# Studies on the natural transmission cycle of West Nile virus and the antibody survey in birds

（ウエストナイルウイルスの自然感染環と
鳥類における抗体調査に関する研究）

**Ryo Murata**

**Contents**

**Abbreviation used in this thesis**

| | |
|---|---|
| antibody | Ab |
| BHK | baby hamster kidney |
| BSL | biosafety level |
| CMC | carboxymethyl cellulose |
| d.a.c. | days after challenge |
| d.p.i. | days post inoculation |
| E | envelope |
| ELISA | enzyme-linked immunosorbent assay |
| EMEM | Eagle's Minimal Essential Medium |
| FCS | fetal calf serum |
| Fig. | Figure |
| $FRNT_{80}$ | 80% focus reduction neutralization test |
| h | hours |
| JE | Japanese encephalitis |
| $LD_{50}$ | 50% lethal dose |
| LP | large plaque |
| MAb | monoclonal antibody |
| MEM | Minimal Essential Medium |
| MOI | multiplicity of infection |
| NA | non-structural |
| NCR | non-cording region |
| NT | neutralization test |
| NY | New York |
| OD | optical density |
| PBS | phosphate-buffered saline |
| PCR | polymerase chain reaction |
| PFU | plaque forming units |
| SP | small plaque |
| WN | West Nile |

## Preface

West Nile (WN) virus is a member of the family *Flaviviridae*, genus *Flaviviruses*. Virions are 40-50 nm in diameter, spherical in shape and contain a core and an envelope (E). Its genome is a single-strand plus-sense RNA encoding three structural proteins (capsid protein, membrane precursor protein and E protein) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5). Most of viruses belonging to the genus *Flavivirus* are arboviruses and cause serious diseases in humans and animals.[7,13] On the basis of serological cross-reactivity and genetic similarity, they are classified into several groups, such as Japanese encephalitis virus group, dengue virus group, yellow fever virus group and mammalian tick-borne encephalitis virus group (Table 1).[7,13]

Table 1. Typical virus species in the genus *Flavivirus*, family *Flaviviridae*.

| Group | Virus | Vector | Geographic distribution |
|---|---|---|---|
| Japanese encephalitis virus | Japanese encephalitis | Mosquito | Japan, Korea China, India, Southeast Asia, Australasia, Russia |
| | Murray Valley encephalitis | Mosquito | Southeast Asia, Australasia |
| | St. Louis encephalitis | Mosquito | America |
| | West Nile | Mosquito | Africa, Europe, Middle East, India, Central Asia, Australia, America |
| Dengue virus | Dengue | Mosquito | Tropical areas of Asia, India, Australasia, Africa, America |
| Yellow fever virus | Yellow fever | Mosquito | Africa, Latin America |
| Mammalian tick-borne virus | Kyasanur Forest disease | Tick | Asia |
| | Langat | Tick | Asia |
| | Louping ill | Tick | British Isles, Ireland |
| | Omsk hemorrhagic fever | Tick | Asia |
| | Powassan | Tick | North America, Russia |
| | Tick-borne encephalitis | Tick | Europe, Russia, Asia |
| Modoc virus | Apoi | Unknown | Japan |

The WN virus is maintained in bird to mosquito transmission cycles in the nature. Mammals are accidental hosts, and are infected by blood sucking activities of the infected mosquitoes. The WN virus causes lethal encephalitis in humans and horses. Until early 1990s, the WN virus was localized geographically in Africa, Europe and Middle East. However in 1999, WN fever appeared in New York (NY) City for the first time in North America, and rapidly spread to North and South American Continents.[14,42] Currently, there is no effective therapy or vaccine for use in humans. Therefore, it is important to clarify the biological properties and ecological characteristics of this virus from the view point of publichealth. WN virus has been still expanding geographically to the non-endemic areas in various parts of the world. Now, there is an increasing possibility of the WN virus invasion into East Asian countries. Therefore, it is urgently needed to develop diagnostic methods of WN viruses.[47]

Until the early 1990s, the WN virus was associated with only mild febrile infection in humans and horses. Contrarily, the WN virus endemic in NY was characterized by high mortality in avian host and severe clinical course in mammals.[20,34] This suggests that the pathogenicity of the NY strain appears to be higher than that of previously identified WN virus strains. Changes of amino acids of viral proteins are reported as responsible determinants for the high pathogenic characteristics of the NY strain.[3,11] The pathogenic characteristics of the NY strain may explain a rapid expansion of virus distributing area in recent years. In experimental infection of WN virus in birds and mosquitoes, viremic levels in bird are reported to influence the infection rates of mosquitoes which fed upon the birds.[28,55] Therefore, viremic level in bird is one of the crucial factors for efficient transmissibility of WN virus in the nature.

The flavivirus E protein is an important structural protein in virus-cell interactions, and is a major target of the host antibody responses.[44,59] All flaviviruses have one or two potential N-linked glycosylation sites on the E protein. This glycosylation status affects viral pathogenicity in mice and growth rate in culture cells in dengue virus and yellow fever

virus.[8,10,25] Some candidates of dengue vaccine strains lack this glycosylation motif.[32] Some WN viruses contain the one N-linked glycosylation motif on the E protein, while others lack this glycosylation site due to amino acid substitutions.[53] It is interesting to note that many of the WN virus isolates associated with significant human outbreaks, including the recent North American epidemic, posses the glycosylation site on the E protein. In experimental infection in mice, viral E protein glycosylation was reported to be a molecular determinant of the neuroinvasiveness of NY strain of WN virus.[4,53] In avian host, the differences in WN virus susceptibility have been reported depending on species. The Corvidae (especially American Crow) show high susceptibility and mortality but some avian species in the orders Galliformes (quail, chickens and pheasants) and Psittaciformes (parakeets) produce low-level of viremias.[5,28,48] However these less-susceptible birds still show good immunological responses to WN virus and are often used as sentinel animals for surveillance of enzootic transmission.[31,49] Young chicks were reported to show higher viremia titer in WN virus infection than adult chickens.[39,55]

To determine how E protein glycosylation affects the interactions between WN virus and avian hosts, the author inoculated young chicks with the NY strains of WN virus containing either glycosylated or non-glycosylated E protein. The glycosylated variants were more virulent and had higher viremic levels than the non-glycosylated variants in young chicks. The glycosylation status of the variant did not affect viral multiplication and dissemination in mosquitoes *in vivo*. Glycosylated variants showed more heat stable propagation than non-glycosylated variants in mammalian (BHK) and avian (QT6) cells, but not in mosquito (C6/36) cells. Thus, E protein glycosylation may be a requirement for efficient transmission of WN virus from avian hosts to mosquito vectors [Chapter I].

In areas such as Japan, where Japanese encephalitis (JE) virus is endemic, discrimination between WN and JE virus infection is critical for the detection of WN virus invasion. However, the JE serocomplex flaviviruses are antigenically cross reactive and are thus not

readily differentiated by serological methods.[7,35]

The author evaluated neutralization test (NT) in young chicks inoculated with JE and WN virus. The information about the extent of WN virus infection in Russia still remains very limited. Therefore, the author performed a seroepidemiological survey of WN virus infection among wild birds in Far Eastern Russia using NT. After the single virus infection, only the specific neutralizing antibody to the homologous virus was detected in chicks. One hundred forty five wild birds were captured in Far Eastern Russia and serum samples were examined for WN virus and JE virus by the NT. Twenty one out of 145 sera showed positive neutralizing antibodies to WN virus and most of them showed specific neutralizing antibody titers to WN virus. These results suggest that WN virus is prevalent among wild birds in Far Eastern region of Russia [Chapter II].

**[Chapter I]**

# Glycosylation of the West Nile Virus Envelope Protein Increases *in vivo* and *in vitro* Viral Multiplication in Birds

## Introduction

The West Nile (WN) virus is a mosquito-borne flavivirus of the Japanese encephalitis (JE) serocomplex group that causes lethal encephalitis in humans and horses. WN virus was first isolated in 1937 from the blood of a febrile patient in the West Nile district of Uganda.[56] WN virus has since been found to be endemic over a wide range of areas in Africa, the Middle East, western Asia, and Australia.[1,18,23] Outbreaks of various magnitudes occurred in Israel in 1941 and 1951-1954, and in Africa in 1974. After that, no large outbreaks were observed for 20 years; however, from 1994 to 2000, WN outbreaks occurred among humans and horses.[14] Specifically, outbreaks occurred in Algeria in 1994, in Morocco in 1996, in Romania in 1996, in Tunisia in 1997, in the Czech republic in 1997, in the Congo in 1998, in Italy in 1998, in Israel in 1997-2000, in Russia in 1999, in France in 2000, and in the United States in 1999 to the present.[42] In the early outbreaks of the 1990s, the WN virus was associated only with mild pathogenicity to avian and mammalian hosts.

However, during the latter half of the 1990s, new strains of WN virus emerged in Europe. Humans and horses infected with those strains frequently suffer from meningitis and encephalitis.[14] Since the outbreak of WN encephalitis in humans and horses in New York City (NY) in late August 1999, the WN virus has spread throughout North America, and has very rapidly expanded to South American countries. Also in other parts of the world endemic areas are still expanding. The WN virus endemic in North America was characterized by large-scale mortality in wild birds, particularly in corvids.[5] A phenomenon that had not been observed prior to the outbreaks in NY and Israel.[14] A single nucleotide change resulting in the

T249P substitution in the NS3 helicase was reported to be associated with large-scale mortality in American Crows.[6]

WN virus is maintained in the nature via an enzootic transmission cycle between avian reservoir hosts and *Culex* mosquito vectors. Viremic levels of the avian host directly affect the infection rates of vector mosquitoes; birds with higher viremia generate more infected mosquitoes after blood feeding.[28] Replication and dissemination characteristics of the virus within the mosquito vectors also affect transmission efficiency.

The flavivirus envelope (E) protein is an important structural protein in virus-cell interactions, and is a major target of the host antibody responses.[51] All flaviviruses have one or two potential N-linked glycosylation sites on the E protein.[9] Some WN viruses contain the N-linked glycosylation motif (N-Y-T/S) at residues 154-156 of the E protein, while others lack this glycosylation site due to amino acid substitutions. It is interesting to note that many of the WN virus isolates associated with significant human outbreaks, including the recent North American epidemic, posses the glycosylation site on the E protein.[53]

In a previous study of the author's laboratory[53], four variants were isolated from two WN virus NY strains using plaque purification on BHK cells. Two of the variants contained glycosylated E proteins, while the others contained non-glycosylated E proteins. To determine the relationship between E protein glycosylation and pathogenicity of the WN virus, mice were inoculated subcutaneously with these four variants. The glycosylated variants caused higher mortality than the non-glycosylated variants in mice, which suggests that E protein glycosylation is a molecular determinant of neuroinvasiveness in the NY strains of WN virus. Other studies also established the importance of glycosylation of Flaviviruses E protein for viral assembly and infectivity *in vitro* and *in vivo*.[4,16,53]

In this chapter, the author examined the effect of E protein glycosylation on the pathogenicity and growth of WN virus in avian hosts and mosquito vectors. Using a young chick infection model, the author examined whether the glycosylated and non-glycosylated

variants exhibit differences in virulence and viremic level in the chicks. The author also examined multiplication and dissemination of WN virus variants in mosquitoes *in vivo*. The author then examined the multiplication characteristics of the variants in *in vitro* tissue culture cells of mosquito, mammalian, and avian origin to examine how E protein glycosylation affects WN virus multiplication in these different cell types. Using the young chick model, the author demonstrated that E protein glycosylation allows the virus to multiply in a heat-stable manner, and therefore has a critical role in enhanced viremic levels and virulence of the NY strain of WN virus.

## Materials and Methods

**Viruses.** Two NY strains of WN virus (NY99-6922 and BC787) were kindly provided by Dr. Duane Gubler, at the Center for Disease Control and Prevention (CDC, Fort Collins, CO, USA; in 2003). The NY99-6922 strain was isolated from mosquitoes and BC787 was isolated from a horse; both strains were isolated in NY in 1999. The two parent viral strains were subjected to plaque purification on BHK cells. The descendent variants were propagated once in the brains of suckling mice, as described previously[53], and working stocks of the viruses were propagated once in C6/36 cell cultures. The plaque-purified variant of strain NY99-6922 that exhibited large-plaque morphology was designated 6-LP, and the variant that exhibited small-plaque morphology was designated 6-SP. The large-plaque variant of strain BC787 was designated B-LP, and the small-plaque variant was designated B-SP. In previous studies, the variants were sequenced and showed that the 6-LP and B-LP variants contain the N-linked glycosylation motif (N-Y-S) at residues 154-156 of the E proteins, whereas the 6-SP and B-SP variants do not. The 6-SP and B-SP variants had mutations at aa156; thus, residues 154-156 were non-glycosylated motifs, NYP in 6-SP, and NYF in B-SP.[53] GenBank accession numbers for the sequences of the plaque-purified virus variants are as follows: 6-LP, AB185914; 6-SP, AB185915; B-SP, AB185916; and B-LP, AB185917. Western blot analysis revealed that the 6-LP and B-LP variants contain glycosylated E proteins, and the 6-SP and B-SP variants do not contain glycosylated E proteins. [53]

WN virus variants with or without N-linked glycosylation sites on the E protein were generated by infectious clone methodology.[33] WN virus NY99-6922 was used to generate recNY/Gly+, which has a glycosylated E protein, and recNY/Gly−, which has a non-glycosylated E protein. The recNY/Gly+ variant produced large plaques in infected cell monolayers and the recNY/Gly− variant produced small plaques. The amino acid sequence analysis of the full-length cDNAs of the recNY/Gly+ and recNY/Gly− variants showed that

they differed only at the N-linked glycosylation site; whereas aa156 of the recNY/Gly+ variant was a S residue, it was a P residue in recNY/Gly−.

**Cells.** Baby hamster kidney cells (BHK-21, ATCC, USA, #CCL-10) were maintained in Eagle's Minimal Essential Medium (EMEM; Nissui Pharmaceutical Co., Japan) supplemented with 8% fetal calf serum (FCS), 2 mM L-glutamine, and 1.5 g/l sodium bicarbonate. Quail fibroblast cells (QT6, ATCC, USA, #CRL-1708) were maintained in Minimal Essential Medium (MEM; Gibco, Invitrogen, USA) supplemented with 5% FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. *Aedes albopictus* mosquito cells (C6/36, ATCC, USA, #CRL-1660) were maintained in MEM supplemented with 10% FCS, 2 mM L-glutamine, 100 U/mL penicillin, and 100 μg/mL streptomycin. BHK, QT6, and C6/36 cells were grown and maintained at the optimum temperature for each cell line; namely, 37°C, 37°C, and 28°C, respectively. Confluent monolayers of each cell line were prepared for infection by seeding on 12-well plates (Falcon, USA) in 1 mL of the appropriate medium, and by incubation at the optimum temperature for two days. Confluent cell monolayers and virus-infected cells were maintained in the appropriate medium for each cell line, supplemented with 2% FCS.

**Viral growth *in vitro*.** Twelve-well plates containing confluent monolayers of BHK, QT6, and C6/36 cells were infected with virus, in triplicate, at a multiplicity of infection (MOI) of 0.01 plaque forming units (PFU)/cell, based on the titer of BHK cells determined in the plaque assay. After 60 minutes of adsorption at various temperatures (BHKs: 34, 37, and 40°C; QT6s: 37, 40, and 42°C; and C6/36s: 28 and 34°C), the viral solution was aspirated, the cells were washed three times with PBS(−), 1 mL of the appropriate medium was added to each well, and the plates were returned to their respective optimum temperatures. Culture fluids were collected at the indicated times and stored at −80°C. All samples were titrated, in

triplicate, by the plaque assay on BHK cells as described bellow, and viral growth curves were constructed, using the mean virus titer for each time point. The detection limit of plaque assay was 100 PFU/mL

The 6-LP and 6-SP variants were diluted in MEM containing 2% FCS without cells, and were incubated at 37 and 42°C, respectively, for 12, 18, or 24 h. Virus titers in the culture medium were measured by plaque assay on BHK cells.

**Viral titration.** Viruses in the working stocks and collected samples were titrated by the plaque assay on BHK cells. BHK cell monolayers were grown in 6-well plates, and inoculated with serial dilutions of the viral solutions. After 60 minutes of viral adsorption, the viral solution was aspirated, and the cells were washed three times with PBS(−). A 2-mL volume of overlay consisting of EMEM 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 $CO_2$ incubator. After five days of cell cultivation, the CMC-EMEM was aspirated, and the cells were fixed and stained under UV light 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 dried, and the plaques were counted. The viral titer was calculated from the viral dilution that produced 20-200 plaques per well, and was expressed as PFU/mL.

**Enzyme-linked immunosorbent assay (ELISA).** At various times post inoculation, culture cell lysates and supernatants were harvested, and the amounts of intracellular and extracellular E protein of the 6-LP and 6-SP variants were measured by antigen detection ELISA. C6/36 cells were incubated at 28°C, and QT6 cells at 42°C. Culture fluids (0.5 mL) were treated with 1% Triton X-100, and added to the anti-E protein mouse MAb F6/16A[41]-coated wells of 96-well microtiter ELISA plates that were blocked with 4x Block Ace (Snow Brand Milk Products, Japan). The viral antigens in the fractions were detected

using biotinylated anti E-protein mouse MAb 4H8[29] and HRP-conjugated streptavidin (Zymed, USA). HRP activity was detected by adding 100 μL of *o*-phenylene-diamine dihydrochloride (Sigma Chemical Co., USA) to the fractions in the presence of 0.03% $H_2O_2$. The plates were read for optical density (OD) at 450 nm on a microplate reader. The cell lysates and culture fluid removed from non-infected cells at various time points were used as negative controls.

The non-infected cell lysate and supernatant at each time points were used as negative control. ELISA OD values were obtained by correcting background values of negative control. Negative control OD values were from 0.03 to 0.4 depending on samples at different hours post inoculation. The % of expressed proteins were (OD values at each time point) / (OD values at 48 hours of LP in A to D or those at 96 hours of LP in E and F). Mean (±S.D.) values are from triplicate cultures.

**Chicks.** Young Boris-Brown chicks (Hokuren, Japan) were housed in a BSL-3 animal facility. The experiments were approved under the guidelines for using experimental animals of Hokkaido University. Two-day-old male chicks (n=4) were inoculated with 100 PFU of WN virus (6-LP, 6-SP, B-LP, or B-SP variant) via subcutaneous injection into the femoral region. All variants were diluted in PBS(−) containing 10% FCS (10% FCS-PBS(−)). At various time points post inoculation, chicks were euthanized by sevoflurane overdose, and blood samples were collected from the heart, and held at room temperature for 60 minutes, and then at 4°C overnight. The blood samples were centrifuged (4,000 rpm, 10 minutes), and sera were removed and stored at −80°C. The viral titers in sera were measured using the plaque assay on BHK cells.

To determine the differences of pathogenicity of the variants, two-day-old male chicks (n=5) were inoculated subcutaneously with $10^{-1}$-$10^{4}$ PFU of the 6-LP variant and with $10^{2}$-$10^{5}$ PFU of the 6-SP variant, both diluted in 10% FCS-PBS(−). The survival rates of the

chicks were scored daily, and tissue samples (brain, heart, spleen, liver, and kidney) were collected for viral titration.

**Mosquitoes.** Infection experiments using mosquitoes were performed in an arthropod containment level-3 facility under a regulation for animal experiment of Oita University. The *Culex pipiens pallens* colony ($516^{th}$ generations) was derived from mosquito larvae which were collected in drains of Osaka prefecture in 1965. Six to Seven-day-old female mosquitoes (n=4) were inoculated intrathoracically with 100 PFU of all variants. Mosquitoes were maintained in cartons at 27°C and 85% humidity, fed a 5% sucrose solution, harvested 0, 3, 5 and 7 days post inoculation, and stored at −80°C. Individual mosquitoes were triturated in mosquito diluents (2% FCS-MEM containing 500 μg/mL streptomycin and 500 U/mL penicillin) using a Mixer Mill apparatus (Qiagen, USA), and the homogenates were centrifuged at 1,000 rpm for 10 minutes. Viruses in the supernatants were titrated on BHK cells using the plaque assay.

Five- to six-day-old female *Culex pipiens pallens* (n=19-21) were fed a blood/virus suspension using an artificial feeding technique. A blood/virus suspension was prepared by mixing stock virus (the 6-LP or 6-SP variant), normal defibrinated human blood, and PBS(−). The mosquitoes were allowed to feed on the blood/virus suspension, containing $10^7$, $10^6$, or $10^5$ PFU/mL of virus, over a 24 h period, were maintained at 27°C and 85% humidity, and were harvested 13 days post inoculation, and stored at −80°C. Individual mosquito legs were triturated and titrated for virus using the plaque assay.

**Pathology and immunohistochemistry.** The young chicks were necropsied in a biosafety level (BSL)-3 containment facility. Tissue samples, including samples from the liver, spleen, kidneys, heart, and brain, were fixed in 20% neutral buffered formalin solution, and embedded in paraffin. The tissue samples were sectioned at a thickness of 4 μm, and stained

with hematoxylin-eosin (HE) stain for light microscopy. For the detection of WN virus antigens, serial sections were stained with the streptavidin-biotin immunoperoxidase complex method (Histofine SAB-PO kit, Nichirei Co., Japan), using mouse anti-WN virus hyperimmune ascitic fluid at a dilution of 1:5,000 as the primary antibody. The sections were counterstained with hematoxylin.

## Results

**Pathogenicity in chicks.** It was shown that the LP variant of the NY strain of WN virus, which has a glycosylated E protein, showed higher neuroinvasive virulence in mice than the SP variant, which has a non-glycosylated E protein.[53] In order to examine whether glycosylation of the E protein affects the pathogenicity of WN virus in avian hosts, young chicks were selected as the experimental infection model. Two-day-old chicks (n=5) were inoculated subcutaneously with $10^{-1}$-$10^{4}$ PFU of the 6-LP variant or with $10^{2}$-$10^{5}$ PFU of the 6-SP variant. The survival rates of chicks were recorded daily, and tissue samples were collected for observing histopathological changes. The survival rates of chicks inoculated with $10^{0}$-$10^{4}$ and $10^{-1}$ PFU of the 6-LP were 0% and 20%, respectively (Fig. 1A). The $LD_{50}$ value of the 6-LP variant in young chicks was less than $10^{-1}$ PFU. All chicks began to show disease symptoms by 3 days post inoculation (d.p.i.). Chicks exhibited depressed signs and wheezing, but neurological symptoms were not observed. In contrast, the 6-SP variant exhibited low virulence in young chicks, and resulted in dose-independent death. Survival rates were 60% with a $10^{2}$, $10^{4}$ and $10^{5}$ PFU inoculation, and 40% with a $10^{3}$ PFU inoculation (Fig. 1B). The clinical symptoms of the chicks were mild.

Histopathological findings of organs of 6-LP inoculated chicks included severe necrosis in the heart (Fig. 2A) and liver (Fig. 3A). But the chicks inoculated with 6-SP showed only mild inflammation in these tissues (Fig. 2B, 3B). WN virus antigens were only detected in the myocytes of 6-LP inoculated chicks (Fig. 2C) but not in 6-SP (Fig. 2D).

These results suggest that LP variants of WN virus, which have glycosylated E proteins, are highly pathogenic to young chicks.

**Viremia in chicks.** In order to investigate whether glycosylation of the E protein influences multiplication of the NY strain of the WN virus in avian hosts, the author

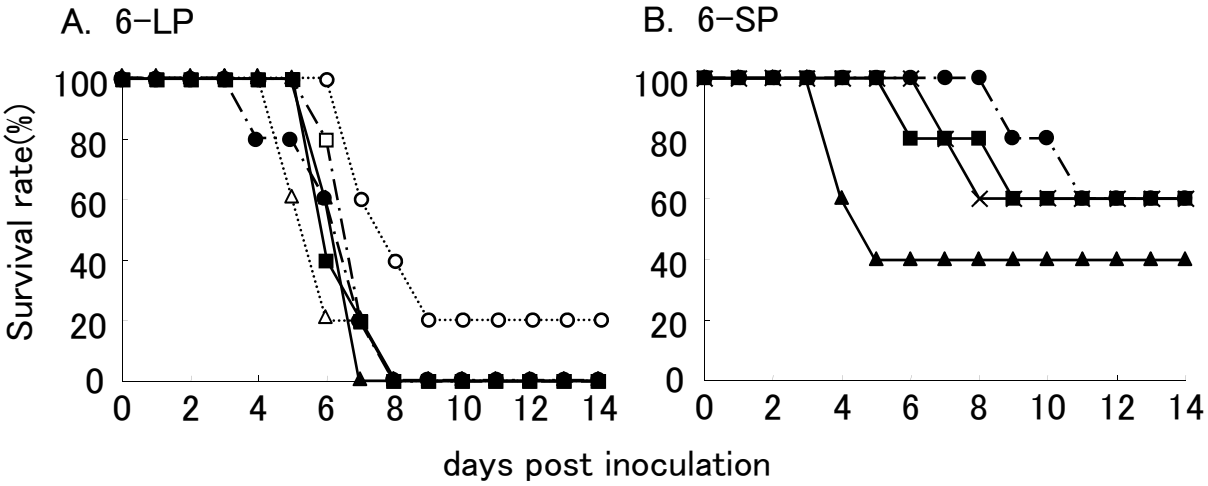


Fig. 1. Survival curves of young chicks subcutaneously inoculated with 6-LP(A) and 6-SP(B) variants of WN virus. Two-day-old male chicks were inoculated with $10^{-1}$(○), $10^{0}$(Δ), $10^{1}$(□), $10^{2}$(●), $10^{3}$(▲), or $10^{4}$(■) PFU of 6-LP, and $10^{2}$(●), $10^{3}$(▲), $10^{4}$(■), or $10^{5}$(×) PFU of 6-SP. Chicks were observed daily for changes in their health. Five chicks of each variant were examined.

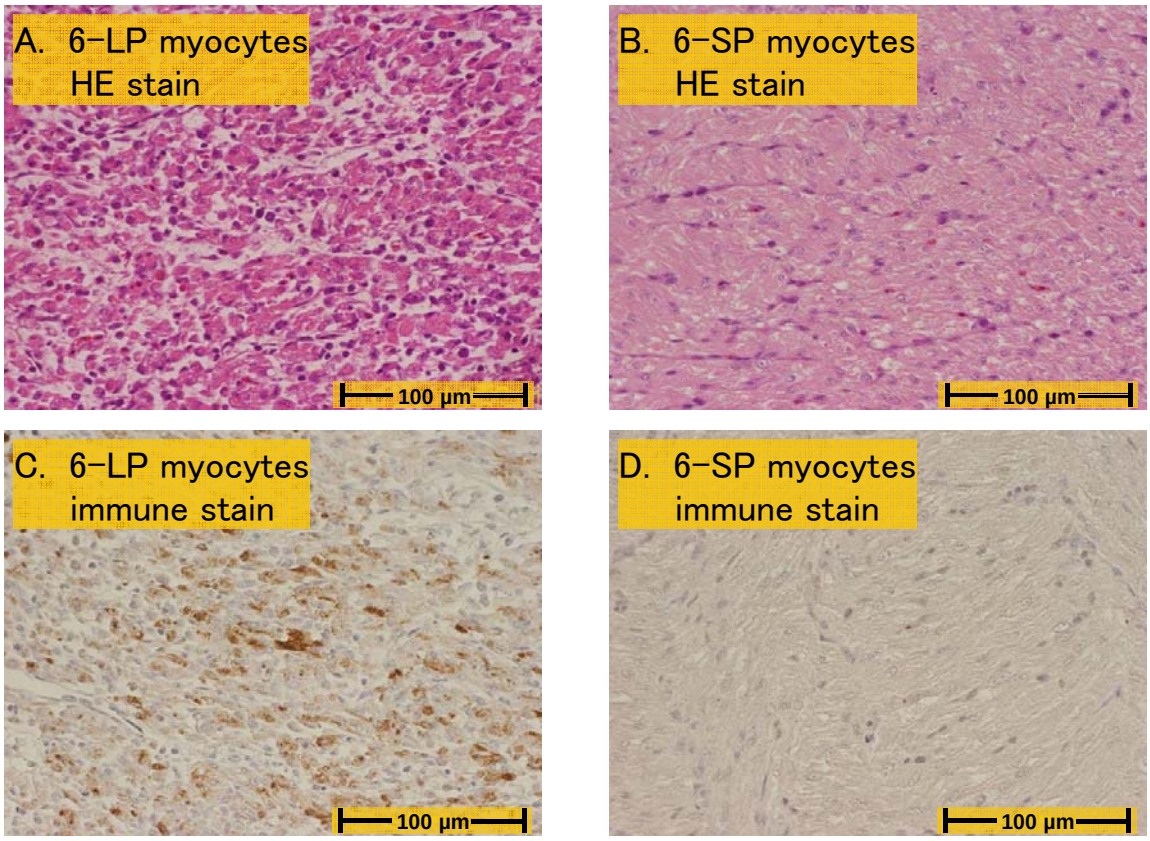


Fig. 2. Histopathological and immunohistochemical characteristics of young chicks inoculated with WN virus variant 6-LP (A, C) and 6-SP (B, D). Myocytes stained with HE stain (A, B) and immune stain (C, D). A) Photomicrograph showing marked necrosis of myocytes from the heart of a young chick infected with WN virus, strain 6-LP. Preparation was stained with HE stain. B) No histological changes were found. C) Myocytes of heart are positively stained for WN virus antigen. D) Myocytes of heart are negatively stained for the antigen.

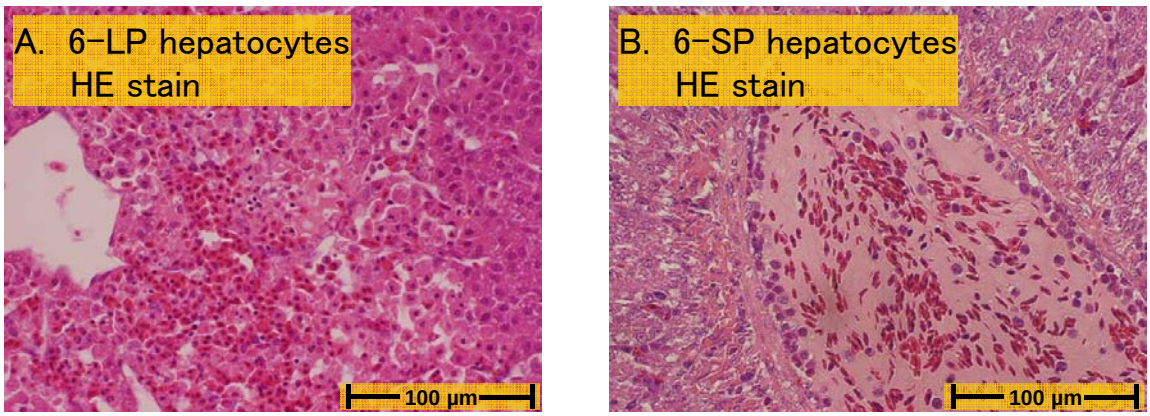


Fig. 3. Histopathological and immunohistochemical characteristics of young chicks inoculated with WN virus variant 6-LP (A) and 6-SP (B). Hepatocytes stained with HE stain. A) Photomicrograph showing marked necrosis of hepatocytes from the liver of a young chick infected with WN virus, strain 6-LP. Preparation was stained with HE stain. B) No histological changes were found.

compared viremia titers in chicks infected with LP and SP variants. Two-day-old chicks (n=4) were subcutaneously inoculated with 100 PFU of both variants, blood samples were collected every 24 h from 0 to 7 d.p.i., and viral titers in the serum were measured.

The viremia titers of the 6-LP variant in chicks were ten or more times greater than those of the 6-SP variant during 1-7 d.p.i. (Fig. 4). The titers of the 6-LP variant exceeded $10^5$ PFU/mL during 2-4 d.p.i., while those of the 6-SP variant did not exceed $10^4$ PFU/mL during the seven day observation period. The titer of the 6-SP variant was below the detection limit (100 PFU/mL) at 7 d.p.i. The viremia titers of B-LP were also found to be higher than those of B-SP. The titers of B-LP exceeded $10^5$ PFU/mL during 2-4 d.p.i., whereas those of B-SP were mostly below $10^4$ PFU/mL.

These results suggest that glycosylation of the WN virus E protein enhances viremia levels in chicks.

**Viral growth in *Culex pipiens pallens*.** To establish whether glycosylation of the E protein influences multiplication of the WN virus in mosquito hosts, seven-day-old female *Culex pipiens pallens* (n=4) were inoculated intrathoracically with 100 PFU of the 6-LP, 6-SP, B-LP, or B-SP variant. The mosquitoes were harvested 0, 3, 5 and 7 d.p.i., and the viral titers in their bodies were measured using the plaque assay. The viral titers in mosquitoes inoculated with LP variants were not significantly different from those inoculated with SP. The titers of all variants increased by up to about $10^5$ PFU/mosquito by 3 d.p.i., and no differences in viral titer were observed between any combination of variants or harvested days (Fig. 5).

Furthermore, to investigate whether glycosylation of the E protein would affect disseminated infection of WN virus in mosquitoes, *Culex pipiens* were fed blood meals containing $10^5$ to $10^7$ PFU/mL of 6-LP or 6-SP variants. Mosquitoes were harvested 13 d.p.i., and mosquito legs were triturated and examined. The infection rates (Table 2) and virus titers

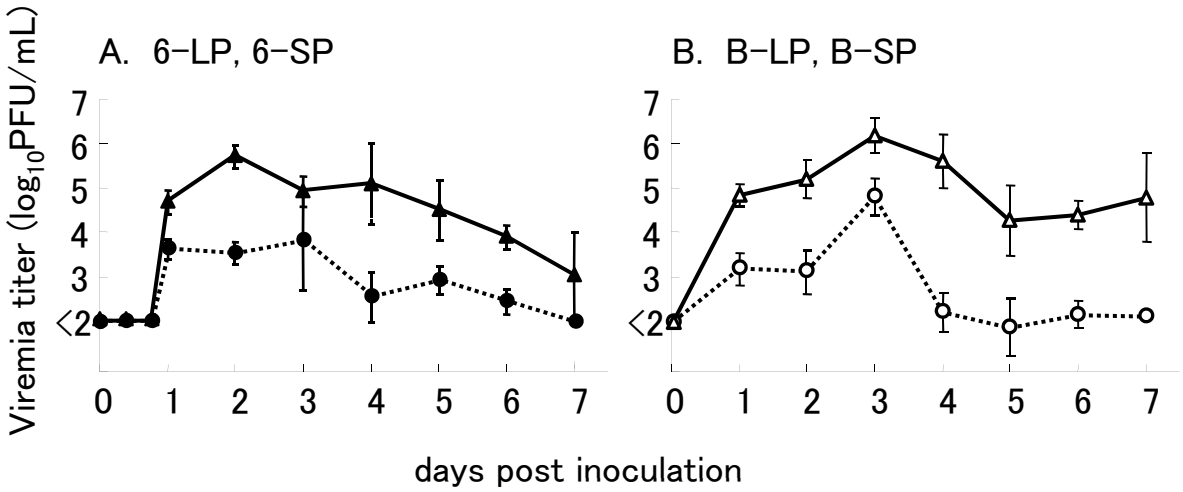


Fig. 4. Viremic levels of young chicks subcutaneously inoculated with WN virus variants. Young chicks were inoculated with WN virus variants 6-LP(▲) and 6-SP(●) in experiment (A), and B-LP(Δ) and B-SP(○) in experiment (B). Two-day- old chicks were inoculated with 100 PFU of the respective variant (n=4). The viral titers in the sera were measured using the plaque assay on BHK cells. Mean (±SD) titers are from triplicate cultures.

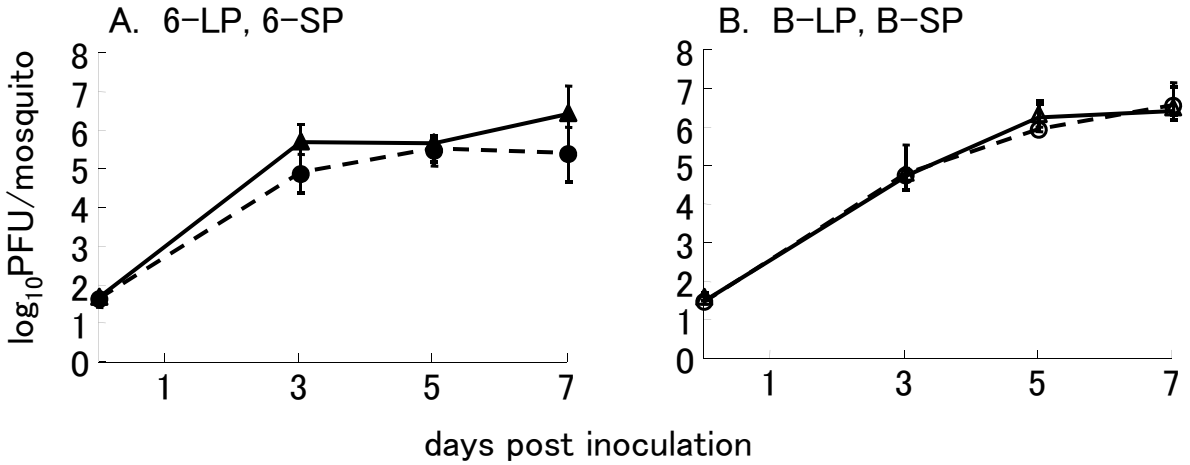


Fig. 5. Viral titer of WN virus variants in *Culex pipiens pallens*. Seven-day-old female mosquitoes (n=4) were inoculated intrathoracically with 100 PFU of variant. The viral titers in mosquito bodies were measured using the plaque assay with BHK cells. The viral titers of 6-LP(▲)and 6-SP(●)are shown in (A) and those of B-LP(Δ) and B-SP(○) are shown in (B). Mean (±S.D.) titers are from average values of 4 individual mosquitoes, each values from triplicate cultures.

Table 2. Disseminated infection rates of *Culex pipiens pallens* during peroral infection experiments with WN virus.

| WN virus variant | Virus dose (PFU) | | |
|---|---|---|---|
| | $10^7$ | $10^6$ | $10^5$ |
| 6-LP | 10/10* | 9/9 | 5/6 |
| 6-SP | 6/6 | 11/11 | 4/10 |

* Number of virus positive mosquitoes / Number of inoculated mosquitoes

Mosquitoes were fed a blood-virus mixture and were maintained at 28°C for 13 days, harvested and titrated for virus in a BHK cell plaque assay.

(data not shown) were not significantly different between mosquitoes fed with 6-LP and 6-SP variants.

The results suggest that glycosylation of the E protein may not influence multiplication and dissemination of WN virus in mosquitoes.

**Viral growth *in vitro*.** In order to determine the effect of glycosylation of the E protein on heat stability of the virus, the growth kinetics of the LP and SP variants of the NY strain of WN virus were examined in three kinds of cell cultures, namely mammalian (BHK), avian (QT6), and mosquito (C6/36) cell lines, at various incubation temperatures.

Mammalian BHK cells were inoculated with the virus, and incubated at 34°C, 37°C or 40°C, and the viral titers were determined in the culture fluids. When incubated at 34°C, there were no differences in viral titers between 6-LP and 6-SP, or between B-LP and B-SP variants (Fig. 6A, B). However, when BHK cells were incubated at 37°C and 40°C, viral titers of 6-SP and B-SP were lower than those of 6-LP and B-LP, respectively (Fig 6C-F).

Avian QT6 cells were also examined for the multiplication of the WN virus variants at different temperatures. When QT6 cells were incubated at 37°C, viral titers of the culture fluids did not differ between those inoculated with 6-LP and 6-SP, or with B-LP and B-SP (Fig. 7A, B). However, at 40°C and 42°C of incubation, viral titers of 6-SP and B-SP were significantly lower than those of 6-LP and B-LP, respectively (Fig. 7C-F).

Next, the multiplication of the variants was tested in mosquito C6/36 cells at different temperatures. Viral titers of 6-LP and B-LP were not significantly different from those of 6-SP and B-SP when incubated at 28°C (Fig. 8A, B) or at 32°C (Fig. 8 C, D).

These results showed that the LP variants of WN virus, which have glycosylated E proteins, multiplied more efficiently in mammalian and avian cell cultures at high temperatures than did the SP variants, which lacked glycosylation of the E protein. However, there was no difference in viral multiplication between the LP and SP variants in mammalian

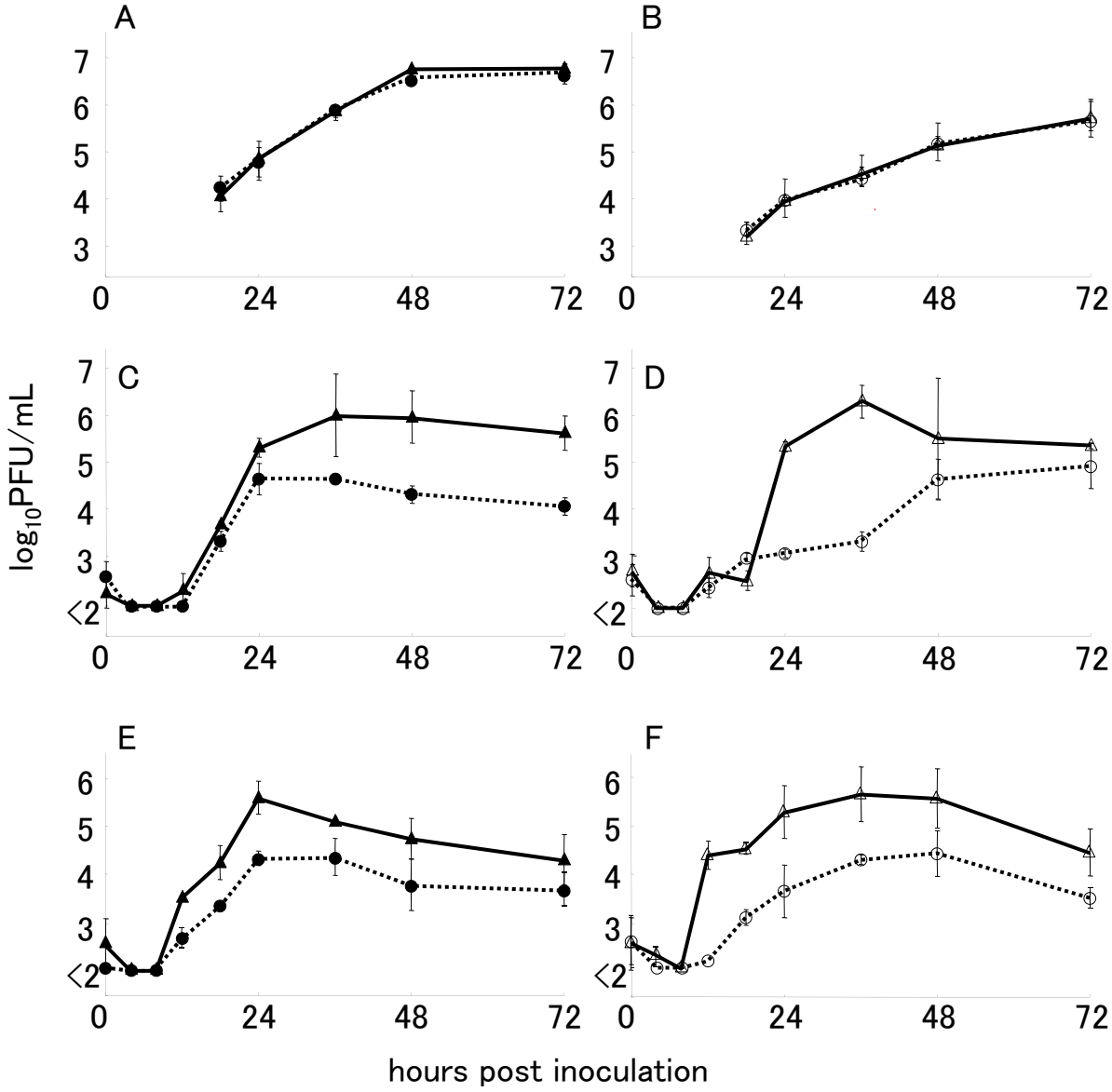


Fig. 6. *In vitro* replication of WN virus variants in hamster (BHK) cells. BHK cells were inoculated with the 6-LP(▲), 6-SP(●), B-LP(Δ), or B-SP(○) variant at an MOI of 0.01. BHK cells were incubated at 34°C (A, B), 37°C (C, D), or 40°C (E, F). The viral titers in culture fluids were measured using the plaque assay on BHK cells. The detection limit was 100 PFU/mL. Mean (±S.D.) titers are from triplicate cultures.

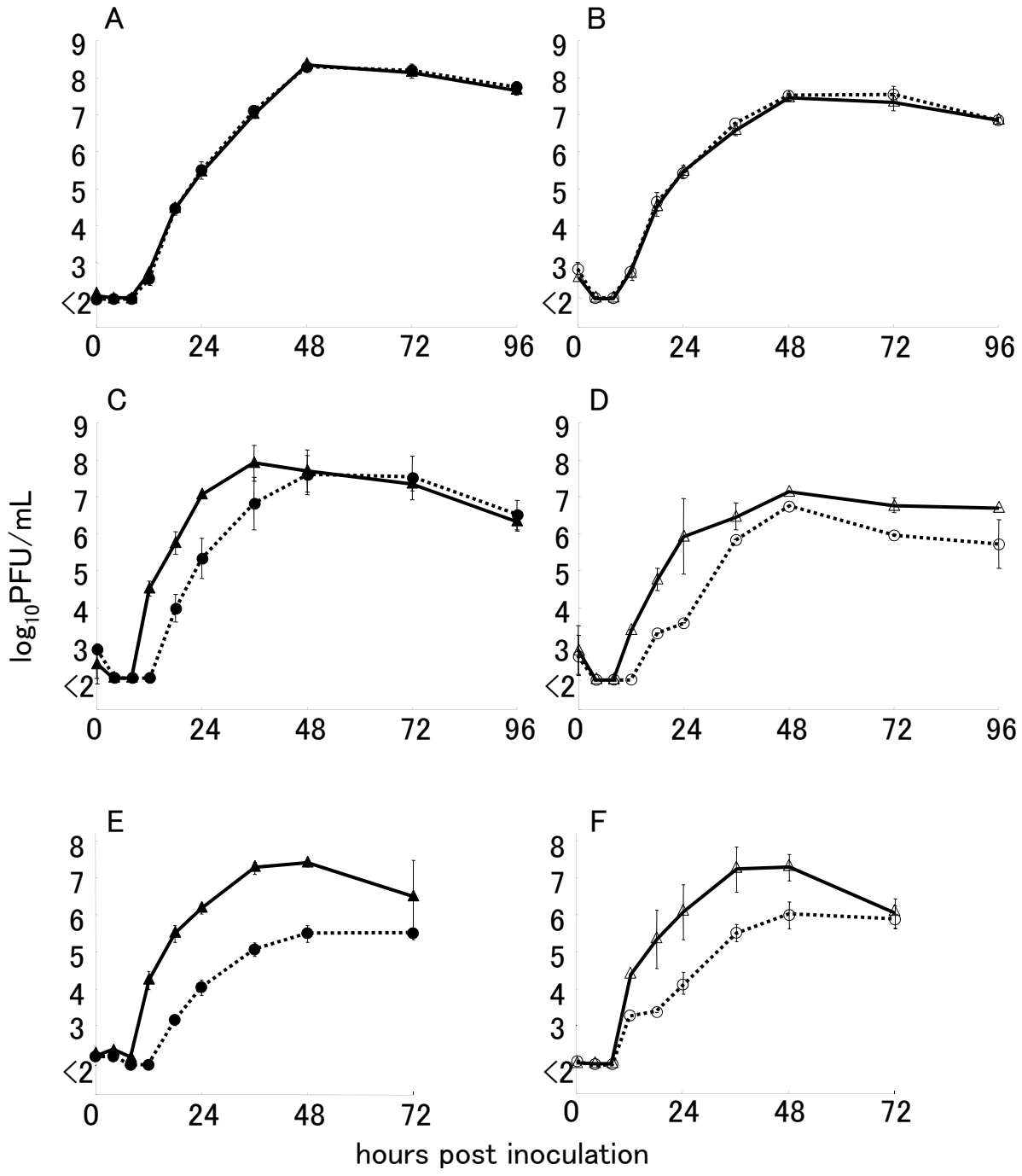


Fig. 7. *In vitro* replication of WN virus variants in quail (QT6) cells. QT6 cells were inoculated with variants 6-LP(▲), 6-SP(●), B-LP(Δ), or B-SP(○) at an MOI of 0.01. QT6 cells were incubated at 37°C (A, B), 40°C (C, D), or 42°C (E,F). The viral titers were measured in the culture fluids using the plaque assay on BHK cells. The detection limit was 100 PFU/mL. Mean (±SD) titers are from triplicate cultures.

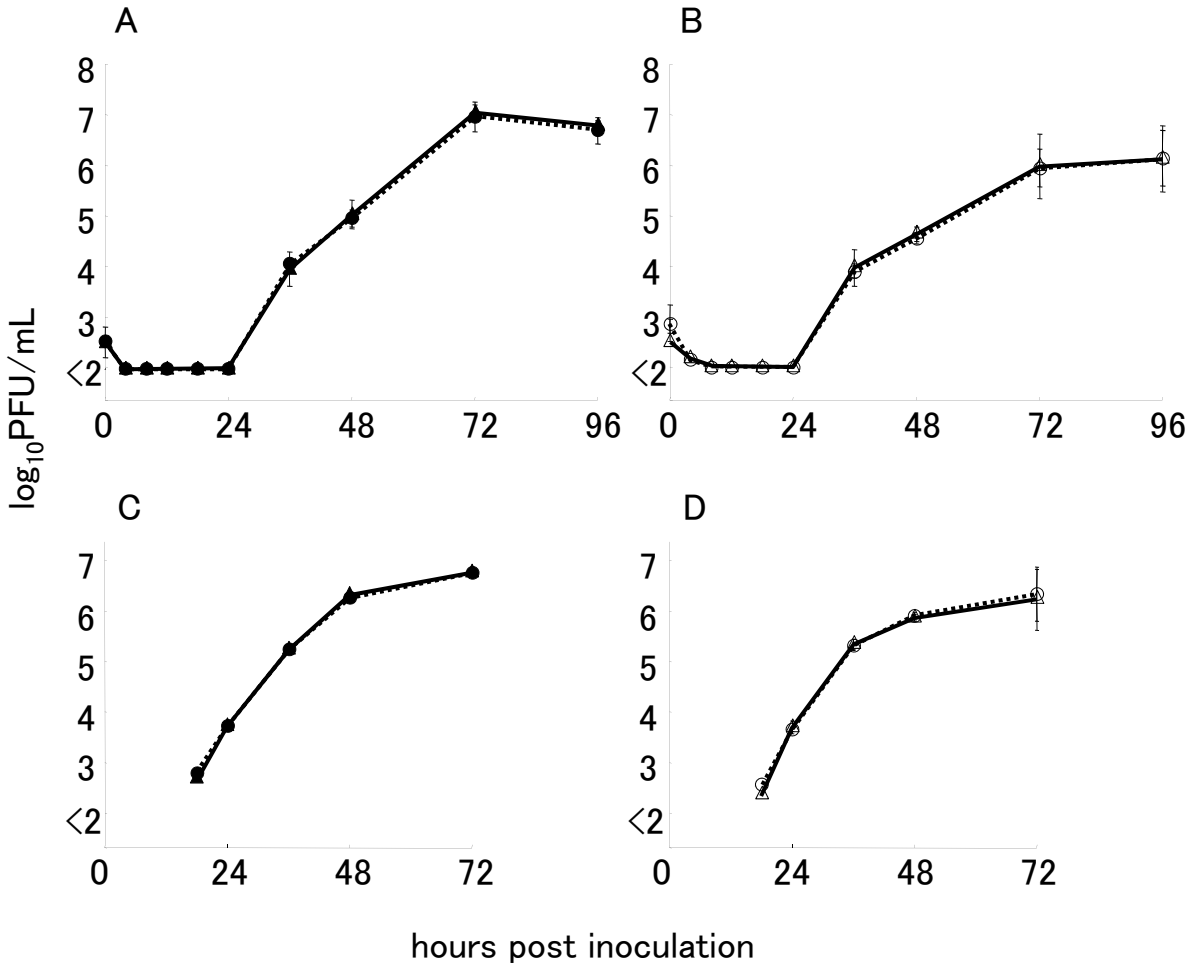


Fig. 8. *In vitro* replication of WN virus variants in *Aedes albopictus* (C6/36) cells. C6/36 cells were inoculated with 6-LP(▲), 6-SP(●), B-LP(Δ), or B-SP(○) at an MOI of 0.01. C6/36 cells were incubated at 28°C (A, B) or 32°C (C, D). The viral titers in the culture fluids were measured using the plaque assay on BHK cells. The detection limit was 100 PFU/mL. Mean (±SD) titers are from triplicate cultures.

and avian cell cultures at low temperatures. The efficiency of viral multiplication did not differ between the LP and SP variants in mosquito C6/36 cells at different temperatures.

In order to examine if the thermostabilities of the variants accounted for the differences in viral multiplication observed in avian and mammalian cells at higher temperatures (i.e., 40°C and 42°C), the variants of WN virus were incubated at different temperatures in culture medium without cells, and the viral titers were determined (Fig. 9). In culture medium, the viral titers declined more rapidly at 42°C than at 37°C. However, there was no difference between the rates of reduction of 6-LP and 6-SP titers, either at 37°C or at 42°C. These results suggest that the difference in viral multiplication among the variants at higher temperatures was not due to the different thermostabilities of the variants.

In order to confirm that N-glycosylation of the E protein is indeed responsible for the above-described differences in multiplication characteristics of WN virus in different cell types, and at various temperatures, WN virus strains with or without the N-glycosylated site were generated from infections cDNA clone and their multiplication patterns were examined in C6/36 and QT6 cells (Fig. 10). When C6/36 cells were infected with WN virus recNY/Gly+, which bears an E protein N-glycosylation site, and WN virus recNY/Gly−, which lacks an E protein glycosylation site (S156P), and incubated at 28°C, no difference in viral multiplication efficiency were observed between the two strains (Fig. 10A). When QT6 cells were infected with both strains and incubated at 40°C, the WN virus recNY/Gly+ strain multiplied more efficiently than WN virus recNY/Gly− (Fig. 10B). This result confirmed that N-glycosylation of the E protein is responsible for the efficient replication of WN virus in QT6 cells at the higher temperature.

**Comparisons of the intracellular and extracellular expression levels of the LP and SP variant viral proteins.** At 42°C, viral titers of the SP variants of WN virus were lower than those of the LP variants in mammalian BHK cells and avian QT6 cells. In order to examine

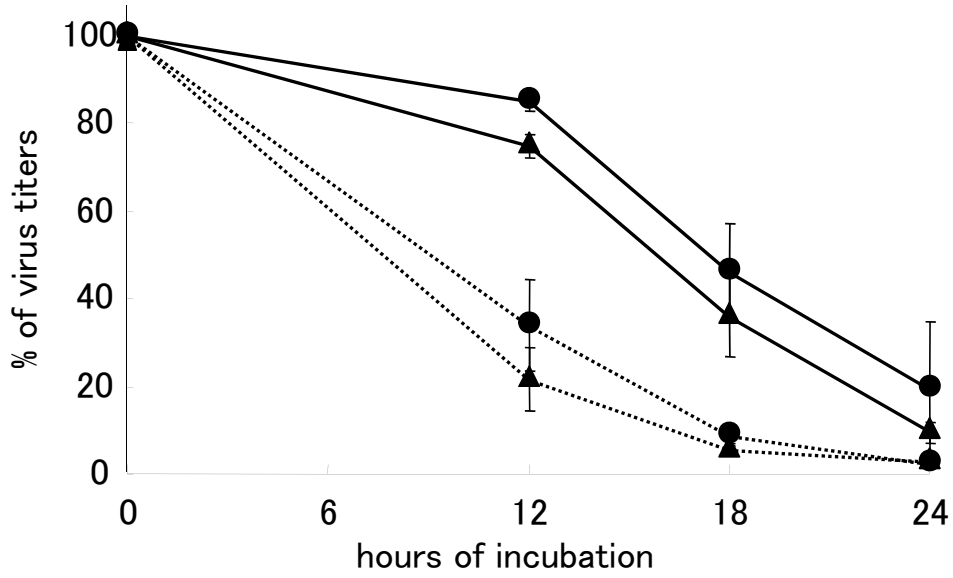


Fig. 9. Thermostability of WN virus variants. Two variants [6-LP(▲) and 6-SP(●)] were incubated at 37°C ( — ) and 42°C (-----) in culture medium without cells, and viral titers in the culture medium were measured using the plaque assay on BHK cells. The % of viral titers were (titers at each time point) / (titers at 0 hour of incubation). Mean (±S.D.) % are from triplicate cultures.

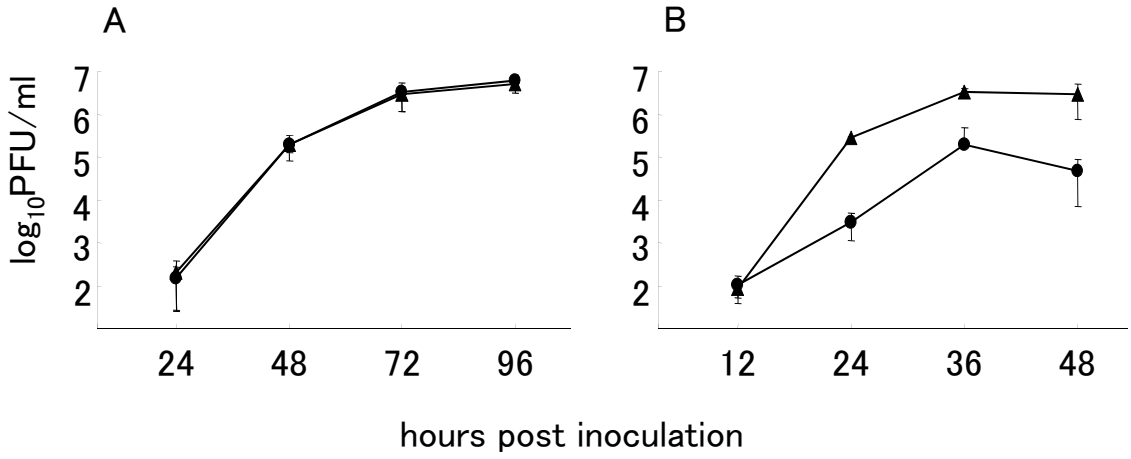


Fig. 10. *In vitro* replication of WN virus strains recNY/Gly+(▲) and recNY/Gly−(●). C6/36(A) and QT6(B) cells were infected with each strain at an MOI of 0.01. C6/36 cells were incubated at 28°C and QT6 cells were incubated at 40°C. The viral titers in culture fluids were measured using the plaque assay on BHK cells. The detection limit was 100 PFU/mL. Mean (±S.D.) titers are from triplicate cultures.

which step of the viral replication cycle is involved in the reduced multiplication of the SP variants, which have non-glycosylated E proteins, at higher temperatures, intracellular and extracellular levels of viral proteins of the LP and SP variants were compared.

QT6 cells were infected with 6-LP or 6-SP variants and incubated at 42°C and 37°C. For comparison, C6/36 cells were also infected with 6-LP or 6-SP variants and incubated at 28°C. The cell lysates and culture fluids were harvested sequentially, and subjected to antigen detection ELISA. The levels of E protein of 6-SP in QT-6 cells and culture fluid were lower than those of 6-LP at 42°C (Fig. 11. A, B), but there was no difference between those of 6-SP and 6-LP at 37°C (Fig. 11. C, D). In contrast, there was no difference in the levels of E protein in either the cell lysates or the culture fluids of C6/36 cells infected with 6-SP or 6-LP, and incubated at 28°C (Fig. 11. E, F). These results suggested that the lack of glycosylation of the E protein affects viral replication at steps before the viral release process.

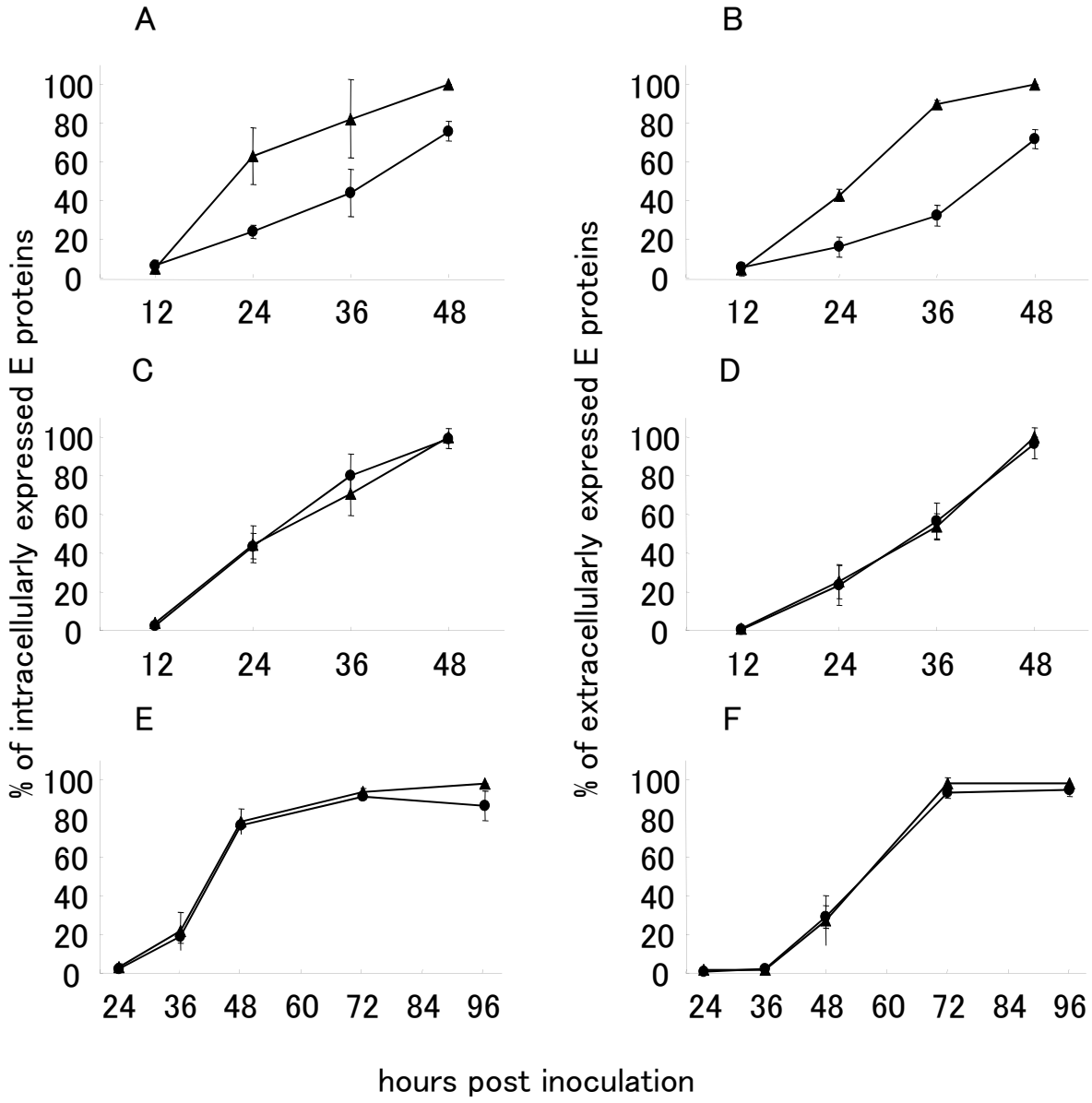


Fig. 11. The Effects of glycosylation of the E protein on intracellular expression and extracellular secretion of the E protein in QT6(A-D) and C6/36(E, F) cells. QT6 cells were incubated at 42°C (A, B) and 37°C (C, D), and C6/36 cells at 28°C. The amounts of intracellular and extracellular E protein of 6-LP(▲) and 6-SP(●) variants were measured by antigen detection ELISA. Culture cell lysates (A, C, E) and supernatants (B, D, F) were harvested sequentially from QT6 and C6/36 cells. The % of expressed proteins were (OD values at each time point) / (OD values at 48 hours of LP in A to D or those at 96 hours of LP in E and F). Mean (±S.D.) values are from triplicate cultures.

## Discussion

Previously, two NY strains of WN virus were plaque-purified, and four variants that had different amino acid sequences at the N-linked glycosylation site in the E protein sequence were isolated. The E protein was glycosylated in two of these strain variants. The glycosylated variants produce large plaques (LPs), and the non-glycosylated variants produce small plaques (SPs) in BHK cells, and the LP variants are more pathogenic in mice than are the SP variants. The gene sequences of WN viruses from various locations around the world were investigated and compared. It was found that most of the strains occurring before the 1990s or that some of the low pathogenic strains do not have the N-glycosylation site, whereas many of the highly pathogenic strains that emerged recently have the N-glycosylation site.[53] A rare WN virus isolated in Mexico lacks the glycosylation site on the E protein, and was shown to have reduced pathogenicity in mice.[2]

N-linked glycosylation of the WN virus E protein was previously shown to be responsible for enhanced neuroinvasiveness of the virus in a mouse model.[4,17,53] However, few studies[38] have been conducted to determine the role of glycosylation of the E protein in WN virus replication and pathogenicity in birds and mosquitoes, both natural hosts of the virus. The author selected young chicks as a model to examine WN virus dynamics, and found that young chicks can serve as a model to study the pathogenicity of WN virus in avian hosts. Subcutaneously injected LP variants resulted in a much higher mortality rate ($LD_{50}$ < 0.1 PFU) than SP variants, suggesting that glycosylation of the E protein of WN virus is a determinant of pathogenicity in chicks that have been peripherally inoculated. Histopathological findings in dead chicks included necrosis in hepatocytes and necrotic myocarditis, and cardiovascular failure was the suspected cause of death in these birds. These histopathological changes were also seen in birds that had been naturally infected with WN virus.[15]

Efficient viral propagation both in avian and mosquito hosts is an important determinant of active viral circulation in the natural transmission cycle. The author tested the viremic levels of LP and SP variants in chicks to reveal the effect of glycosylation of the WN virus E protein on the magnitude of viremic levels. The viremic levels of chicks inoculated with LP variants were higher than those inoculated with SP variants. The viremic titers of chicks inoculated with LP variants exceeded $10^5$ PFU/mL blood during 2-4 d.p.i. Previous studies demonstrated that avian viremic levels higher than $10^5$ PFU/mL are crucial for the efficient infection of vector *Culex tritaeniorhynchus* mosquitoes.[28] These results demonstrated that N-linked glycosylation of WN virus E protein is a determinant of high viremic levels in young chicks, and suggest that glycosylated WN virus variants may be more efficiently transmitted to vector mosquitoes than non-glycosylated variants due to higher viremia in infected birds.

To explain the differences in viremic titers of chicks inoculated with the two variants, growth characteristics of the LP variant, which is glycosylated, and the SP variant, which is not glycosylated, were examined in tissue culture cells at different temperatures. The results suggest that glycosylation of the E protein imparted heat stability to WN virus during propagation in cells at high temperature. The author tested three kinds of cultured cells, namely BHK cells from a mammalian host, QT6 cells from an avian host, and C6/36 cells from mosquitoes, each representing an important host in the natural transmission cycle of WN virus. Viral growth characteristics were examined by culturing the cells at different temperatures. Compared with LP variants, SP variants showed a remarkable reduction in viral growth in BHK cells at 37°C and 40°C, and in QT6 cells at 40°C and 42°C. Reduction rates of viral titers in the culture media without cells were not significantly different between SP and LP variants. Collectively, differences in the heat-stable characteristics of the LP variants and the heat-labile characteristics of the SP variants in BHK cells and QT-6 cells at high temperatures depended on the glycosylation status of the E protein of the variants, which

affected the viral replication steps within the cells.

In contrast, The author did not detect a significant difference in viral titers between the LP and SP variants when *Culex pipiens* mosquitoes were inoculated intrathoracically with each variant, and the amount of virus was measured in the mosquitoes. The disseminated infection rates of mosquitoes orally infected with the variants did not show any difference between the LP and SP variants. Moreover, there were no differences in the propagation of the two variants in C6/36 cells, at various temperatures. The results suggest that the glycosylation status of the E protein may not affect viral propagation and dissemination in mosquitoes. Recently, Moudy et al. reported that WN virus E protein glycosylation is required for efficient viral transmission by *Culex* mosquitoes.[38] This contradiction might have been caused by the difference of the mosquito strains or method of viral measurement assays. In the present study, a relatively small number of mosquitoes were examined as compared with that of their experiment, which might also cause this contradiction. In addition, they mutated the E protein glycosylation site from NYS to IYS in a full-length clone of the NY99 strain, which resulted in a virus that lacked the glycan at aa154. In the present study, the author generated non-glycosylated variants by mutating aa156; the NYS glycosylation site was changed to NYP for 6-SP, and to NYE for B-SP. It is interesting to establish how these differences in amino acid sequence among the variants affect viral propagation and dissemination in vector mosquitoes.

Furthermore, the author conducted the same multiplication experiment using variants of the NY strain of the WN virus generated by infectious cDNA clone technology.[33] This experiment was performed to exclude the possibility that some silent mutations in the coding regions other than the N-glycosylation site and the 3'NCR contribute to the differences between the SP and LP variants. Since the recNY/Gly+ and the recNY/Gly− strains only differed in the N-linked glycosylation site (S156P), and the recNY/Gly+ strain, with a glycosylated E protein, showed stable multiplication in QT6 cells at higher temperature.

Collaborators of the author previously examined the role of the N-linked glycans of E protein in the secretion of tick-borne encephalitis virus particle secretion using subviral particles.[16] The secretion of virus particles was greatly reduced in culture cells transfected with mutant vectors that have an amino acid substitution of T156A in the E protein, and the study also suggested that the reduced particle secretion is due to glycan loss, rather than to the amino acid substitution per se. The amino acid substitution of T156A is similar to that of S156P in our current study in terms of amino acid characteristics, and both mutations altered the protein such that it would not be recognized by oligosaccharyl-transferase.[26] Collectively, the observed differences between LP and SP variants are most likely due to glycan loss on the E protein, rather than to the amino acid substitutions.

When the E proteins in samples from QT6 cells were quantified by ELISA, the E protein levels were found to be lower both in the cell lysates and the supernatants of cells infected with the 6-SP variant than in those infected with 6-LP. This result suggests that glycosylation participates in the multiplication of WN virus at an earlier stage than the viral release process. There were several reports of temperature sensitive, attenuated WN virus variants and those temperature sensitive phenotypes were due to the amino acid substitutions at the NS genes, which might reduce the efficiency of RNA replication.[24,27,60] However, there was no report studying the relationship among temperature sensitivity of WN virus, the glycosylation of envelope protein and the intracellular viral protein maturation and trafficking. A previous study[16] using a subviral system of tick-borne encephalitis virus showed that a mutant lacking E protein glycosylation has a large reduction in the level of secretion of the E protein, and the E protein is retained at the endoplasmic reticulum, and is rarely present in the Golgi complex. In the dengue virus, this glycosylation at aa154 occurs in E protein domain I, close to the center of the fusion peptide of E protein domain II, and glycosylation of the E protein is considered to increase the stability of the protein.[37,50] Glycosylation of the E protein of WN virus may also be important for the folding and stability of the viral protein at high

temperatures.

Mutations of NS3 or NS4B of the NY strain of WN virus were reported to be responsible for the increased pathogenicity and viremic level in avian or mammalian hosts.[6,60] Importantly, the introduction of a T249P amino acid in NS3 helicase was shown to be crucial for the above-mentioned viral characteristics. The author demonstrated that N-glycosylation of the E protein facilitated efficient multiplication of the NY strain of WN virus at high temperatures in an avian cell culture, and was responsible for the higher viremic level in an avian host. The observation that most recent isolates of lineage I WN virus carry the N-glycosylation site on the E protein,[53] suggests that glycosylation of the E protein is a prerequisite for the stable circulation of WN virus in the avian-mosquito transmission cycle, and may be one of the multiple determinants for efficient transmission.

**Summary**

Many West Nile (WN) virus isolates associated with significant outbreaks possess a glycosylation site on the envelope (E) protein. E protein glycosylated variants of New York (NY) strains of WN virus are more neuroinvasive in mice than the non-glycosylated variants. To determine how E protein glycosylation affects the interactions between WN virus and avian hosts, the author inoculated young chicks with NY strains of WN virus containing either glycosylated or non-glycosylated variants of the E protein. The glycosylated variants were more virulent and had higher viremic levels than the non-glycosylated variants. The glycosylation status of the variant did not affect viral multiplication and dissemination in mosquitoes *in vivo*. Glycosylated variants showed more heat stable propagation than non-glycosylated variants in mammalian (BHK) and avian (QT6) cells, but not in mosquito (C6/36) cells. Thus, E protein glycosylation may be a requirement for efficient transmission of WN virus from avian hosts to mosquito vectors.

**[Chapter II]**

## Evaluation of a Neutralization Test for West Nile Virus in Chicks and the Seroprevalence of West Nile Virus in Wild Birds in Far Eastern Russia

### Introduction

Since an outbreak of WN encephalitis in humans and horses in New York City in 1999, the WN virus has spread throughout the North American continents very rapidly.[14] In Russia, the WN virus was first isolated from humans and ticks in 1963 in European Russia. In 1999, 318 confirmed cases of human infection with WN virus were reported in the Volgograd Region, resulting in 40 deaths.[45,46] In 2004, the WN virus was reported in patients in Novosibirsk in the southwest region of Siberia.[57] The WN virus has a tendency to spread eastward through Russia. It is possible that migratory birds carry WN virus from Far East Russia to East Asian countries during migration.

The JE virus, which belongs to the same serocomplex group as WN virus, is endemic to East Asia. Although pigs are well known to be the amplifier host of the JE virus, wild birds have been reported to serve as the reservoir host. JE and WN viruses are closely related to each other and often show serological cross-reactivity.[30,35] The geographical distribution of JE and WN viruses rarely overlap; however, as the epidemic region of WN virus continues to expand, both viruses may infect wild birds, which are a common host. Therefore, a diagnostic test that can distinguish between WN or JE virus infection is required.

In this chapter, the author investigated whether infection in birds with JE and/or WN virus could be diagnosed using the neutralization test (NT). Chicks were inoculated with JE and WN viruses at 2-day and 3-week-old, and the titers of neutralizing antibodies against both viruses were measured. To investigate the cross-reaction to heterologous virus infection, a double infection experiment with JE and WN virus strains was also performed. Finally, in

order to clarify whether WN virus is prevalent in the Far Eastern region of Russia, the author used the NT to investigate the seroprevalence of WN virus among wild birds in the region.

## Materials and Methods

**Viruses.** To determine the serological cross-reactivity between JE and WN viruses, the author 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 #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 Center for Disease Control and Prevention (CDC, Fort Collins, CO., USA; in 2003). The NY99-6922 strain was isolated from mosquitoes in NY in 1999. The author used the plaque-purified variant of strain NY99-6922, 6-SP (#AB185915)[53], 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 under the guidelines for the use of experimental animals of Hokkaido University.

To determine the primary neutralizing antibody responses in chicks to viruses, 2-day-old chicks were inoculated with 100 PFU of the JE or WN virus, and 3-week-old chicks with 1,000 PFU of each virus via subcutaneous injection into the femoral region. All viruses were diluted in PBS(−) containing 10% 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 minutes, and then kept at 4°C overnight. The blood samples were then centrifuged at 4,000 rpm for 10 minutes, and the sera were decanted and stored at −80°C until use.

To examine the neutralizing antibody responses after secondary challenge with heterologous

viruses, the chicks were inoculated with both of the viruses. First, 2-day-old chicks (n=4) were inoculated with 100 PFU of either the JE or WN virus. After 3 weeks, the chicks (23-day-old) were inoculated again, this time with 1,000 PFU of heterologous secondary virus (WN virus in chicks previously inoculated with JE virus or the JE virus in chicks previously inoculated with WN virus).

**Viremia titration.** To confirm that the chicks were successfully infected with JE and WN viruses, the viremia titers of 2 to 9-day-old chicks were measured. The viral titers in the sera were measured by plaque assay on BHK cells (BHK-21, ATCC, USA, #CCL-10). BHK cell monolayers were grown in 12-well plates and inoculated with serial dilutions of the sera from infected chicks. After 60 minutes of viral adsorption, the sera were 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 $CO_2$ incubator. After five 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 dried, and the plaques were counted. The viral titer was expressed as the number of plaque forming units (PFU)/mL. The minimum threshold for virus detection was 50 PFU/mL.

**Antibody detection by neutralization test.** The sera of chicks and wild birds were tested for the presence of neutralizing antibody with the 80% focus reduction neutralization test ($FRNT_{80}$) using the fluorescent antibody technique. The test sera were diluted serially in two-fold steps from 1:20 to 1:2560 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 approximately 50 focus-forming units per well. The serum-virus mixtures were incubated for

60 min at 37°C in a $CO_2$ 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 hr 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, USA). Each incubation lasted 60 min and was followed by three washes with PBST. The neutralizing antibody titer was expressed as the reciprocal of the highest dilution that reduced the number of foci to 80% or less of the control value.

**Serological analysis of wild birds in Far Eastern Russia.** To determine the prevalence of WN virus in the Far Eastern region of Russia, the author analyzed the seroprevalence of WN virus among wild birds in the region. 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.[21,22] RNA was extracted from the kidneys of all 152 birds and the presence of the WN virus gene was determined by the TaqMan Real-Time PCR method.[54]

The 145 sera which were able to separate from the blood of 152 birds were tested for the presence of neutralizing antibody by $FRNT_{80}$ and the cut-off titer was set at 1:160.

## Results

**Viremia in chicks.** To confirm that the chicks were successfully infected with the JE and WN viruses, the viremia titers of the chicks were measured after inoculation at 2-day-old. The viremia profiles of young chicks infected with JE and WN virus are shown in Fig. 12. The chicks inoculated with JE virus produced measurable viremia (>50 PFU/mL) at 1-5 days post inoculation (d.p.i.). The highest titer of the JE virus exceeded $10^4$ PFU/mL at 2 d.p.i. The viremia titers of chicks inoculated with the WN virus were higher than those inoculated with the JE virus at 1, 3, 5, and 6 d.p.i., and below the threshold level at 7 d.p.i. These chicks showed maximum levels of viremia at 2, or 3 d.p.i., with levels that reached $10^4$ PFU/mL. These results confirmed that the chicks were successfully infected with JE and WN viruses.

**Neutralizing antibody responses in single virus infection.** In order to evaluate the applicability of the $FRNT_{80}$ 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 d.p.i. to 14 d.p.i. At 10 d.p.i., the maximum JE virus neutralizing antibody titer in chicks inoculated with the virus at 2-day-old exceeded >1:320 (Fig. 13A), and the maximum WN virus neutralizing antibody titer in chicks inoculated with WN virus at 2-day-old exceeded >1:1280 (Fig. 13B). Neutralizing antibody titers to heterologous virus were significantly lower (<1/4) than those to homologous virus (Fig. 13A, B). When the viruses were inoculated into 3-week-old chicks, the specific neutralizing antibody titers measured at 7 d.p.i. were lower than the titers measured at 7 d.p.i. in chicks inoculated at 2-day-old (Fig. 13C, D). The maximum JE virus neutralizing antibody titer in chicks inoculated with the JE virus at 3-week-old was 1:40 (Fig. 13C) at 14 d.p.i., and the maximum WN virus neutralizing antibody titer in chicks inoculated with WN

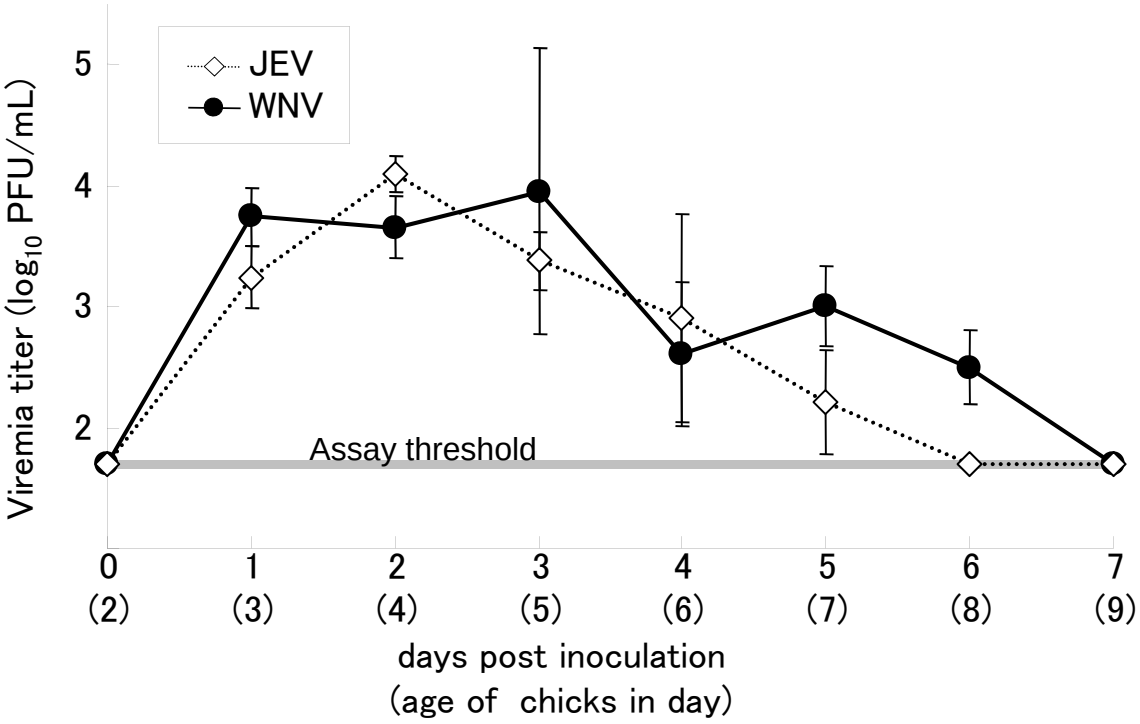


Fig. 12. Viremia levels in young chicks subcutaneously inoculated with JE virus (◊) and WN virus (●). Two-day-old chicks were inoculated with 100 PFU of each virus (n=4). The viral titers in the sera were measured using plaque assay on BHK cells. Mean (±SD) titers are from triplicate cultures. The minimal threshold for virus detection was 50 PFU/mL, shown by the gray line.

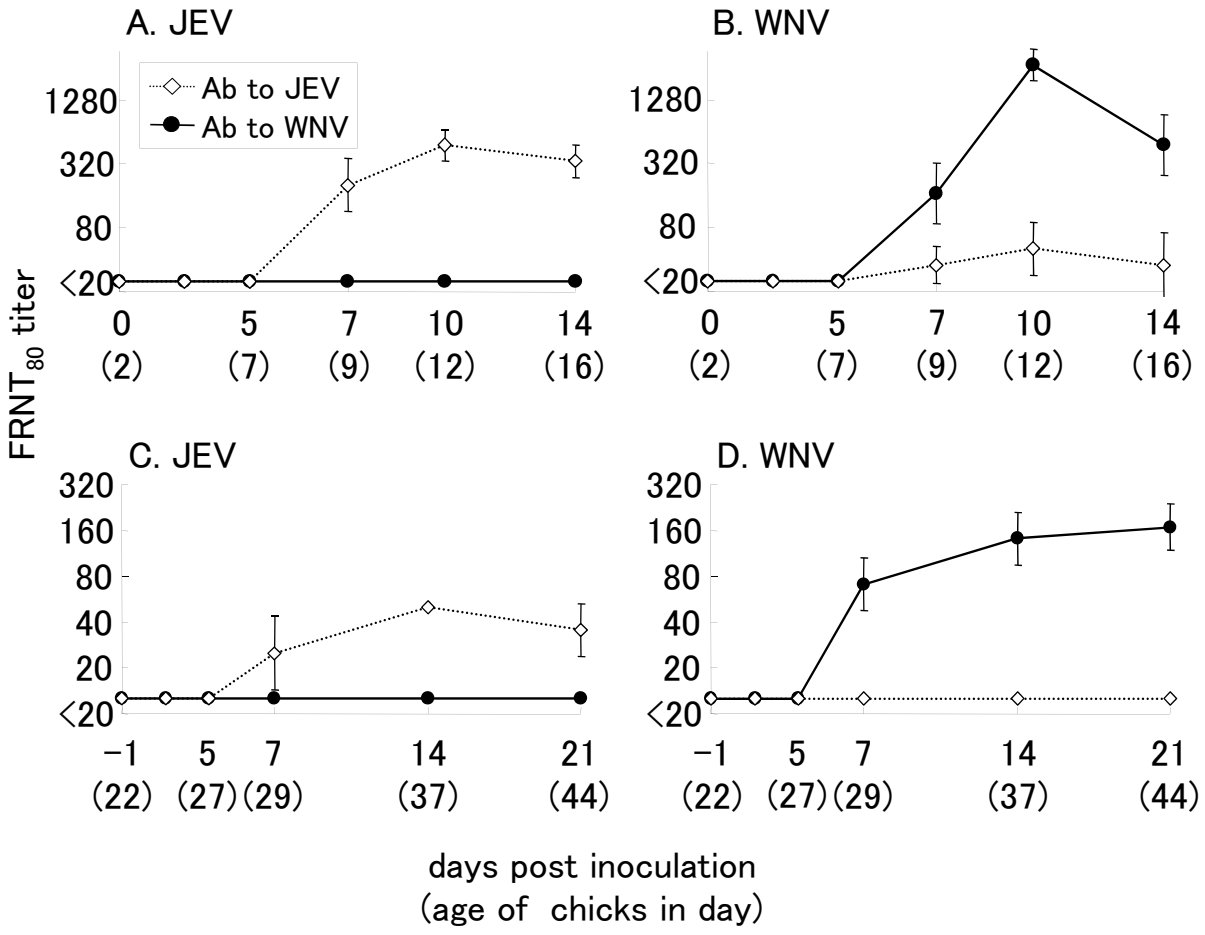


Fig. 13. Primary neutralizing antibody (Ab) responses in chicks inoculated with JE and WN viruses. Two-day-old chicks (n=4) were inoculated with 100 PFU of JE virus (A) and WN virus (B), and 3-week-old chicks (n=4) with 1,000 PFU of JE virus (C) and WN virus (D). JE virus (◊) and WN virus (●) neutralizing antibody titers were measured by $FRNT_{80}$ and expressed as the means (±SD).

virus at 3-week-old was approximately 1:160 (Fig. 13D).

**Neutralizing antibody responses after secondary challenge with heterologous virus.** In order to investigate the cross-reaction to heterologous virus infection, a double infection experiment was conducted. In this procedure, 2-day-old chicks were inoculated initially with JE virus (or WN virus) and challenged by inoculation with WN virus (or JE virus) after three weeks (Fig. 14A). In chicks inoculated with JE virus first and challenged with WN virus after three weeks, neutralizing antibodies to WN virus were measurable beginning at 3 day after challenge (d.a.c.). A detectable JE virus neutralizing antibody titer was not seen until 7 d.a.c., as in the single infection experiment (Fig. 13C). In chicks that were inoculated first with the WN virus and challenged with the JE virus after three weeks (Fig. 14B), WN virus neutralizing antibody titers were always higher than the JE virus titers. For example, although the maximum WN virus antibody titer was approximately 1:1280, 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 (Fig. 14B) than those in chicks that received a single inoculation (Fig. 13C, D). These results indicated that the neutralizing antibody responses are due to the booster effect that is typical of the secondary antibody response.

**Seroprevalence of WN virus in wild birds in Far Eastern Russia.** In order to clarify whether WN virus is prevalent in wild birds in Far Eastern Russia, samples collected from 152 wild birds captured in 2005-2006 were analyzed. TaqMan Real-Time PCR did not detect WN virus RNAs in the kidneys of any of the birds analyzed (data not shown). However, WN virus neutralizing antibody was detected in 21/145 (14.5%) of the captured birds. The birds in which neutralizing antibody to WN virus was detected belonged to the Anseriformes, Charadriiformes, Columbiformes, and Pelecaniformes orders (Table 3), and particularly high

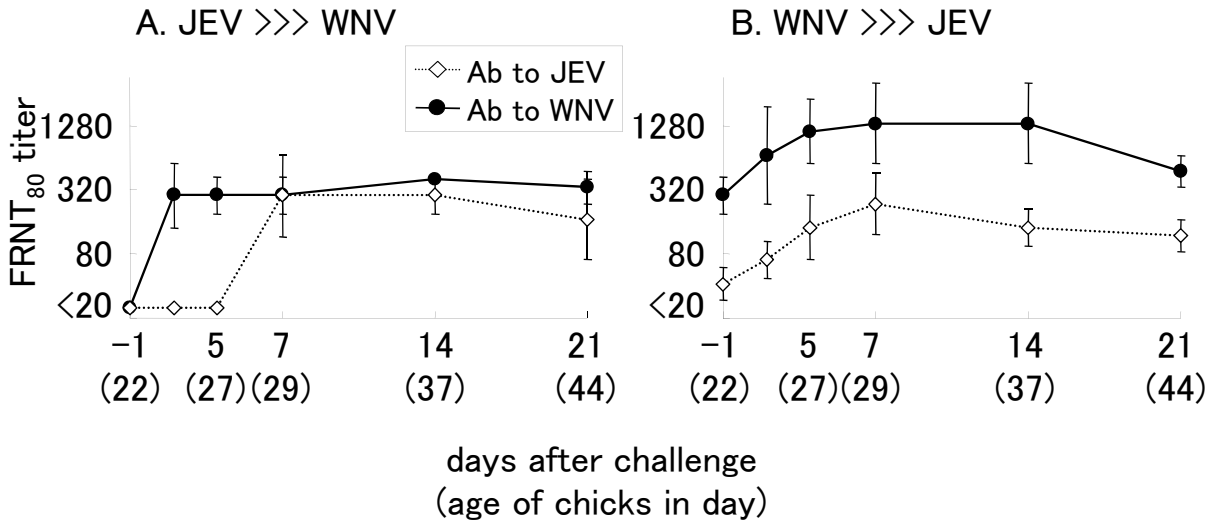


Fig. 14. Neutralizing antibody (Ab) responses in chicks after secondary challenge with heterologous viruses. Two-day-old chicks (n=4) were inoculated with 100 PFU of primary viruses; (A) with JE virus, (B) with WN virus. After 3 weeks, the chicks (23-day-old) were inoculated again with 1,000 PFU of heterologous virus; (A) with WN virus, (B) with JE virus. JE virus (♢) and WN virus (●) neutralizing antibody titers were measured by $FRNT_{80}$ and expressed as the mean (±SD).

Table 3. Seroprevalence of wild birds collected in Far Eastern 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 | *Anas poecilorhyncha* (Anseriformes) | 1 / 1 | 100 | 160 | 40 |
| | *Larus ridibundus* (Charadriiformes) | 1 / 1 | 100 | 160 | 80 |
| | *Streptopelia orientalis* (Columbiformes) | 1 / 1 | 100 | 1280 | <40 |
| | Five other species | 0 / 23 | 0 | ≤80 | NT† |
| Anyui River/2005 | *Histrionicus histrionicus* (Anseriformes) | 3 / 13 | 23.1 | 160-320 | 40 |
| | Four other species | 0 / 11 | 0 | ≤80 | NT |
| Khanka Lake/2006 | *Anas poecilorhyncha* (Anseriformes) | 1 / 2 | 50.0 | 160 | 40 |
| | *Mergus serrator* (Anseriformes) | 1 / 8 | 12.5 | 160 | <40 |
| | *Sterna hirundo* (Charadriiformes) | 2 / 13 | 15.4 | 160, 320 | 40, 320 |
| | *Columba livia* (Columbiformes) | 1 / 1 | 100 | 320 | 80 |
| | *Streptopelia orientalis* (Columbiformes) | 4 / 9 | 44.4 | 1280-2560 | 80 |
| | Three other species | 0 / 8 | 0 | ≤80 | NT |
| Chor River/2006 | *Anas poecilorhyncha* (Anseriformes) | 2 / 9 | 22.2 | 160 | 40, 80 |
| | *Mergus serrator* (Anseriformes) | 2 / 22 | 9.1 | 160, 640 | 40, 80 |
| | *Phalacrocorax carbo* (Pelecaniformes) | 2 / 9 | 22.2 | 160 | 40 |
| | Twelve other species | 0 / 14 | 0 | ≤80 | NT |
| | Total | 21/ 145 | 14.5 | ≤80-2560 | <40-320 |

*$FRNT_{80}$, 80% focus reduction neutralization test; WNV, West Nile virus; JEV, Japanese encephalitis virus.
†NT, Not tested.

neutralizing antibody titers (≥ 1:1280) were detected in four Eastern Turtle Doves (*Streptopelia orientalis*). Because JE virus which is endemic to East Asia and WN virus often show serological cross-reactivity[30,35], the sero-positive samples to WN virus were also tested for neutralizing antibodies to JE virus. Most of the sera showed significantly higher titers of neutralizing antibodies to the WN virus (≥4 fold) than to the 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, WN virus has rapidly expanded its geographic distribution to various parts of the world.[14] 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.[30,35] In this study, the author evaluated a NT for effective differential sero-diagnosis of JE and WN virus infection in birds. Furthermore, analysis of blood samples from wild birds using this NT 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.[28,31,43,52] Although wild birds are known to be a natural host of JE virus, similar to WN virus, few instances of JE virus infection in birds have been reported.[19,36] The author evaluated the effectiveness of a NT in chicks inoculated with JE and WN viruses. First, the author measured the viremia titers in the chicks to ensure that infection had taken place. Viremia was measurable in all chicks that had been inoculated with JE virus or WN virus, and the maximum viremia titer reached $10^4$ PFU/mL. These results suggested that the young chicks infected with JE virus or WN virus were an effective infection animal model for both viruses.

Next, the author 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. It is known that adult Galliformes have low susceptibility to WN virus, and viremia titers in these birds are reported to be lower than those of the young birds.[39,55] Because 3-week-old chicks were older, the antibody response to JE virus infection in these birds was lower than 2-day-old chicks,

but in this study, antibody titers sufficient for evaluation of the NT were obtained.

In order to investigate 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 homologous and heterologous virus were observed after challenge inoculation. However, it was difficult to judge which virus was infected first or how many times the chicks were exposed to the viruses based on the NT. These results agree with a previously published report of combined infection with WN virus and St. Louis encephalitis virus[12,40,58], in which differential diagnosis of the closely related viruses was shown to be very difficult.

The author investigated the seroprevalence of WN virus among wild birds in the Far Eastern region of Russia using the NT, $FRNT_{80}$. The neutralizing antibody to the WN virus was identified in 21 serum samples taken from 145 wild birds (14.5%) using $FRNT_{80}$. The orders of the birds that were positive for antibodies to WN virus are as follows: Anseriformes, Charadriiformes, and Columbiformes. 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.[28] The JE virus is endemic to East Asia, and is closely related to the WN virus. These viruses often show antigenic cross-reactivity in serological tests.[30,35] 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 antibodies against JE virus. These data indicate that the positive results of the $FRNT_{80}$ for WN virus were due to antibodies specific to WN virus infection and not to cross-reactivity with antibodies produced by JE virus-infection.

All of the tested Rock Doves (*Columba livia*) and some Eastern Turtle Doves (*Streptopelia orientalis*), which are sedentary birds, had WN virus antibodies and were probably infected with the virus near Khanka Lake. Because Khanka Lake lies far to east of where WN was

first isolated in Russia, WN virus appears to have been transmitted among wild birds in Far Eastern Russia. Because the other WN virus-positive birds in the study, Spotbills (*Anas poecilorhyncha*), Harlequin Ducks (*Histrionicus histrionicus*), Red-breasted Mergansers (*Mergus serrator*), Black-headed Gulls (*Larus ridibundus*), and Common Terns (*Sterna hirundo*), are migratory birds, it is possible that these birds were infected with WN virus in Far Eastern Russia and carried the virus into other region of East Asia.

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. In order to prepare for the introduction of WN virus to East Asia, development of a diagnostic test that can accurately differentiate between WN and JE virus infection is needed. In addition, continued epidemiological evaluation of WN virus infection among birds and humans in Far Eastern Russia and East Asia is important for monitoring the spread of the disease.

**Summary**

West Nile (WN) virus has been expanding geographically to non-endemic areas in various parts of the world. However, little information about the extent of WN virus infection in Russia is available. Japanese encephalitis (JE) virus, which is closely related to WN virus, is prevalent throughout East Asia. The author evaluated the effectiveness of a neutralization test (NT) in young chicks inoculated with JE and WN viruses, and performed a seroepidemiological survey of WN virus infection among wild birds in Far Eastern Russia using the NT. Following single virus infection, only neutralizing antibodies specific to the homologous virus were detected in the chicks. The NT was then used to analyze serum samples from 145 wild birds for WN and JE virus. Of these, 21 were positive for neutralizing antibodies to WN virus and showed neutralizing antibody titers specific to WN virus. These results suggest that WN virus is prevalent among wild birds in the Far Eastern region of Russia.

## Conclusion

In this thesis, the effect of the glycosylation of E protein of Wes Nile (WN) virus New York (NY) strain on the virus multiplication was examined and antibody survey of WN virus was performed among wild birds in Far Eastern Russia.

Many of the WN virus isolates associated with significant human outbreaks, including the recent North American epidemic, possess the glycosylation site on the E protein. In experimental infection in mice, the glycosylated variants caused higher mortality than the non-glycosylated variants, which suggests that E protein glycosylation is a molecular determinant of neuroinvasiveness of the NY strain of WN virus. In the chapter I, the author examined the effect of E protein glycosylation on the interaction between WN virus and avian hosts and mosquito vectors. Using a young chick infection model, the author examined whether the glycosylated (LP) and non-glycosylated (SP) variants exhibit differences in virulence and viremic level in the birds. The survival rate of chicks inoculated with LP variant was 0-20%. And pathology in these chicks included severe necrosis in the liver and heart. In contrast, the LP variant exhibited low virulence in young chicks. The viremia titers of the LP variant in chicks were ten or more times greater than those of the SP variant. However, the glycosylation status of the variants did not affect viral multiplication and dissemination in *Culex* mosquitoes. The author then examined the multiplication characteristics of the variants *in vitro* tissue culture cells of mammalian, avian and mosquito origin to establish how E protein glycosylation affects WN virus multiplication in these different cell types. LP variants showed more heat stable propagation than SP variants in mammalian (BHK) and avian (QT6) cells, but not in mosquito (C6/36) cells. These results suggested that high viremic titer in avian host was related with glycosylation of E protein. Viremic titer in avian host is known to be crucial for the WN virus transmissibility to the mosquitoes. Therefore, N-linked glycosylation of E protein may be essential for an efficient

transmission of WN virus NY strain in nature.

In areas where Japanese encephalitis (JE) virus is endemic, discrimination between WN and JE viruses is critical for the detection of WN virus invasion. However, the JE serocomplex flaviviruses are antigenically cross reactive and are thus not readily differentiated by serological methods. In the chapter II, the author evaluated neutralization test (NT) in young chicks inoculated with JE and WN viruses. After the single virus infection, only the specific neutralizing antibody to the homologous virus was detected in chicks. In order to investigate 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 homologous and heterologous virus were observed after challenge inoculation. However, it was difficult to judge which virus infected first or how many times the chicks were exposed to the viruses based on the NT. The information about the extent of WN virus infection in Russia still remains very limited. Therefore, a seroepidemiological survey of WN virus infection was performed among wild birds in Far Eastern Russia using NT. One hundred forty five wild birds were captured in Far Eastern Russia and serum samples were examined for WN and JE viruses by the NT. Twenty one out of 145 sera showed positive neutralizing antibodies to WN virus and most of these showed specific neutralizing antibody titers to WN virus. These results suggest that WN virus is prevalent among wild birds in Far Eastern region of Russia.

**Acknowledgements**

I wish to express my sincere gratitude to my chief supervisor, Prof. Ikuo Takashima, Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University, for his support and encouragement throughout the experimental work and the preparation of this thesis.

I also appreciate the kind guidance and advice of the other supervisors, Prof. Kazuhiko Ohashi, Laboratory of Infectious Diseases, Department of Diseases Control and Associate Prof. Akihiko Maeda, Department of Prion Disease and Associate Prof. Hiroaki Kariwa, Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University.

I express special thanks to Dr. Kentaro Yoshii (Instructor), Mr. Kazuaki Hashiguchi and all the members of Laboratory of Public Health, Department of Environmental Veterinary Sciences, Graduate School of Veterinary Medicine, Hokkaido University.

Finally my sincere thanks are go to my “good brothers”, Nur Hardy bin Abu Daud and Daisuke Miyashita, and my 12 classmates of the doctoral course for their constant supports physically and mentally. The greatest treasure will be friendship with them among many other things achieved in the graduate school.

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## 和文要旨

# ウエストナイルウイルスの自然感染環と鳥類における抗体調査に関する研究

ウエストナイルウイルス（WNV）は蚊によって媒介される人獣共通感染症の原因ウイルスである。自然界では野鳥と蚊の間でウイルスの感染環が維持されている。ヒトやウマは髄膜炎や脳炎を発症し、重篤な症例では死に至る。

1999 年、ニューヨーク（NY）市で北米では初めて WNV が確認され、その後わずか数年でアメリカ合衆国全域に流行が拡大した。WNV は 1990 年代前半まで病原性の低いウイルスであると考えられてきたが、近年北米で流行している株はヒトやウマだけでなく自然宿主である鳥類に対しても強い病原性を示す。ヒト用の効果的なワクチンや治療法は未だ開発されておらず、WNV の生物学的・生態学的特性を明らかにすることが公衆衛生上重要である。現在 WNV の分布域は北米大陸だけでなく、南米大陸およびロシアにおいても拡大しており、ウイルスが渡り鳥や物流を介して日本や東アジア諸国に侵入する危険がある。日本国内での WNV の流行はまだ報告されていないが、日本には WNV を媒介可能な蚊と増幅動物となる鳥類が多く生息し、また WNV に近縁で血清学的に交差反応を示す日本脳炎ウイルス（JEV）が常在している。JEV は豚だけでなく野鳥も宿主とすることから、日本や東アジアに WNV が侵入した場合、両ウイルスが野鳥に重感染する可能性がある。鳥類における両ウイルスの感染を鑑別可能な診断法の確立も急務である。これらの背景から以下の研究を行った。

第一章では、WNV のエンベロープ（E）蛋白質上への糖鎖付加がウイルスの増殖に与える影響を調べた。多くの病原性の弱い WNV 株は E 蛋白質上に N 型糖鎖付加部位を欠損しており、WNV NY 株を含む近年の病原性の強いウイルス株は糖鎖付加部位を持つ。このことから、WNV の強毒化には糖鎖付加が関連している可能性がある。以前の研究で、WNV NY 株から E 蛋白質上に N 型糖鎖付加部位を持つ LP 株と糖鎖付加部位を持たない SP 株を単離した。LP 株は SP 株に比べてマウス末梢での増殖性が高く、神経侵襲性毒力も強いことが判明している。本研究では、WNV の E 蛋白質上への糖鎖付加がウイルスの増殖および伝播に与える影響を明らかにすることを目的とした。

自然宿主内での LP 株と SP 株の増殖性や病原性を調べるために、鶏雛およびアカイエカを用いて感染実験を行った。LP 株を接種した鶏雛ではほぼ全ての個体が死亡したが、SP 株接種群では半数以上が生き残った。また、LP 株を接種した個体にのみ重度の壊死性心筋炎が観察されたことから、LP 株は鶏雛に対して SP 株に比べて高い病原性を有することが判明した。鶏雛血清中のウイルス量を経時的に測定したところ、接種後 1～7 日目まで、LP 株は SP 株に比べて常に 10 倍以上高いウイルス血症を示した。一方でアカイエカに LP 株と SP 株を胸腔内接種または吸血感染させたところ、両株の間に増殖性の差は認められなかった。

次に、各ウイルス株の増殖性を調べるために、WNV の宿主となる哺乳類、鳥類および蚊に由来する培養細胞を用いて経時的なウイルスの増殖性を調べた。哺乳類由来細胞（BHK）および鳥類由来細胞（QT6）において、高温培養条件下では LP 株が SP 株より 10 倍以上高い増殖性を示した。しかし蚊由来細胞（C6/36）においては両株の増殖性に差は見られなか

った。

これらの結果から、WNV の E 蛋白質上糖鎖付加はウイルスの増殖性、特に鳥類宿主における高いウイルス血症に関与していることが示唆された。鳥類内でのウイルス血症が高ければ、蚊は高率に WNV に感染するため、この糖鎖付加が自然界における効率的な WNV 感染環成立に寄与しているのではないかと考えられた。

第二章では、ウエストナイルウイルスの極東ロシアの野鳥における抗体調査を行った。アメリカ大陸だけでなくロシアでも WNV は検出されており、近年その分布域が拡大している。極東ロシアでの WNV 流行状況は良く調べられていないが、もしこれらの地域にも WNV が分布しているならば、近接する東アジア諸国へとウイルスが侵入してくる危険性がある。日本を含む東アジアには WNV に近縁で同じ日本脳炎ウイルス血清型群に属する JEV が常在している。両ウイルスは抗原的に交差反応性を示すため、血清診断による鑑別が難しい。本研究では、信頼性の高い血清診断法である中和試験を用いて両ウイルスの交差反応性を評価した。また、日本に近接する極東ロシアにおいて野鳥の疫学調査を行い、中和試験による血清中の抗 WNV 抗体の検出を試みた。

中和試験の特異性を検討するため、2 日齢の鶏雛に JEV もしくは WNV を皮下接種し、一部の個体には 3 週間後に他方のウイルスを重感染させた。JEV または WNV を単独感染させた鶏雛血清についてフォーカス減少法による中和試験を実施したところ、それぞれのウイルスに対する中和抗体を特異的に検出することができた。また JEV と WNV を重感染させた鶏雛では、どちらのウイルスを先に接種しても両ウイルスに対する中和抗体が検出されることが判った。

次に、極東ロシアにおける WNV の浸淫状況を把握するため、野鳥における血清疫学調査を行った。野鳥が多く生息し、渡り鳥の中継地となるハンカ湖やアニュイ川、ホル川で 2005 年 8 月と 2006 年 8 月に合計 152 羽の野鳥を捕獲した。回収した野鳥の腎臓から RNA を抽出し、Real-Time PCR 法によって WNV 遺伝子の検出を試みたが全て陰性であった。一方、中和試験を用いて野鳥血清中の抗体測定を行ったところ、145 検体中 21 検体（14.5%）で WNV に対する中和抗体が検出された。WNV に対する抗体が検出された鳥類種はカモ目やチドリ目、ハト目に属し、WNV 感染によって高いウイルス血症を生じることが知られているものであった。WNV 抗体陽性検体については JEV に対する中和試験も実施したが、ほとんどの検体で JEV に対する中和抗体価よりも WNV 中和抗体価が 4 倍以上高く、この中和試験の結果は JEV に対する交差反応によるものではないことが判った。これら WNV 抗体陽性の野鳥には、留鳥であるドバトやキジバトが含まれ、極東ロシアの野鳥間で WNV が流行していることが示唆された。また、その他の野鳥は全て渡り鳥であるため、日本や東アジア諸国への WNV 侵入の危険性が高まっていると思われる。これらの結果から、今後もロシアやロシアに隣接する地域における疫学調査を継続していくことが重要であると考えられた。