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Antibodies to Flaviviruses in Wild Ducks Captured in Hokkaido, Japan: Risk Assessment of Invasive Flaviviruses

Mika Saito,[1] Yuichi Osa,[2] and Mitsuhiko Asakawa[3]

Abstract

Recently, the distribution of Japanese encephalitis virus (JEV) and West Nile virus (WNV) has expanded into new territories. The invasion of WNV into Japan is of great concern. The migration of birds is suggested to be involved in the expanded distribution of these flaviviruses. In this study, 92 wild ducks—20 *Anas poecilorhyncha* (migratory breeders), 50 *Anas platyrhynchos* (undetermined), 16 *Anas acuta* (winter visitors), and 6 *Anas penelope* (winter visitors)—were captured in autumn of 2005 and 2006, in the central part of Hokkaido, a low JEV activity area. A seroepidemiologic analysis of flavivirus infections was conducted with 90% and 50% focus reduction neutralization tests ($FRNT_{90}$ and $FRNT_{50}$). Of the 92 serum samples, 1 (1.1%) and 5 (5.4%) tested positive for WNV-specific and JEV-specific antibodies, respectively, in the $FRNT_{90}$, and 61 (66.3%) and 79 (85.9%) tested positive for WNV and JEV, respectively, in the $FRNT_{50}$. These results indicate that wild ducks in this study had been exposed to flaviviruses. The results together with the recognized distribution of flaviviruses and migratory routes of individual duck species strongly suggested that the birds captured in this study had been exposed to flaviviruses, including WNV, on the flyway, not in Hokkaido.

Key Words: Emerging—Flavivirus—West Nile virus—Japanese encephalitis virus—Infectious disease—Bird—Migration—Prevention—Control.

Introduction

Flaviviruses such as Japanese encephalitis virus (JEV) and West Nile virus (WNV) are recognized as emerging and re-emerging pathogens (Mackenzie et al. 2004). Recently, the distribution of JEV and WNV has expanded into new territories. JEV emerged in the Torres Strait in 1995 (Williams et al. 2000) and on the mainland of Australia in 1998 (van den Hurk et al. 2006). JEV genotype 1, distributed geographically limited areas from northeast Thailand to Cambodia before the 1990s (Chen et al. 1990), has recently been isolated in Vietnam, China, the Republic of Korea, Japan, and Australia (Chung et al. 1996, Pyke et al. 2001, Ma et al. 2003, Nga et al. 2004, Yoshida et al. 2005, Nerome et al. 2007, Saito et al. 2007, Wang et al. 2007). WNV is distributed in Africa, Europe, the Middle-East, and Central and Western Asia. Since WNV emerged in 1999 and became established in North America, the disease has continued to be a great threat to public health (Mackenzie et al. 2004). Around the same time, WNV was detected in Siberia, including far-east Russia (Alexander 2001, Ternovoi et al. 2006). The invasion of WNV into Japan is of great concern to public health and wildlife conservation. The mechanisms behind the expanded distribution of these flaviviruses are unknown, although natural factors such as bird migration and wind-blown mosquitoes, and human factors such as the transport of infected animals and mosquitoes are suggested to be involved (Lanciotti et al. 1999, Ritchie and Rochester 2001, Reed et al. 2003, Mackenzie et al. 2004, Nga et al. 2004).

Hokkaido is the northern-most prefecture of Japan, approximately 850 km from Tokyo (Fig. 1). JEV activity in Hokkaido is low, as indicated by a low HI antibody prevalence rate (less than 10%) in pigs (Infectious Disease Surveillance Center 2006), although JEV and tick-borne encephalitis (TBE) virus (belonging to different flavivirus sero-complexes) have been isolated in the past, both locally in the southern part of Hokkaido (Takashima et al. 1988, 1997). The immunization of human residents with the Japanese encephalitis vaccine has never been recommended in Hokkaido, and no cases of

[1]Division of Molecular Virology and Oncology, Department of Microbiology, Graduate School of Medicine, University of the Ryukyus, Okinawa, Japan.
[2]Wildlife Section, Nature Conservation Department, Hokkaido Institute of Environmental Sciences, Hokkaido, Japan.
[3]Department of Pathobiology, School of Veterinary Medicine, Rakuno Gakuen University, Hokkaido, Japan.

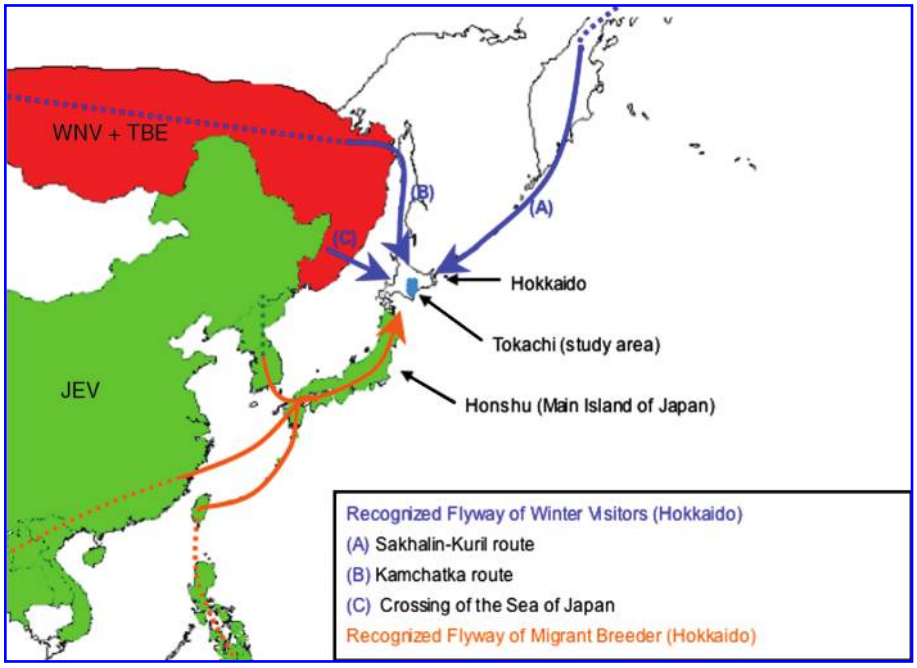

FIG. 1. Map of East Asia, the distribution of flaviviruses, and flyways of migratory Anatidae. The red area is where the distribution of WNV and TBE virus overlap. The green area is where the JEV is distributed. The light blue area is the capture area in this study. The dark blue lines are recognized migrational routes of winter visitors. The orange lines are the recognized migrational routes of migratory breeders.

Japanese encephalitis have been reported there. In addition, Hokkaido is an important area for wildlife including waterfowls and endangered birds.

We report the results of a sero-survey of flavivirus infections in wild, migratory ducks, captured in Hokkaido and discuss the antibodies to flaviviruses; WNV and JEV.

Materials and Methods

Serum samples from wild ducks

A total of 92 wild ducks were captured in the Tokachi-area, Hokkaido (Fig. 1) in October and November 2005 and October and November 2006 with permission from the Ministry of Environment, Japan. The climate of Tokachi is sub-arctic with an annual mean temperature of about 6.1°C, and no mosquitoes were found during the season of capture. All ducks were marked with numbered rings and released after blood sampling. Most of those captured were adults. The season of capture was autumn, so there were no infant ducks but there were young ducks. Twenty Spot-billed Ducks (*A. poecilorhyncha*), 50 Mallards (*A. platyhynchos*), 16 Northern Pintails (*A. acuta*), and 6 Eurasian Wigeons (*A. penelope*) were subjected to a seroepidemiologic test for flavivirus infections. Collected serum samples were stored at −30°C before use. Before the serologic testing was performed, diluted sera were heat inactivated at 56°C for 30 min.

Cell culture

The mosquito *Aedes albopictus* clone C6/36 cell line was grown at 28°C with Eagle's MEM supplemented with seven nonessential amino acids and 8% heat-inactivated fetal bovine serum (FBS). BHK-21 cell clones were grown at 37°C in 10% FBS Eagle's MEM as growth medium. All of the cell clones were maintained with 2% FBS Eagle's MEM as maintenance medium when the neutralization test was being conducted.

Serologic test

Focus reduction neutralization tests (FRNTs) were conducted to measure neutralizing antibody titers against WNV Eg-101 (prototype strain) and JEV Nakayama (prototype strain). The testing was done on BHK-21 cells in 96-well microplates with a slight modification of the peroxidase-anti-peroxidase staining method (Okuno et al. 1985, Saito et al. 2007). This method is a modified version of the plaque reduction neutralization test (Okuno et al. 1978, Beaty et al. 1995). The duck sera were diluted threefold from 1:10 to 1:270 and reacted with aliquots of virus solution (50 μL) containing 100 focus forming unit/12.5 μL at 37°C for 90 min. Twenty-five microliters of each dilution of serum/virus mixture was inoculated in triple-wells of BHK cell monolayers and incubated at 37°C for 90 min. One hundred microliters of maintenance medium was added to each well and incubated at 37°C for 24–26 h for JEV and for 30–32 h for WNV. Monolayers were fixed with methanol for 15 min, washed three times with phosphate-buffered saline (PBS), and incubated for 40 min with diluted hyper immune rabbit anti-JEV (Beijing-1 strain) serum as primary antibody for both JEV and WNV. After one wash with PBS, peroxidase-conjugated goat anti-Rabbit IgG (heavy and light chains) (American Qualex, San Clemente, CA) was added as secondary antibody and incubated for 40 min. After three washes with PBS, diaminobenzidine (Sigma, St. Louis, MO) and hydrogen peroxide were added and the cells were incubated until the infected cells were properly stained as foci. Plates were washed and

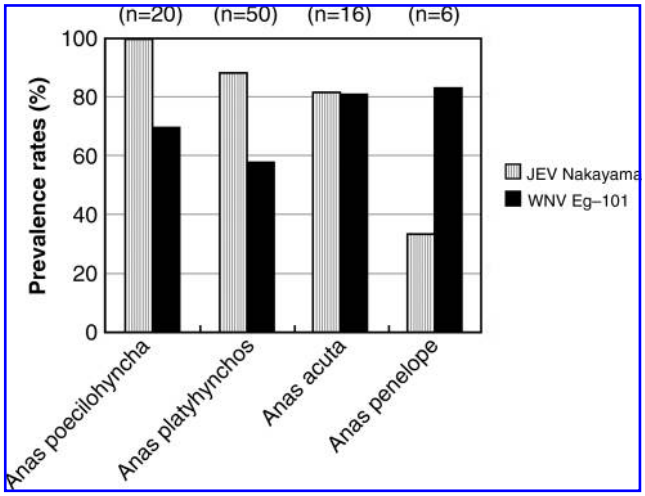

FIG. 2. $FRNT_{50}$ antibody positive rates in sera of wild ducks captured in Hokkaido. Positive rates for JEV Nakayama and WNV Eg-101 in each species of duck are shown.

dried, and the foci were counted. To confirm the reliability of the testing, positive and negative human control sera for JEV and WNV infections were used in all tests, as duck controls were not available. The neutralization test using the 90% threshold method is the standard method that has been used to identify WNV-neutralizing antibodies in dead and dying bird in the USA (Beaty et al. 1995, Farfan-ale et al. 2006). As focal deaths and debility were not reported among birds in the study area, and the studied ducks were healthy, analysis at both high and low stringency ($FRNT_{90}$ and $FRNT_{50}$) were applied in this study (Buckey et al. 2003). The percent reduction in foci at each dilution was plotted on a probit chart, and the serum dilution giving 90% and 50% reductions from the probit regression line was defined as the $FRNT_{90}$ and $FRNT_{50}$, respectively (Saito et al. 2008). A neutralizing antibody titer less than 10 was considered negative in each analysis. The passage history of the strains was unknown. WNV Eg-101 was passed twice on Vero cells then once on C6/36 after a final passage of unknown history. JEV Nakayama was passed six times on C6/36 after a final passage on suckling mouse brain.

Results

Serologic survey

In the high stringency $FRNT_{90}$, WNV-specific antibody (titer of 20) was detected in serum of a single *A. platyhynchos*, while JEV-specific antibodies were detected in low titers (10 to 30) in two *A. poecilorhyncha*, two *A. platyhynchos*, and one *A. acuta*. Although the proportion of samples that tested positive using the $FRNT_{90}$ was low, this provided evidence that both JEV and WNV had previously infected migratory ducks captured in Hokkaido.

Of the 92 serum samples examined, 61 (66.3%) and 79 (85.9%) tested positive for WNV and JEV, respectively, in the $FRNT_{50}$. Among the 20 samples from *A. poecilorhyncha*, the positive rate was 70.0% and 100%; among the 50 samples from *A. platyhynchos*, it was 58.0% and 88.0%; among the 16 samples from *A. acuta*, it was 81.3% and 81.3%; among the six samples from *A. penelope*, it was 83.3% and 33.3%, respectively (Fig. 2). Surprisingly high positive rates in the $FRNT_{50}$ were obtained for all four species of wild ducks captured in Hokkaido, an area low in JEV activity.

Analysis of antibody titers

Neutralizing antibody titers against JEV and WNV using the $FRNT_{50}$ in all 92 samples from all ducks and samples from individual species were plotted in correlation graphs (Fig. 3). A neutralizing titer less than 10 was calculated as 5 throughout. The correlation graphs revealed differences in neutralization reactivity among species. Most serum samples from *A. poecilorhyncha*, considered migrant breeders, had a higher titer for JEV than WNV and six samples had JEV-specific, but none had WNV-specific, antibodies. Sera from *A. platyhynchos*, undetermined, tended to react with both strains with less bias: 17 samples had JEV-specific and four had WNV-specific antibodies. Of sera from *A. acuta*, considered winter visitors, three had JEV-specific and three had WNV-specific antibodies. Sera from *A. penelope*, considered winter visitors, had a higher titer for WNV, and three samples had WNV-specific antibodies, but none had JEV-specific antibodies. The difference in neutralizing reactivity between species may reflect migratory flyway and suggests that these $FRNT_{50}$ positive ducks had been exposed to flaviviruses on the flyway, not in Hokkaido.

The coefficient of the correlation in antibody titers between JEV and WNV was as low as −0.00638 in the total of 92 samples. This result suggests no association in antigenic cross-reactivity between JEV and WNV, and that each antibody might reflect a specific infection.

Geometric mean titers (GMTs) in the four species of ducks are shown in Table 1. The GMT was the average of $\log_{10}$ titers and an $FRNT_{50}$ of less than 10 was calculated as 5 throughout. Migrant breeders, *A. poecilorhyncha*, had the highest GMT against JEV among the four species, while winter visitors, *A. acuta* and *A. penelope*, had higher GMTs against WNV than the other species. The GMTs against JEV in *Anas pecilohyncha* and *A. platyhynchos* were higher than those against WNV. This result is consistent with the different neutralizing reactivity indicated in Figure 3.

Discussion

We provided serological evidence that there were migratory birds in this study that had been exposed to flaviviruses, probably on the flyway and not in the capture area where JEV activity is low.

As a gold standard in the USA, the existence of virus-specific antibodies in neutralization test using a 90% threshold is evidence of a specific infection by both JEV and WNV in migratory ducks.

Buckley and others (2003) concluded that results of neutralization tests using a 50% threshold were valuable and robust for healthy birds in Europe. In this study, we precisely analyzed the results of the $FRNT_{50}$, a low stringency test, because there is no evidence of WNV and evidence of only low JEV activity in the study area, and the ducks studied were healthy. The results of the $FRNT_{50}$ showed surprisingly high antibody positive rates for flaviviruses that reflect the history of exposure of the migratory birds in this study.

At present, basic problems remain regarding the validity of FRNTs. Positive and negative controls of duck sera could not

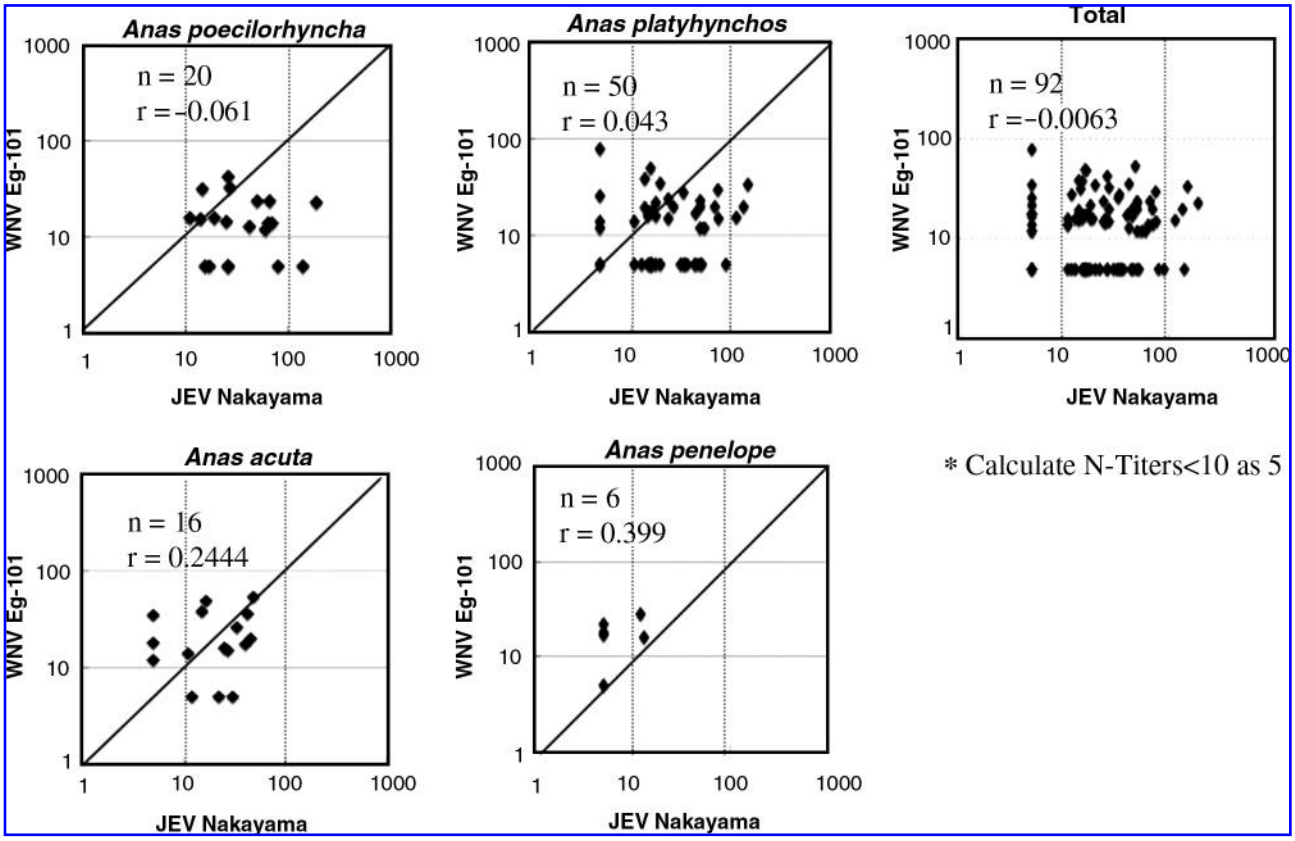

FIG. 3. Correlation graphs of titers of $FRNT_{50}$ in log_{10} scales in individual species for JEV Nakayama and WNV Eg-101. Each plot expresses the antibody titers of the individual. The correlation of the coefficient is described as an *r*-value.

be obtained for the $FRNT_{50}$; therefore, a careful assessment of the reliability of the testing is necessary. In addition, antigenic reactivity differed within strains of JEV, and within those of WNV (Buckley et al. 2003, Saito et al. 2007). For the sero-survey of healthy birds, volumes of sera were limited; therefore, the selection of strains such as the far-east Russian isolate of WNV and other flaviviruses, including TBE, was an issue.

Because of cross-neutralizing reactions between flaviviruses, especially within the JEV sero-complex including WNV, some studies used the criterion for etiologic diagnosis that the neutralization titer to the respective virus be at least fourfold greater than that to other flaviviruses (Farfan-ale et al. 2006). However, we were not able to confirm whether these antibodies indicate infections with specific flaviviruses, because we conducted FRNTs for only JEV and WNV. There is also the possibility of multi-infections that does not follow the criterion (Makino et al. 1994), and infections with other flaviviruses including unrecognized flaviviruses. Even with these problems and difficulties in interpretation, the analysis with $FRNT_{50}$ gave great insight into the exposure of migratory ducks to flaviviruses together with the flyways and geographic distribution of flaviviruses.

Table 1. GMT Measured with $FRNT_{50}$ for JEV and WNV in Ducks

	Nakayama (JEV)	*Eg-101 (WNV)*
A. poecilorhyncha	1.55	1.11
A. platyhynchos	1.38	1.06
A. acuta	1.27	1.25
A. penelope	0.83	1.20
All ducks	1.36	1.11

Hokkaido is an important site in the East Asian flyway of *Anatidae*; however, flyways in nature remain unclear because of individual variety (Miyabayashi and Mundkur 1999). In general, *A. acuta* and *A. penelope* are considered winter visitors in Hokkaido, found between November and April. They migrate from Eastern Siberia, where WNV and TBE virus distribute, partly to mainland Japan through Hokkaido by (A) the Sakhalin-Kurile route, (B) the Kamchatka route, and (C) the Crossing of the Sea of Japan (Fig. 1). *A. poecilorhyncha* are considered migrant breeders, found from April to October in Hokkaido. They migrate from South-East Siberia, other parts of Japan, the Korean Peninsula, South-east China, and Taiwan, where JEV distributes (Fig. 1), and are also partly resident breeders. *A. platyhynchos* are most commonly observed in Hokkaido, and difficult to classify. Most are considered resident breeders. Some are considered winter visitors from Siberia and partly migrant breeders, from Eastern and North-Eastern Asia (personal communication).

The results of neutralization tests (prevalence rates and GMTs) together with the recognized distribution of flaviviruses (Fig. 1) and the recognized migrational routes of wild

ducks (Fig. 1) suggested that winter visitors (*A. acuta* and *A. penelope*) were infected by WNV, possibly TBE virus, in Siberia including far-east Russia in summer, while migrant breeders (*A. poecilorhyncha*) were infected by JEV in Asia where JEV distributes.

In *A. platyhynchos*, generally considered resident breeders in Hokkaido, surprisingly high prevalence rates and GMTs for JEV and WNV antibodies were obtained in the $FRNT_{50}$, with 34.0% and 8.0% of sera having JEV- and WNV-specific antibodies, respectively. The breeding sites of *A. platyhynchos* were widely distributed overlapping with where JEV, WNV, and TBE virus distribute. This result might indicate that *A. platyhynchus* are winter visitors and migrant breeders rather than resident breeders, and migrate short distances between East Asia during the summer season.

The study of bird migration is now a hot topic in research into infectious diseases like avian influenza virus and has revealed that some birds can migrate several hundred kilometers without resting. In this study, virus isolation was attempted unsuccessfully. The high sero-prevalence in this study may not mean the viral invasion, and virus isolation was attempted unsuccessfully. However, it indicates that Hokkaido is located on the flyway of birds that have been infected by flaviviruses.

Environmental destruction and global warming might change the flyways and timing of migration. JEV (Dhanda et al. 1977), WNV (Hutcheson et al. 2005), and TBE virus (van Tongeren 1983) are able to cause viremia in ducks, and viremia ends when antibody is detected. Birds with viremia may carry viruses to Hokkaido and other parts of Japan.

A recent study of mosquitoes in the eastern part of Hokkaido revealed the most abundant species to be *Culex pipiens* (Higa et al. 2006), a potentially efficient vector of WNV, which can establish the natural transmission cycle between wild birds and mosquitoes (Center for Disease Control 2005). *Culex tritaeniorhynchus*, the main vector mosquito of JEV, was not found (Higa et al. 2006), which supports the low JEV activity in Hokkaido.

If WNV was to be introduced into Hokkaido or other parts of Japan in the appropriate season for vectors, the transmission cycle could be established. In addition, most human residents of Hokkaido as dead-end hosts have never been exposed to flaviviruses, and there are an abundance of animals including endangered species susceptible to WNV. WNV may therefore cause epidemics in humans and animals in Hokkaido.

Further well-organized prospective and retrospective studies should be conducted to assess the risk, and prevent and control invasive flaviviruses such as WNV and JEV.

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Address correspondence to:
Mika Saito
Division of Molecular Virology and Oncology
Department of Microbiology
Graduate School of Medicine
University of the Ryukyus
207 Uehara, Nishihara
Okinawa 903-0215
Japan

E-mail: mikas@med.u-ryukyu.ac.jp

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