NOTE *Public Health*

Cross-Reactivity of Japanese Encephalitis Virus-Vaccinated Horse Sera in Serodiagnosis of West Nile Virus

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ABSTRACT. Flavivirus-infected sera are known to show cross-reactions in serodiagnoses of heterologous flavivirus infections. Japanese encephalitis virus (JEV) is endemic in Asia and, in Japan, many horses are vaccinated against JEV. However, the cross-reactivity level of JEV-vaccinated horse sera in the serodiagnosis of West Nile virus (WNV) has not been clarified. The antibody cross-reactivity of JEV-vaccinated horse sera in WNV serological tests, such as the plaque reduction neutralization test (PRNT), IgG indirect ELISA (IgG-ELISA) and hemagglutination inhibition (HI) test, was examined. All JEV-vaccinated horse sera were positive for JEV antibodies with JEV PRNT at both 90% and 50% plaque reductions. In WNV PRNT, 16.7% of the horses were positive at 90% plaque reduction, and 50% of the horses were positive at 50% plaque reduction. All the JEV-vaccinated horse sera showed positive-to-negative (P/N) ratios of over 2.0 with JEV IgG-ELISA, and half of them had P/N ratios of over 2.0 with WNV IgG-ELISA. There was little difference between the JEV HI and WNV HI titers in individual horses. These results indicate that in serosurveillance of WNV, JEV-vaccinated horses can produce false-positive results in WNV IgG-ELISA, HI and PRNT.
KEY WORDS: arthropod-borne viruses, diagnosis, equine, *Flavivirus*, horse.

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West Nile virus (WNV) is an RNA-enveloped virus of the genus *Flavivirus*, family *Flaviviridae*, and belongs to the Japanese encephalitis virus (JEV) serocomplex group [16]. In the last decade, WNV has re-emerged as an important pathogen of humans, birds and horses. The endemic area of WNV is currently expanding into east Russia [23], and the risk of WNV invasion into East Asia is increasing.

Serodiagnoses have been used in WNV surveillance of horses because of the short-term viremia and low virus titer in WNV-infected horses [3, 5, 7, 9, 14, 20]. This viremia precedes the clinical onset of disease accompanying with only a few clinical symptoms [7]. In fact, WNV RNA can be detected using RT-PCR from extracts of the internal organs of dead animals but is not easily identified in the blood or sera of living animals [12, 13, 17]. Therefore, serodiagnoses are mainly used to diagnose WNV infections in living animals [1, 17]. Indeed, IgG indirect ELISA (IgG-ELISA) is used as a first screening test for WNV infections in horses in Europe [1, 17]. In addition, hemagglutination inhibition (HI) tests [25] and virus neutralization test [3, 9, 14] are also used in flavivirus surveillance of horses.

However, cross-reactions in serodiagnosis frequently occur in heterologous flavivirus-infected sera. The cross-reaction in serodiagnosis is caused by high amino acid sequence homology of the envelope E protein with flaviviruses belonging to the same serocomplex group [16]. The envelope E protein is a major component of the virus surface, and neutralization and hemagglutinin antibodies mainly react with envelope E protein [16]. Actually, IgG-ELISA [2] and HI [2, 4, 11, 24] are known to show cross-reactivity in heterologous flavivirus-infected sera in birds, hamsters, humans and monkeys. Additionally, in virus neutralization tests, cross-reactions have been reported using hyperimmune mouse ascitic fluids and hyperimmune rabbit serum [6, 10]. These cross-reactions could be a cause of misdiagnosis.

JEV is endemic in Asia, and many horses are vaccinated against JEV in Japan. Anti-JEV antibodies could be cross-reactive in serodiagnoses of WNV infection because JEV is in the same serocomplex group as WNV. However, the cross-reactions of JEV-vaccinated horse sera in serodiagnoses of WNV are not known.

The objective of this study was to investigate the level of cross-reactivity of anti-JEV horse antibody from JEV-vaccinated horses in plaque reduction neutralization tests (PRNT), IgG-ELISA and HI of WNV.

Horse sera: Serum samples were obtained from 18 adult horses at a riding club in Tokyo that had been vaccinated with a commercially available inactivated JEV vaccine. The average age of the horses was 13.5 years old, and their ages ranged from 8 to 20 years old; they were vaccinated twice every year. As negative controls, serum samples were

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Table 1. Results of PRNT

Horse ID	90% plaque reduction			50% plaque reduction		
	JEV	WNV	JEV titer/ WNV titer	EV	WNV	JEV titer/ J WNV titer
No. 1	1:200	1:40	5	1:1000	1:160	6
No. 2	1:200	1:20	10	1:625	1:80	8
No. 3	1:400	1:10	40	1:625	1:40	16
No. 4	1:100	<1:10	–	1:400	1:80	5
No. 5	1:200	<1:10	–	1:625	1:20	31
No. 6	1:200	<1:10	–	1:800	1:10	80
No. 7	1:100	<1:10	–	1:400	1:10	40
No. 8	1:100	<1:10	–	1:200	1:10	20
No. 9	1:50	<1:10	–	1:200	1:10	20
No. 10	1:20	<1:10	–	1:80	<1:10	–
No. 11	1:400	<1:10	–	1:625	<1:10	–
No. 12	1:200	<1:10	–	1:400	<1:10	–
No. 13	1:200	<1:10	–	1:400	<1:10	–
No. 14	1:100	<1:10	–	1:400	<1:10	–
No. 15	1:100	<1:10	–	1:400	<1:10	–
No. 16	1:100	<1:10	–	1:200	<1:10	–
No. 17	1:50	<1:10	–	1:200	<1:10	–
No. 18	1:40	<1:10	–	1:125	<1:10	–

Table 2. Results of IgG-ELISA

Horse ID	JEV P/N ratio	WNV P/N ratio
No. 1	5.86	3.18
No. 2	4.92	3.03
No. 3	7.67	3.81
No. 4	3.41	1.97
No. 5	5.59	2.83
No. 6	3.93	1.99
No. 7	5.05	3.62
No. 8	4.70	2.84
No. 9	3.97	2.01
No. 10	2.02	1.64
No. 11	3.60	1.59
No. 12	2.62	1.16
No. 13	2.57	1.54
No. 14	2.46	1.66
No. 15	7.88	6.30
No. 16	4.61	2.92
No. 17	2.04	1.62
No. 18	2.49	1.61

Table 3. Results of HI

Horse ID	JEV	WNV	JEV titer / WNV titer
No. 1	1:80	1:20	4
No. 2	1:40	1:20	2
No. 3	1:160	1:40	4
No. 4	1:20	1:20	1
No. 5	1:40	1:40	1
No. 6	1:20	1:20	1
No. 7	1:20	1:20	1
No. 8	1:40	1:10	4
No. 9	1:40	1:20	2
No. 10	1:20	1:20	1
No. 11	1:40	1:20	2
No. 12	1:40	1:20	2
No. 13	1:40	1:10	4
No. 14	1:40	1:10	4
No. 15	1:20	1:10	2
No. 16	1:20	1:10	2
No. 17	1:40	1:40	1
No. 18	1:20	1:20	1

collected from 9 horses that were less than 1 year old before vaccination for JEV in Hokkaido, Japan, where JEV infection is rare. These sera were confirmed as negative by JEV-PRNT, WNV-PRNT, JEV-HI and WNV-HI.

Virus: The NY99-A301 strain of WNV and JaGAr01 strain of JEV were utilized for PRNT. Both of these strains produce clear plaques in plaque assays using Vero cells. Virus antigens for IgG-ELISA and HI were constructed using the NY99-A301 strain of WNV and Nakayama strain of JEV with Vero cells. All experiments using infectious viruses were performed in a biosafety level 3 laboratory and were approved by the Biosafety Committee of the National Institute of Animal Health in Japan.

PRNT: PRNT was performed as described in the OIE Manual for WNV using a 6-well plate format with Vero cells [19]. Briefly, test sera were heat-inactivated at 56°C for 30 min. The sera were then diluted and mixed with an equal volume of virus preparation (10^3 plaque forming unit/ 1 m*l*) in minimal essential medium containing 10% guinea pig serum and kept for 1 hr at 37°C. Aliquots of 100 μl of the mixtures were inoculated into each well of a Vero cell monolayer and kept at 37°C for 1 hr in a CO_2 incubator with occasional agitation. Nutrients containing tragacanth gum (Nacalai Tesque Inc., Kyoto, Japan) were then added, and the cells were kept in the CO_2 incubator for 3–5 days before fixing with formalin and staining with crystal violet (Nacalai Tesque). The highest serum dilution with plaque reductions of 90% and 50% was defined as the titration end

point. Serum samples that showed 90% or 50% plaque reduction at more than 1:10 dilution were considered to be positive.

IgG-ELISA: The WNV and JEV IgG-ELISA tests were performed as described previously with minor modifications [21]. The ELISA antigens of both viruses were prepared by sucrose gradient purification from β-propiolactone (Nacalai Tesque)-inactivated virus culture supernatant. Each virus antigen was used at protein concentrations of 100 *n*g/well. The positive-to-negative (P/N) ratios were determined as follows. The ODs of test sera were divided by the mean OD of the negative control sera. The mean ODs for the negative controls (n=9) were 0.197 ± 0.069 (OD range: 0.12–0.33, mean ± standard deviation [SD]) for JEV-ELISA and 0.227 ± 0.070 (OD range: 0.12–0.33, mean ± SD) for WNV-ELISA. Samples with a P/N value ≥ 2 were considered positive.

HI: HI was performed as previously described using a 96-well plate format [8]. The HI antigens of JEV and WNV were extracted from the virus culture supernatant by concentration using ultrafiltration. Acetone extraction and adsorption with goose erythrocytes were performed on the serum samples, and the treated sera were heat-inactivated at 56°C for 30 min. The diluted sera were then mixed with 8 hemagglutination units of each virus at optimal pH and incubated overnight at 4°C. Goose erythrocytes were then added to the mixture, and the plates were incubated for 1 hr at 37°C. The HI titer of each serum sample was determined at the highest dilution of serum that showed complete inhibition of erythrocyte agglutination. Serum samples that inhibited erythrocyte agglutination at more than 1:10 dilution were considered to be positive.

All of the JEV-vaccinated horse sera had JEV neutralization titers of between 1:20 and 1:400 at 90% plaque reduction and between 1:40 and 1:1000 at 50% plaque reduction. At 90% plaque reduction, 3 vaccinated horse sera had WNV neutralization titers of between 1:10 and 1:40, and the other 15 horse sera did not show any titer. At 50% plaque reduction, 9 horse sera had WNV neutralization titers of between 1:10 and 1:160, and the other 9 horse sera did not show any titer (Table 1). The virus neutralization test is one of the most specific serodiagnosis assays of flavivirus infection and is considered to be the "gold standard" for diagnosis of flavivirus infection. In this study, 16.7% of the JEV-vaccinated horses were positive at 90% plaque reduction, and 50% of the JEV-vaccinated horses were positive at 50% plaque reduction. These results indicate that in serosurveillance of WNV, JEV-vaccinated horses can produce false-positive results in WNV PRNT, even at 90% plaque reduction. The difficulty in diagnosing WNV infection of JEV-vaccinated horses using PRNT has been reported previously [22]. Based on these facts, diagnosis of WNV infection in areas where horses are vaccinated against JEV requires a cautious approach. Given that the plaque-neutralization antibody titer is closely correlated with protection against a lethal infection of flavivirus [15], the fact that half of the JEV-vaccinated horses we tested had no neutralization antibody against WNV in PRNT, even at 50% plaque reduction, suggests that these horses may not have been protected from WNV infection.

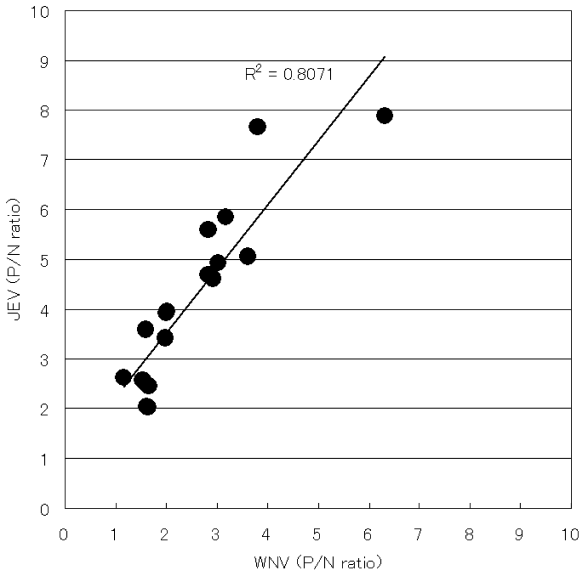

Fig. 1. Relationship between the JEV IgG-ELISA P/N and WNV IgG-ELISA P/N ratios of JEV-vaccinated horse sera.

All of the JEV-vaccinated horse sera showed P/N ratios of over 2.0, ranging from 2.02 to 7.88 with JEV IgG-ELISA. With WNV IgG-ELISA, half of the sera also had P/N ratios of over 2.0, and all ranged from 1.16 to 6.30 (Table 2). As shown in Fig. 1, all of the JEV-vaccinated horse sera showed comparatively higher P/N ratios on JEV IgG-ELISA than on WNV IgG-ELISA. There was a correlation between the JEV IgG-ELISA P/N and WNV IgG-ELISA P/N ratios of each JEV-vaccinated horse serum (r^2=0.807; Fig. 1). IgG-ELISA is particularly valuable for serosurveillance, as it is sufficiently sensitive, can be applied to numerous samples in a short period of time and requires only small amounts of serum and antigen. However, half of JEV-vaccinated horse sera are cross-reactive in WNV IgG-ELISA, and therefore, the screening test for WNV cannot be based solely on WNV IgG-ELISA in areas where horses are vaccinated against JEV.

All of the JEV-vaccinated horse sera showed a JEV HI titer of between 1:20 and 1:160. They also reacted in WNV HI with titers between 1:10 and 1:40 (Table 3). HI has been used to diagnose flavivirus infections, including JEV and WNV, for many decades [18, 19]. About 40% of the JEV-vaccinated horses showed the same titers in JEV HI and WNV HI. JEV-vaccinated horse sera are highly cross-reactive in WNV HI.

In summary, all of the JEV-vaccinated horse sera were cross-reactive in WNV HI, and half of the JEV-vaccinated horse sera were cross-reactive in WNV IgG-ELISA. In WNV PRNT, 16.7% of the JEV-vaccinated horses were positive at 90% plaque reduction, and 50% of the JEV-vac-

cinated horses were positive at 50% plaque reduction.

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