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Cross-Reactivity of Chicken Anti-Japanese Encephalitis Virus Serum and Anti-West Nile Virus Serum in Serological Diagnosis

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ABSTRACT. The cross-reactivity of Japanese encephalitis virus (JEV)-immunized chicken sera and West Nile virus (WNV)-immunized chicken sera in serological tests, such as the IgG indirect ELISA (IgG-ELISA), hemagglutination inhibition test (HI) and plaque reduction neutralization test (PRNT), for JEV and WNV were examined. The mean JEV/WNV ELISA ratio in IgG-ELISA of JEV-immunized sera was significantly higher than that of WNV-immunized sera ($P<0.01$). JEV-immunized chicken sera did not cross-react in the WNV HI. However, all the WNV-immunized chicken sera cross-reacted in the JEV HI. JEV-immunized chicken sera did not show the WNV neutralization titer at 90% plaque reduction, and WNV-immunized chicken sera did not showed the JEV neutralization titer at 90% plaque reduction. Therefore, it is possible that chicken JEV serum can be distinguished from WNV serum by comparing the titers of IgG-ELISA, HI or PRNT respectively.

KEY WORDS: chicken, cross-reaction, diagnosis, Japanese encephalitis virus, West Nile virus.

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West Nile virus (WNV) and Japanese encephalitis virus (JEV) are RNA-enveloped viruses of the genus *Flavivirus*, family *Flaviviridae*, in the JEV serocomplex group [10]. Birds are known to play an important role in amplification of JEV [3] and WNV [10]. In particular, birds are infected more frequently and earlier with WNV than humans and horses. Therefore, chickens are used as sentinel animals for WNV surveillance, and serological diagnoses are used to check for WNV infection in chicken sentinel surveillance. However, cross-reactions in serological diagnoses frequently occur in heterologous flavivirus-infected animal sera. Generally, IgG indirect ELISA (IgG-ELISA) and the hemagglutination inhibition test (HI) are known to show cross-reaction in heterologous flavivirus-infected sera [1]. Cross-neutralization of hyperimmunized mouse ascitic fluids or rabbit sera against heterologous-flaviviruses has been reported [4, 6]. JEV-vaccinated sera from horses showed cross-reactivity in WNV serological tests such as the IgG-ELISA, HI and plaque reduction neutralization test (PRNT) [8]. In birds, cross-reaction in the HI has been reported in herons experimentally infected with Murray Valley encephalitis and Kunjin virus [2]. Cross-neutralization between St. Louis encephalitis virus-infected and WNV-infected house finches has been reported [7]. These cross-reactions complicate the serological surveillance of flavivirus. Chickens are known to be infected with both JEV [11, 15] and WNV [9]. To perform serological surveillance of WNV in a JEV endemic area, it is important to understand the degree of cross-reaction of anti-JEV and anti-WNV sera in serological diagnoses. The specificity of a neutralization test using JEV- and WNV-infected chicken sera had been reported previously [12]. But, the cross-reactions of anti-JEV serum and anti-WNV serum in the HI and IgG-ELISA in chicken still remain unknown. In this paper, we compared the cross-reactions of chicken anti-JEV serum and anti-WNV serum in the IgG-ELISA, HI and PRNT for JEV and WNV.

Chicken sera: Sixteen specific-pathogen-free chickens (5 weeks old) were used (Nisseiken Co., Ltd., Ome, Japan). Ten chickens were immunized with a commercially available JEV-inactivated vaccine (Kyoto Biken Laboratories, Inc., Kyoto, Japan) 3 times intramuscularly. The other 6 chickens were immunized with sucrose gradient-purified inactivated WNV (NY99-A301 strain, 25 μg/chick/time; protein concentration) with alum adjuvant 3 times intramuscularly. Blood samples were collected from all chickens 2–4 weeks after the final immunization, coagulated at room temperature, kept overnight at 4°C and then centrifuged at 900 × *g* for 20 min. The separated sera were frozen at −80°C until use.

Virus: Virus antigens for ELISA and HI were made using JEV (Nakayama strain) and WNV (NY99-A301 strain). The ELISA antigens of both viruses were prepared by sucrose gradient purification from β-propiolactone-inactivated virus culture supernatant. The HI antigens of JEV and WNV were made from virus culture supernatant by concentration using ultrafiltration. Viruses used for PRNT were JEV (JaGar strain) and WNV (NY99-A301 strain).

IgG indirect ELISA: The JEV and WNV IgG-ELISA tests were performed as described previously with minor modification [8, 14]. The virus antigen quantity, expressed as the protein concentration, was 100 *n*g/well. The P/N ratio was obtained with the following formula: sample serum (optical density) OD value divided by mean negative serum

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Table 1. Results of the IgG indirect ELISA, hemagglutination inhibition test and plaque reduction neutralization test

	ID of samples	IgG indirect ELISA			Hemagglutination inhibition test		Plaque reduction neutralization test			
		P/N ratio*		JEV/WNV ELISA ratio**	JEV	WNV	90% plaque reduction		50% plaque reduction	
		JEV	WNV				JEV	WNV	JEV	WNV
JEV-immunized chickens	No. 1	12.36	7.77	1.59***	1:160	<1:10	1:80	<1:10	1:640	<1:10
	No. 2	8.10	2.64	3.06	1:40	<1:10	1:20	<1:10	1:160	<1:10
	No. 3	13.00	4.41	2.95	1:320	<1:10	1:40	<1:10	1:320	<1:10
	No. 4	13.81	5.94	2.32	1:80	<1:10	<1:10	<1:10	1:20	1:20
	No. 5	3.11	1.67	1.87	<1:10	<1:10	1:40	<1:10	1:160	<1:10
	No. 6	9.51	2.75	3.45	1:40	<1:10	1:10	<1:10	1:40	<1:10
	No. 7	9.43	3.05	3.09	1:160	<1:10	1:80	<1:10	1:320	1:10
	No. 8	9.99	3.69	2.71	1:160	<1:10	1:160	<1:10	1:640	<1:10
	No. 9	14.77	8.61	1.72	1:640	<1:10	1:160	<1:10	1:1280	<1:10
	No. 10	7.04	5.55	1.27	<1:10	<1:10	1:40	<1:10	1:640	<1:10
WNV-immunized chickens	No. 11	22.25	22.31	1.00***	1:20	1:20	<1:10	1:40	<1:10	1:80
	No. 12	23.25	22.46	1.04	1:10	1:20	<1:10	1:20	<1:10	1:160
	No. 13	26.25	24.00	1.09	1:40	1:80	<1:10	1:40	<1:10	1:320
	No. 14	27.25	25.85	1.05	1:20	1:160	<1:10	1:40	<1:10	1:320
	No. 15	24.25	21.08	1.15	1:10	1:20	<1:10	1:40	<1:10	1:320
	No. 16	26.50	24.15	1.10	1:20	1:20	<1:10	1:20	<1:10	1:80

*:P/N ratio: Sample serum optical density (OD) value divided by mean negative serum OD value. The mean OD for the negative controls (n = 4) was 0.03 (OD range: 0.02 to 0.04) for the JEV IgG indirect ELISA (IgG-ELISA) and 0.04 (OD range: 0.02 to 0.07) for the WNV IgG-ELISA, respectively.

**:The JEV/WNV ELISA ratio was calculated with the following formula; P/N ratio of JEV IgG-ELISA divided by P/N ratio of WNV IgG-ELISA.

***:The JEV/WNV ELISA ratio of JEV-immunized sera (2.40 +/– 0.75 (mean +/– SD)) was significantly higher than that of WNV-immunized sera (1.07 +/– 0.05) (Welch's *t* test, $P<0.01$).

OD value. The mean ODs for the negative controls (n=4) were 0.03 (range: 0.02 to 0.04) for JEV IgG-ELISA and 0.04 (range: 0.02 to 0.07) for WNV IgG-ELISA, respectively. The JEV/WNV IgG-ELISA ratio was calculated with following formula: P/N ratio of JEV IgG-ELISA divided by P/N ratio of WNV IgG-ELISA.

Hemagglutination inhibition test: The HI was performed as previously described using a 96-well plate format [5, 8]. Serum samples were treated by acetone extraction and adsorption with goose erythrocytes and then heat-inactivated at 56°C for 30 min.

Plaque reduction neutralization test: The PRNT was performed as described in the OIE Manual for WNV using a 6-well plate format with Vero cells [8, 13].

All the JEV-immunized sera showed P/N ratios exceeding 3.11 in the JEV IgG-ELISA and P/N ratios exceeding 1.67 in the WNV IgG-ELISA. All the WNV-immunized sera showed P/N ratios exceeding 22.25 in the JEV IgG-ELISA and P/N ratios exceeding 21.08 in the WNV IgG-ELISA. The mean JEV/WNV ELISA ratio of JEV-immunized sera (2.40 +/− 0.75 (mean +/− SD)) was significantly higher than that of WNV-immunized sera (1.07 +/− 0.05) (Welch's *t*-test, $P<0.01$) (Table 1). Thus, it would be possible that chicken JEV or WNV serum was distinguished by comparing the JEV/WNV ELISA ratio in IgG-ELISA.

In the HI results, 8 of the JEV-immunized sera had a JEV HI titer between 1:40 and 1:640, although they had no WNV HI titer. All the WNV-immunized sera had a WNV HI titer between 1:20 and 1:160. All also had a JEV HI titer between 1:10 and 1:40 (Table 1). HI is commonly used in serodiagnosis of flavivirus infection, including JEV and WNV [13]. In this study, all the WNV-immunized sera cross-reacted in the JEV HI; however, none of the JEV-immunized sera cross-reacted in the WNV HI. This result indicates the possibility that JEV or WNV chicken sera were distinguished by comparing the titers of JEV HI and WNV HI in JEV endemic areas.

In the PRNT, all the JEV-immunized sera had a JEV neutralization titer between 1:20 and 1:1280 at 50% plaque reduction, with 9 showing a JEV neutralization titer of 1:10 to 1:160 at 90% plaque reduction. None of the JEV-immunized sera had a WNV neutralization titer at 90% plaque reduction. Two of the JEV-immunized sera had WNV neutralization titers of 1:10 and 1:20 at 50% plaque reduction. All the WNV-immunized sera had WNV neutralization titers exceeding 1:80 at 50% plaque reduction, and they also showed a WNV neutralization titer of 1:20 to 1:40 at 90% plaque reduction. None of the WNV-immunized sera had JEV neutralization titers at either 50% or 90% plaque reduction (Table 1). Previous studies using hyperimmune mice and rabbits showed cross-neutralization in the same serocomplex flavivirus [4, 6]. Furthermore, JEV-vaccinated horse sera showed cross-reactivity in the WNV serological test [8]. However, cross-neutralizations between anti-JEV and anti-WNV sera were much lower in the chicken than in the mouse, rabbit and horse. In chicken sera, there were no cross-reactions at 90% plaque reduction. The PRNT can thus be used to distinguish anti-JEV serum from anti-WNV serum by applying the 90%

plaque reduction criterion.

It has been figured whether the infected serum is same as the immunized serum or not. In previous reports, JEV-infected and WNV-infected chickens did not cross-react in a neutralizing test [12]. This finding corresponds with our data in the PRNT. This would compensate that JEV- or WNV-immunized sera can be used as a substitute for JEV- or WNV-infected sera, respectively.

In summary, the mean JEV/WNV IgG-ELISA ratio of JEV-immunized sera was significantly higher than that of WNV-immunized sera. Anti-JEV sera did not cross-react in the WNV HI. The PRNT can be used to distinguish anti-JEV sera from anti-WNV sera by use of the 90% plaque reduction criterion. Therefore, it is possible to distinguish chicken JEV serum from WNV serum by comparing the titers of the IgG-ELISA, HI or PRNT respectively.

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