Seroprevalence of West Nile Virus in Wild Birds in Far Eastern Russia Using a Focus Reduction Neutralization Test

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Abstract. West Nile (WN) virus has been spreading geographically to non-endemic areas in various parts of the world. However, little is known about the extent of WN virus infection in Russia. Japanese encephalitis (JE) virus, which is closely related to WN virus, is prevalent throughout East Asia. We evaluated the effectiveness of a focus reduction neutralization test in young chicks inoculated with JE and WN viruses, and conducted a survey of WN infection among wild birds in Far Eastern Russia. Following single virus infection, only neutralizing antibodies specific to the homologous virus were detected in chicks. The neutralization test was then applied to serum samples from 145 wild birds for WN and JE virus. Twenty-one samples were positive for neutralizing antibodies to WN. These results suggest that WN virus is prevalent among wild birds in the Far Eastern region of Russia.

INTRODUCTION

West Nile (WN) virus belongs to the genus *Flavivirus* in the family *Flaviviridae*, and is a member of the Japanese encephalitis (JE) virus serocomplex group. In nature, WN virus is transmitted between avian hosts and mosquito vectors.¹ Humans can become infected when bitten by a mosquito and the infection can result in the development of fatal encephalitis.²

Since an outbreak of WN encephalitis in humans and horses in New York City in 1999, WN virus has spread throughout North America very rapidly.³ In European Russia, WN virus was first isolated from humans and ticks in 1963. In 1999, 318 confirmed cases of human infection with WN virus were reported in the Volgograd Region, resulting in 40 deaths.^{4,5} In 2004, WN virus was reported in patients in Novosibirsk in the southwest region of Siberia.⁶ West Nile virus has shown a tendency to spread eastward through Russia. It is possible that migratory birds have carried the virus from Far East Russia to East Asian countries during migration.

The JE virus is endemic to East Asia. Although pigs are the amplifier hosts of the JE virus, wild birds may serve as the reservoir host. Japanese encephalitis and WN viruses are closely related and often display serological cross-reactivity.^{7,8} The geographical distributions of JE and WN viruses rarely overlap; however, as WN virus continues to spread, both viruses may infect wild birds, which are a common host. Therefore, a diagnostic test that can distinguish between WN and JE virus infections is required.

In the current study, we investigated whether JE and/or WN virus infection in birds can be diagnosed using the focus reduction neutralization test (FRNT). The FRNT method has several advantages over the plaque reduction neutralization test; a large number of serum samples can be handled at once and the test can be performed on a small volume (15 µL) of serum. Chicks that were 2 days and 3 weeks old were inoculated with JE and WN viruses and the titers of neutralizing antibodies

against both viruses were measured. To investigate cross-reactivity to heterologous virus infection, an infection experiment was performed. We investigated the seroprevalence of WN virus among wild birds in the Far Eastern region of Russia using the FRNT.

MATERIALS AND METHODS

Viruses. We used the genotype-1 strain of JE virus and the New York (NY) strain of WN virus. The JE virus Mie strain (Sw/Mie/40/2004, DNA Data Bank of Japan [DDBJ] accession no. AB241118.1) was kindly provided by Dr. T. Takasaki of the National Institute of Infectious Diseases (Tokyo, Japan). The WN virus NY strain (NY99-6922) was kindly provided by Dr. D. Gubler of the Centers for Disease Control and Prevention (CDC, Fort Collins, CO; in 2003). We used the plaque-purified variant of strain NY99-6922, 6-SP (DDJB AB185915),⁹ which does not contain the N-linked glycosylation motif (N-Y-S) at residues 154–156 of the E proteins, and is associated with only mild febrile infections in chicks. The 6-SP variant was used for these experiments to ensure that the chicks would remain alive during the observation period.

Chicks. Young male Boris-Brown chicks (Hokuren, Japan) were housed in a BSL-3 animal facility. All experiments were conducted in accordance with the guidelines for the use of experimental animals of Hokkaido University.

To determine the primary neutralizing antibody responses to viruses in chicks, 2-day-old chicks were inoculated with 100 plaque forming units (PFU) of JE or WN virus, and 3-week-old chicks were inoculated with 1,000 PFU of each virus by subcutaneous injection into the femoral region. All viruses were diluted in phosphate-buffered saline (PBS[–]) containing 10% fetal calf serum (FCS) (10% FCS-PBS[–]). At various time points after inoculation, the chicks were euthanized by sevoflurane overdose. Blood samples were collected from the heart and held at room temperature for 60 min, and then kept at 4°C overnight. The blood samples were then centrifuged at 4,000 rpm for 10 min, and the sera were decanted and stored at –80°C until use.

To examine the neutralizing antibody responses after secondary challenge with heterologous viruses, 2-day-old chicks (N = 4) were inoculated with 100 PFU of either JE or WN

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virus. After 3 weeks, the chicks (23 days old) were inoculated again, this time with 1,000 PFU of heterologous virus (WN virus in chicks previously inoculated with JE virus or JE virus in chicks previously inoculated with WN virus).

Viremia titration. To confirm that the chicks were infected, the viremia titers of 2- to 9-day-old chicks were measured by plaque assay using baby hamster kidney cells (BHK-21, ATCC #CCL-10). The BHK cell monolayers were grown in 12-well plates and inoculated with serial dilutions of the viral solutions. After 60 min of viral adsorption, the viral solution was aspirated, and the cells were washed three times with PBS(-). A 1 mL volume of overlay consisting of Eagle's minimal essential medium (EMEM; Nissui Pharmaceutical Co., Japan) containing 1.5% carboxymethyl cellulose (CMC; Wako, Japan) and 2% FCS (CMC-EMEM) was added to the cells, and the plates were incubated at 37°C in a CO₂ incubator. After 5 days of culture, the CMC-EMEM was aspirated, and the cells were fixed and stained with a solution of 0.1% crystal violet and 10% formalin in PBS(-). After staining for 2 h, the cells were washed with water and then dried, and the plaques were counted. The viral titer was expressed as the number of PFUs per mL. The minimum threshold for virus detection was 50 PFU/mL.

Antibody determination. The sera of chicks and wild birds were tested for the presence of neutralizing antibody by the 80% FRNT using the fluorescent antibody technique used previously for tick-borne encephalitis virus.¹⁰ The test sera (15 µL) were diluted serially in 2-fold steps from 1:20 to 1:2,560 in a 96-well plate. Each serum dilution was then combined with an equal volume of WN or JE virus, adjusted to give a final count of ~50 focus-forming units per well. The serum-virus mixtures were incubated for 60 min at 37°C in a CO₂ incubator. After incubation, the mixtures were transferred to the wells of 96-well plates containing a monolayer of BHK cells. The plates were incubated for 60 min at 37°C to allow for virus adsorption. After removing the mixture, the cells were covered with CMC-EMEM. After incubation for 24 h at 37°C, the medium was removed and the cells were washed with PBS(-) three times and fixed with absolute methanol at room temperature for 20 min. Focus staining was performed by the fluorescent antibody technique. Fixed BHK cells were treated consecutively with anti-WN virus mouse hyperimmune ascitic fluid (1:500) or anti-JE virus mouse hyperimmune ascitic fluid (1:800) and Alexa Fluor 555 goat anti-mouse IgG (1:400, Invitrogen, Carlsbad, CA). Each incubation lasted 60 min and was followed by three washes with PBS with Tween 20(T) (PBS-T). The neutralizing antibody titer was expressed as the reciprocal of the highest dilution that reduced the number of foci to ≤ 80% of the control value. The cutoff titer was set at 1:20 and 1:160 for wild birds.

We tested chick sera for WN virus using a plaque reduction neutralization test (PRNT). Briefly, a BHK cell monolayer was prepared on a 12-well plate and plaques were visualized by staining with crystal violet solution, as described previously for virus titration. Other procedures were the same as those used for the FRNT. The neutralizing test (NT) antibody titers by plaque reduction neutralizing test (PRNT) were similar to those obtained by FRNT (S. Totani and I. Takashima, unpublished data).

Serological analysis of wild birds in Far Eastern Russia. To determine the prevalence of WN virus in the Far Eastern region of Russia, we analyzed the seroprevalence of WN virus among

wild birds. A total of 152 wild birds were captured at Khanka Lake, Anyuy River, and Chor River in Far Eastern Russia in August 2005 and 2006, and blood and kidneys were collected. These areas are known to be resting points for migratory birds.^{11,12} The presence of WN virus RNA was determined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR) (TaqMan method).¹³ The RNA was extracted from the kidneys (50 mg) of all 152 birds using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. First-strand complementary DNA (cDNA) was synthesized using Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase (Takara Bio Inc., Shiga, Japan) and a random primer (Invitrogen). TaqMan assay was performed using TaqMan universal PCR Master Mix (Applied Biosystems, Carlsbad, CA), WN virus-specific forward (5'-GCACGAAGATCTCGATGTCTAAG-3') and reverse (5'-ATTCCGCGTTTTAGCATATTGAC-3') primers, and a probe (FAM-5'-ACCAGGAGGGCCCGG-3'-MGB) common to WN and JE viruses. Real-time PCR was performed using an ABI Prism 700 (Applied Biosystems). The detection limit of the assay was 10 PFU/g tissue or blood from WN virus-infected mice.

The 145 sera that were separable from the blood of 152 birds were tested for the presence of neutralizing antibody by FRNT.

RESULTS

Viremia in chicks. To confirm that the chicks had been successfully infected, the viremia titers of the chicks were measured after inoculation. The chicks inoculated with JE virus produced measurable viremia (> 50 PFU/mL) 1–5 days post inoculation (dpi). The highest titer of JE virus exceeded 10⁴ PFU/mL at 2 dpi. The viremia titers of chicks inoculated with WN virus were higher than those inoculated with the JE virus at 1, 3, 5, and 6 dpi, and below the threshold level at 7 dpi. These chicks showed maximum levels of viremia at 2 or 3 dpi, with levels that reached 10⁴ PFU/mL. These results confirmed that the chicks had been successfully infected with the JE and WN viruses.

Neutralizing antibody responses to single virus infection. To evaluate the FRNT for determining infection with JE or WN virus, 2-day-old and 3-week-old chicks were inoculated with the viruses, and the titers of neutralizing antibody to each virus were measured. When the JE or WN virus was inoculated into 2-day-old chicks, neutralizing antibody titers were detected from 7 to 14 dpi. At 10 dpi, the maximum JE virus neutralizing antibody titer in chicks inoculated with the virus at 2 days old exceeded 1:320 (Figure 1A), and the maximum WN virus neutralizing antibody titer in chicks inoculated with WN virus at 2 days old exceeded 1:1,280 (Figure 1B). Neutralizing antibody titers following heterologous virus infection were significantly lower (< 1/4) than those resulting from homologous virus infection (Figure 1A and B). When the viruses were inoculated into 3-week-old chicks, the specific neutralizing antibody titers measured at 7 dpi were lower than those measured at 7 dpi in chicks inoculated at 2 days old (Figure 1C and D). The maximum JE virus neutralizing antibody titer in chicks inoculated with the JE virus at 3 weeks old was 1:40 (Figure 1C) at 14 dpi, and the maximum WN virus neutralizing antibody titer in chicks inoculated with WN virus at 3 weeks old was ~1:160 (Figure 1D).

FIGURE 1. Primary neutralizing antibody responses in chicks inoculated with Japanese encephalitis (JE) and West Nile (WN) viruses. Two-day-old chicks ($N = 4$) were inoculated with 100 plaque forming units (PFU) of (A) JE virus and (B) WN virus, and 3-week-old chicks ($N = 4$) were inoculated with 1,000 PFU of (C) JE virus and (D) WN virus. Japanese encephalitis virus ($\diamond$) and WN virus ($\bullet$) neutralizing antibody titers were measured by FRNT₈₀ and are expressed as the mean $\pm$ SD.

Neutralizing antibody responses after secondary challenge with heterologous virus. To investigate the cross reaction to heterologous virus infection, 2-day-old chicks were inoculated initially with JE virus (or WN virus) and challenged by inoculation with WN virus (or the JE virus) after 3 weeks (Figure 2A). In chicks inoculated with JE virus, neutralizing antibodies to the WN virus were first detected 3 days after challenge (dac). A detectable JE virus neutralizing antibody titer was not observed until 7 dac, as in the single infection experiment (Figure 1C). In chicks that had been inoculated first with WN virus and challenged with the JE virus after 3 weeks (Figure 2B), the WN virus neutralizing antibody titers were always higher than the JE virus titers. For example, although the maximum WN virus antibody titer was $\sim 1:1,280$, the JE virus antibody titer was significantly lower ($< 1:320$). However, the antibody titers for both viruses were higher and increased more rapidly in chicks that were doubly infected (Figure 2B) compared with those in chicks that received a single inoculation (Figure 1C and D). These results indicate that the neutralizing antibody responses are caused by the

FIGURE 2. Neutralizing antibody responses in chicks after a secondary challenge with heterologous viruses. Two-day-old chicks ($N = 4$) were inoculated with 100 plaque forming units (PFU) of primary viruses: (A) Japanese encephalitis (JE) virus, (B) West Nile (WN) virus. After 3 weeks, the chicks (23 days old) were inoculated again with 1,000 PFU of heterologous virus: (A) WN virus, (B) JE virus. JE virus ($\diamond$) and WN virus ($\bullet$) neutralizing antibody titers were measured by FRNT₈₀ and are expressed as the mean $\pm$ SD.

booster effect, which is typical of the secondary antibody response.

Seroprevalence of WN virus in wild birds in Far Eastern Russia. To clarify WN virus prevalence in wild birds in Far Eastern Russia, samples collected from 152 wild birds captured in 2005–2006 were analyzed. Real-time PCR did not detect WN virus RNA in the kidneys of any of the birds analyzed. However, WN virus neutralizing antibody was detected in 21 of 145 (14.5%) of the birds captured. These birds belonged to the orders Anseriformes, Charadriiformes, Columbiformes, and Pelecaniformes (Table 1), and particularly high neutralizing antibody titers ($\geq 1:1280$) were detected in four eastern turtle doves (*Streptopelia orientalis*). Because the WN and JE viruses endemic to East Asia often show serological cross-reactivity,^{5,6} WN virus-positive samples were tested for JE virus neutralizing antibodies. Most of the sera showed significantly higher titers of neutralizing antibodies to WN virus (≥ 4 -fold) than to JE virus, with the exception of one sample from a common tern (*Sterna hirundo*). Thus, 20 of 21 positive sera had WN virus-specific antibodies.

DISCUSSION

In recent years, the geographic distribution of WN virus has expanded rapidly to various parts of the world.³ When WN virus spreads to a non-endemic area, a differential diagnosis with a closely related flavivirus is required. The JE virus, which belongs to the same serocomplex as WN virus, is distributed throughout East Asian countries, and the viruses are serologically cross-reactive.^{7,8} In this study, we evaluated an FRNT for effective differential sero-diagnosis of JE and WN virus infection in birds. Furthermore, an analysis of blood samples from wild birds using this FRNT indicated that WN virus is prevalent among wild birds in Far East Russia.

Young chicks were used for the WN virus infection experiment, as a model of wild birds.^{14–17} Although wild birds are natural hosts of JE virus, similar to WN virus, few instances of JE virus infection in birds have been reported.^{18,19} We evaluated the effectiveness of FRNT in chicks inoculated with JE and WN viruses. First, we measured the viremia titers in the chicks to ensure that infection had taken place. Viremia was measurable in all inoculated chicks, and the maximum viremia titer reached 10^4 PFU/mL. These results suggest that the young chicks infected with JE virus or WN virus were an effective animal model for infection by both viruses.

Next, we inoculated 2-day-old and 3-week-old chicks with JE or WN virus and measured the antibody response. After single-virus infection, only neutralizing antibodies specific to the homologous virus were detected in the chicks. In 3-week-old chicks, the antibody responses were low compared with those of the 2-day-old chicks. Adult Galliformes have a low susceptibility to WN virus, and viremia titers in these birds have been reported to be lower than those of young birds.^{20,21} Because the 3-week-old chicks were older, the immunological response to JE virus infection in these birds was weaker than in 2-day-old chicks, but in this study, antibody titers sufficient for evaluation of the FRNT were obtained.

To study the effect of heterologous virus infection, a double-infection experiment was conducted. Two-day-old chicks were inoculated with JE or WN virus, and challenged with the other virus after 3 weeks. Regardless of which virus was inoculated first, booster immune responses to both

TABLE 1
Seroprevalence of wild birds collected in Far East Russia (2005–2006) with WNV and/or JEV neutralizing antibodies.

Area/Year	Bird species (order)	No. of WNV-positive/tested sera	Positive for anti-WNV antibodies %	FRNT ₈₀ titer* range	
				WNV	JEV
Khanka Lake/2005	<i>Anas poecilorhyncha</i> (Anseriformes)	1/1	100	160	40
	<i>Larus ridibundus</i> (Charadriiformes)	1/1	100	160	80
	<i>Streptopelia orientalis</i> (Columbiformes)	1/1	100	1,280	< 40
	Five other species	0/23	0	< 160	NT†
Anyui River/2005	<i>Histrionicus histrionicus</i> (Anseriformes)	3/13	23.1	160–320	40
	Four other species	0/11	0	≤ 80	NT
Khanka Lake/2006	<i>Anas poecilorhyncha</i> (Anseriformes)	1/2	50.0	160	40
	<i>Mergus serrator</i> (Anseriformes)	1/8	12.5	160	< 40
	<i>Sterna hirundo</i> (Charadriiformes)	2/13	15.4	160, 320	40, 320
	<i>Columba livia</i> (Columbiformes)	1/1	100	320	80
	<i>Streptopelia orientalis</i> (Columbiformes)	4/9	44.4	1,280–2,560	80
	Three other species	0/8	0	< 160	NT
Chor River/2006	<i>Anas poecilorhyncha</i> (Anseriformes)	2/9	22.2	160	40, 80
	<i>Mergus serrator</i> (Anseriformes)	2/22	9.1	160, 640	40, 80
	<i>Phalacrocorax carbo</i> (Pelecaniformes)	2/9	22.2	160	40
	Twelve other species	0/14	0	< 160	NT
	Total	21/145	14.5	< 160–2560	< 40–320

* FRNT₈₀ = 80% focus reduction neutralization test; WNV = West Nile virus; JEV = Japanese encephalitis virus.

† NT = not tested.

homologous and heterologous virus were observed after challenge inoculation. However, it was difficult to judge which virus had infected first, or how many times the chicks were exposed to the viruses, based on the NT. These results are in agreement with a previous report of combined infections with WN virus and St. Louis encephalitis (SLE) virus,^{22–24} in which the differential diagnosis of those closely related viruses was demonstrated to be very difficult.

Fang and Reisen²³ reported that infection with SLE virus after recovery from WN virus infection in house finches elicited a consistent and significant rise in WN virus PRNT titers, but not SLE virus PRNT titers, perhaps because protective immunity prevented the immunologic response associated with a second viremia episode (“original antigenic sin”). This description fits well with our results for infection with JE virus after recovery from WN virus infection. In contrast, they mentioned that infection with WN virus after recovery from SLE virus produced very high antibody titers and a non-specific response that was highly variable among individual birds within this treatment group. These differences might be attributable to differential virulence associated with SLE virus and WN virus infection. Their data were not inconsistent with our result of infection with WN virus after recovery from JE virus, except for the fact that our titers were lower, which might be explained by the differential virulence among JE, SLE, and WN viruses to two different bird species, chicks, and house finches.

We investigated the seroprevalence of WN virus among wild birds in Far East Russia using FRNT. Neutralizing antibody to WN virus was identified in 21 serum samples taken from 145 wild birds (14.5%) using FRNT. Birds that were positive for antibodies to WN virus were in the orders Anseriformes, Charadriiformes, Columbiformes, and Pelecaniformes. Birds in these orders are known to support WN virus propagation with high levels of viremia, and to serve as efficient amplifying hosts for the transmission of WN virus to mosquitoes.¹⁴ The JE virus is endemic to East Asia, and is closely related to WN virus. These viruses often show antigenic cross-reactivity in serological tests.^{7,8} Therefore, WN virus-positive samples were further

tested for the neutralizing antibody to JE virus. The majority of WN virus-positive sera were negative for neutralizing antibody against the JE virus. These data indicate that the positive results of the FRNT for WN virus were caused by antibodies specific to WN virus infection and not because of cross-reactivity with antibodies produced by JE virus infection.

All of the rock doves (*Columba livia*) tested and some eastern turtle doves (*Streptopelia orientalis*), which are resident birds, had WN virus antibodies and were probably infected with the virus near Khanka Lake. Because Khanka Lake lies far to the east of where WN was first isolated in Russia, the WN virus appears to have been transmitted among wild birds in Far Eastern Russia. The other WN virus-positive birds identified in this study were spotbills (*Anas poecilorhyncha*), harlequin ducks (*Histrionicus histrionicus*), red-breasted mergansers (*Mergus serrator*), black-headed gulls (*Larus ridibundus*), and common terns (*Sterna hirundo*), which are all migratory birds, therefore it is possible that these birds were infected with the WN virus in Far Eastern Russia and carried the virus into other regions of East Asia.

In the Asia-Pacific region, migratory water birds typically display north-south flying patterns.²⁵ Long-distance migratory birds use three flyways, the Central Asian-Indian, East Asian-Australasian, and West Pacific flyways. Among the WN virus antibody-positive bird species, the common tern (*Sterna hirundo*) is a long-distance migratory species that may migrate between Far East Russia and Australasia. The possibility that the WN virus-positive antibodies in common tern might be the result of the Kunjin virus infection could not be excluded because of the limitation of the neutralization test. Recent studies of migration routes of mallard (*Anas platyrhynchos*) determined by satellite telemetry have shown that besides the northward flyway from Japan to Russia, a northwestward flyway also exists in Far East Russia.²⁶

In Japan, WN virus activity has not yet been detected. In the metropolitan area of Tokyo from 2002 to 2006, a total of 7,281 mosquitoes and 139 crow samples (blood, brain, kidney, and spleen) were tested for WN virus RNA, and none of them were positive.²⁷ In Hokkaido, we also collected about 100 individual

wild birds, including crows and sparrows, which were found dead. Kidneys and brains of these birds were tested for WN virus RNA using real-time PCR and none of them were positive (I. Takashima and H. Saito, unpublished data).

The results of this study suggest that WN virus is distributed throughout Far East Russia and that it may spread to East Asian countries with the migration of wild birds. To prepare for the introduction of WN virus to East Asia, the development of a diagnostic test that can accurately differentiate between WN and JE virus infection is needed. In addition, continued epizootiological evaluation of WN virus infection among birds and humans in Far East Russia and East Asia will be important for monitoring the spread of the disease.

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