Studies on the viral proliferation and pathogenicity of flaviviruses isolated from mosquitoes and wild animals in Zambia

（ザンビアの蚊および野生動物から単離された
フラビウイルスの増殖機構と病原性解析）

Hiroko Kobayashi

CONTENTS

ABBREVIATIONS

ATCC: American Type Culture Collection

bp: base pairs

BSA: bovine serum albumin

BW: body weight

C: core

COVID-19: Coronavirus disease 2019

Croc110: strain Croc110/2019/1/ZM

CPE: cytopathic effect

DMEM: Dulbecco's modified eagle medium

dpi: days post infection

E: envelope

EDTA: ethylenediaminetetraacetic acid

FAM: fluorescein

FBS: fetal bovine serum

hpi: hours post infection

IC: intracerebral

ID: intradermal

IP: intraperitoneal

JCRB: Japanese Collection of Research Biosources

MEM: minimum essential media

MGB: minor groove binder

MOI: multiplicity of infection

NGS: next generation sequencing

NS: non-structural protein

PBS: phosphate-buffered saline

PCR: polymerase chain reaction

PFU: plaque forming units

prM: precursor membrane

RT-PCR: reverse transcription PCR

RT-qPCR: reverse transcription quantitative PCR

SARS−CoV-2: severe acute respiratory syndrome coronavirus 2

SC: subcutaneous

$TCID_{50}$: 50% Tissue Culture Infectious Dose

UTR: untranslated region

Vero cells: Vero 9013 cells

WNV: West Nile virus

Zmq16: strain Zmq16m11

NOTES

Chapter 1 and 2

Kobayashi H, Chambaro H, Tabata K, Ariizumi T, Phongphaew W, Ndashe K, Ndebe J, Fandamu P, Kobayashi S, Ito N, Sasaki M, Hang'ombe BM, Simulundu E, Orba Y, Sawa H. African lineage 1a West Nile virus isolated from crocodiles exhibits low neuroinvasiveness in mice. J Gen Virol, 105 (11), 002051, 2024.

GENERAL INTRODUCTION

In recent years, coronavirus disease 2019 (COVID-19) pandemic, caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has threatened human society, and the society has experienced the fear of infectious diseases transmitted by animals (1). Most of the infectious diseases for human are derived from animals, and it is estimated that around 60% of these pathogens for human are zoonotic in nature (2, 3). Countermeasure against the zoonosis is complicated, because comprehensive approach is necessary targeting for human, domestic and wild animals, atmosphere, climate and so on (4). Vector-borne diseases account more than one-sixth of infectious diseases, and mosquitoes are considered as most dangerous creature in the world, because they transmit many pathogens, such as protozoa and viruses (5, 6). Orthoflaviviruses are the well-known viruses transmitted by mosquitoes. In addition to humans and mosquitoes, orthoflaviviruses can infect to many animal species such as birds and reptiles, and global warming affects on prevalence of orthoflaviviruses infection (7). The representative mosquito-borne orthoflaviviruses are dengue virus, Zika virus, Japanese encephalitis virus, West Nile virus (WNV) and yellow fever virus (8). Especially, it is estimated that 100 to 400 million infection cases were caused by dengue virus each year (9). However, the treatment of orthoflaviviruses is lacking and patients are treated symptomatically (10).

WNV belonging to orthoflaviviruses was first discovered in 1937 in Uganda and now spreads out around the world. WNV outbreaks in human and animals have been reported in all continents except Antarctica (11). The emergence of WNV disease was noted in 1999 when a WNV strain entered North America and caused an outbreak in New York City. During the epidemic, 59 hospitalized and 7 fetal cases due to WNV meningoencephalitis were reported (12). The transmission cycle of WNV consists of mosquitoes, birds and other animals, including humans and horses which are dead-end hosts. Several studies of simulation models of climate change and vector distribution have predicted the spread of WNV infection. One study suggests that the risk of WNV can increase approximately 18-fold in North America in 2050 (13). In Europe, its risk is thought to increase maximum 5-fold from 2040 to 2060 (14). Given the recent situation

and the potential of future spread, epidemiological surveillance and characterization of WNV are important for control of public health.

CHAPTER 1

In vitro characterization of WNV strain isolated from crocodiles in Zambia

Introduction

WNV is a single-stranded RNA virus belonging to the genus *Orthoflavivirus* within the family *Flaviviridae.* The WNV genome is approximately 11 kb and contains three structural proteins [core (C), precursor membrane (prM) and envelope (E)], seven non-structural proteins [non-structural protein (NS) 1, 2A, 2B, 3, 4A, 4B, and 5] and two untranslated regions (UTRs) at the 3′- and 5′- end of the genome (15). This virus circulates between mosquitoes, especially *Culex* species, and wild birds in the field, and it can be transmitted to humans and animals through mosquito bites (11, 12). In humans, approximately 80% of infected individuals are asymptomatic, whereas the rest can present with fever, headache, fatigue, and gastrointestinal signs. In severe cases, WNV infection causes neuroinvasive diseases, such as encephalitis and meningitis (16-18). Birds are considered as the amplifier and WNV transmitter is mosquitoes (19). *Culex* mosquitoes represented by *Culex pipiens* is well known to be the important vector of WNV. However, it has been reported that the efficient transmission has been experimentally demonstrated in various mosquito species in addition to *Culex spp.* (20). The range of WNV infection target is very wide, including humans, houses, birds, crocodilians and other wild animals.

WNV infection has been previously reported in farmed crocodilians (i.e., *Crocodylus niloticus*, *Crocodylus moreletii*, *Alligator mississippiensis*) (21-25). Crocodiles infected with WNV can develop neurological disorders such as swimming in circles and lymphohistiocytic proliferative cutaneous lesions, called “pix,” leading to significantly worse commercial skin quality (25, 26). Previous reports suggested that WNV can be transmitted to crocodiles through mosquito bites, the consumption of infected birds, and horizontal transmission through water in the habitat (27, 28).

Although no human clinical case of West Nile fever has been reported in Zambia, previous studies revealed the circulation of two different WNV lineages in Zambia (21,

29). The lineage 2 WNV strain Zmq16m11 (Zmq16) was isolated from *Culex* mosquitoes captured in the Western Province (29). On the contrary, a lineage 1a WNV was detected by reverse transcription-polymerase chain reaction (RT-PCR) in whole-blood samples from farmed crocodiles (*Crocodylus niloticus)* in the Southern Province in Zambia. Its protein cording sequence was reported as Croc110/2019/ZM; however, its whole genome sequence was not determined yet (21).

In this study, a crocodile-derived WNV strain in Zambia was successfully isolated, and its whole genome sequence was determined. Additionally, its *in vitro* growth kinetics was investigated in comparison with two prototype strains, a highly pathogenic (NY99) and a low pathogenic (Eg101) strain (30-32).

Materials & Methods

Ethical statement

The whole blood samples from the occipital sinus of the spinal vein in crocodiles were collected, related to the previous study, which was approved by the Department of National Parks and Wildlife (Act no. 14; 2015) and Crocodile Producers Consortium, Zimbabwe (21). WNVs were used following the Legislation on Handling of Pathogens and Other Hazardous Agents in Hokkaido University (Act no. 10 (1))

Cells and viruses

Unless otherwise stated, cells were cultured in medium supplemented with 10% fetal bovine serum (FBS) and an antibiotic mixture (penicillin, 100 unit/mL; streptomycin, 100 µg/mL). Mosquito-derived C6/36 cells were cultured in Eagle's Minimum Essential Medium (MEM, Wako, Osaka, Japan) supplemented with non-essential amino acids solution (Thermo Fisher Scientific, Waltham, MA, USA). Vero 9013 cells (Japanese Collection of Research Bioresources Cell Bank (JCRB cell bank), Ibaraki, Japan), SH-SY5Y human neuroblastoma cells (health protection agency culture collections, UK) and NA mouse neuroblastoma cells (kindly provided from Dr. Ito in Gifu university) were

cultured in Dulbecco's Modified Eagle's Medium (DMEM, Wako), DMEM F12 (Thermo Fisher Scientific) and MEM, respectively. MEM containing 2% FBS and an antibiotic mixture was used during viral infection. C6/36 cells (American Type Culture Collection (ATCC), Manassas, VA, USA) were incubated at 28℃, and the other cell lines were incubated at 37℃, both in the presence of 5% CO_2. The NY99 (accession no. AB185914), Eg101 (accession no. AF260968), Zmq16 (accession no. LC318700), and Croc110 (Croc110/2019/1/ZM, accession no. LC817237) were propagated in C6/36 cells at 28℃.

Virus titration

Virus titration was assessed in Vero cells using the plaque assay. Briefly, Vero cells were cultured in six-well plates and infected with 10-fold serially diluted WNV. After 1 h of incubation with agitation every 10 min, the supernatant was removed, and cells were overlaid with MEM supplemented with 2% FBS, 1% methylcellulose, and 1% antibiotic mixture and incubated at 37℃. At 4 days post-infection (dpi), Vero cells were formalin-fixed and stained with 1% crystal violet. The plaque number was expressed as plaque-forming units (PFU) and finally used to calculate the viral multiplicity of infection (MOI). MOI was defined as 1 PFU/cell in this study.

Virus isolation

The Croc110 strain, previously reported by Simulundu *et al.*, was isolated from crocodile blood as previously described with slight modifications (21, 33). Blood (n = 22) was collected in ethylenediaminetetraacetic acid (EDTA) tubes from juvenile crocodiles *via* the post-occipital sinus of the spinal vein. RNA was extracted using the QIAamp viral RNA mini Kit (QIAGEN, Venlo, Netherlands) according to the manufacturer's instructions. WNV RNA was screened using an in-house primer pair and probe (probe, 5′- fluorescein (FAM)-GATCTCGATGTCTAAGAAACCAGGAGG-3′- minor groove binder (MGB)-Eclipse; F, 5′-GCGAGCTGTTTCTTRGCAC-3′; R, 5′-AGRCTCARCATWGCCCTCTT-3′) in the ABI Step One real-time PCR-system (ABI, Warrington, UK) using the Thunderbird probe one-step qRT-PCR kit (TOYOBO, Osaka, Japan). Four samples were used to identify the sequence which Ct value was lower than

37 on reverse transcription-quantitative polymerase chain reaction (RT-qPCR) for each sample. The partial sequences of the structural protein-coding region were identified by conventional PCR and Sangar sequencing with specific primers (21), and a 100% match was observed in all samples. Each blood sample was diluted 100-fold in MEM supplemented with MEM non-essential amino acids solution (Thermo Fisher Scientific) and 2% FBS and filtrated twice. The filtrate was serially diluted (1:100–1:10,000) and inoculated onto a C6/36 cell monolayer under biosafety level 3 containment. The cytopathic effects (CPEs) of cells inoculated with filtrates were examined each day for 7 days. One of four samples was used as the Croc110 isolate.

Electron microscopic examination of the Croc110 isolate

The supernatant from C6/36 cells inoculated with Croc110 (MOI of 0.01) for 5 days was collected and fixed with paraformaldehyde (final concentration of 2.5%) overnight at 4℃. After fixation, the supernatant was centrifuged to remove the cellular debris, filtrated using 0.22-μm filters, and then ultracentrifuged at 110,000 × *g* on a 20% sucrose cushion at 4℃ for 3 h. The precipitant after ultracentrifugation was suspended in phosphate-buffered saline (PBS) overnight at 4℃. Dissolved samples (20 μL) were spotted onto elastic carbon grids (Okenshoji Co., Ltd., Tokyo, Japan) for 5 min. Thereafter, the grids were washed with PBS three times. The samples on the grids were stained with 2% phosphotungstic acid (pH 5.8) and observed using a transmission electron microscope (TEM HT7800, HITACHI, Tokyo, Japan).

***In vitro* viral growth assay**

NY99, Eg101, Croc110, and Zmq16 were inoculated into C6/36 cells at MOI of 0.01 and Vero, NA, and SH-SY5Y cells at MOI of 0.001 in 24-well plates for 1 h. Then, the cells were washed with PBS and maintained in medium. The supernatant was collected from each cell line at 1, 2, 3, and 4 dpi. Virus titration in the supernatant was assessed using the 50% Tissue Culture Infectious Dose ($TCID_{50}$) assay with Vero cells. Briefly, WNV-inoculated cell supernatants were serially diluted 10-fold in 96-well plates, and this dilution was repeated four times in each sample. The diluted samples were

inoculated into Vero cells in 96-well plates. The CPEs in each well were monitored for 7 days. The Reed and Muench method was used to calculate the viral titers as $TCID_{50}$/mL (34). Three independent experiments were performed under identical conditions. The mean of three viral titers at each time point is presented in the results.

Immunofluorescent staining and focus-forming assay

Croc110 (MOI of 0.01) was inoculated into C6/36 cells in six-well plates and incubated with agitation at 28℃ for 1 h. After infection, cells were overlaid with MEM supplemented with 2% FBS, 1% methylcellulose, and 1% antibiotic mixture and incubated at 28℃. At 3 dpi, cells were fixed with formalin, permeabilized with 0.2% Triton X-100, and blocked with 1% bovine serum albumin (BSA) in PBS. Cells were stained with an anti-flavivirus NS1 antibody (4G4) (1:500, Mozzy Mabs, kindly provided by Prof. Roy Hall, University of Queensland, Australia) or with an anti-flavivirus E antibody (4G2) (1:50, Mozzy Mabs, kindly provided by Prof. Roy Hall, University of Queensland, Australia) (35, 36) for 1 h, stained with Alexa 488-labeled anti-mouse IgG (1:1,000, Invitrogen, Carlsbad, CA, USA), and counterstained with Hoechst 33342 for 1 h. Fluorescent images were evaluated using an Olympus IX-73 microscope (Olympus, Tokyo, Japan).

To analyze intracellular viral propagation by the focus-forming assay, each WNV strain was inoculated into Vero, NA, and SH-SY5Y cells in six-well plates as previously described. Following incubation period (Vero, 36 h; NA, 72 h; SH-SY5Y, 30 h), cells were fixed in formalin and stained with 4G4 antibody. The stained focus area (n = 50) was measured using Cell Sens software (Evident, Tokyo, Japan).

Whole-genome sequencing and phylogenetic analysis

Viral RNA was extracted from Croc110-infected cell supernatant using the High Pure Viral RNA Kit (Roche, Basel, Switzerland) and reverse-transcribed using the PrimeScript Double Standard cDNA Synthesis Kit (Takara, Shiga, Japan). DNA libraries were prepared using the Nextera XT DNA Library Preparation Kit (Illumina, San Diego, CA, USA) and sequenced using the Illumina iSeq platform (Illumina). Reads were

processed and mapped to a reference strain (accession number: KU588135.1) using the CLC Genomics Workbench (QIAGEN). The mapped reads were extracted and used for *de novo* assembly to determine the full-length Croc110 sequence. Since some sequences in the 3′-UTR could not be determined, Sanger sequencing of the 3′-UTR region was performed using the primers designed on the basis of a lineage 1a strain (accession no. AF404757; F, 5′-GAGGACATGCTGGAGGTTTGGAAC-3′; R, 5′-AGATCCTGTGTTCTCGCACCACC-3′). To confirm the absence of the genomes of other pathogens in the isolated virus sample, unmapped reads were also used for *de novo* assembly and contigs more than 500 nucleotides in length were extracted and analyzed by Multi-BLAST (https://ftp.ncbi.nlm.nih.gov/blast/executables/blast+/LATEST/).

Phylogenetic analysis of the polyprotein amino acids sequences of WNV was performed by the maximum likelihood method. Each sequence used in the phylogenetic analysis was obtained from GenBank and aligned with ClustalW (37). The evolutionary history was inferred by the maximum likelihood method and JTT+G+I model using MEGA11 (38, 39).

Statistical analysis

All statistical analyses were performed using GraphPad Prism 10.1.1. For comparison of three or more groups, one-way ANOVA with Dunnett's test was used. $P < 0.05$ was considered statistically significant.

Repositories

The complete genome sequence of the Croc110/2019/1/ZM (accession no. LC817237) has been deposited in the DDBJ/EMBL/ NCBI GenBank database.

Results

Virus isolation and phylogenetic analysis of Croc110/2019/1/ZM

WNV was isolated from the blood samples of diseased juvenile crocodiles that showed clinical signs, including anorexia, weakness, swimming in circles, bloody diarrhea, and scoliosis (21). Of the 16 samples positive for WNV (16/22), four samples with Ct values lower than 37 were used for virus isolation. CPEs in C6/36 cells were evident at 4 dpi in all inoculated samples. Negative-staining electron microscopy of the cell supernatant identified virus particles of approximately 50 nm in diameter, consistent with orthoflaviviruses (Fig. 1A). In C6/36 cells, as foci positive for flavivirus antigen were observed at 4 dpi, isolated virus showed proliferative potential in mosquito-derived cell (Fig. 1B). Next-generation sequencing (NGS) of total RNA from the supernatant from virus-infected cells and Sanger sequencing of the 3'-UTR were performed to determine the entire genome of WNV. Compared to the previously reported polyprotein sequence of Croc110/2019/ZM (accession number: LC489409) (21), which was obtained by RT-PCR from one of the blood samples used in this study, the genome sequence of our isolated WNV (Croc110/2019/1/ZM, Croc110, accession number: LC817237) had four nucleotide substitutions. Nonetheless, our isolate and Croc110/2019/ZM were 100% identical at the amino acid level. Phylogenetic analysis based on the amino acid sequence of WNV polyproteins illustrated that Croc110 belongs to lineage 1a, and it is most closely related to a WNV strain detected from a camel in United Arab Emirates (accession no. KU588135) (40) (Fig. 1C). Although no whole-genome sequence data have been deposited for other WNV isolates from crocodilians, molecular phylogenetic data suggest that WNV circulating in crocodiles does not form a unique lineage. To further investigate amino acid substitutions characteristic of the Croc110 strain, sequences between Croc110 and representative lineage 1a and lineage 2 WNVs were compared (Table 1). In the coding sequences, 13 amino acid differences were detected in the Croc110 strain compared with the other strains, and notably, four unique substitutions were found in NS4B.

Comparative analysis of the viral growth kinetics in mosquito-derived C6/36 cells

The propagation of Croc110 in mosquito-derived C6/36 cells was compared with that of another Zambian strain isolated from mosquito (Zmq16) and lineage 1a reference strains (NY99 and Eg101). The cellular supernatant was collected every day after

infection, and the viral titer was investigated by $TCID_{50}$ assay using Vero cells (Fig. 2). In C6/36 cells, the viral titer of Croc110 in the supernatant increased to $1 \times 10^{6.5}$ $TCID_{50}$/mL at 2 dpi. Croc110 proliferation was comparable with Zmq16 and NY99 and significantly higher than Eg101 at 2 and 3 dpi. This data indicated that Croc110 propagate well in C6/36 cells similarly to NY99 and Zmq16 strains.

Comparative analysis of the viral growth kinetics in mammalian cells

The propagation of Croc110 in Vero cells, mouse neuroblastoma-derived NA cells, or human neuroblastoma-derived SH-SY5Y cells was compared with that of Zmq16, NY99, and Eg101 (Fig. 3A-C). The cellular supernatant was collected every day after infection, and the viral titer was analyzed by $TCID_{50}$ assay. In Vero cells, the viral titer of Croc110 in the supernatant increased to 1×10^{8} $TCID_{50}$/mL within 2 dpi (Fig. 3A). In NA cells, the viral titer of Croc110 was 1×10^{7} $TCID_{50}$/mL at 3 dpi, indicating slower proliferation than in other cell lines. There was no distinct difference in the titers among four WNV isolates in NA cells (Fig. 3B). In SH-SY5Y cells, the viral titer of Croc110 increased to $1 \times 10^{7.5}$ $TCID_{50}$/mL at 3 dpi and was comparable to that of other strains except at 1 dpi, when the Croc110 titer was significantly higher than that of Eg101 (Fig. 3C). These results clarified that proliferation of Croc110 in mammalian cells was comparable to other WNV strains.

Comparative analysis of the viral propagation in mammalian cells by focus-forming assay

The viral propagation of Croc110 was investigated by focus-forming assay at each time points when the titer of Croc110 in supernatant showed around 1×10^{7} $TCID_{50}$/mL. Croc110 formed significantly smaller foci in Vero and SH-SY5Y cells than other strains in focus-forming assay by NS1 staining (Fig. 4A and 4C). In NA cells, only Eg101 showed a significantly larger focus size than Croc110 (Fig. 4B). Similar results were confirmed by assays using E protein staining, as staining of secreted NS1 may affect focus size. These results indicated that the foci size at the early stage of infection with

Croc110 was lower than that of other strains. The tendency of foci size of WNV strains was varied in cell types.

Discussion

In the present study, a new WNV strain derived from naturally infected crocodiles was successfully isolated, providing valuable insights into the biology of WNV derived from reptilian hosts. Phylogenetic analysis demonstrated that Croc110 belongs to lineage 1a and is closely related to a strain isolated from camel in the United Arab Emirates, thus, this lineage may have a broader geographic and host range (40). Croc110 and Croc110/2019/ZM did not form a cluster genetically close D66 strain (accession no. MN954648), a lineage 1b WNV experimentally adapted to crocodiles (27). Considering that Croc110 was isolated from a whole blood sample from a crocodile, additional information about whole genome sequence on WNV isolated from naturally infected crocodiles is needed to determine whether the WNV infection and amplification in crocodiles is accidental or whether crocodiles are potential reservoirs of WNV.

Viral growth kinetics in mosquito-derived C6/36 cells revealed robust proliferation of Croc110 comparable to other strains. Interestingly, in mammalian cells, Croc110 exhibited smaller focus size compared to other WNV strains at the early stage of infection. Despite comparable viral titers in the cell supernatants, the reduced spread of Croc110 in neighboring cells may imply a lower efficiency in facilitating intercellular transmission in mammalian hosts. Previous studies have shown that amino acid mutation, particularly in E protein, are involved in the intracellular transmission of WNV (41). N-glycosylation at the amino acids 154-156 position (asparagine-tyrosine-serine) in E protein is one of the sites associated with activity of transcellular transport *in vitro* and neurovirulence in mice *in vivo* (42-44). In addition, the amino acid change at 159 position in E protein from valine to isoleucine decreased the WNV replication activity *in vitro* and in mice brain *in vivo* (45). Croc110 has E protein with the amino acid sequence at 154-156 position which may be potentially N-glycosylated but has isoleucine at the 159

position in E protein. As shown in Table 1, 13 unique amino acids were identified in Croc110 that differ from other representative WNV strains, and 10 of the 13 substitutions were observed in NS proteins (NS1, NS3, NS4B, NS5). In the case of Croc110, amino acid substitutions in the E protein or other regions may interact to balance the propagative activity *in vitro*. As these NS proteins have been suggested to be involved in modulating viral replication and host immune evasion (46-48), amino acid substitutions in the NS proteins of Croc110 may affect the growth of Croc110 in mammals. To clarify the *in vivo* pathogenicity of Croc110, further experiments were conducted using a mouse model in Chapter 2.

Summary

WNV is a mosquito-borne orthoflavivirus that causes encephalitis in humans and infects crocodiles, resulting in rashes and neurological signs. In Zambia, two distinct lineages of WNV have been detected in neighboring areas: lineage 2 in mosquitoes and lineage 1a in farmed crocodiles with clinical symptoms. In this study, lineage 1a WNV was successfully isolated from naturally infected farmed crocodiles (Croc110/2019/1/ZM, Croc110). The isolated virus was then analyzed the whole genome sequence and viral proliferation in cells in comparison to a WNV isolate from mosquitoes captured in Zambia (Zmq16) and two reference strains, including a highly pathogenic (NY99) and a low pathogenic (Eg101) strain. Croc110 well propagated in mosquito-derived C6/36 cells and in mammalian cells, including Vero and mammalian neuronal cells. Although viral proliferation was comparable among the strains, Croc110 exhibited small focus in mammalian cells, which suggested that Croc110 has low intracellular transmission efficiency at the early stage of infection. This study provided genomic information and *in vitro* characterization of WNV isolated from naturally infected crocodiles in Zambia.

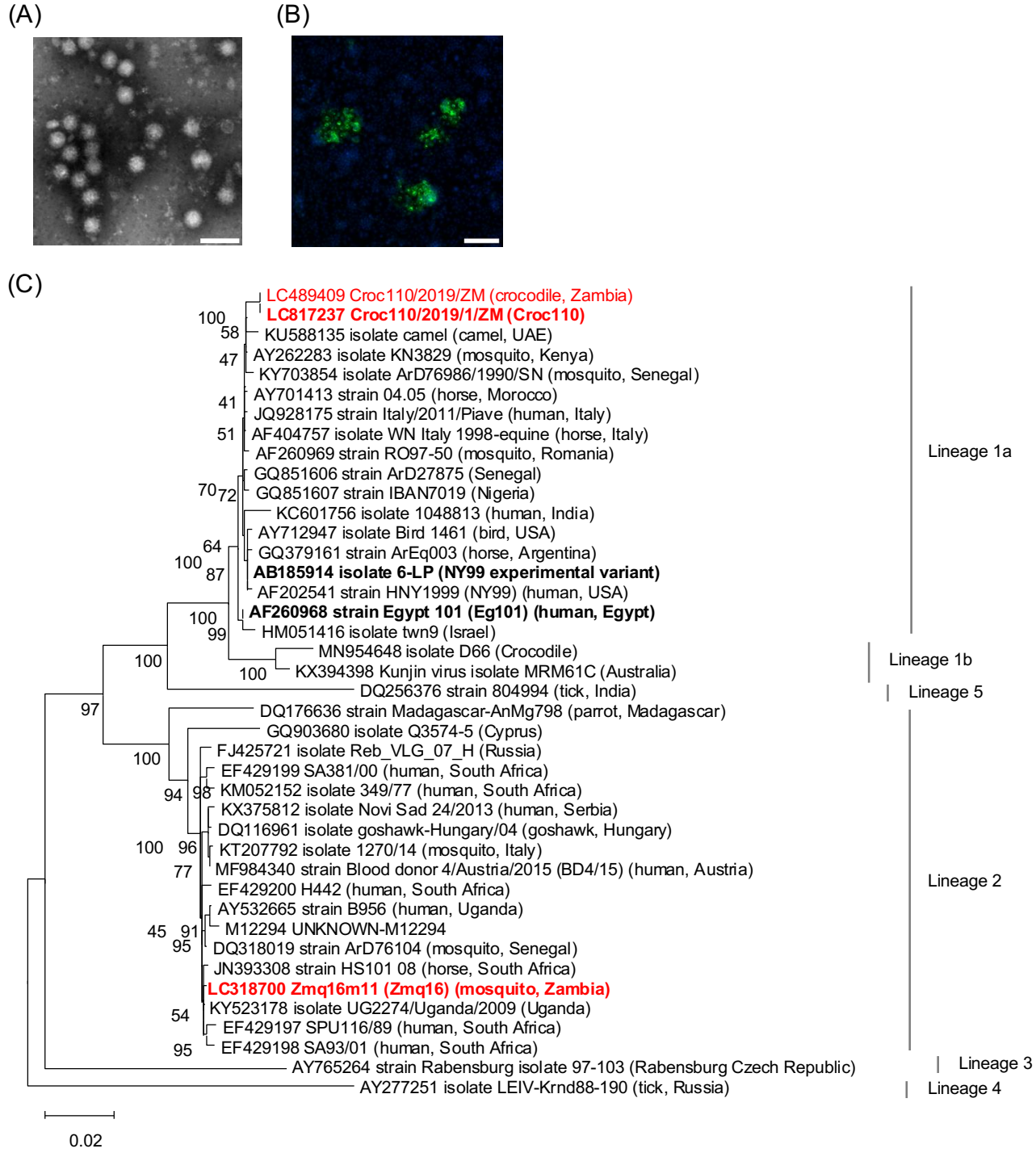

Fig. 1. Characterization of the WNV Croc110 strain isolated from crocodiles

(A) Electron microscopic image of negatively stained Croc110 particles. Scale bar, 100 nm. (B) Focus formation by Croc110 in C6/36 cells. Cells were infected with Croc110 at

an MOI of 0.01 and stained with 4G4 antibody at 4 dpi (green). Cell nuclei were counterstained with Hoechst (blue). Scale bar, 100 μm. (C) Phylogenetic tree of Croc110 and other representative WNV strains. Phylogenetic analysis was performed using the full-length WNV polyprotein amino acid sequences by the maximum likelihood method with 1,000 bootstrap replicates. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The isolated Croc110, Croc110/2019/1/ZM, previously identified Croc110/2019/ZM and Zmq16 are indicated in red text. The WNV strains used in this study are denoted in bold.

Table 1. The amino acid differences characteristic of Croc110 compared to other WNV strains

	Protein	prM	E		NS1	NS3			NS4B				NS5	
	Amino acid position	35	7	44	96	157	334	368	8	31	72	102	42	791
Lineage 1a	Croc110 LC817237	V	G	R	L	A	F	T	R	G	V	S	Y	D
	Camel KU588135	I	S	K	M	P	S	M	K	E	I	C	H	N
	KN3829 AY262283	I	S	K	M	P	S	M	K	E	I	C	H	N
	NY99 AF202541	I	S	K	M	P	S	M	K	E	I	C	H	N
	Eg101 EU081844	I	S	K	M	P	S	M	K	E	I	C	H	N
Lineage 2	SA93/01 EF429198	I	S	K	M	P	S	M	K	S	I	C	H	N
	Zmq16 LC318700	I	S	K	M	P	S	M	K	S	I	C	H	N

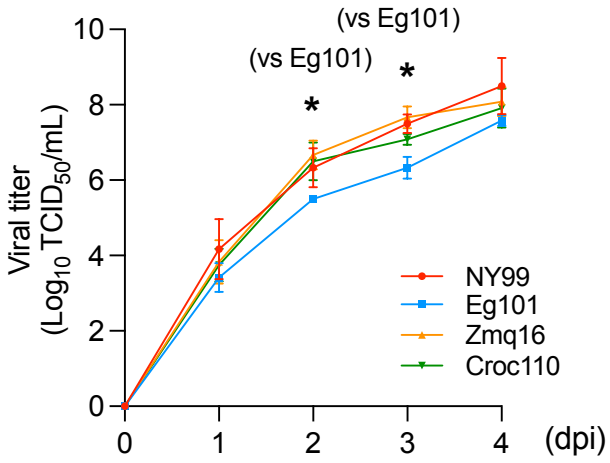

Fig. 2. Comparative analysis of viral growth kinetics in mosquito-derived C6/36 cells

The viral growth kinetics of Croc110 compared with NY99, Eg101 and Zmq16 in C6/36 cells. The viral titer in the supernatant was determined by the $TCID_{50}$ assay using Vero cells. The log-transformed values of viral titer are presented on a $\log_{10}$ scale as the mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA and Dunnett's multiple-comparison test in comparison to the result for Croc110 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

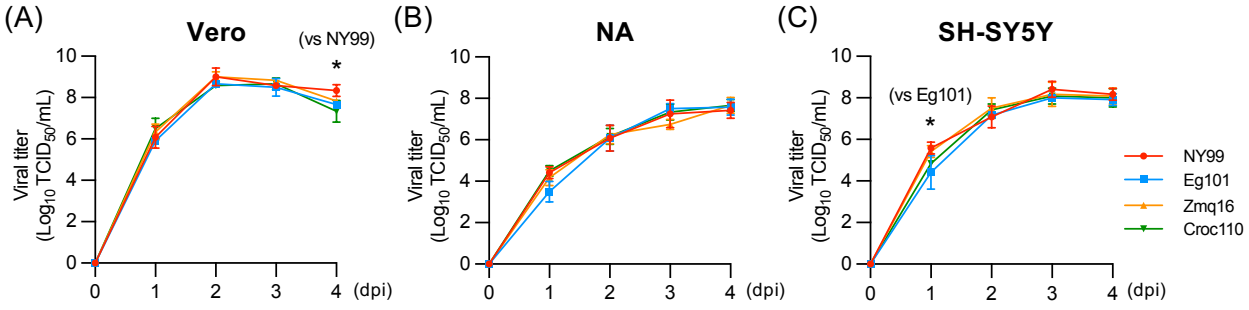

Fig. 3. Comparative analysis of the viral growth kinetics *in vitro*

Viral proliferation of Croc110, Zmq16, NY99, and Eg101 in Vero (A), NA (B), and SH-SY5Y cells (C). The viral titer in the supernatant was determined by the $TCID_{50}$ assay using Vero cells. The log-transformed values of viral titer are presented on a $\log_{10}$ scale as the mean ± SD of three independent experiments. Statistical analysis was performed by one-way ANOVA and Dunnett's multiple-comparison test in comparison to the result for Croc110 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

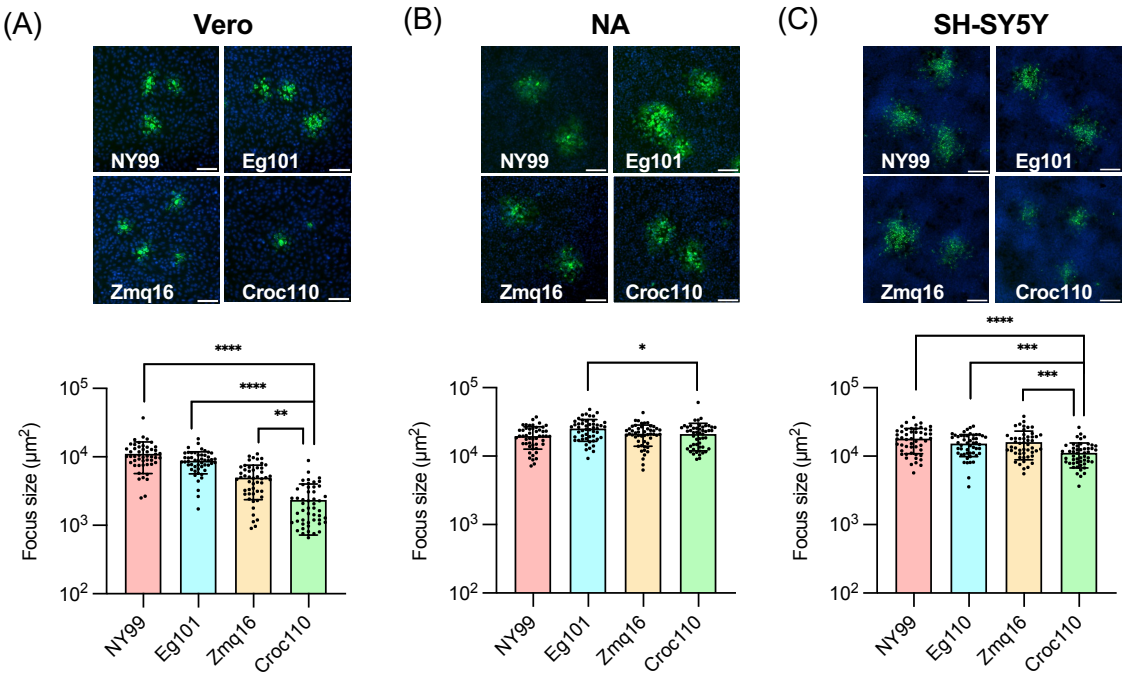

Fig. 4. Comparative analysis of viral proliferation *in vitro* by focus-forming assay

Differences of focus size in Vero (A), NA (B), and SH-SY5Y cells (C). Focus formation was visualized with anti-flavivirus NS1 antibody (green) and nuclear staining (blue) at 36 h post-infection in Vero cells, 3 dpi in NA cells, and 30 h post-infection in SH-SY5Y cells. Representative focus images of each WNV strain are presented. Scale bar, 100 μm. Foci (n = 50) were randomly selected to measure the focus area. The values are presented as the mean ± SD of 50 foci from one experiment. Statistical analysis was performed by one-way ANOVA and Dunnett's multiple-comparison test in comparison to the result for Croc110 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$).

CHAPTER 2

Investigation of pathogenicity of WNVs isolated from crocodiles and mosquitoes in Zambia

Introduction

WNV infection causes many symptoms in humans. Following an incubation period within two weeks, patients experience fever, headache, fatigue, diffuse maculopapular rash and myalgias. Gastrointestinal complaints, including nausea and vomiting, were reported in many cases (50). In severe cases, encephalitis and meningitis may occur, and even patients who have recovered have been observed to develop aftereffects such as depression (51). WNV infection, which is considered an endemic disease in many regions, is well monitored in the USA and Europe, and a lot of previous reports investigated the WNV infection in Africa as well (52-54). In order to take countermeasures against the transmission of WNV infection from wild animals to humans, it is necessary to conduct epidemiological surveys and correctly evaluate the pathogenicity of WNVs found in various wild animals (55).

The pathogenicity of WNV can be assessed in rodent models, and C57BL/6 mouse has been used to understand WNV pathogenicity and immune response toward WNV infection. In addition to C57BL/6, C3H/HeN has been also used as immunocompetent mouse models. The mouse models exhibit lethal neurological signs following Intraperitoneal (IP) or intradermal (ID) WNV infection, providing insight into the characterization of WNV pathogenicity (18, 41, 56, 57). In order to determine the pathogenicity of WNV, various inoculation methods have been used, such as IP, ID, intracerebral (IC) and subcutaneous (SC) inoculation. IC inoculation is useful to examine WNV proliferation in brains (58-60). ID inoculation *via* footpad or auricular skin have been used to mimic transmission by mosquito-bites and has often been used in the research of WNV pathogenicity (61-64). It has been reported that survival rate of WNV infected mice was varied in different inoculation methods. For example, NY99 showed almost 100% lethal rate in IP inoculation under various conditions (65, 66), but the

survival rate was increased under footpad-ID and SC inoculation (32, 67). It remains unclear how the viral inoculation route affects the pathogenicity of WNV.

In this study, the first *in vivo* analysis was conducted using C57BL/6 mice to determine the pathogenicity of Zambian strains (Croc110 and Zmq16) in comparison with NY99 and Eg101. Moreover, to investigate the differences in pathogenicity manifested by different inoculation routes, the clinical manifestations of these WNV strains were examined by IC, IP, and ID inoculation routes.

Materials & Methods

Cells and viruses

Unless otherwise stated, cells were cultured in medium supplemented with 10% FBS and an antibiotic mixture (penicillin, 100 unit/mL; streptomycin, 100 μg/mL). Mosquito-derived C6/36 cells were cultured in MEM (Wako) supplemented with non-essential amino acids solution (Thermo Fisher Scientific). Vero cells were cultured in DMEM (Wako). MEM containing 2% FBS and an antibiotic mixture was used during viral infection. C6/36 cells were incubated at 28℃, and the Vero cells were incubated at 37℃, both in the presence of 5% CO_2. The NY99 (accession no. AB185914), Eg101 (accession no. AF260968), Zmq16m11 (accession no. LC318700), and Croc110 (Croc110/2019/1/ZM, accession no. LC817237) were propagated in C6/36 cells at 28℃.

Virus titration

Virus titration was assessed in Vero cells using the plaque assay. Briefly, Vero cells were cultured in six-well plates and infected with 10-fold serially diluted WNV. After 1 h of incubation with agitation every 10 min, the supernatant was removed, and cells were overlaid with MEM supplemented with 2% FBS, 1% methylcellulose, and 1% antibiotic mixture and incubated at 37℃. At 4 dpi, Vero cells were formalin-fixed and stained with 1% crystal violet. The plaque number was expressed as PFU.

Animal experiments

Animal experiments were approved by the Institutional Animal Care and Use Committee of Hokkaido University (19-0019 and 23-0060) and performed according to the committee's guidelines.

Groups (n = 6) of female C57BL/6J mice (6 weeks old) were IC (10 PFU/20 μL), IP (100 PFU/100 μL), or ID (100 PFU/50 μL) infected with NY99, Eg101, Croc110, or Zmq16 or mock-infected with virus-free medium. The survival rate and body weight (BW) changes of mice were monitored for 13 days after IC inoculation and for 22 days after ID or IP inoculation. Experiments were replicated twice. Clinical signs were monitored every day and evaluated using a clinical score as follows: 0, healthy; 1, 7.5% BW loss versus the maximum BW; 2, unsteady gait; 3, kinetic tremors and severe ataxia; 4, paralysis; and 5, death or humane endpoint euthanasia. The criteria for the humane endpoint were 25% BW loss versus maximum BW and a loss of spontaneous drinking or feeding by considering the severe neurological symptoms in each mouse.

The serum samples of surviving mice inoculated ID or IP were collected for further neutralizing antibody testing. After heat inactivation of serum at 56℃ for 30 min, samples were first diluted 5-fold and then diluted serially 2-fold in medium. Diluted sera were mixed 1:1 with 100 $TCID_{50}$ of each virus strain and incubated at 37℃ for 1 h. Following incubation, mixed samples were inoculated into Vero cells and observed for 7 days. Serum concentrations that inhibited CPEs by 50% were taken as neutralizing antibody titers.

To evaluate viral RNA levels in tissues, WNV-infected mice by ID or IP inoculation were sacrificed *via* humane euthanasia under deep anesthesia. Serum, spleen, brain, and small intestine samples were collected from mice by ID inoculation at 3, 6, and 9 dpi, and brain samples were collected from mice by IC inoculation at 2, 4, and 6 dpi. Half of the brain or intestine and the entire spleen were used and homogenized in PBS (800 μL for brain samples, 500 μL for spleen samples, and 3 mL for small intestine samples). The clarified supernatant (100 μL) and serum samples were used for RNA extraction. Viral RNA levels in whole tissue were determined by RT-qPCR as previously

mentioned, corrected according to the tissue volume, and expressed as PFU in whole tissues using a calibration curve generated using a previously titrated sample.

Statistical analysis

All statistical analyses were performed using GraphPad Prism 10.1.1. Mann-Whitney *U* test was performed to compare viral RNA amounts of Croc110 and each of the other strains. For comparison of three or more groups, one-way ANOVA with Dunnett's test was used. $P < 0.05$ was considered statistically significant.

Results

Differences in the pathogenicity of WNV isolates in C57BL/6 mice

To compare the pathogenicity of Zambian WNV isolates in mice with that of NY99 and Eg101, each WNV was inoculated into C57BL/6 mice by IC, IP, or ID route. The ID inoculation was performed on the skin of the auricle to mimic transmission by mosquito bites (68). As for IC inoculation, the survival rate of mice inoculated with Croc110 was 25%, which was significantly higher than that for mice inoculated with Eg101, and BW loss was observed in all the strain groups (Fig. 5A and B). However, the survival rates of mice inoculated with Croc110 were 91.7% and 100% following ID and IP inoculation, respectively, similar to those observed for Eg101 (100% following ID inoculation and 91.7% following IP inoculation, Fig. 5A). The mortality rate was 100% by IC inoculation of Zmq16, and high mortality rates were observed in mice inoculated by ID or IP with the Zmq16 and NY99 strains (Fig. 5A). Most of the mice inoculated by ID or IP with Croc110 did not show obvious BW loss, and small number of mice recovered from weight loss after 10 dpi (Fig. 5B). Some of the mice that recovered from Croc110 infection showed BW loss of up to about 20%. In addition, the survival rate of mice inoculated by ID with Zmq16 (16.7%) was significantly lower than that of mice inoculated with NY99 (41.7%). These results suggest that the pathogenicity of Croc110

was comparable to that of the low pathogenic strain Eg101, whereas Zmq16 was highly pathogenic following IP inoculation.

Sera from surviving mice inoculated by ID or IP were collected and assayed for neutralizing antibodies to confirm establishment of WNV infection in those mice (Fig. 5C and 5D). For Croc110 infection, the neutralizing antibody titer was 1:160 - 1:320 by ID inoculation and 1:80 - 1:320 by IP inoculation. Neutralizing antibodies were detected in all surviving mice, indicating that infections occurred also in surviving mice.

Differences in the clinical signs of WNV isolates in C57BL/6 mice

Clinical signs such as BW loss, lethargy, unsteady gait, kinetic tremor, severe ataxia, and paralysis were scored on a five-point scale following the previous study (69) (Fig. 6). The severity of clinical signs was consistent with the BW changes. Croc110-infected mice showed the BW loss (score 1) and neurological symptoms (score 2-4) by IC inoculation. However, by ID inoculation, only one mouse showed kinetic tremors and severe ataxia (score 3) and died. In the case of Zmq16 infection, most of infected mice showed neurological symptoms (score 2-4) by all inoculation routes. As Zmq16-infected mice showed neurological symptoms from 7 dpi by ID and IP inoculation, the earliest appearance of neurological signs was found in Zmq16-infected mice by ID and IP inoculations, and NY99-infected mice developed the neurological symptoms after a delay of 2-3 days from the case of Zmq16 infection.

Replication of WNV isolates in sera, spleen, small intestine and brain

From Fig. 5A and Fig. 6, the pathogenicity of Croc110 was almost similar among ID and IP inoculation, and Zmq16-infected mice showed lower survival rate than NY99 by ID inoculation, unlike IP inoculation. Therefore, in order to investigate the propagation of each WNV strain, ID inoculation was applied to C57BL/6 mice. The viral replication in peripheral tissues after ID inoculation was evaluated in sera, and spleen and small intestine at 3, 6 and 9 dpi (Fig. 7A-C). When viral RNA levels were analyzed by RT-qPCR, low amounts of viral genome were detected in the serum and viremia was observed at 3 dpi for all strains, and the RNA amount of Croc110 was significantly lower than that

of NY99 and Zmq16 (Fig. 7A). An increase in viral RNA was observed in the small intestine from 3 to 6 dpi for all strains, indicating productive infection in the intestine following ID inoculation (Fig. 7C). The RNA levels of Croc110 were significantly lower than those of the highly pathogenic strains NY99 and Zmq16 in the spleen at 3, 6 and 9 dpi and small intestine at 6 and 9 dpi (Fig. 7B and 7C). In the spleen at 3 dpi and in small intestine at 6 dpi, Zmq16 infected mice showed higher RNA amount than NY99 infected mice. These data indicated that Croc110 proliferated less efficiently in peripheral tissues compared with high pathogenic WNV strain, NY99 and Zmq16 strain, whereas the proliferation of Zmq16 was similar to or greater than that of NY99.

The viral replication in the brain was further analyzed in each WNV-infected mice at 3, 6 and 9 dpi for ID inoculation (Fig. 7D) and 2, 4 and 6 dpi for IC inoculation (Fig. 7E). In the brain, the viral RNA of Zmq16, NY99, and Eg101 were detectable at 6 dpi by ID inoculation, whereas Croc110 RNA was below the detection limit in the majority of brain samples (Fig. 7D). By contrast, IC inoculation resulted in comparable viral growth in the brain among all examined WNV isolates, suggesting that Croc110 could replicate similarly as NY99 and Zmq16 in the brain (Fig. 7E). Zmq16 proliferation in the brain by IC inoculation was significantly higher than that of NY99 at 4 dpi (Fig. 7E). Thus, it was suggested that Croc110 has a comparable proliferation ability in brain but is less neuroinvasiveness than other strains.

Taken together, these results indicate that the efficiency of virus replication in the periphery before brain entry differs between WNV isolates and that Croc110 is less proliferative than NY99 and Zmq16 in the periphery, and the difference of pathogenicity by ID inoculation might be related to viral proliferation in peripheral tissues.

Discussion

There was no detailed information on WNV isolates derived from naturally infected crocodiles and their pathogenicity in mammals. In this study, Croc110, belonging to lineage 1a of WNVs circulating in Africa, Middle East, and Europe, was isolated from

crocodiles with neurological signs in chapter 1. The results of virus propagation in the brain after IC inoculation indicated that the efficiency of Croc110 replication in neuronal cells was comparable to that of the highly pathogenic strain NY99. However, when mice were peripherally infected with these WNV strains, the initial proliferation of Croc110 in peripheral tissues was lower than that of NY99, which might reduce its neuroinvasiveness and contribute to its low pathogenicity in mice.

On the contrary, mice infected with the lineage 2 WNV Zmq16, which was isolated from *Culex* mosquitoes in Zambia, displayed a comparable phenotype as those infected by the highly neuroinvasive NY99 strain. Zmq16 is genetically related to the South African isolates HS101_08, SPU116/89, and SA93/01, which were originally isolated from horse or human cases, and they have been demonstrated to be highly pathogenic in mice (59, 70). Thus, the pathogenicity results of Zmq16 in mouse are consistent with the highly pathogenic characteristics exhibited by the closely related South African strains.

Previous studies on the pathogenicity of WNV in mice suggested that both lineage 1a and lineage 2 have highly and less neuroinvasive phenotypes and that pathogenicity is related to genetic differences, but not to lineages or geographic distributions (59, 70). Several genetic determinants involved in WNV pathogenicity have been identified in the viral non-coding and protein-coding regions (41). This study showed that Croc110 was less neuroinvasiveness and replicated less in peripheral tissues than other strains. Croc110 had several amino acid substitutions in the polyprotein that have been reported to be associated with low pathogenicity. As discussed in chapter 1, Croc110 has isoleucine at 159 position in E protein, which has been suggested to decrease the WNV replication activity *in vitro* and in mice brain *in vivo* (45). However, as this amino acid substitution was suggested not to affect viral neuroinvasiveness but decrease the viral replication in brain, it might not be related to low-neuroinvasive Croc110 pathogenicity. It is also noted that Croc110 possesses a serine residue at position 102 in NS4B protein (C102S). The C102S substitution in NS4B has been reported to attenuate viral proliferation at 41°C and causes less neuroinvasive phenotypes in NY99-infected mice (71). This C102S substitution in NS4B might be associated with the attenuated

pathogenicity of Croc110 in mice. In addition, the high temperature-sensitive phenotype of the C102S mutant also suggests that Croc110 replicates well in ectothermic crocodiles but not in mice. Further experiments are required to elucidate the factors associated with the differences in pathogenicity between crocodilians and mammals.

The ID inoculation of mice has normally been performed *via* the footpad in WNV infection experiments. In this study, ID inoculation *via* the ear skin was established in WNV infections in mice. The ear skin infection is useful for evaluation of WNV infection dynamics in mice, considering that it can mimic a mosquito-bite and is relatively easy to collect samples from the initial infection site than the inoculation *via* the footpad. The mice infected with WNV *via* the ear skin showed the neurological symptoms. Macroscopic changes were observed in the small intestines, including paralysis of the small intestines presumed from faces retention and intestinal distension or atrophy. In summary, the mammalian reactivity to acute WNV infection can be validly assessed *in vivo* using conventional mice, and ID inoculation *via* the ear skin allows systemic infection.

Summary

Considering the risk of direct WNV transmission from mosquitoes and direct or vector-mediated WNV transmission from crocodiles to mammals, it is necessary to elucidate the pathogenicity of WNV strains. In Chapter 2, the pathogenicity and viral proliferation of two Zambian WNV strains isolated from mosquito (Zmq16) and crocodile (Croc110) were investigated in a mouse model and compared with highly pathogenic (NY99) and low pathogenic (Eg101) strains. In addition to intracerebral (IC) and intraperitoneal (IP) inoculation routes, intradermal (ID) inoculation was used to mimic the mosquito-bites. As a result, more than 70% of mice after IC inoculation with Croc110 displayed neurological signs, and Croc110-infected mice exhibited similarly high mortality rates as NY99- and Zmq16-infected mice. Meanwhile, comparable virus growth was observed among the strains in the brain. However, the virulence of Croc110 was

significantly lower than that of Zmq16 and NY99 following ID and IP inoculation. Consistently, Croc110 displayed lower growth than Zmq16 and NY99 in the brain and peripheral tissues after ID inoculation. These results revealed that the crocodile-derived WNV strain is less neuroinvasive in mice, and it exhibits distinct pathogenicity from the highly pathogenic mosquito-derived WNV strain circulating in Zambia.

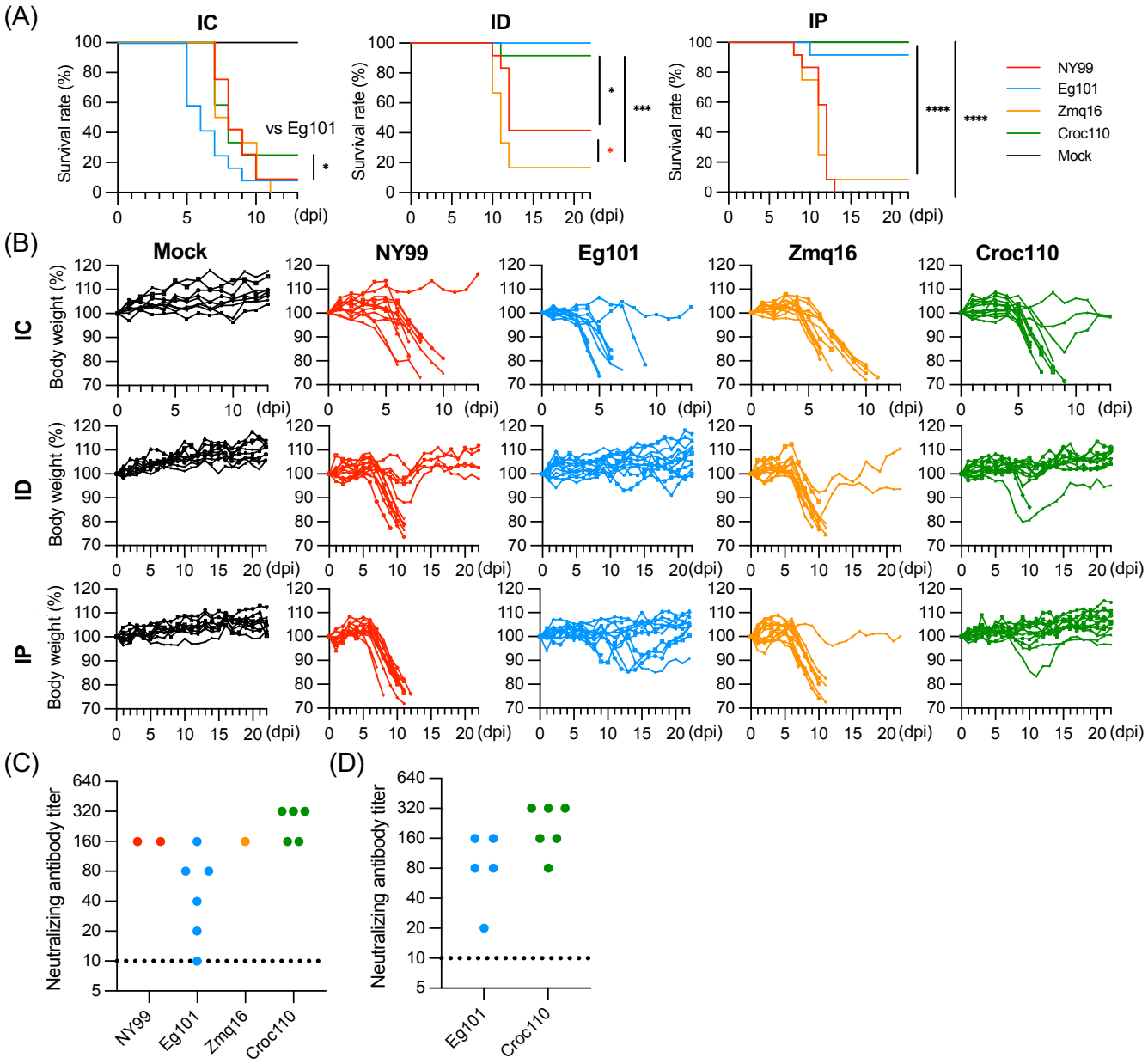

Fig. 5. Differences in the pathogenicity of WNV isolates in C57BL/6 mice

C57BL/6 mice were infected with Croc110, Zmq16, NY99, or Eg101 or mock-infected intracerebrally (IC), intradermally (ID), or intraperitoneally (IP). Survival rates (A) and body weight changes (B) were monitored every day. In total, 12 (for each strain) or 9 mice (mock infection) were used in this study (A, B). BW at 0 dpi was set as 100% (B). Statistical analysis was performed using one-way ANOVA and the log-rank test in

comparison to the result for Croc110 (*, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$). The result of statistical analysis between Zmq16 and NY99 is indicated by red letters. (C, D) Serum samples were collected from the surviving mice in (A) in one experiment. The neutralizing antibody titer was measured using Vero cells, and the dilution that inhibited CPEs by 50% was taken as the titer (C, ID inoculation; D, IP inoculation). Samples with a 10-fold dilution or greater were considered as WNV neutralizing antibody-positive.

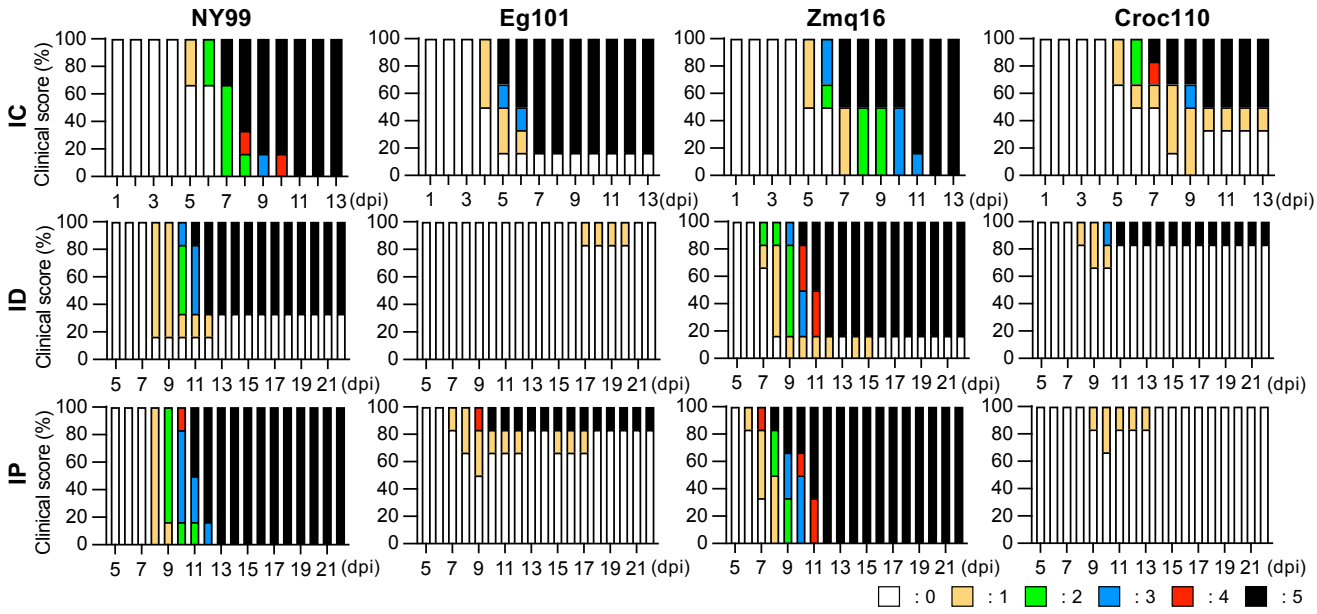

Fig. 6. Differences in the clinical signs of WNV isolates in C57BL/6 mice

C57BL/6 mice were infected with Croc110, Zmq16, NY99, or Eg101 or mock-infected intracerebrally (IC), intradermally (ID), or intraperitoneally (IP), and clinical signs were monitored every day. 6 mice were monitored and scored as follows: 0, healthy; 1, body weight loss; 2, unsteady gait; 3, kinetic tremors and severe ataxia; 4, paralysis; and 5, death. The percentage of each clinical score is presented as a bar graph.

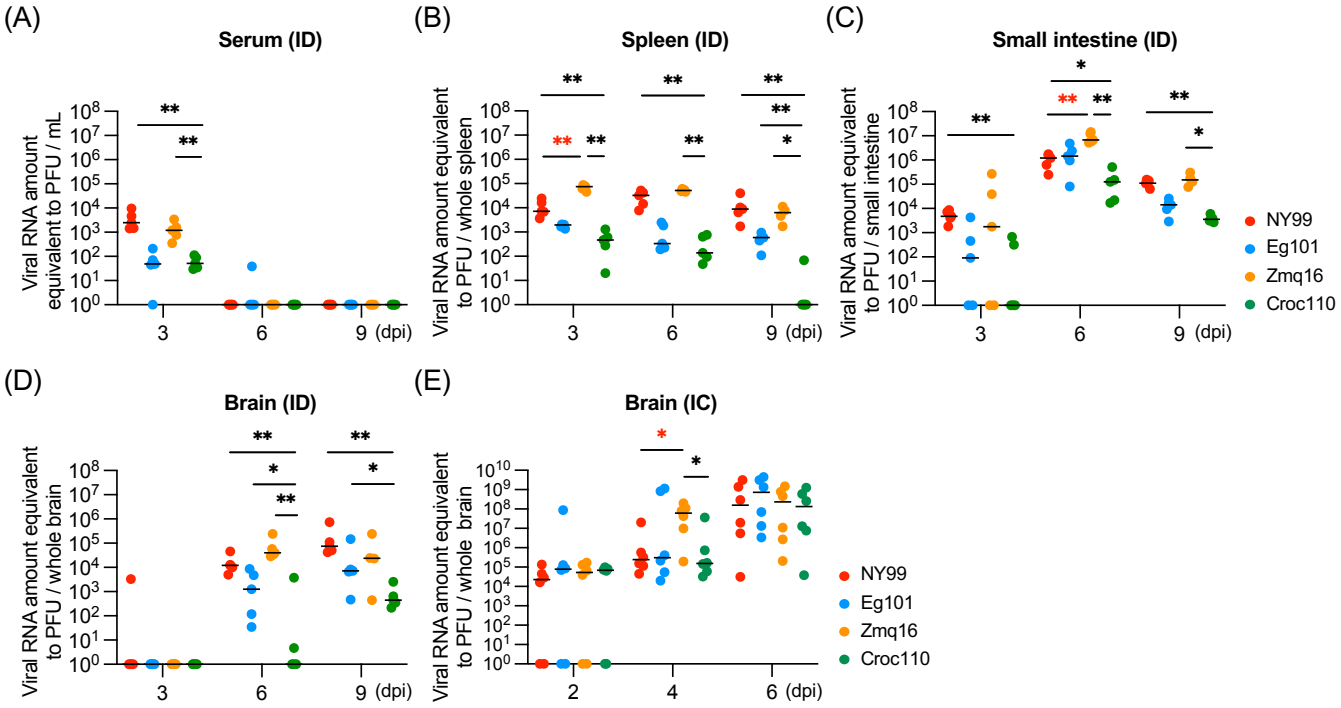

Fig. 7. Replication of WNV isolates in sera, spleen, small intestine and brain

The viral RNA levels in the serum (A), spleen (B), small intestine (C), brain (D) of C57BL/6 mice intradermally (ID) infected with each WNV strain (n = 5). Each sample was collected at 3, 6 and 9 dpi. The viral RNA levels in brain of C57BL/6 mice intracerebral (IC) infected with each WNV strain (n = 6) (E). Each sample was collected at 2, 4 and 6 dpi. In the Zmq16-infected group at 9 dpi, the analysis results from four mice are shown, as one mouse died before analysis. The amount of viral RNA was quantified by RT-qPCR and presented as PFU equivalents, i.e., corrected as the amount in whole tissue samples. Individual values were presented as each plot, with black lines indicating the median for each group. Statistical analysis was performed using Mann-Whitney *U* test in comparison to the result for Croc110 (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$). The result of statistical analysis between Zmq16 and NY99 is indicated by red letters.

CONCLUSION

WNV is one of the mosquito-borne orthoflaviviruses and known to infect a wide range of organisms, including mosquitoes, birds, reptiles, and mammals, spreading through mosquito-mediated transmission. Although WNV has been detected in crocodiles in some countries, there have been no report on the characterization of WNV isolated from naturally infected crocodiles. In this study, a novel WNV strain, Croc110, was isolated from farmed crocodiles that showed neurological symptoms. *In vitro* and *in vivo* analyses of Croc110 compared with another Zambian strain, Zmq16, and two reference strains demonstrated that Croc110 has lower intercellular proliferation in neuronal cells and exhibits low neuroinvasiveness in mice model. On the other hand, mosquito-derived Zmq16 showed high neuroinvasiveness and propagation in mice model. This study revealed that there are two different WNV strains with distinct pathogenicity in Zambia. To assess the WNV pathogenicity in mammal, the conventional C57BL/6 mice model and ID inoculation *via* the ear skin were used in this study. C57BL/6 mice are commonly used mice model for WNV infection, and this mice model allows for the evaluation of acute infection dynamics of Croc110 and Zmq16. The data in this study also showed that ID inoculation *via* the ear skin established systemic WNV infection.

In conclusion, this study is the first report about characteristics of WNV strain isolated from naturally infected crocodile. This study also elucidated the characteristics and pathogenicity of new WNV strains detected in Zambia and provides novel foundational insights into WNV research. Our findings suggest that differences in pathogenicity between animal species should be considered in preventive measures against zoonotic WNV diseases and in the development of WNV vaccines for humans and crocodiles. A previous study reported the detection of WNV antibodies in human serum samples from Zambia, highlighting the need for further epidemiological studies on WNV prevalence in the region. Thus, continued monitoring for the infection status of each WNV strain, including its transmission route, is required to prevent West Nile fever epidemics in humans and crocodiles in Zambia.

ACKNOWLEDGEMENTS

I would like to appreciate my supervisors, Prof. Hirofumi Sawa (Division of Biological Response Analysis, Institute for Vaccine Research and Development (IVReD), Hokkaido University), Prof. Yasuko Orba, Asso. Prof. Michihito Sasaki (Division of Molecular Pathobiology, International Institute for Zoonosis Control (IIZC), Hokkaido university). I am very grateful to all the supervisors for teaching me how to plan and carry out the research, how to use new research techniques, how to write manuscript and how to approach research as an academic researcher. They gave me many advises also on my private difficulties and health concerns. I think that I could not complete my Ph.D. except their big helps. I would like to continue the research in next step with keeping their guidance in my mind.

I would like to appreciate Prof. Yoshihiro Sakoda (Laboratory of Microbiology, Faculty of Veterinary Medicine, Hokkaido University) and Asso. Prof. Shintaro Kobayashi (Laboratory of Public Health, Faculty of Veterinary Medicine, Hokkaido University). Their critical review and insightful comments are greatly improved this manuscript. I would like to say thank you also for all collaborators of my manuscript. Their accumulation of important data and kind co-operation enabled me to publish an article. I also would like to express my gratitude for all lab members. They gave me new insights toward science, mental supports and happy memories through my Ph.D. life.

From Prof. Masayuki Horie (Laboratory of Veterinary Microbiology, school of veterinary science, Osaka Metropolitan University), I could lean the bioinformatics methods. I would like to appreciate for using his resources and kind helps. I would like to continue my effort to publish our manuscript.

I also would like to appreciate Prof. Carla Maria Saleh (Saleh lab., RNAi Unit, Pasteur Institut, France). She gave me courage to do science following my interests and cheer me up to continue the research. Her passion for science and advice has given me back my confidence. In connection with this encounter, I am grateful to the support from Asso. Prof. Keita Matsuno (Division of Risk Analysis and Management, International Institute for Zoonosis Control, Hokkaido University).

My previous supervisors, Prof. Satoshi Taharaguchi and Asso. Prof. Kan Fujino (Laboratory of Microbiology, Faculty of Veterinary medicine, Azabu University) helped me with completion of another my manuscript and with advice on my career path.

Finally, I would like to express my deepest gratitude to my family. They entirely supported me throughout 4 years on my Ph.D. I could not continue my Ph.D. without their supports.

My Ph.D. works and life were financially supported by Hokkaido University World-Leading Innovative and Smart Education (WISE) program (2021), Hokkaido University DX Doctoral Fellowship (2021-2022), Research fellowship for young scientists from the Japanese Society for the Promotion of Science fellows (2022-2025, project number: JP22J21842)

REFERENCES

1. Holmes EC. COVID-19—lessons for zoonotic disease. Science, 375 (6585), 1114-1115, 2022.
2. Thompson A, Kutz S. Introduction to the special issue on 'Emerging Zoonoses and Wildlife'. Int J for Parasitol Parasites Wildl, 9, 322, 2019.
3. Rahman MT, Sobur MA, Islam MS, Ievy S, Hossain MJ, El Zowalaty ME, Rahman AT, Ashour HM. Zoonotic Diseases: Etiology, Impact, and Control. Microorganisms, 8 (9), 1405, 2020.
4. Wang LF, Crameri G. Emerging zoonotic viral diseases. Rev Sci Tech, 33 (2), 569-581, 2014.
5. Campbell-Lendrum D, Manga L, Bagayoko M, Sommerfeld J. Climate change and vector-borne diseases: what are the implications for public health research and policy? Philos Trans R Soc B Biol Sci, 370 (1665), 20130552, 2015.
6. Conway MJ, Colpitts TM, Fikrig E. Role of the vector in arbovirus transmission. Annu rev virol, 1 (1), 71-88, 2014.
7. Pandit PS, Doyle MM, Smart KM, Young CC, Drape GW, Johnson CK. Predicting wildlife reservoirs and global vulnerability to zoonotic Flaviviruses. Nat commun, 9 (1), 5425, 2018.
8. Rückert C, Ebel GD. How Do Virus–Mosquito Interactions Lead to Viral Emergence? Trends Parasitol, 34 (4), 310-321, 2018.
9. Paz-Bailey G, Adams LE, Deen J, Anderson KB, Katzelnick LC. Dengue. The Lancet, 403 (10427), 667-682, 2024.
10. Bifani AM, Chan KWK, Borrenberghs D, Tan MJA, Phoo WW, Watanabe S, Goethals O, Vasudevan SG, Choy MM. Therapeutics for flaviviral infections. Antiviral Res, 210, 105517, 2023.
11. Habarugira G, Suen WW, Hobson-Peters J, Hall RA, Bielefeldt-Ohmann H. West Nile Virus: An Update on Pathobiology, Epidemiology, Diagnostics, Control and "One Health" Implications. Pathogens, 9 (7), 589, 2020.

12. Campbell GL, Marfin AA, Lanciotti RS, Gubler DJ. West Nile virus. Lancet Infect Dis, 2 (9), 519-529, 2002.
13. Chen CC, Jenkins E, Epp T, Waldner C, Curry PS, Soos C. Climate change and West Nile virus in a highly endemic region of North America. Int J Environ Res Public Health, 10 (7), 3052-3071, 2013.
14. Farooq Z, Sjödin H, Semenza JC, Tozan Y, Sewe MO, Wallin J, Rocklöv J. European projections of West Nile virus transmission under climate change scenarios. One Health, 16, 100509, 2023.
15. Pierson TC, Diamond MS. The continued threat of emerging flaviviruses. Nat Microbiol, 5, 796-812, 2020.
16. Rossi SL, Ross TM, Evans JD. West Nile virus. Clin Lab Med, 30:47-65, 2010.
17. Sejvar JJ. West Nile Virus Infection. Microbiol Spectr, 4 (3), 10.1128, 2016.
18. Byas AD, Ebel GD. Comparative Pathology of West Nile Virus in Humans and Non-Human Animals. Pathogens, 9 (1), 48, 2020.
19. Heidecke J, Lavarello Schettini A, Rocklöv J. West Nile virus eco-epidemiology and climate change. PLOS Clim, 2 (5), e0000129, 2023.
20. Ciota AT. West Nile virus and its vectors. Curr Opin Insect Sci, 22, 28-36, 2017.
21. Simulundu E, Ndashe K, Chambaro HM, Squarre D, Reilly PM, Chitanga S, Changula K, Mukubesa AN, Ndebe J, Tembo J, Kapata N, Bates M, Sinkala Y, Hang'ombe BM, Nalubamba KS, Kajihara M, Sasaki M, Orba Y, Takada A, Sawa H. West Nile Virus in Farmed Crocodiles, Zambia, 2019. Emerg Infect Dis, 26 (4), 811-814, 2020.
22. Machain-Williams C, Padilla-Paz SE, Weber M, Cetina-Trejo R, Juarez-Ordaz JA, Loroño-Pino MA, Ulloa A, Wang C, Garcia-Rejon J, Blitvich BJ. Antibodies to West Nile virus in wild and farmed crocodiles in southeastern Mexico. J Wildl Dis, 49 (3), 690-693, 2013.
23. Steinman A, Banet-Noach C, Tal S, Levi O, Simanov L, Perk S, Malkinson M, Shpigel N. West Nile virus infection in crocodiles. Emerg Infect Dis, 9 (7), 887-889, 2003.

24. Dahlin CR, Hughes DF, Meshaka WE, Jr., Coleman C, Henning JD. Wild snakes harbor West Nile virus. One Health, 2, 136-138, 2016.
25. Jacobson ER, Ginn PE, Troutman JM, Farina L, Stark L, Klenk K, Burkhalter KL, Komar N. West Nile virus infection in farmed American alligators (Alligator mississippiensis) in Florida. J Wildl Dis, 41 (1), 96-106, 2005.
26. Isberg SR, Moran JL, De Araujo R, Elliott N, Davis SS, Melville L. First evidence of Kunjin strain of West Nile virus associated with saltwater crocodile (Crocodylus porosus) skin lesions. Aust Vet J, 97 (10), 390-393, 2019.
27. Habarugira G, Moran J, Colmant AMG, Davis SS, O'Brien CA, Hall-Mendelin S, McMahon J, Hewitson G, Nair N, Barcelon J, Suen WW, Melville L, Hobson-Peters J, Hall RA, Isberg SR, Bielefeldt-Ohmann H. Mosquito-Independent Transmission of West Nile virus in Farmed Saltwater Crocodiles (Crocodylus porosus). Viruses, 12 (2), 198, 2020.
28. Byas AD, Gallichotte EN, Hartwig AE, Porter SM, Gordy PW, Felix TA, Bowen RA, Ebel GD, Bosco-Lauth AM. American alligators are capable of West Nile virus amplification, mosquito infection and transmission. Virology, 568, 49-55, 2022.
29. Orba Y, Hang'ombe BM, Mweene AS, Wada Y, Anindita PD, Phongphaew W, Qiu Y, Kajihara M, Mori-Kajihara A, Eto Y, Sasaki M, Hall WW, Eshita Y, Sawa H. First isolation of West Nile virus in Zambia from mosquitoes. Transbound Emerg Dis, 65 (4), 933-938, 2018.
30. Lanciotti RS, Roehrig JT, Deubel V, Smith J, Parker M, Steele K, Crise B, Volpe KE, Crabtree MB, Scherret JH, Hall RA, MacKenzie JS, Cropp CB, Panigrahy B, Ostlund E, Schmitt B, Malkinson M, Banet C, Weissman J, Komar N, Savage HM, Stone W, McNamara T, Gubler DJ. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. Science, 286 (5448), 2333-2337, 1999.
31. Melnick JL, Paul JR, Riordan JT, Barnett VH, Goldblum N, Zabin E. Isolation from human sera in Egypt of a virus apparently identical to West Nile virus. Proc Soc Exp Biol Med, 77 (4), 661-665, 1951.

32. Kumar M, O'Connell M, Namekar M, Nerurkar VR. Infection with non-lethal West Nile virus Eg101 strain induces immunity that protects mice against the lethal West Nile virus NY99 strain. Viruses, 6 (6), 2328-2339, 2014.
33. Brien JD, Lazear HM, Diamond MS. Propagation, quantification, detection, and storage of West Nile virus. Curr Protoc Microbiol, 31, 15d.3.1-15d.3.18, 2013.
34. REED LJ, MUENCH H. A SIMPLE METHOD OF ESTIMATING FIFTY PER CENT ENDPOINTS. Am J Epidemiol, 27 (3), 493-497, 1938.
35. Clark DC, Lobigs M, Lee E, Howard MJ, Clark K, Blitvich BJ, Hall RA. In situ reactions of monoclonal antibodies with a viable mutant of Murray Valley encephalitis virus reveal an absence of dimeric NS1 protein. J Gen Virol, 88 (4), 1175-1183, 2007.
36. Tabata K, Itakura Y, Toba S, Uemura K, Kishimoto M, Sasaki M, Harrison JJ, Sato A, Hall WW, Hall RA, Sawa H, Orba Y. Serological characterization of lineage II insect-specific flaviviruses compared with pathogenic mosquito-borne flaviviruses. Biochem Biophys Res Commun, 616, 115-121, 2022.
37. Larkin MA, Blackshields G, Brown NP, Chenna R, McGettigan PA, McWilliam H, Valentin F, Wallace IM, Wilm A, Lopez R, Thompson JD, Gibson TJ, Higgins DG. Clustal W and Clustal X version 2.0. Bioinformatics, 23 (21), 2947-2948, 2007.
38. Tamura K, Stecher G, Kumar S. MEGA11: Molecular Evolutionary Genetics Analysis Version 11. Mol Biol Evol, 38 (7), 3022-3027, 2021.
39. Jones DT, Taylor WR, Thornton JM. The rapid generation of mutation data matrices from protein sequences. Comput Appl Biosci, 8 (3), 275-282, 1992.
40. Joseph S, Wernery U, Teng JL, Wernery R, Huang Y, Patteril NA, Chan KH, Elizabeth SK, Fan RY, Lau SK, Kinne J, Woo PC. First isolation of West Nile virus from a dromedary camel. Emerg Microbes Infect, 5 (6), e53, 2016.
41. Fiacre L, Pagès N, Albina E, Richardson J, Lecollinet S, Gonzalez G. Molecular Determinants of West Nile Virus Virulence and Pathogenesis in Vertebrate and Invertebrate Hosts. Int J Mol Sci, 21 (23), 9117, 2020.

42. Beasley DWC, Whiteman MC, Zhang S, Huang CY-H, Schneider BS, Smith DR, Gromowski GD, Higgs S, Kinney RM, Barrett ADT. Envelope Protein Glycosylation Status Influences Mouse Neuroinvasion Phenotype of Genetic Lineage 1 West Nile Virus Strains. J Virol, 79 (13), 8339-8347, 2005.
43. Scherret JH, Mackenzie JS, Khromykh AA, Hall RA. Biological Significance of Glycosylation of the Envelope Protein of Kunjin Virus. Ann N Y Acad Sci, 951, 361-363, 2001.
44. Hasebe R, Suzuki T, Makino Y, Igarashi M, Yamanouchi S, Maeda A, Horiuchi M, Sawa H, Kimura T. Transcellular transport of West Nile virus-like particles across human endothelial cells depends on residues 156 and 159 of envelope protein. BMC Microbiol, 10, 165, 2010.
45. Kobayashi S, Kaneko C, Kawakami R, Hasebe R, Sawa H, Yoshii K, Kariwa H. Amino acid 159 of the envelope protein affects viral replication and T-cell infiltration by West Nile virus in intracranial infection. Sci Rep, 10 (1), 7168, 2020.
46. Wang Y, Xie X, Shi PY. Flavivirus NS4B protein: Structure, function, and antiviral discovery. Antiviral Res, 207, 105423, 2022.
47. Goh JZH, De Hayr L, Khromykh AA, Slonchak A. The Flavivirus Non-Structural Protein 5 (NS5): Structure, Functions, and Targeting for Development of Vaccines and Therapeutics. Vaccines, 12 (8), 865, 2024.
48. Serman T, Chiang C, Liu G, Sayyad Z, Pandey S, Volcic M, Lee H, Muppala S, Acharya D, Goins C, Stauffer SR, Sparrer KMJ, Gack MU. Acetylation of the NS3 helicase by KAT5γ is essential for flavivirus replication. Cell Host Microbe, 31 (8), 1317-1330.e10, 2023.
49. Fiacre L, Pagès N, Albina E, Richardson J, Lecollinet S, Gonzalez G. Molecular Determinants of West Nile Virus Virulence and Pathogenesis in Vertebrate and Invertebrate Hosts. Int J Mol Sci, 21 (23), 9117, 2020.
50. Sejvar JJ. Clinical Manifestations and Outcomes of West Nile Virus Infection. Viruses, 6 (2), 606-623, 2014.
51. Murray KO, Resnick M, Miller V. Depression after infection with West Nile virus. Emerg Infect Dis, 13 (3), 479-481, 2007.

52. Ronca SE, Ruff JC, Murray KO. A 20-year historical review of West Nile virus since its initial emergence in North America: Has West Nile virus become a neglected tropical disease? PLoS Negl Trop Dis, 15 (5), e0009190, 2021.
53. Lu L, Zhang F, Oude Munnink BB, Munger E, Sikkema RS, Pappa S, Tsioka K, Sinigaglia A, Dal Molin E, Shih BB, Günther A, Pohlmann A, Ziegler U, Beer M, Taylor RA, Bartumeus F, Woolhouse M, Aarestrup FM, Barzon L, Papa A, Lycett S, Koopmans MPG. West Nile virus spread in Europe: Phylogeographic pattern analysis and key drivers. PLoS Pathog, 20 (1), e1011880, 2024.
54. Mencattelli G, Ndione MHD, Rosà R, Marini G, Diagne CT, Diagne MM, Fall G, Faye O, Diallo M, Faye O, Savini G, Rizzoli A. Epidemiology of West Nile virus in Africa: An underestimated threat. PLOS Negl Trop Dis, 16 (1), e0010075, 2022.
55. Runstadler JA, Lowen AC, Kayali G, Tompkins SM, Albrecht RA, Fouchier RAM, Stallknecht DE, Lakdawala SS, Goodrum FD, Casadevall A, Enquist LW, Alwine JC, Imperiale MJ, Schultz-Cherry S, Webby RJ. Field Research Is Essential to Counter Virological Threats. J Virol, 97 (5), e0054423, 2023.
56. Kimura T, Sasaki M, Okumura M, Kim E, Sawa H. Flavivirus encephalitis: pathological aspects of mouse and other animal models. Vet Pathol, 47 (5), 806-818, 2010.
57. Graham JB, Swarts JL, Lund JM. A Mouse Model of West Nile Virus Infection. Curr Protoc Mouse Biol, 7 (4), 221-235, 2017.
58. Lim SM, Koraka P, van Boheemen S, Roose JM, Jaarsma D, van de Vijver DA, Osterhaus AD, Martina BE. Characterization of the mouse neuroinvasiveness of selected European strains of West Nile virus. PLoS One, 8 (9), e74575, 2013.
59. Pérez-Ramírez E, Llorente F, Del Amo J, Fall G, Sall AA, Lubisi A, Lecollinet S, Vázquez A, Jiménez-Clavero M. Pathogenicity evaluation of twelve West Nile virus strains belonging to four lineages from five continents in a mouse model: discrimination between three pathogenicity categories. J Gen Virol, 98 (4), 662-670, 2017.
60. Prow NA, Edmonds JH, Williams DT, Setoh YX, Bielefeldt-Ohmann H, Suen WW, Hobson-Peters J, van den Hurk AF, Pyke AT, Hall-Mendelin S, Northill JA,

Johansen CA, Warrilow D, Wang J, Kirkland PD, Doggett S, Andrade CC, Brault AC, Khromykh AA, Hall RA. Virulence and Evolution of West Nile Virus, Australia, 1960-2012. Emerg Infect Dis, 22 (8), 1353-1362, 2016.

61. Daffis S, Suthar MS, Szretter KJ, Gale M, Jr., Diamond MS. Induction of IFN-β and the Innate Antiviral Response in Myeloid Cells Occurs through an IPS-1-Dependent Signal That Does Not Require IRF-3 and IRF-7. PLoS Pathog, 5 (10), e1000607, 2009.
62. Roe K, Kumar M, Lum S, Orillo B, Nerurkar VR, Verma S. West Nile virus-induced disruption of the blood–brain barrier in mice is characterized by the degradation of the junctional complex proteins and increase in multiple matrix metalloproteinases. J Gen Virol, 93 (6), 1193-1203, 2012.
63. Turvey ME, Uppu D, Mohamed Sharif AR, Bidet K, Alonso S, Ooi EE, Hammond PT. Microneedle-based intradermal delivery of stabilized dengue virus. Bioeng Transl Med, 4 (2), e10127, 2019.
64. Secundino NFC, Chaves BA, Orfano AS, Silveira KRD, Rodrigues NB, Campolina TB, Nacif-Pimenta R, Villegas LEM, Silva BM, Lacerda MVG, Norris DE, Pimenta PFP. Zika virus transmission to mouse ear by mosquito bite: a laboratory model that replicates the natural transmission process. Parasit Vectors, 10 (1), 346, 2017.
65. Frost MJ, Zhang J, Edmonds JH, Prow NA, Gu X, Davis R, Hornitzky C, Arzey KE, Finlaison D, Hick P, Read A, Hobson-Peters J, May FJ, Doggett SL, Haniotis J, Russell RC, Hall RA, Khromykh AA, Kirkland PD. Characterization of virulent West Nile virus Kunjin strain, Australia, 2011. Emerg Infect Dis, 18 (5), 792-800, 2012.
66. Kobayashi S, Kawakami R, Takeda C, Maezono K, Thammahakin P, Eguchi H, Hang'ombe BM, Orba Y, Sawa H, Yoshii K, Kariwa H. Ubiquitin accumulation induced by the finger and palm sub-domains of NS5 modulates the replication of West Nile virus. Virology, 588, 109902, 2023.

67. Rothan HA, Arora K, Natekar JP, Strate PG, Brinton MA, Kumar M. Z-DNA-Binding Protein 1 Is Critical for Controlling Virus Replication and Survival in West Nile Virus Encephalitis. Front Microbiol, 10, 2089, 2019.
68. Schmid MA, Kauffman E, Payne A, Harris E, Kramer LD. Preparation of Mosquito Salivary Gland Extract and Intradermal Inoculation of Mice. Bio Protoc, 7 (14), e2407, 2017.
69. Wu YH, Tseng CK, Lin CK, Wei CK, Lee JC, Young KC. ICR suckling mouse model of Zika virus infection for disease modeling and drug validation. PLoS Negl Trop Dis, 12 (10), e0006848, 2018.
70. Beasley DW, Li L, Suderman MT, Barrett AD. Mouse neuroinvasive phenotype of West Nile virus strains varies depending upon virus genotype. Virology, 296 (1), 17-23, 2002.
71. Wicker JA, Whiteman MC, Beasley DW, Davis CT, Zhang S, Schneider BS, Higgs S, Kinney RM, Barrett AD. A single amino acid substitution in the central portion of the West Nile virus NS4B protein confers a highly attenuated phenotype in mice. Virology, 349 (2), 245-253, 2006.

SUMMARY IN JAPANESE

本研究の背景

蚊は様々な病原体をヒトへ媒介することにより、現在も世界各地で多くの感染症を起こしていることから、最もヒトに有害な生物であると考えられている。昨今の地球温暖化による蚊の生息域の拡大から、今まで熱帯、亜熱帯地域でのみ発生が確認されていた感染症が、新たな地域で発生するリスクが高まり、蚊媒介ウイルス感染症は公衆衛生学的に重要な問題となっている。ウエストナイルウイルス（WNV）は、デングウイルス、ジカウイルス、日本脳炎ウイルス等とともに蚊媒介性のフラビウイルス科オルソフラビウイルス属のRNAウイルスに分類される。WNV は 1937 年にウガンダで発見されて以来、現在では南極大陸を除く全ての大陸で流行しており、特に病原性の高い lineage 1a と 2 が近年公衆衛生学上問題となっている。WNV は約 80%の感染症例でヒトに不顕性感染を起こし、残りの 20%は発症し、熱性疾患や発疹、消化器症状を呈する。脳炎・髄膜炎等の重篤な症状を発症する患者は感染者のうち 1%未満で、重症患者の死亡率は 3〜15%である。WNV の病原性は、C57BL/6 マウスを含むマウスモデルを用いて解析されている。現在ヒト WNV 感染症に有効なワクチン、及び特異的な治療法は確立されておらず、WNV の増殖性と病原性に関する基礎的知見の蓄積は、WNV 感染症対策に必須である。WNV は広い宿主域を有しており、自然界では蚊と鳥類で感染環を形成するが、他にもヒト、爬虫類、ウマ、その他様々な野生動物が蚊の刺咬により感染する。WNV はワニに感染して pix と呼ばれる発疹や神経学的症状を生じさせ、ワニ皮革産業に打撃を与えている。

本研究は、第一章で、ザンビアの養殖ワニから検出された新規 WNV 株を対象に、ウイルス分離と各種培養細胞を用いたウイルス性状解析を実施した。第二章では、ワニからの分離株に加えて、以前にザンビアの蚊から分離された別の WNV 株に関して、実験室株と比較して、感染モデルマウスを用いた病原性解析を実施し、ザンビアの動物から単離された WNV に関する増殖性と病原性を解析することを目的とした。

第一章 ザンビアのワニ、及び蚊から分離された２つのウエストナイルウイルス株の *in vitro* における性状解析

これまでに、アメリカ、メキシコ、イスラエル等で、ワニから WNV が検出されているが、自然感染したワニから単離された WNV 株の全ゲノム解析や細胞を用いた性状解析の報告はない。ザンビア共和国では、2016 年に採集された蚊のサンプルから初めて WNV (Zmq16m11 株、Zmq16)が単離され、さらに、2019 年にはザンビア南部のワニ養殖場において神経症状等の臨床症状を示した個体から、蚊から分離された WNV とは異なる lineage の WNV が検出された。本研究は、同養殖ワニから、WNV（Croc110/2019/1/ZM : Croc110）株の分離に成功し、その全ゲノム配列を同定した。次に、Zmq16、及び実験株である高病原性株（NY99）と低病原性株（Eg101）と比較して、Croc110 の培養細胞内増殖性を複数の細胞株で比較した。その結果、蚊由来 C6/36 細胞では、Croc110 は Eg101 と比べて、2, 3 dpi で有意に増殖性が高いこと、NY99 や Zmq16 と同等の増殖性を有することを明らかにした。また、哺乳類動物由来非神経細胞である Vero 細胞、及び哺乳類動物神経由来細胞株である NA 細胞におけるウイルス増殖性は、上記の株間で同程度であること、SH-SY5Y 細胞では Eg101 と比較して、1 dpi で有意に増殖性が高いことを明らかにした。一方、培養細胞におけるプラークサイズを比較した結果、NA 細胞では Croc110 は Eg101 より有意に小さいプラークサイズを示し、NY99 と Zmq16 株と同程度のプラークサイズを示した。Vero 細胞と SH-SY5Y 細胞においては、Croc110 は他の 3 株と比較して有意にプラークサイズが小さかった。

第二章 ザンビアのワニおよび蚊から分離されたウエストナイルスウイルスのマウスにおける病原性解析

ザンビアのワニ、及び蚊から単離された WNV 株の哺乳類動物での病原性を明らかにするため、第二章では、ワニ由来 Croc110 と蚊由来 Zmq16 株のマウス（C57BL/6）における病原性とウイルス増殖性を、NY99、及び Eg101 と比較解析した。ウイルス接種経路として、脳内接種、腹腔内接種、耳介からの皮内接種を使用した。その結果、脳内接種した際には、Croc110 感染マウスの 70%程度が神経学的症状を呈し、死亡率は Eg101 感染マウスより有意に低く、NY99、

及び Zmq16 感染マウスと同等であった。しかし、皮内、及び腹腔内接種では、Croc110 感染マウスの死亡率は、低病原性株の Eg101 と同程度であり、NY99、及び Zmq16 に比べて有意に低いことが明らかになった。Zmq16 は脳内接種と腹腔内接種において NY99 と同程度の死亡率と臨床症状を呈し、皮内接種では NY99 に比べて有意に高い死亡率を呈した。マウスにおける接種実験において、Croc110 は末梢接種において Eg101 と同程度の低い病原性、脳内接種では NY99 と同程度の病原性を示した。また、Zmq16 は脳内接種と腹腔内接種では NY99 と同等の高病原性であり、皮内接種では NY99 より高い病原性を示すことがわかった。この結果に一致して、Croc110 皮内接種後の感染マウス脳内、及び末梢組織内におけるウイルス増殖は、高病原性である Zmq16、及び NY99 感染マウスと比較して有意に低いことが確認された。本研究により、Croc110 は、末梢接種したマウスにおいて各種臓器での増殖性、及び神経侵襲性が低く、lineage 2 に属する Zmq16 とは異なる病原性を示すことが明らかとなった。

本研究の結論

本研究では、ザンビアのワニから lineage 1a に属する新たな WNV を分離し、その全ゲノム配列を同定したほか、ワニ、及び蚊から検出された、異なる 2 株の細胞での増殖性、感染マウスでの病原性と増殖性を解析した。本研究は、自然感染ワニから分離された WNV 株に関する初めての性状解析である。本研究で分離、解析をした Croc110 は哺乳類動物由来培養細胞において他の株と比べてプラークサイズが小さく、マウスへの末梢接種で各種臓器において増殖性が他の株よりも低く、低神経侵襲性と低病原性を示した。対して、Zmq16 は細胞での増殖性が他の株と同程度で、末梢接種におけるマウスでの病原性は高く NY99 と同程度以上であり、各種臓器で高い増殖性を示した。また、本研究では、マウスへの WNV 接種において脳内接種、腹腔内接種、耳介の皮膚を介した皮内接種を検討した。耳介を介した皮内接種は腹腔内接種同様全身性の WNV 感染を確立した。接種部位の採材の容易さを加味すると、耳介を介した皮内接種は、既報で汎用されている foodpad を介した皮内接種よりも、WNV の皮内接種に有用であると考えられた。

以前には、ELISA を用いた血清学的調査により、ザンビアのヒト血清中に、抗 WNV 抗体が検出されたという先行研究が報告されている。本研究によ

り、ザンビアには、隣接する地域において異なる lineage と病原性を有する WNV が存在することが示された。そのため、ザンビアにおけるヒトや動物に対して、Croc110 や Zmq16 の感染状況のサーベーランスを継続することは、ザンビアにおける WNV 循環を評価するために必要であると考えられた。

以上、本研究は、ザンビアで単離された 2 つの WNV 株の性状を解析することにより、自然感染ワニ由来 WNV に関する遺伝的特徴と細胞内増殖性を特徴づけるとともに、ザンビア由来 WNV 株のマウスでの病原性とウイルス増殖性を決定した。本基礎的知見に基づいて、Croc110 の配列を先行研究で特徴づけられた他の株と配列を比較することで、WNV における細胞内増殖性、及びマウスでの病原性と神経侵襲性に関与するアミノ酸変異の候補を提示する事が可能になる。