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NOTE

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Seroconversion of anti-Getah virus antibody among Japanese native Noma horses around 2012

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ABSTRACT. Getah virus (GETV), an arthropod-borne virus transmitted by mosquitoes, has been isolated from several animals. GETV infection in horses shows clinical signs such as fever, rash, and edema in the leg. Noma horses are one of the eight Japanese native horses. The present study aimed to clarify the occurrence of GETV infection in Noma horses. Serum samples collected from Noma horses were analyzed using a virus neutralization test and enzyme-linked immunosorbent assay and showed that the anti-GETV antibody titers in the samples collected in 2017 were significantly higher than those collected in 2012. We concluded that a seroconversion of anti-GETV antibodies was occurred in the Noma horse population around 2012, providing evidence of the GETV epidemic in Japan circa 2012.

KEYWORDS: ELISA, Getah virus, Japanese native horse, virus neutralization test

Getah virus (GETV), which belongs to the genus *Alphavirus* of the family *Togaviridae*, order *Martellivirales*, is a positive-sense single-stranded RNA virus [7]. It is an arthropod-borne virus (arbovirus) transmitted by various species of mosquitoes, and pigs are one of its main amplifiers [5]. GETV has been isolated from domestic animals such as horses, pigs, and cattle [1, 16, 19, 28], and anti-GETV antibodies have also been detected in the other animal species including wild boars, chickens, cattle, ducks, and humans [4, 14, 15]. GETV infection is related to depression, tremor, yellowish-brown diarrhea, and death in piglets as well as reproductive disorders in pregnant sows [9, 28]. Horses also act as amplifiers of GETV, showing clinical signs such as fever, rash, and edema in the leg [3, 5]. GETV multiplies in lymphoid tissues and is thought to cause a rash through an immunological response, but it is not highly pathogenic to horses and does not cause any sequelae [5, 27]. GTEV can be shed from the nasal cavity of horses, but the shed virus titer is low and the natural aerosol transmission is thought to be rare [12]. Clinical signs of GETV infection have also been observed in other animals, including fever, anorexia, depression, neurological sign, and death in blue foxes as well as fever, loss of appetite, and depression in cattle [16, 23].

In Japan, GETV outbreak was first recognized among thoroughbred racehorses in 1978 at the Miho Training Center of the Japan Racing Association, located in the Kanto district, central Japan. Furthermore, subsequent outbreaks occurred in 1979 and 1983 [11, 22, 24]. At that time, an inactivated GETV vaccine was developed and has been used in some horse facilities in Japan since 1979 [1]. Over the subsequent 30 years, no GETV outbreaks were observed among racehorses in Japan. However, GETV outbreaks occurred suddenly from 2014 to 2016 at the Miho Training Center among racehorses which had been vaccinated against GETV [2, 18, 20].

In Japan, eight Japanese native horses, namely Hokkaido, Kiso, Misaki, Noma, Taishu, Tokara, Miyako, and Yonaguni, have been bred to date [8]. Noma horses are one of the Japanese native horses that have been maintained in Ehime Prefecture on Shikoku Island. These are the smallest among the eight Japanese native horses, which stand around 110 cm in wither height. The origin of the Noma horse is thought to be back to the 17th century, and it was used as a pack animal on steep mountainsides of Setouchi remote islands [8]. However, the population of Noma horses drastically reduced in the 19th century due to modernization. Through the efforts of

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Noma horse conservation activities, the population has recovered in approximately 50 horses [21]. Since Noma horses have no history of vaccination against GETV, their serum samples could be evaluated by natural infection with GETV. The present study aimed to clarify the occurrence of GETV infection in Japanese native Noma horses for the estimation of potential risk of disease caused by GETV in this population.

Animal experiment procedures were carried out in compliance with the regulations outlined in Guide for the Care and Use of Laboratory Animals of the Okayama University of Science, and were reviewed and approved by the Institutional Animal Care and Use Committee at the Okayama University of Science (approval number; Jitsu 2021-049). Sequential serum samples from 39 horses kept at the Nomauma Highland Park in Imabari City, Ehime Prefecture, were analyzed in this study. The samples were collected in June–September 2012, June–September 2017, September 2018, October 2019, and September 2020. All of these horses were individually identifiable, and each individual was examined chronologically from 2012 to 2020. However, some samples were included without serum due to poor body condition or difficulty collecting blood.

The GETV strain 14-I-605-C1, which was isolated from the blood of a diseased racehorse in 2014 [1], was used. No antigenic differences were observed between the GETV 14-I-605 and vaccine strains in a cross-neutralization test [19]. Vero and BHK-21 cells were cultured in minimum essential medium (Sigma Aldrich Inc., St. Louis, MO, USA) supplemented with 10% fetal calf serum (JR Scientific Inc., Woodland, CA, USA), 2% non-essential amino acids (Thermo Fisher Scientific Inc., Waltham, MA, USA), and 1% penicillin-streptomycin (Life Technologies Corp., Grand Island, NY, USA) at 37°C under 5% CO_2. An 80% plaque-reduction neutralization test ($PRNT_{80}$) was used to measure anti-GETV antibody titer in the serum samples of Noma horses. First, 77 serum samples collected in October 2019 (n=39) and September 2020 (n=38) were diluted at 1:10 and screened positive or negative using $PRNT_{80}$ to estimate the seroprevalence of GETV infection and calculate the enzyme-linked immunosorbent assay (ELISA) cut-off value. Furthermore, 38 serum samples collected in September 2020 were prepared by two-fold serial dilution from 1:10 to 1:5,120 to measure the anti-GETV antibody titer. For the virus neutralization test, the serum samples were inactivated for 30 min at 56°C. Vero cells were seeded in 12-well plates (TPP Techno Plastic Products AG, Trasadingen, Switzerland) at a concentration of 2.0×10^5 cells/well, and the virus-neutralizing (VN) test was performed to determine the titer for the anti-GETV antibody.

In total, 174 sequential samples collected from 39 horses were subjected to ELISA. ELISA was performed using extracts from GETV- and mock-infected BHK cells as the antigens. This was performed using the method described in our previous study [14]. ELISA antigen diluted to 5 µg/mL with absorption buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) was dispensed into 96-well microplates (MaxiSorp; Nunc, Roskilde, Denmark) with 100 µL per well. After blocking with 1% Block Ace (DS Pharma Biomedical UK-B40) in PBS, 100 µL of serum samples diluted 100-fold with 0.4% Block Ace in PBS-T were added in each well as the primary antibody. Peroxidase-conjugated recombinant protein A/G (Thermo Fischer Scientific) diluted in 0.4% Block Ace in PBS-T was used as a secondary antibody. Further, 100 µL of ABTS® 2-Component Microwell Peroxidase Substrate Kit (SeraCare Life Sciences, Inc., Milford, MA, USA) was added in each well and shaken for 30 min at room temperature. The absorbance was measured using a spectrophotometer (Bio-Rad, Hercules, CA, USA) with a 405-nm filter. All statistical analyses were performed using the EZR statistical software version 1.54 [10]. Statistical significance was set at $P<0.001$. The correlation between ELISA absorbance and VN titer was analyzed using Spearman's correlation test to assess the accuracy of the ELISA. The ELISA cut-off value, sensitivity, and specificity were analyzed using receiver operating characteristic (ROC) analysis. Differences in the antibody titers for each year between 2012 and 2017–2020 were tested using Friedman's test with the Bonferroni correction for group comparisons.

To estimate the seroprevalence of GETV infection among Japanese native Noma horses, 77 Noma horse sera collected in October 2019 (n=39) and September 2020 (n=38) were diluted at 1:10 and tested for $PRNT_{80}$. The positive ratio of anti-GETV antibodies among Noma horses was 69.2% (27/39) in October 2019 and 65.8% (25/38) in September 2020, indicating that Noma horses had previously been infected with GETV. Titers for anti-GETV antibodies in 38 Noma horse sera samples collected in September 2020 were serially diluted 2-fold from 1:10 to 1:5,120 and examined for $PRNT_{80}$; 13 horses were found with an antibody titer of <1:10; 25 horses, with an antibody titer of ≥1:320; and no horses, with an antibody titer of 1:20, 1:40, 1:80, and 1:160 (Fig. 1). The comparison between VN titers and ELISA absorbance revealed that the ELISA results correlated with the VN titer with a significant positive correlation (Spearman correlation coefficient, r=0.79, $P<0.001$) (Fig. 1). This result indicates that this ELISA was useful for the evaluation of anti-GETV antibody titer in Japanese native Noma horses. ROC analysis indicated that the ELISA cut-off value was calculated to be 0.268 with a sensitivity and specificity of 1.000 and 1.000, respectively.

A total of 174 Noma horse sera samples collected from 2012 and 2017–2020 was analyzed using ELISA (cut-off value of 0.268). The seropositivity of anti-GETV antibodies was 37.9% (11/29) in 2012, 75.0% (24/32) in 2017, 69.4% (25/36) in 2018, 69.2% (27/39) in 2019, and 65.8% (25/38) in 2020 for the sera samples collected. We found anti-GETV antibodies were detected in serum sample collected in 2012, indicating that GETV had been distributed in Ehime prefecture before 2012. Friedman test with Bonferroni correction was performed on the ELISA results for each year. The results showed that the anti-GETV antibody titers in the samples collected in 2017 were significantly ($P<0.001$) higher than those collected in 2012 (Fig. 2), indicating seroconversion of anti-GETV antibody titers in the Noma horse population from 2012 to 2017. In addition, we found that the kinetics of anti-GETV antibody titers in Noma horses could be classified into three groups (Fig. 3); in group A, 8 horses showed high antibody titers continuously from 2012 to 2020; in group B, 18 horses had low titers in 2012, which increased after 2012 (n=16 were between 2012 to 2017, and n=2 were between 2012 to 2018 because no serum samples in 2017 for two horses) and remained high until 2020; in group C, 13 horses showed low antibody titers continuously from 2012 to 2020. This result indicated that Noma horses classified as group A had already been infected with GETV before 2012, those in group B were infected with GETV between 2012 and 2017, and those in group C were not infected with GETV (Fig. 3).

The present study demonstrated that Japanese native Noma horses were exposed to GETV infection. This result is the second

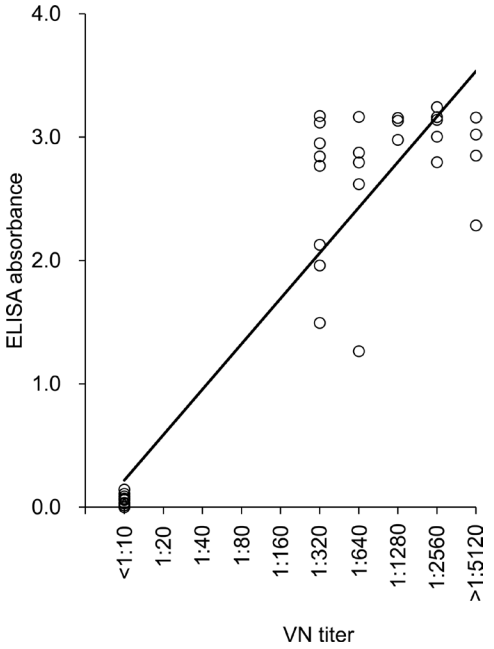

Fig. 1. Comparison between enzyme-linked immunosorbent assay (ELISA) absorbance and virus-neutralizing (VN) antibody titers against Getah virus in Japanese native Noma horses. The correlation between ELISA and VN test using 38 Noma horse sera samples was analyzed. Dot plots in the X and Y axes indicate the VN titer and the ELISA absorbance, respectively. Spearman's correlation test was performed between the ELISA absorbance and the VN titer; r=0.79, $P<0.001$.

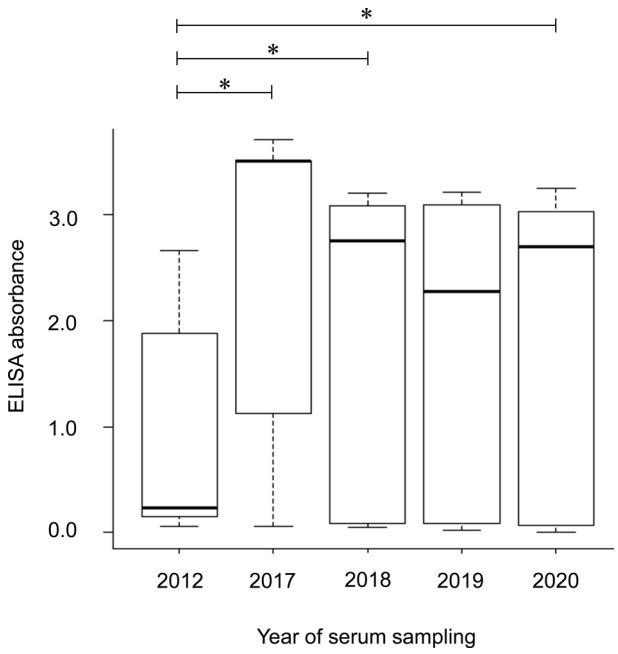

Fig. 2. Box plot of ELISA absorbance for anti-Getah virus antibody of Japanese native Noma horse sera by year. The number of horses were n=29 (2012), n=32 (2017), n=36 (2018), n=39 (2019), and n=38 (2020). The median is marked by the bold line inside the box, the ends of the box show the upper and lower quartiles, and the whiskers show the minimum to maximum range. Data were analyzed using Friedman test with Bonferroni multiple comparison test; *$P<0.001$.

report for Japanese native horses that were infected with GETV after the case of Hokkaido horse [17]. Japanese native horses are more closely related to Mongolian horses than to thoroughbred horses and several horse populations in Japan are genetically similar to each other than imported thoroughbred horses [26]. These facts suggest the possibility of GETV infection in other Japanese native horses. Although the clinical signs of GETV infection in Japanese native horses are unclear, it is necessary to make further efforts for grasping the risk of GETV infection, avoid virus-infected mosquito bites, and introduction of vaccination program if necessary, for protecting the health and preserving the Japanese native horses.

The ELISA results of Noma horses in group B showed seroconversion of anti-GETV antibody titers between 2012 and 2017, while those in group C showed no increase in antibody titer during the study even though they are all raised in the same condition (Fig. 3). This suggests that an epidemic of GETV infection occurred in the Noma horse population between 2012 and 2017, and no GETV epidemic probably occurred after 2017. In addition, 26 Noma horses in groups A and B maintained high antibody titers from 2017 to 2020 (Fig. 3). These results suggest that anti-GETV antibodies in Noma horses in groups A and B were likely acquired before 2017, after which antibody titers may have been maintained for at least three years. Furthermore, examination of the births of the 39 Noma horses revealed that all horses in groups A and B were born before October 2011, while all but three horses in group C were born in February 2014 or later (Fig. 4). This indicates that Noma horses born after 2014 were not infected with GETV infection, further limiting the duration of the GETV epidemic in the Noma horse population until 2014.

Several retrospective studies of GETV infections in Japan have been reported. One study reported that GETV was unexpectedly isolated from *Culex tritaeniorhyncus* mosquitoes at high minimum infection rates in Nagasaki Prefecture in 2012 [13]. This GETV strain 12IH26 isolated from mosquitoes in Nagasaki Prefecture is phylogenetically related to the GETV strains that caused an epidemic among racehorses in 2014 and 2016 [20], being also closely related to Chinese and South Korean strains, rather than the Kochi/01/2005 strain reported in 2005, which is considered a Japanese domestic strain [25]. Another study reported an epidemic of GETV infection among wild boar populations in Japan in 2012 [14]. These studies suggested that the invasion of GETV that appeared to originate from mainland China or the Korean Peninsula, occurred in Japan in 2012 or earlier, and the virus spread from western to eastern Japan, and caused the epidemic of GETV among thoroughbred racehorses in 2014. The result of this study supports two previous retrospective studies reporting possible incursion of GETV and its spread in Japan around 2012 [13, 14], and further suggests that the duration of anti-GETV antibody is probably from 2014 to 2020. Experimental studies have shown that anti-GETV antibody titers are maintained for at least 12 months in thoroughbred horses [6], and our study suggests that the duration could be even longer.

Shikoku Island, where Noma horses are bred, is geographically separated from mainland Honshu by an ocean. It is not likely that wild animals, infected with GETV and developed viremia in Honshu around 2012, will play a major role in introducing GETV to

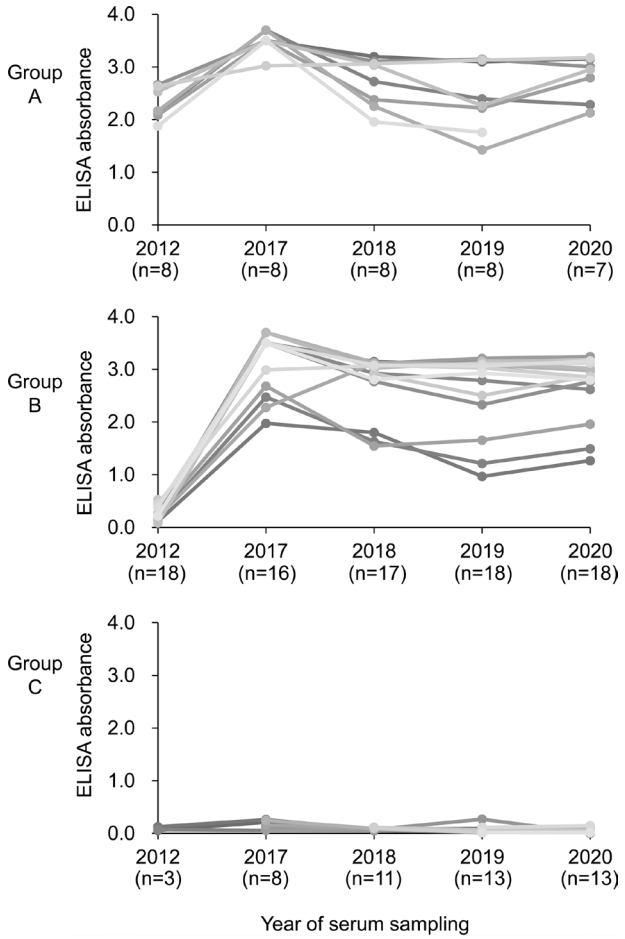

Fig. 3. Kinetics of anti-Getah virus antibody titers in Noma horse sera. Noma horse population was grouped by pattern of chronological changes of anti-Getah virus antibody titers. 174 sera from 39 Noma horses were collected and analyzed using ELISA. The vertical axis indicates ELISA absorbance, and the horizontal axis indicates the year for serum collection. 39 Noma horses were grouped as **A** (n=8), **B** (n=18), and **C** (n=13), and the numbers above the line indicate the number of sera for each year.

Fig. 4. Year of birth distribution among Noma horses. Year of birth of the 39 Noma horses in the three groups. The horses were grouped as **A** (n=8), **B** (n=18), and **C** (n=13) by kinetics of anti-GETV antibody titers in Fig. 3. The numbers above the dots indicate the number of horses born in the same year.

Shikoku Island across the sea in a short period of time. Although wild boars have been implicated in the recent spread of GETV in Japan [14], it is unlikely that there was any wild boar interaction between Honshu and Shikoku in the short term. Unfortunately, reverse transcription polymerase chain reaction amplification did not yield genetic information about GETV strain from Noma horse sera (data not shown). Even if the GETV epidemic in the Noma horse population is related to an epidemic of GETV that occurred in the wild boar population of Yamaguchi Prefecture in southwestern Japan around 2012 [14], it is likely that the introduction of GETV into Shikoku Island was not by wild boars but by virus-infected mosquitoes or birds flying across the sea. Mosquitoes would play a major role in the short-term spread of arboviruses and the epidemics of arboviral diseases.

The present study exhibited a seroconversion of anti-GETV antibodies among Japanese native Noma horses around 2012, providing additional evidence for the GETV epidemic in Japan around 2012, as reported by some previous studies [13, 14]. Surveillance of the horse, vector mosquitoes, and other animals involved in the circulation of GETV will be required to determine the status of GETV infection in each region bred Japanese native horses in further study. The case of GETV epidemic spreading around the breeding sites of Japanese native horses, future efforts to reduce the risk of arbovirus infection from vector arthropods by using insect repellents and mosquito nets, and if necessary, vaccination against GETV for at least antibody-negative horses would help us to conserve Japanese native horses.

CONFLICT OF INTEREST. The authors declare no conflicts of interest.

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