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NOTE

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Seroepidemiology of non-primate hepacivirus (NPHV) in Japanese native horses

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ABSTRACT. Non-primate hepacivirus (NPHV) is recently identified as a closely related homologue of hepatitis C virus. The previous studies showed a high prevalence of NPHV infection among Japanese domestic horses originated from abroad. The historical distribution of NPHV among horses in Japan, therefore, is still unknown. In this study, seroepidemiological study of NPHV was conducted using 335 sera from five breeds of Japanese native horses. These horses are maintained as the pedigree and are reared apart from other horse breeds. The detection of antibodies against NPHV were conducted by western blot analysis using the recombinant protein of the NPHV core protein. The antibodies against NPHV were detected in all five breeds, 83 out of 335 (23.4%) horses. These results suggested that NPHV was circulating among Japanese native horses.

KEY WORDS: Japanese native horse, non-primate hepacivirus, seroepidemiology

It is estimated that hepatitis C virus (HCV) chronically infects approximately 3% of the world's population, and is a major cause of chronic liver diseases of humans [19]. HCV belongs to the genus *Hepacivirus* of the *Flaviviridae* family together with three other genera, *Flavivirus*, *Pestivirus*, and *Pegivirus*. Only two species, HCV and GB virus B, had been known species of the genus *Hepacivirus* until 2010 [2, 10, 20]. Recently, new non-primate hepacivirus (NPHV) was identified in dogs, horses, rodents, bats and cattle [1, 3–5, 7, 9, 11, 12, 16, 18]. The genes of this virus showed the high homology to the HCV genes by phylogenetic analysis [8]. NPHV infections were circulating among horses, occurred vertical and horizontal transmission [6]. After a primary NPHV infection, horses were protected against experimentally reinfection with the homologous viruses [15]. On the other hand, there was the frequent occurrence of NPHV infections among thoroughbreds [17]. The high occurrence of NPHV infections in breeding thoroughbreds revealed the association of age and international transportation as risk factor for NPHV infections.

In Japan, a seroepidemiological study revealed that 33.55% of the horses were carrying the NPHV antibodies when 435 serum samples taken from racehorses and riding horses were examined [14]. The majority of horses tested in that study were domestic thoroughbreds. Another study also showed that 22.6% of Japanese-born domestic horses tested positive for NPHV [21]. Because breed horses such as thoroughbreds were originally imported from abroad, NPHV could have been introduced from abroad and have spread among the horses in Japan. In contrast, there are some horses indigenous to Japan. They have been prevented from being hybridized with foreign-bred horses, and have maintained their genetic traits unique to Japanese native horses. In this study, we intended to obtain seroprevalence of NPHV in Japanese native horses for further understanding of the natural history of NPHV.

A total of 355 serum samples was collected from five breeds of healthy Japanese native horses, 148 of Kiso-Uma (2010–2013), 66 of Hokkaido-Washuba (2014), 63 of Misaki-Uma (2014), 43 of Yonaguni-Uma (2012) and 35 of Miyako-Uma (2013) (Fig. 1 and Table 1). Their ages ranged from one to over 20 years but the ages of 61 horses were unknown. These sera had been previously collected for routine health check and have been stored at −80°C. The human embryonic kidney cell line 293T cells were maintained in Dullbecco's modified Eagle's minimal essential medium supplemented with 100 U/ m*l* penicillin, 100 μg/ m*l* streptomycin (Life technologies, Grand Island, NY, U.S.A.) and 10% fetal bovine serum and was cultured at 37°C under the condition of a humidified atmosphere and 5% CO_2. Plasmid encoding the NPHV core protein (pCAG-F-EHcVc-HA) was obtained in a previous study [21]. 293T cells were cultured in 6-well-plates and transfected with the plasmid using FuGene HD Transfected Reagent (Promega, Madison, WI, U.S.A.) according to the manufacturer's instruction. The transfected cells were harvested at 48 hr posttransfection, washed with cold phosphate-buffered-saline, and suspended in 60 μ*l* of the lysis buffer consisting of 20

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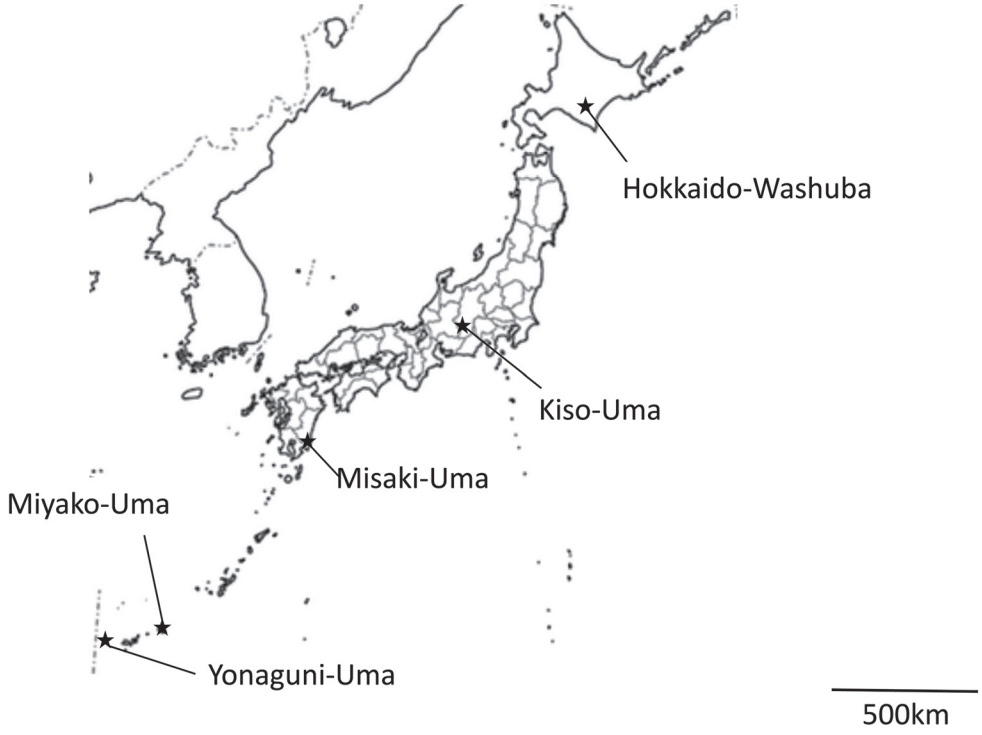

Fig. 1. Map of Japan where serum samples were collected from Japanese native horses in this study.

Table 1. The detection of antibodies against NPHV core protein in Japanese native horses

Age (years)	Hokkaido-Washuba	Kiso-Uma	Misaki-Uma	Miyako-Uma	Yonaguni-Uma	Total (%)
<2	7/11	0/0	0/15	0/5	0/0	7/31 (22.6)
3–6	9/16	0/0	2/16	2/12	0/0	13/44 (29.5)
7–10	10/12	0/1	2/13	0/8	0/0	12/34 (35.3)
11–15	7/9	4/24	1/14	1/7	0/0	13/54 (24.1)
>16	12/17	5/106	1/5	0/3	0/0	18/131 (13.7)
Unkown	0/1	0/17	0/0	0/0	20/43	20/61 (32.8)
Total	45/66 (68.2)	9/148 (6.1)	6/63 (9.5)	3/35 (8.6)	20/43 (46.5)	83/355 (23.4)

mM Tris-HCl (pH 7.5), 125 mM NaCl, 10% glycerol, 1% Triton X-100, protease inhibitor cocktail I (Wako, Osaka, Japan), and 1% lysozyme (Wako). After centrifugation at 15,000 rpm for 10 min at 4°C, 16 μl of the supernatants were mixed with 800 μl of sample buffer consisting of 62.5 mM Tris-HCl (pH 6.8), 25% Glycerol, 2% Sodium dodecyl sulfate, 0.5% Bromophenol blue and 5% 2-mercaptethanol and then boiled at 96°C for 10 min. The resulting mixtures were subjected to 12% SDS-PAGE. The developed proteins were then transferred to a ployvinylidene difluoride (PVDF) membrane. The membrane was incubated with TBS (10 mM Tris, 150 mM NaCl, pH 7.5) containing 1% Tween 20 (TBS-T) followed by blocking with 5% skim milk (Wako) at 4°C for overnight. After blocking, the membrane was incubated with TBS-T containing 1% skim milk (1% TBS-T) at room temperature (RT) for 15 min, and then washed three times with TBS-T. Horse sera diluted to 1:25 in 1% TBS-T were applied to the PVDF membrane and left at RT for 1 hr. The membrane was then washed with TBS-T and immersed in a 1:5,000 dilution of goat anti-horse IgG-AP (Santa Cruz Biothechnology Inc., Santa Cruz, CA, U.S.A.) at RT for 1 hr. After washing, the immunocomplexes were visualized with Western Blue Substrate (Promega). For the detection of the positive band size, the serum from rabbit immunized by the NPHV core protein was used (Fig. 2).

The NPHV antibodies were detected in a total of 83 horse sera (83/355, 23.9%). Forty-five out of 66 Hokkaido-Washuba sera (68.2%) and 20 out of 43 Yonaguni-Uma (46.5%) were seropositive for NPHV, while 6 out of 63 (9.5%) Misaki-Uma, 3 out of 35 Miyako-Uma (8.6%), and 9 out of 148 Kiso-Uma (6.1%) were found to be seropositive. There was no significant difference between Hokkaido-Washuba and Yonaguni-Uma by Ryan test. On the other hand, there were significant differences Hokkaido-Washuba and Yonaguni-Uma compared with other three breeds, Misaki-Uma, Miyako-Uma and Kiso-Uma, respectively. In addition, there were no significant differences in age and sex by logistic regression analysis using Epi Info7 software. Although the rates of positive sera were wide range, 6.1% to 68.2% between five breeds, these results suggested that NPHV is widespread

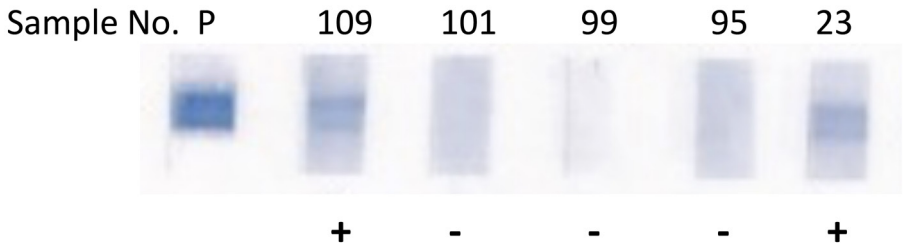

Fig. 2. The strips of PVDF membranes of typical results in western blot analysis. P is the control band size using immunized rabbit serum. Numbers are sample ID of Hokkaido-Washuba.

among Japanese native horses that have been kept in the restricted areas. In Germany, statistical analysis revealed the association of age and international transportation as risk factor for NPHV infection, whereas there was no association between age and NPHV infection among domestic horses [17]. The Japan Equine Affairs Association reported that there are eight breeds of Japanese native horses in Japan, a total of 1,749 horses in 2016, 1,106 of Hokkaido-Washuba, 150 of Kiso-Uma, 53 of Noma-Uma, 39 of Tsushima-Uma, 102 of Misaki-Uma, 123 of Tokara-Uma, 46 of Miyako-Uma and 130 of Yonaguni-Uma. Especially, Miyako-Uma and Yonaguni-Uma are reared in small southern remote islands over 100 years without breeding and transportation. Since previous studies showed that NPHV infections were occurred by vertical and horizontal transmission pasture herds [6], NPHV might be maintained among Japanese native horses for quite a long time. However, the contact between Japanese native horses and wildlife is unknown. The seroprevalence of *Toxoplasma gondii* in Japanese native horses was 4/254 (1.57%) from 2 of Kiso-Uma, one each of Miyako-Uma and Yonaguni-Uma [13]. NPHV and other infectious pathogens might be transferred to Japanese native horses from not the other horse breeds but wildlife and maintained among Japanese native horses. Further studies involving Japanese native horses are required to address the origin by genetic analysis and epidemiological survey of NPHV.

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