection in racehorses is about 40% (12) and the clinical signs in horses are generally mild, infected horses have febrile responses with a lack of appetite and depression lasting a short period of time (20). Other clinical signs such as edema of the lower limbs, swelling of the submandibular lymph nodes, rashes on the skin of the neck, and stiff gait have been reported (12, 13, 20). Most infected horses recover spontaneously within one week.

For diagnosis, serum antibodies against GETV can be measured using hemagglutination inhibition (HI) tests, complement fixation tests, enzyme-linked immunosorbent assays, and virus neutralization (VN) tests (12, 13, 20, 21). However, the procedures for HI tests are complicated. For HI tests, sera should be treated with kaolin to eliminate nonspecific inhibitors of hemagglutination, and HI tests must be performed on inactivated whole virus as an agglutination antigen in the presence of goose red blood cells. The most specific test for differentiating a GETV infection from other antigen-related alphaviruses is the VN test (22).

Clinical sign of GETV is generally mild and infection of GETV has been reported in both pigs and horse. However, international movement and populations of horses have been increased every year. Because global warming has a relation to the prevalence of vector-borne disease, infection of GETV as one of emerging disease is important. In addition, isolation of the GETV was reported in racehorse in Korea

in 1991 (23). Although racehorses are an important part of the horse industry, nationwide sero-epidemiological surveillance of GETV infections in Korea has not been performed recently. In this study, we screened horse sera for the presence of antibodies against GETV using VN tests.

MATERIALS AND METHODS

Serum sample collection

For the sero-surveillance of GETV in horses, a total of 1,182 serum samples were collected from jugular vein of Thoroughbred racehorses using serum separate tube. To estimate influence of activity of mosquitoes, 375 samples were obtained from Thoroughbred racehorses in four provinces (Gyeongnam, Gyeonggi, Jeonbuk, Jeju) in April and 474 sera samples were collected from the horses in the same provinces in September in 2013. Among them, 47 samples were obtained from the same Thoroughbred racehorses. In 2014, 360 serums were collected from the horses in the same province and 45 samples were collected from the same horses in 2013. The distribution of the horse age was ranged from 1-year-old to 23-year-old. About 70% of the racehorses tested in this study were produced in Korea; the others were imported from various countries, including the United States of America, Australia and Japan, *etc.* As GETV vaccination has been only used in Japan, most of the Korean Thoroughbred racehorses were not immunized with the GETV vaccine. Two horses imported from Japan had no information about GETV vaccines. The serum samples were heated at 56°C for 30 min and stored at -20°C until use.

Virus and cells

GETV strain QIAG9301 (NCBI accession number: KR-081238), isolated from swine blood in 1993, was used in this study. Passaging of the QIAG9301 strain was conducted in Vero cells (ATCC® CCL-81™) derived from green monkey kidney cells. The maintenance medium used was α -MEM containing 10% fetal bovine serum (FBS), 100 IU/ml penicillin, 100 μ g/ml streptomycin, and 0.25 μ g/ml amphotericin B (Gibco, Carlsbad, CA, USA). For the propagation of GETV, monolayered Vero cells were rinsed

Table 1. Distribution of virus neutralizing titers against GETV among Korean Thoroughbred horses between 2013 and 2014

| Year | No. of samples | No. of positive samples (%) | Virus neutralizing titer | | | | | | | |
|-------|----------------|-----------------------------|--------------------------|------|------|------|------|------|-------|-------|
| | | | 1: 2 | 1: 4 | 1: 8 | 1:16 | 1:32 | 1:64 | 1:128 | 1:256 |
| 2013 | 822 | 102 (12.4) | 40 | 26 | 27 | 6 | 0 | 3 | 0 | 0 |
| 2014 | 360 | 44 (12.2) | 0 | 9 | 2 | 8 | 5 | 9 | 5 | 6 |
| Total | 1,182 | 146 (12.4) | 40 | 35 | 29 | 14 | 5 | 11 | 5 | 6 |

Table 2. Seasonal seropositivity rate of the Korean Thoroughbred horses in 2013

| Month of collection | No. of samples | No. of positive | Seropositivity rates (%) | No. of the same samples | No. of positive | No. of high titer |
|---------------------|----------------|-----------------|--------------------------|-------------------------|-----------------|-------------------|
| April | 348 | 30 | 8.6 | 47 | 13 | 2 |
| September | 474 | 72 | 15.2 | | | 11 |

twice with PBS (pH 7.2) and then inoculated with strain QIAG9301 and incubated in a 5% CO₂ incubator for 1 h. After rinsing, the monolayered Vero cells were incubated at 37°C under 5% CO₂ until cytopathic effects (CPEs) were observed. After three freeze-thaw cycles, the virus was harvested and clarified by centrifugation (3,000 × g, 15 min) to remove cell debris. The titer of GETV was measured using the Reed-Muench method (24). Briefly, 100 µl of a ten-fold serial dilution of the virus was mixed same volume of Vero cells (2 × 10⁵ cells/ml) in α-MEM containing 10% FBS. The microplates were incubated for five days at 37°C under 5% CO₂ until cytopathic effects (CPEs) were observed. Fifty percent of tissue culture infectious dose of the virus was calculated based on Reed-Muench method.

Virus neutralization test

A VN test was performed to estimate the prevalence of anti-GETV antibodies in the serum samples. The test was conducted using the microneutralization test technique. Briefly, a 50-µl aliquot of a two-fold serial dilution of heat-inactivated serum was mixed with an equal volume of 200 TCID₅₀/0.1 ml. After incubation for 1 h at 37°C, a total of 100 µl of Vero cells in α-MEM containing 10% FBS were added to each well at a concentration of 2 × 10⁵

cells/ml. The microplates were incubated for five days at 37°C under 5% CO₂. The titers of the serum samples are expressed as the reciprocal of the highest serum dilution completely inhibiting CPEs, from 1:2 to 1:128. An antibody titer ≥ 1:2 was considered positive.

Statistical analysis

The chi-square test was used to analyze differences in sero-prevalence by age, sex, and region. A *p*-value < 0.05 was deemed to indicate statistical significance.

RESULTS

The results of our analysis are shown in Tables 1, 2, 3 and 4 and in Fig. 1. The seropositivity rate for GETV was 12.4% (146/1182) among 1,182 serum samples collected from Korean Thoroughbred horses. The seropositivity rates according to year were found to be 12.4% (102/822) and 12.2% (44/360) in 2013 and 2014, respectively. The distribution of VN titers against GETV in Thoroughbred horses ranged from 1:2 to 1:64 and from 1:2 to 1:256 in 2013 and 2014, respectively. Most of the serum samples showed GETV antibody titers of less than 1:2. However, six horses showed high VN antibody titers of 1:256 against GETV in

Table 3. Regional distribution of GETV antibodies among Koran Thoroughbred horses between 2013 and 2014

| Province | 2013 | | | 2014 | | | Total |
|----------|----------------|-----------------|---------------------------|----------------|-----------------|---------------------------|-------|
| | No. of samples | No. of positive | Sero-positivity rates (%) | No. of samples | No. of positive | Sero-positivity rates (%) | |
| GG* | 378 | 32 | 8.5 | 130 | 17 | 13 | 9.6 |
| GN | 175 | 39 | 22.3 | 100 | 15 | 15 | 19.6 |
| JB | 40 | 2 | 5.0 | 20 | 0 | 0 | 3.3 |
| JJ | 229 | 29 | 12.7 | 110 | 12 | 10.9 | 12.1 |
| Total | 822 | 102 | 12.4 | 360 | 44 | 12.2 | 12.4 |

*GG: Gyeonggi Province, GN: Gyeongnam Province, JB: Jeonbuk Province, JJ: Jeju Province.

Table 4. Distribution of seropositivity rates according to horse between 2013 and 2014

| Age | 2013 | | | 2014 | | |
|-------|----------------|-----------------|--------------------------|----------------|-----------------|--------------------------|
| | No. of samples | No. of positive | Seropositivity rates (%) | No. of samples | No. of positive | Seropositivity rates (%) |
| 1 | 0 | 0 | – | 1 | 0 | 0 |
| 2 | 192 | 7 | 3.6 | 67 | 6 | 9 |
| 3 | 261 | 27 | 10.3 | 108 | 2 | 1.9 |
| 4 | 143 | 20 | 14 | 81 | 9 | 11.1 |
| 5 | 61 | 11 | 18 | 32 | 7 | 21.9 |
| ≥ 6 | 165 | 37 | 22.4 | 71 | 20 | 28.2 |
| Total | 822 | 102 | 12.4 | 360 | 44 | 12.2 |

2014. The seasonal prevalence of the antibodies to GETV showed in Table 2. The seropositivity rates in April and September in 2013 turned out to be 8.6% and 15.2%, respectively. Thirteen sera samples among 47 samples showed seropositivity rates and the antibody titers of racehorses sera collected in September were higher than those of sera collected in April in 2013. The regional distribution of seropositivity was 22.3% (39/175) in Gyeongnam (GN), 12.7% (29/229) in Jeju (JJ), 8.5% (32/378) in Gyeonggi (GG), and 5.0% (2/40) in Jeonbuk (JB) in 2013. In comparison, the regional distribution of VN titers was 15.0% (15/100) in GN, 13.1% (17/130) in GG, 10.9% (12/110) in JJ, and 0% (0/20) in JB in 2014. The prevalence rates according to horse age showed that horses over 6 years of age had the highest GETV seropositivity rates (22.4% and 28.2%, respectively),

and that the seropositivity rates increased with increasing racehorse age ($p < 0.05$). The incidence of GETV was higher in geldings than in males and females in 2013 and 2014 ($p < 0.05$ in 2013; Fig. 1).

DISCUSSION

Since GETV was first isolated from mosquitoes in Malaysia, GETV infections in horses have been reported in Japan, India and Korea (12, 13, 23). In addition, the serosurveillance of GETV in horses has been reported in Japan (47.8%), China (25%), and Hong Kong (25%) (9, 14, 16). Antibodies against GETV have also been detected in pigs, with prevalence rates ranging from 47.8% to 15% (16, 25).

When serological surveys of GETV in Korean horses

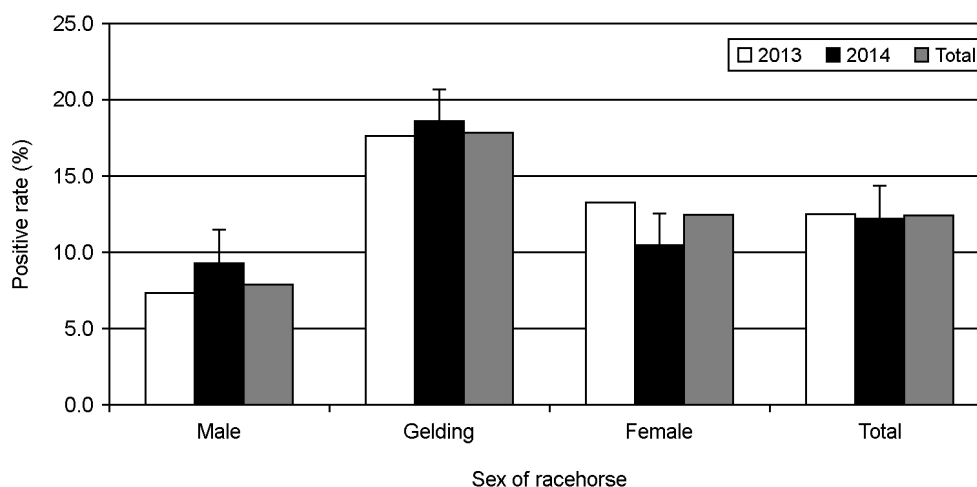


Figure 1. Seropositivity rates for GETV gender among 1,182 Korean Thoroughbred horses between 2013 and 2014.

were performed in 1986, the results showed that 41.5% of horses were positive for GETV (15). Our sero-prevalence data show that the overall seropositivity rate for GETV in racehorses was moderately low (12.4%); in addition, the regional sero-prevalence against GETV ranged from 22.3% to negative. Compared with previous data, the seropositivity rate for GETV decreased. It remains unclear why Thoroughbred racehorses exhibited such a low sero-prevalence compared to the horses tested in 1986. However, according to a recently published analysis of 371 sample pools of culicine mosquitoes, only two pools of *Aedes vexans nipponii* collected in June 2010 were positive for GETV (10), indicating that the prevalence of the vector transmitting GETV has been decreasing in Korea. In addition, since an aggressive treatment program for the eradication of mosquitoes was performed, the mosquito density has decreased significantly. The use of mosquito repellents and insecticides has resulted in decreased animal-vector contact (26). Samples were collected from Thoroughbred racehorses bred by the Korea Racing Authority (KRA). However, the blood was collected from Thoroughbred racehorses managed by the KRA and Jeju ponies bred by farmers in 1986. It is possible that the breeding conditions and hygienic practices of the KRA were better than those found on common horse farms.

Our sero-prevalence data showed that the seropositivity

rate for GETV in sera obtained in September was higher than that of serum sample collected in April in 2013. In addition, the antibody titers of racehorses sera collected in September were higher than those of horses serum collected in April in 2013. It suggests that the activity of mosquitoes influence seropositivity rate. Also an analysis of the distribution of VN titers against GETV according to horse age showed the highest seropositivity rate in horses over 6 years of age. It indicated that the horses were repeatedly exposed to GETV. There was no GETV vaccine for horses in Korea, and the seropositivity rate for GETV indicates that horses were bitten by mosquitoes carrying GETV. Moreover, our distribution data were similar in 2014 (12.4%) and 2013 (12.2%). However, six horses showed a high antibody titer against GETV (1:256) in 2014, suggesting that natural infections still occur each year in a small number of Korean racehorses.

It is known that *A. vexans nipponii*, which transmits both GETV and Japanese encephalitis virus (JEV), is distributed widely in Korea; it was first found in Gyeongnam province during the early summer season (27, 28). According to a recently paper, *Aedes vexans nipponii* collected in June 2010 was positive for GETV (10). According to our sero-surveillance data, Gyeongnam province showed the highest seropositivity rate for GETV (22.3%) among the other prov-

inces. Although our serological data indicate no statistically significant differences among the provinces, our results suggest that GETV infections are closely associated with mosquito (*A. vexans nipponii*) activity in Korea.

No differences in the seropositivity rate for JEV were observed among female horses, male horses, and geldings (29). However, our results show that the incidence of GETV was higher in geldings than in males and females. The reason for this remains unclear, but it is possible that geldings secrete large amounts of materials that attract mosquitoes.

In conclusion, antibodies against GETV were identified in Korean racehorses, indicating that the horses were infected to GETV without any symptoms. It is supposed that serosurveillance of GETV in racehorses should perform annually in Korea. Also it is necessary to predict the transmission of vector-borne viral diseases between horses and mosquitoes and prepare vaccine about GETV in the near future.

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