The West Nile Virus-Like Flavivirus Koutango Is Highly Virulent in Mice due to Delayed Viral Clearance and the Induction of a Poor Neutralizing Antibody Response

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ABSTRACT

The mosquito-borne West Nile virus (WNV) is responsible for outbreaks of viral encephalitis in humans, horses, and birds, with particularly virulent strains causing recent outbreaks of disease in eastern Europe, the Middle East, North America, and Australia. Previous studies have phylogenetically separated WNV strains into two main genetic lineages (I and II) containing virulent strains associated with neurological disease. Several WNV-like strains clustering outside these lineages have been identified and form an additional five proposed lineages. However, little is known about whether these strains have the potential to induce disease. In a comparative analysis with the highly virulent lineage I American strain (WNV_{NY99}), the low-pathogenicity lineage II strain (B956), a benign Australian strain, Kunjin (WNV_{KUN}), the African WNV-like Koutango virus (WNV_{KOU}), and a WNV-like isolate from Sarawak, Malaysia (WNV_{Sarawak}), were assessed for neuroinvasive properties in a murine model and for their replication kinetics *in vitro*. While WNV_{NY99} replicated to the highest levels *in vitro*, *in vivo* mouse challenge revealed that WNV_{KOU} was more virulent, with a shorter time to onset of neurological disease and higher morbidity. Histological analysis of WNV_{KOU}- and WNV_{NY99}-infected brain and spinal cords demonstrated more prominent meningoencephalitis and the presence of viral antigen in WNV_{KOU}-infected mice. Enhanced virulence of WNV_{KOU} also was associated with poor viral clearance in the periphery (sera and spleen), a skewed innate immune response, and poor neutralizing antibody development. These data demonstrate, for the first time, potent neuroinvasive and neurovirulent properties of a WNV-like virus outside lineages I and II.

IMPORTANCE

In this study, we characterized the *in vitro* and *in vivo* properties of previously uncharacterized West Nile virus strains and West Nile-like viruses. We identified a West Nile-like virus, Koutango virus (WNV_{KOU}), that was more virulent than a known virulent lineage I virus, WNV_{NY99}. The enhanced virulence of WNV_{KOU} was associated with poor viral clearance and the induction of a poor neutralizing antibody response. These findings provide new insights into the pathogenesis of West Nile virus.

West Nile virus (WNV) is a mosquito-transmitted, single-stranded, positive-sense flavivirus that has emerged as an important causative agent of viral encephalitis in humans and horses in many parts of the world. Outbreaks of potentially fatal neurological syndromes traditionally have been documented in Europe and Africa (1). However, in recent times strains of WNV have caused large outbreaks of encephalitis in the New World, involving humans and equines in the United States and equines in Australia (2–10). There have also been recent incursions of new, virulent strains in Europe (8–10). In the summer of 2012, the United States saw the second highest number of WNV cases on record with concurrent outbreaks in several European countries, highlighting the continuing public health threat of WNV to humans (11). In Australia, an indigenous strain of WNV, WNV Kunjin (WNV_{KUN}), historically has caused only infrequent and mild symptoms in humans and horses. However, a large outbreak of encephalitis in horses in 2011 saw the emergence of the first virulent strain of WNV_{KUN} in Australia, associated with the acquisition of at least two known molecular markers of WNV virulence not found in the prototype WNV_{KUN} (4), demonstrating ongoing evolution even among low-virulence WNV strains.

Phylogenetic analysis has suggested that WNV emerged in Africa and subsequently dispersed through avian migration and can

be separated into two main lineages (I and II), with an additional 5 lineages proposed (12). Lineage I contains WNV_{KUN} isolates and WNV isolates from north, west, and central Africa, southern and eastern Europe, India, the Middle East, and North America. Lineage I can be further divided into 3 clades, with clade 1a containing WNV isolates from around the world, the Australian WNV_{KUN} isolates forming clade 1b, and clade 1c containing isolates from India (previously described as lineage V [13]). Lineage II comprises WNV isolates from west, central, and east Africa and Madagascar (14, 15). Historically, lineage II strains were associated with fever and mild symptoms until 2008, when the emergence of lineage II strains was responsible for outbreaks of neurological disease in Greece, Hungary, and Italy (6, 8, 10, 16).

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TABLE 1 Summary of West Nile virus and West Nile-like viruses included in this study

Virus	Proposed lineage	Yr of isolation	Place of isolation	Source of isolation	Known neurovirulence property(ies)
NY99-4132	Ia	1999	New York City, NY	Human, mosquito	Highly virulent in humans, mice, birds ^b
MRM16	Ib	1960	Mitchell River Mission, Australia	Mosquito (<i>Culex annulirostris</i>)	Low virulence in humans, mice ^d
Hu6774	Ib	1991	Southern NSW, Australia	Human, mosquito	Low virulence in humans ^d
Sarafend	II	Unknown	Unknown	Unknown	Virulent in mice ^c
B956 ^a	II	1937	Uganda	Human	Low virulence in humans, mice ^e
Sarawak (MP502-66)	VI	1966	Sarawak, Borneo, Malaysia	Mosquito (<i>Culex pseudovishnui</i>)	None
Koutango-DakAad 5443 ^f	VII	1968	Senegal, Africa	Rodent (<i>Tatera kempi</i>)	None

^a Prototype strain B956.^b Data are from references 2 and 22.^c Data are from reference 30.^d Data are from references 12 and 33.^e Data are from reference 35.^f Accession number EU082200.

Studies comparing the virulence of various WNV strains in mice have identified several viral motifs residing in both structural and nonstructural genes as well as in the 5'- and 3'-untranslated regions that were associated with enhanced invasion of the central nervous system (CNS) and onset of neurological disease in this species (17–23). One example of these virulence determinants is N-linked glycosylation at a conserved site in the E protein (residues 154 to 156) of WNV that has been shown to increase virulence of lineage I WNV strains (19, 24) and which likely is mediated via enhanced assembly and/or secretion of virus particles (21, 25). However, the biological influence of N-linked glycosylation on viruses that branch outside lineages I and II has not been investigated.

WNV infection remains subclinical in most humans, but ~20% may develop symptoms of disease ranging from a mild flu-like illness, known as West Nile fever, to more serious neurological complications, including meningitis and encephalitis. Postneurologic sequelae are common (26). In both humans and mice, WNV encephalitis is characterized by the reaction of resident cells in the CNS and infiltration of inflammatory leukocytes, including monocytes and T cells, in the perivascular space and parenchyma. Although increased age and immunosuppression are risk factors for severe WNV infection in humans, little is known about the mechanisms accountable for this (27, 28). A mouse model of infection is widely used in studies of WNV pathogenesis, and various components of the innate and adaptive immune response, including antibodies, T cells (particularly CD8⁺ T cells), monocytes/macrophages, and gamma delta T cells, have been identified as being critical for viral clearance. In this model, CD4⁺ and CD8⁺ T cells, as well as natural killer cells and infiltrating monocyte/macrophages, accumulate within the CNS in aggregates that colocalize primarily with WNV-infected neurons (29). The majority of murine studies have focused on the virulent lineage I North American strain of WNV, namely, NY99 (WNV_{NY99}), or the lineage II Sarafend strain (30–33), and little is known about the pathogenesis of other strains of WNV, especially those viruses that branch outside lineages I and II.

This study aimed to characterize the pathogenic properties of multiple WNV strains and West Nile-like viruses to identify both virus and host factors leading to disease development. In particular, two previously uncharacterized West Nile-like viruses, WNV_{Sarawak}, isolated in 1966 from Borneo, and Koutango virus

(WNV_{KOU}), isolated in 1968 from Senegal, were investigated to determine phenotypic characteristics *in vitro* and virulence *in vivo*. Aside from phylogenetic data on these two viruses, little information was known prior to this study. Replication kinetics in cells confirmed the importance of N-linked glycosylation in the total amount of infectious virus at different time points. Studies using an adult mouse model of disease demonstrated for the first time that a West Nile-like virus, WNV_{KOU}, branching outside the main lineages, was more virulent than the North American lineage I strain, with the ability to induce more prominent neuropathology. The enhanced virulence of WNV_{KOU} was associated with poor viral clearance in extraneural tissues and a delayed and poor neutralizing antibody response.

MATERIALS AND METHODS

Cell culture and virus production. African green monkey kidney (Vero) cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2% fetal bovine serum (FBS), 50 U ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin, and 2 mM L-glutamine. Human brain microvascular endothelial cells (HBMECs) were cultured in RPMI 1640 medium supplemented with 20% FBS and nonessential amino acids (Invitrogen, Victoria, Australia) (34). HBMECs were plated on collagen-coated 24-well plates for growth kinetics studies. Cells were cultured at 37°C with 5% CO₂. *Aedes albopictus* cells (C6/36) were cultured in RPMI 1640 supplemented with 10% FBS, 50 U ml⁻¹ penicillin, 50 µg ml⁻¹ streptomycin, and 2 mM L-glutamine. These cells were cultured at 28°C.

West Nile virus and West Nile-like viruses used in this study are listed with their sources of isolation in Table 1 and included B956, NY99 (GenBank accession number HQ596519), Sarafend, Koutango (accession number EU082200), Sarawak, MRM16, and Hu6774 viruses. These viruses were selected to represent the two main lineages of WNV (I and II), as well as those proposed to branch outside these lineages, namely, WNV_{KOU} (proposed to represent lineage VII) and WNV_{Sarawak} (proposed to represent lineage VI). Strains representing lineage I include the North American strain NY99 (WNV_{NY99}), known to be highly neurovirulent in humans, horses, birds, and mice, as well as two strains of WNV_{KUN}, the only known human isolate of WNV_{KUN}, Hu6774, and a mosquito isolate, MRM16, both thought to have low virulence (Table 1). Lineage II was represented by the prototype strain B956 and Sarafend viruses (31, 35). WNV strain NY99-4132 was obtained from the Division of Vector Borne Infectious Diseases, Centers for Disease Control, Fort Collins, CO. Virus stocks were produced by infecting a subconfluent monolayer of C6/36 cells with virus at a multiplicity of infection (MOI) of 0.1 to 1 in RPMI containing 2% FBS (2% FBS-RPMI). Culture supernatant was harvested

and clarified at 72 to 96 h postinfection and stored at -80°C . The concentration of infectious virus in stocks was determined by titration on Vero cells in 96-well plates by serial 10-fold dilutions in 2% FBS-DMEM. After 5 days of incubation, wells exhibiting cytopathic effects (CPE) were identified and viral titers determined by the method of Reed and Muench and expressed as a 50% tissue culture infectious dose (TCID_{50}) (36).

Viral growth kinetics. Cells were cultured in 24-well plates, allowing one well for each sample collected. All time points were collected in triplicate. When the cells were 70% confluent they were infected with virus at an MOI of 0.1 and diluted in 2% FBS-DMEM. After 1 h of incubation at 37°C , virus was removed and the cells were washed with phosphate-buffered saline (PBS) before addition of 1 ml 2% FBS-DMEM. Samples were removed every 12 or 24 h postinfection (p.i.) and stored at -80°C . The virus titer in each sample was determined as TCID_{50} s on Vero cells by the method of Reed and Muench (36).

Plaque assays. Vero cells in 6-well plates were infected with a series of 10-fold dilutions as described above for TCID_{50} assays. Cells were overlaid with 0.75% low-melting-point agarose in DMEM containing 2% FBS and incubated at 37°C . After 4 days, monolayers were fixed with 10% formaldehyde and stained with 0.2% crystal violet. The diameter of plaques of each virus tested were measured and compared to each other.

Assessment of glycosylation status by enzyme digestion and nucleotide sequence analysis. Glycosylation of the E protein was examined by digestion with N-glycosidase F (PNGase F; Roche) using the protocol described by Adams and colleagues (37). Proteins in the viral supernatant were denatured with 10% (vol/vol) SDS at 98°C for 3 min and were digested with 1.2 μl of 0.5 M EDTA and 3 μl of 20% n-octyl- β -D-glucopyranoside. The mixture was digested overnight at 37°C with 1 U PNGase F. Proteins were separated and analyzed by Western blotting. Samples were loaded with nonreducing SDS-PAGE NuPage LDS sample buffer (Invitrogen) on a 12% NuPage gel (Invitrogen). Electrophoresed proteins were electroblotted onto nitrocellulose paper (Hybond C; Amersham) and immunostained with monoclonal antibody (MAb) 4G2 (anti-E) at a dilution of 1:5 as previously described (37). The genomic sequence encompassing amino acid residues 154 to 156 was confirmed by sequence analysis using reverse transcription-PCR (RT-PCR) and Sanger sequencing. Briefly, viral RNA was extracted using the RNeasy minikit (Qiagen) by following the manufacturer's instructions, followed by RT-PCR using a SuperScript III OneStep RT-PCR system with Platinum *Taq* DNA polymerase (Invitrogen) with a degenerate primer set (WNEglyF, 5'-GRAGCATYGACACATG-3'; WNEglyR, 5'-GTTCYCKGTTCTCCA-3'). The following RT-PCR cycling conditions were carried out: 1 cycle of 45°C for 20 min; 1 cycle of 94°C for 2 min; 40 cycles of 94°C (30 s), 45°C (1 min), and then 68°C (1 min); and a final extension at 68°C for 5 min. The expected band (400 bp) was resolved, and gel was purified from a 2% agarose gel using a NucleoSpin gel and PCR cleanup kit (Macherey-Nagel). Sequencing was completed at the Australian Genome Research Facility, The University of Queensland.

Mouse virulence. All animal procedures received prior approval from The University of Queensland Animal Ethics Committee and, where necessary, were performed under ketamine-xylazil anesthesia. Four- to 5-week-old Swiss outbred mice CD-1 (Animal Resources Centre, Murdoch, Western Australia, Australia) were challenged intraperitoneally (i.p.) with a range of doses of WNV strains and West Nile-like viruses (described in Table 1). Mice were kept on clean bedding and given food and water *ad libitum*. Infected animals were monitored daily for the onset of disease and culled when the first signs of encephalitis (hunching, lethargy, eye closure, or hind-leg flaccid paralysis) were apparent. Surviving mice were bled by cardiac puncture at the end of the experiment (day 21), and the sera were tested for evidence of seroconversion to WNV using a fixed-cell ELISA as previously described (38). To assess differences in the induction of neutralizing antibodies postinfection between viral strains, 6-week-old Swiss outbred mice were injected i.p. with a sublethal dose of 1 infectious unit (IU) and bled on days 7, 14, and 21 days postinfection.

Blood was separated and assayed for neutralizing antibodies in a virus neutralization assay.

Titration of virus in tissue samples. To measure the amount of infectious virus in peripheral (spleen and sera) and CNS (brain and spinal cord) tissues, samples were extracted, weighed, snap-frozen on dry ice, and stored at -80°C until viral titration assays were performed. At the time of these titrations, 10% (wt/vol) homogenates of each sample were prepared in 2% FBS-DMEM, and serial 10-fold dilutions of each homogenate were assayed by TCID_{50} on monolayers of Vero cells. The results presented are the means $\pm$ standard errors of the means (SEM) of the $\log_{10}$ IU per gram of tissue derived from three animals at each time point. The amount of infectious virus in serum also was determined. In this case, blood samples were collected via cardiac puncture, stored on ice until the blood had separated and serum could be collected, and stored at -80°C . Infectious virus in serum samples was assayed on Vero cells as described above.

Virus neutralization assay. Heat-inactivated test sera were titrated in doubling dilutions from 1:10 to 1:10,240 in DMEM without fetal bovine serum (FBS) in wells of a 96-well microtiter plate (Costar, Corning). Approximately 100 IU of WNV_{NY99} or WNV_{KOU} diluted in DMEM with 2% FBS was added to each well containing diluted serum, and plates were incubated at 37°C for an hour. Test sera were tested in a virus neutralization assay against both the homologous virus and heterologous virus. Low-passage-number Vero cells (passage 14 to 15) then were added to each well at a density of 2×10^5 cells/plate, and the plates were incubated for 5 days at 37°C in a humidified CO_2 incubator before microscopic examination for CPE. Cells also were added to a 1:10 dilution of each serum in the absence of virus to ensure that the samples were not toxic to the cells. The neutralization titer was expressed as the reciprocal of the highest serum dilution where CPE was recorded (39). The cells were fixed *in situ* in the plates and subjected to an ELISA protocol using an anti-flaviviral antibody, MAb 6B6C1 conjugated to horseradish peroxidase (HRP) (40), to confirm viral replication.

Assessment of inflammatory mediators. A commercial BD cytometric bead array (CBA) mouse inflammation kit (catalog no. 552364; BD Biosciences Pharmingen, San Diego, CA) was used to determine the levels (pg/ml) of interleukin-6 (IL-6), IL-10, monocyte chemoattractant protein-1 (MCP-1), gamma interferon ($\text{IFN-}\gamma$), tumor necrosis factor (TNF), and IL-12p70 in serum samples and brain homogenates of mice infected with 100 IU and terminated on days 1, 3, 5, and 7 postinfection. Fluorescence was analyzed using a flow cytometer (BD Accuri C6; BD Biosciences, San Jose, CA), and cytokine levels were determined using BD Accuri C6 software.

Histology. Animals infected with 100 IU and designated for histological analyses sequentially were perfused with chilled PBS and 4% paraformaldehyde in PBS via a transcardial approach. Brain and spinal cord then were postfixed in 4% paraformaldehyde overnight at 4°C , after which they were routinely processed for paraffin embedding. Five-micron sections were stained with hematoxylin and eosin (HE) and Luxol fast blue (FLB) and examined on a Nikon Eclipse 51E microscope equipped with a Nikon DS-Fi1 camera with a DS-U2 unit and NIS elements F software. Microphotographs are reproduced without manipulation other than cropping and adjustment of light intensity.

IHC. Immunohistochemistry (IHC) detection of flavivirus antigen was carried out as previously described (41), with a modification of the antigen retrieval protocol. Briefly, 5- μm sections of the paraformaldehyde-fixed, paraffin-embedded brain and spinal cord specimens were collected onto charged slides, heat treated, and deparaffinized. The sections were subjected to antigen retrieval by heating in an EDTA-Tris solution, pH 9.0 (Target Retrieval; Dako, Carpinteria, CA, USA), at 96°C for 25 min and allowed to cool to room temperature, followed by three blocking steps at room temperature: (i) 10-min incubation with Dako hydrogen peroxidase block, (ii) 15-min incubation with 0.1 M glycine in PBS, and (iii) 30-min incubation with Dako antibody dilution agent (Dako), with brief Tris-buffered saline-Tween 20 (TBST) rinses between each step. This

TABLE 2 Envelope protein glycosylation and plaque phenotype of West Nile virus and West Nile-like viruses

Virus	Sequence	Glycosylation status ^b	Avg plaque size ^a (mm)
MRM16	NYF	—	1.3
Hu6774	NYS	+	2.2
Sarawak	NYP	—	1.0
NY99	NYS	+	3.0
Koutango	NFP	—	1.2
B956	Deletion	—	0.8
Sarafend	NYS	+	2.2

^a Plaque size represents the diameter at 4 days postinfection.
^b Glycosylation motif located at amino acid positions 154 to 156 of the envelope protein. Glycosylation was determined by PNGase F digestion and sequence analysis.

was followed by 2 h of incubation with neat 4G4-hybridoma culture supernatant (flavivirus anti-nonstructural protein 1) (38) at room temperature. After multiple TBST rinses over a 10- to 15-min period, antibody binding was visualized using a Dako Envision anti-mouse kit. IHC for the apoptosis marker activated caspase 3 was performed as previously described (42). The slides were examined and micrographed as described above.

Data analysis. Levels of infectious virus in growth kinetics were assessed using analysis of variance (ANOVA). Mean virus titers were compared between viruses using the Tukey method for pairwise multiple comparisons. The significance of clinical differences *in vivo* between groups was calculated by Kaplan-Meier analysis and analyzed by log-rank test. Differences in viral burden were analyzed by Mann-Whitney test. All statistical analysis was performed in Graphpad Prism 5.0 (GraphPad Software Inc., San Diego, CA).

RESULTS

Glycosylation status of WNV strains and West Nile-like viruses. N-linked glycosylation of the envelope protein has been established as an important determinant of WNV neuroinvasion in murine models (19). Therefore, the presence of a carbohydrate molecule on the E protein, indicative of a glycosylated virus, was tested for all WNV strains and West Nile-like viruses included in this study by both sequence analysis over this region (residues 154 to 156) (Table 2) and PNGase F digestion of infected cell lysates (results not shown). The viral strains, NY99, Hu6774, and Sarafend, contained an NYS sequence at residues 154 to 156 of the E protein, which exhibited a band shift in Western blotting following PNGase F digestion indicative of a glycosylated E protein. Other viruses contained either an NYF (MRM16), NYP (Sara-

wak), or NFP (Koutango) sequence, and the E protein in lysates from these viruses did not exhibit a band shift following PNGase F digestion, consistent with an unglycosylated E protein. The B956 strain contained a deletion across residues 154 to 156, which also leads to an unglycosylated E protein (Table 2).

Glycosylated viral strains grow more efficiently *in vitro*. To determine the ability of WNV and West Nile-like viruses to replicate *in vitro*, growth kinetics of all viral strains were assessed in Vero cells (Fig. 1A) and a clinically relevant cell line derived from human brain microvascular endothelial cells (HBMECs) (Fig. 1B). In general, the glycosylated strains (WNV_{NY99}, WNV_{Sarafend}, and WNV_{Hu6774}) grew more efficiently than the unglycosylated viruses (WNV_{B956}, WNV_{KOU}, and WNV_{MRM16}), with the most notable difference in replication observed between 12 and 36 h postinfection. During this time period, glycosylated viruses replicated in Vero cells to approximately 7 logs IU/ml, whereas the unglycosylated virus replication levels were significantly lower, at approximately 4 logs IU/ml ($P < 0.05$ at 24 h postinfection). The exception was the unglycosylated virus WNV_{Sarawak}, which appeared to grow as efficiently as the glycosylated strains in both Vero cells and HBMECs. Glycosylated viruses also exhibited a large-plaque phenotype (2.2 to 3 mm) compared to unglycosylated viruses (0.8 to 1.3 mm) (Table 2). Overall, glycosylated viruses were able to grow more efficiently in cells *in vitro*.

Virulence properties of WNV strains and West Nile-like viruses in mice. We have previously demonstrated that a young adult mouse model allows differentiation between virulent and attenuated strains of WNV (4). Four- to five-week-old mice were injected with a variety of doses, from 0.1 to 10,000 IU of each viral strain, to assess their virulence phenotype (Table 3). Interestingly, WNV_{KOU} was consistently the most virulent of all strains tested, with the shortest time to death of 6.8 days and inducing 100% mortality at 100 IU after three independent experiments (Table 3 and Fig. 2). WNV_{KOU}-infected mice appeared to succumb to seizures prior to humane endpoints. This symptom was observed repeatedly from day 4 postinfection and always in mice that ultimately were euthanized due to the onset of CNS symptoms (e.g., hind-limb paralysis). Seizures were observed in routine health checks, and occasionally WNV_{KOU}-infected mice experienced severe seizures leading to death. Despite WNV_{KOU} being significantly more virulent than WNV_{NY99} ($P < 0.05$ at 100 IU), producing more morbidity in a shorter time, further experiments testing additional doses determined the viruses had similar LD₅₀ values of 0.5 and 0.7, respectively (Table 3). WNV_{NY99} and

FIG 1 Growth kinetics of WNV strains in Vero (A) and human brain microvascular endothelial cells (HBMECs) (B). Cells were infected at an MOI of 0.1, and the titer of the virus in the supernatant was determined as TCID₅₀ on Vero cells every 12 h until 72 h p.i. Growth curves were statistically different ($P < 0.05$) in HBMECs between NY99 and Koutango, NY99 and B956, Koutango and Sarafend, Koutango and Hu6774, and B956 and Hu6774 viruses.

TABLE 3 Virulence of West Nile virus and West Nile-like viruses included in the study in 4- to 5-week-old Swiss mice following i.p. injection

Virus and dose (IU ^a)	Mortality (no. died/total no. [%])	AST ^b (days)	LD ₅₀ ^c
NY99			
1,000	7/10 (70)	11.8	0.49
100	30/30 (100)	8.2 ^d	
10	10/10 (100)	8.9	
1	7/10 (70)	12.9	
0.1	8/20 (40)	17	
MRM16			
10,000	3/10 (30)	17.2	>10,000
1,000	2/10 (20)	19	
100	2/10 (20)	18.7	
Hu6774			
10,000	2/10 (20)	18.2	>10,000
1,000	3/10 (30)	18.2	
100	1/10 (10)	19.5	
Sarafend			
1,000	8/10 (80)	11.2	1
100	10/10 (100)	9.2	
10	9/10 (90)	10.9	
1	5/10 (50)	15.4	
B956			
10,000	0/10 (0)	21	>10,000
1,000	0/10 (0)	21	
100	2/10 (20)	19.6	
Sarawak			
10,000	2/10 (20)	19	>10,000
1,000	0/10 (0)	21	
100	1/10 (10)	19.6	
Koutango			
1,000	10/10 (100)	7.6	0.72
100	30/30 (100)	6.8 ^d	
10	10/10 (100)	7.6	
1	8/10 (70)	11.4	
0.1	6/20 (30)	18.4	

^a IU, infectious units.^b AST, average survival time.^c LD₅₀, 50% lethal dose.^d *P* = 0.01 (comparison between NY99 and Koutango strains at 100 IU).

WNV_{Sarafend} both were virulent in this mouse model, causing 100% mortality at a dose of 100 IU (Fig. 2A) and 70% and 80% mortality, respectively, at a dose of 1,000 IU (Fig. 2B), in line with what has been reported previously (17, 35). WNV_{Sarafend} was comparatively less virulent than WNV_{NY99}, as demonstrated by a longer time to death of 9.2 days at 100 IU (Table 3). WNV_{KUN} strains MRM16 and Hu6774, WNV_{Sarawak}, as well as WNV_{B956} all were attenuated in this model at all doses tested (Table 3 and Fig. 2). However, all viruses that displayed an attenuated phenotype in the adult model were neuroinvasive in the more sensitive weanling mouse model (18 to 19 days old; data not shown), consistent with our previous observations with attenuated WNV strains (4). Since the West Nile-like virus WNV_{Sarawak} was not virulent in the young adult model, further characterization of this strain was not pur-

sued. Instead, we focused on WNV_{KOU} to determine a mechanism(s) for its enhanced virulence. Collectively, these data have identified a virulent strain of WNV outside the two major lineages.

Viral clearance of WNV_{KOU} is delayed in extraneural tissues.

To investigate the contribution of virus replication to overall virulence, 4- to 5-week-old mice were challenged with selected viruses at a dose of 100 IU in order to determine levels of virus replication and clearance in CNS and peripheral tissues (Fig. 3). WNV_{NY99} caused a detectable viremia from day 1 postinfection that peaked at day 3, with virus cleared from the blood by day 5 postinfection (Fig. 3A). A similar pattern was observed for WNV_{Sarafend}; however, levels of virus were lower than those of WNV_{NY99}, consistent with WNV_{Sarafend} exhibiting comparatively longer average survival times (Table 3). The attenuated prototype strain WNV_{B956} was included as a nonneuroinvasive control, and no virus was detected at any time point assessed. Interestingly, WNV_{KOU} caused a delayed but prolonged viremia which peaked at day 5 postinfection, but virus was still present at the last time point sampled, day 7 postinfection (Fig. 3A). A similar pattern also was seen in the spleen, where similar levels of virus were present on both days 3 and 5 postinfection for WNV_{KOU} (Fig. 3C), indicating that there was delayed viral clearance from this organ. Conversely, while virus was present on day 3 in both WNV_{NY99}- and WNV_{Sarafend}-challenged mice, levels of virus were significantly reduced by day 5 in the case of WNV_{NY99} or were no longer detectable in the case of WNV_{Sarafend}. These results suggest an inability of WNV_{KOU}-infected mice to clear the virus, in contrast to WNV_{NY99}- or WNV_{Sarafend}-infected mice, indicative of different virus-host interactions between these virulent viruses.

Viral replication in the central nervous system is similar for both WNV_{NY99} and WNV_{KOU}. Virus replication in the CNS was investigated to determine whether differences in pathogenesis also extend to this immune-privileged site (Fig. 3B and D). High levels of virus were detected for WNV_{NY99} on days 5 and 7 postinfection, with little reduction in viral load between these days. While levels for WNV_{KOU} were slightly lower, significant differences were not observed between the two viruses. For WNV_{Sarafend}, virus was not detected until day 7 postinfection. The prototype strain, WNV_{B956}, did not have detectable virus in the CNS, suggesting this virus was unable to invade the CNS. Collectively, these data suggest that differences in virulence between WNV_{NY99} and WNV_{KOU} involve mechanisms that function not only in the CNS but also in the periphery.

WNV_{KOU} induces poor neutralizing antibody responses.

Neutralizing antibody responses play an essential role in the control of severe WNV infection and can be detected as early as day 4 postinfection for WNV (43, 44). Therefore, we investigated differences in the levels of neutralizing antibodies produced following infection with WNV_{NY99} and WNV_{KOU} at 7, 14, and 21 days postinfection using a sublethal dose of 1 IU to ensure enough survivors at each time point for testing. There were no detectable neutralizing antibodies for mice infected with WNV_{KOU} at 7 or 14 days postinfection, whereas mice infected with WNV_{NY99} had detectable neutralizing antibody responses of 67 and 533 at days 7 and 14, respectively (Table 4). Low levels of neutralizing antibodies were detected for WNV_{KOU}-infected mice at 21 days postinfection (average, 40) compared to WNV_{NY99}-infected mice, which had relatively high levels of neutralizing antibodies (average, 2,560). Thus, while WNV_{KOU} infection did elicit an antibody response in mice, reaching a titer of 533 by day 21 postinfection, this

FIG 2 Survival curves of 4- to 5-week-old Swiss mice following intraperitoneal infection with 100 IU (A) or 1,000 IU (B) per mouse ($n = 10$ per group). Mice were monitored for 21 days after injection for signs of encephalitis and then euthanized. Survival differences at the dose of 1,000 IU were statistically significant between NY99 and MRM16 as well as NY99 and Sarawak, NY99 and B956, Sarafend and MRM16, and Sarafend and Hu6774 ($P < 0.01$); and Koutango and Sarawak, Koutango and B956, Koutango and MRM16, and Koutango and Hu6774 ($P < 0.0001$) viruses. Survival differences at the dose of 100 IU were statistically significant between NY99 and Sarawak, NY99 and B956, NY99 and MRM16, NY99 and Hu6774, Koutango and Sarafend, Koutango and Sarawak, Koutango and B956, Koutango and MRM16, Koutango and Hu6774, Sarafend and B956, Sarafend and MRM16, and Sarafend and Hu6774 ($P < 0.0001$); and NY99 and Koutango ($P < 0.05$) viruses.

response was still considerably lower than that in WNV_{NY99}-infected mice at the same time point (average, 5,120). Also interesting was the observation that antibodies produced in WNV_{NY99}-infected mice were able to neutralize WNV_{KOU}, albeit to a lower titer than for its cognate virus, while no WNV_{NY99}-neutralizing

activity was found in the sera of the WNV_{KOU}-infected animals (Table 4). Collectively, the data obtained on antibody responses postinfection suggest that WNV_{KOU} is less able to induce critical neutralizing antibodies that are essential for the control of infection.

FIG 3 Replication of selected WNV and WNV-like strains (NY99, Sarafend, B956, and Koutango viruses) were compared in the periphery (spleen and sera) and in the central nervous system (brain and spinal cord). Each point represents the means $\pm$ SEM of the log₁₀ IU per gram of tissue or per ml for sera from three animals. The exception is day 7 postinfection, where only 2 animals were available for titration for WNV_{NY99}- and WNV_{KOU}-infected mice. An asterisk indicates differences that are statistically significant ($P < 0.05$).

TABLE 4 Average antibody responses following viral infection

Infecting virus ^a	Day p.i.	Ab response to ^b :			
		NY99 (avg total Ab)	NY99 (avg neutralizing Ab)	Koutango (avg total Ab)	Koutango (avg neutralizing Ab)
NY99	7	426	67	10	20
NY99	14	960	533	40	40
NY99	21	5,120	2,560	170	160
Koutango	7	20	<10	20	<10
Koutango	14	53	<10	87	<10
Koutango	21	266	<10	533	40

^a Mice were infected with 1 IU of virus. Three mice were bled per time point.

^b Total antibody responses were determined by fixed-cell ELISA, and neutralizing antibody responses were determined by virus neutralization assay.

Cytokine responses, histopathology, and viral antigen distribution. To further investigate differences in pathology between WNV_{NY99}- and WNV_{KOU}-infected mice, tissues were harvested on days 3 and 7 postinfection with 100 IU of virus to study levels of

viral antigen and development of pathology. Mice terminated on day 3 p.i. were devoid of gross and histological changes in the CNS. However, by day 7 p.i., meningoencephalitis was evident in both the WNV_{NY99}- and WNV_{KOU}-infected mice, with more severe changes seen in the latter group (Fig. 4 and 5). Both groups of animals exhibited meningeal and perivascular infiltration of lymphocytes, monocytes, some neutrophils, and mild to moderate gliosis, but these features were more exaggerated in the WNV_{KOU}-infected animals than the WNV_{NY99}-infected animals, with the former also featuring marked neuropil infiltration of leukocytes (compare Fig. 4C to D). Moreover, while the spinal cord was histologically unremarkable in the WNV_{NY99}-infected animals, there was marked loss of motor neurons and axonal degeneration in the spinal cord of the WNV_{KOU}-infected mice (Fig. 5). However, apoptosis did not appear to be a feature of this loss, as no cells positive for activated caspase 3 were detected by IHC (data not shown).

Regional distribution of viral antigen was similar in WNV_{NY99}- and WNV_{KOU}-infected mice, but the immunolabeling was notably more intense and widespread in WNV_{KOU}-infected mice than

FIG 4 Meningoencephalitis in mice infected with WNV_{NY99} (A and C) and WNV_{KOU} (B and D). While meningeal infiltration, perivascular cuffing, and neuropil infiltration of lymphocytes and monocytes are present in mice infected with either virus, the inflammatory infiltrates are more severe in the WNV_{KOU}-infected animals. HE staining was used. Original magnifications are $\times 40$ for panels A and B and $\times 200$ for panels C and D.

FIG 5 WNV_{KOU} causes notable myelitis (B and D) compared to WNV_{NY99} (A and C), with loss of motor neurons and mild demyelination leaving clear spaces in the neuropil (B and D). Panels A and B show HE stain at an original magnification of $\times 100$. Panels C and D show Luxol fast blue stain at an original magnification of $\times 100$.

in WNV_{NY99}-infected mice, indicating greater viral protein accumulation (compare Fig. 6A and C to B and D), particularly in the thalamus and basal ganglia (Fig. 6C and D). Since the antibody used for the IHC, 4G4, binds to cultured cells infected at a similar MOI for these two viruses, WNV_{NY99} and WNV_{KOU}, and with similar affinity (data not shown), it is unlikely that the difference in intensity seen during IHC analysis is due to differences in the affinity of monoclonal antibody binding to a particular virus.

The marked differences in histopathology and viral clearance in extraneural tissues between WNV_{NY99}- and WNV_{KOU}-infected mice prompted us to examine the cytokine profile in serum and brain of a small number of animals infected with 100 IU of either virus (three animals per virus on days 1, 3, and 5 postinfection and two animals per virus on day 7 postinfection), using a multiplex Luminex assay for IL-6, IL-10, IL-12p70, TNF- α , IFN- γ , and MCP-1 (CCL2). Expression of MCP-1 increased steadily to an average of 5.4×10^3 pg/ml of brain homogenate and 1.48×10^3 pg/ml in serum in the WNV_{NY99}-infected mice on day 7 postinfection. In contrast, the MCP-1 levels in WNV_{KOU}-infected animals remained low or undetectable in serum and peaked at $1.27 \times$

10^3 pg/ml in brain homogenate on day 5 postinfection. Similar patterns of expression were noted for IL-10, IL-12, and IFN- γ , although overall the levels were lower, while there was no detectable TNF- α in WNV_{KOU}-infected animals at any time postinfection and only a single WNV_{NY99}-infected animal had TNF- α in brain homogenate (165.3 pg/ml) on day 7 postinfection. In contrast, the WNV_{KOU}-infected animals had a very marked IL-6 response on day 5 postinfection (average, 2,529 pg/ml), exceeding the response in WNV_{NY99}-infected animals (average, 152 pg/ml). These data suggest that mice infected with WNV_{KOU} are less responsive overall with respect to innate defense mechanisms than WNV_{NY99}-infected mice.

Sequence differences between WNV_{NY99} and WNV_{KOU} genomes. To identify whether known virulence determinants also were present in the WNV_{KOU} genome that could contribute to the enhanced virulence phenotype, comparisons between published sequences WNV_{NY99} (accession number [HQ596519](#)) and WNV_{KOU} (accession number [EU082200](#)) were performed. Sequence comparison between WNV_{NY99} and WNV_{KOU} revealed 89.2% identity at the amino acid level, with a total of 371 amino

FIG 6 Immunolabeling for WNV antigen in WNV_{NY99} (A and C)- and WNV_{KOU} (B and D)-infected mice. Paraformaldehyde-fixed, paraffin-embedded tissues were immunolabeled with the flavivirus-NS1-specific MAb 4G4, and binding was visualized with the Envision reagents, leaving virus-antigen-positive cells with a reddish-brown coloration. While distribution of virus antigen was similar for the two viruses, the labeling intensity and number of positive cells were more pronounced in the WNV_{KOU}-infected mice (B and D) than in the WNV_{NY99}-infected mice (A and C). Images in panels A and B are hippocampus at an original magnification of $\times 40$. Images in panels C and D are thalamus at an original magnification of $\times 100$.

acid substitutions with 173 nonconservative changes (Table 5). The structural genes accounted for 103 amino acid changes (54 nonconservative), while the remaining 268 changes (119 nonconservative) were located in the nonstructural region. Interestingly, known virulence determinants for WNV_{NY99} (19–21) were not shared with WNV_{KOU}, including amino acids at positions 72 in prM (S in WNV_{NY99} and M in WNV_{KOU}), 154 to 156 in E (NYS in WNV_{NY99} and NFP in WNV_{KOU}), 249 in NS3 (P in WNV_{NY99} and T in WNV_{KOU}), and 653 in NS5 (F in WNV_{NY99} and S in WNV_{KOU}). Particular areas of the genome with accumulated amino acid changes between WNV_{NY99} and WNV_{KOU} that may represent important virulence motifs include E domain III (10 nonconservative changes) and the prM protein (13 nonconservative changes).

DISCUSSION

With the increasing global activity of West Nile virus, including the emergence of lineage II strains, causing widespread disease across Europe (8, 9), and the emergence of the first virulent strain

of WNV to cause an outbreak in Australia (4), there is a growing need to better understand the ability of different WNV strains to cause disease. This knowledge will lay the foundation for more effective therapeutics or vaccine candidates. While an extensive body of work exists on the pathogenesis of lineage I and II strains in mice, particularly the North American strains, little is known about the capacity for disease induction for WNV strains proposed to branch outside these major lineages. In this study, we have investigated the capacity of a proposed lineage VI strain (WNV_{Sarawak}) and a proposed lineage VII strain (WNV_{KOU}) to cause disease in an established murine model of WNV disease.

WNV_{KOU} was first isolated in 1968 from the blood of a gerbil (*Tatera kempi*) in the Koutango region of Senegal and later from gerbils in Somalia (45). WNV_{KOU} also has been found in *Mastomys* species and *Lemmyscomys* species in Senegal and the Central African Republic (46). *Aedes aegypti* mosquitoes have been confirmed as a competent vector for WNV_{KOU} (47); therefore, it is expected that WNV_{KOU} can be transmitted by vectors similar to those of other WNV strains. However, since the only isolations of

TABLE 5 Amino acid differences between viruses NY99-4132 and Koutango Dak ArD 5443

Gene and aa position in polyprotein (corresponding protein position)	NY99 4132 virus ^a amino acid	Koutango Dak ArD 5443 virus ^b amino acid	No. of substitutions in each gene (no. of nonconservative changes), % total changes
C			13 (6), 46
11 (11)	S	N	
25 (25)	L	P	
63 (63)	R	K	
71 (71)	G	K	
100 (100)	S	T	
108 (108)	K	T	
111 (111)	I	L	
113 (113)	V	C	
114 (114)	M	I	
115 (115)	I	L	
117 (117)	L	M	
118 (118)	I	L	
120 (120)	S	T	
prM			27 (15), 56
126 (3)	L	F	
128 (5)	N	S	
132 (9)	K	R	
141 (18)	D	E	
143 (20)	T	A	
147 (24)	T	S	
150 (27)	T	I	
152 (29)	A	S	
156 (33)	L	Q	
158 (35)	I	T	
162 (39)	M	I	
181 (58)	A	P	
193 (70)	T	D	
195 (72)	S	M	
197 (74)	V	I	
205 (82)	T	I	
206 (83)	K	R	
207 (84)	T	G	
208 (85)	R	K	
209 (86)	H	N	
210 (87)	S	P	
235 (112)	M	L	
264 (141)	I	L	
270 (147)	S	G	
273 (150)	M	T	
279 (156)	V	A	
280 (157)	V	I	
E			63 (33), 52
313 (23)	L	I	
321 (31)	V	I	
333 (43)	V	I	
336 (46)	M	L	
337 (47)	N	S	
339 (49)	E	S	
341 (51)	A	T	
354 (64)	T	A	
355 (65)	V	I	
358 (68)	L	P	
378 (88)	P	S	
380 (90)	F	Y	
383 (93)	R	K	

TABLE 5 (Continued)

Gene and aa position in polyprotein (corresponding protein position)	NY99 4132 virus ^a amino acid	Koutango Dak ArD 5443 virus ^b amino acid	No. of substitutions in each gene (no. of nonconservative changes), % total changes
410 (120)	A	T	
414 (124)	K	Q	
416 (126)	I	V	
418 (128)	R	K	
419 (129)	T	I	
421 (131)	L	Q	
430 (140)	A	S	
439 (149)	V	L	
445 (155)	Y	F	
446 (156)	S	P	
449 (159)	V	R	
450 (160)	G	T	
456 (166)	R	T	
457 (167)	F	I	
459 (169)	I	V	
460 (170)	T	S	
462 (172)	A	S	
466 (176)	Y	S	
472 (182)	E	D	
478 (188)	V	I	
486 (196)	I	V	
489 (199)	N	S	
498 (208)	T	S	
500 (210)	T	S	
516 (226)	S	A	
518 (228)	A	P	
520 (230)	S	A	
521 (231)	T	S	
522 (232)	V	T	
530 (240)	M	L	
543 (253)	I	V	
566 (276)	S	N	
568 (278)	T	N	
600 (310)	K	Q	
602 (312)	L	H	
604 (314)	T	H	
618 (328)	Q	K	
651 (361)	F	Y	
653 (363)	S	A	
654 (364)	V	S	
655 (365)	A	S	
656 (366)	T	A	
660 (370)	K	I	
680 (390)	E	D	
700 (410)	T	A	
702 (412)	L	V	
724 (434)	V	I	
728 (438)	V	L	
757 (467)	A	V	
769 (479)	R	K	
NS1			43 (21), 49
800 (9)	S	T	
820 (29)	M	V	
825 (34)	Y	F	
826 (35)	Y	H	
837 (46)	I	V	
842 (51)	K	A	

(Continued on following page)

TABLE 5 (Continued)

Gene and aa position in polyprotein (corresponding protein position)	NY99 4132 virus ^a amino acid	Koutango Dak ArD 5443 virus ^b amino acid	No. of substitutions in each gene (no. of nonconservative changes), % total changes
845 (54)	V	I	32 (14), 44
848 (57)	L	I	
861 (70)	A	S	
862 (71)	V	I	
884 (93)	Q	H	
896 (105)	T	P	
897 (106)	A	S	
904 (113)	I	M	
914 (123)	L	I	
937 (146)	Q	S	
938 (147)	N	R	
944 (153)	L	M	
960 (169)	L	M	
965 (174)	S	I	
983 (192)	L	Q	
985 (194)	I	V	
999 (205)	R	G	
1010 (219)	E	D	
1025 (234)	D	E	
1026 (235)	G	S	
1027 (236)	I	V	
1031 (240)	D	E	
1036 (245)	V	I	
1042 (251)	R	K	
1052 (261)	K	M	
1066 (275)	I	L	
1075 (284)	T	K	
1078 (287)	L	V	
1080 (289)	E	A	
1081 (290)	S	L	
1084 (293)	H	R	
1106 (315)	S	T	
1117 (326)	D	E	
1129 (338)	Q	T	
1134 (343)	K	R	
1140 (349)	Q	K	
1142 (351)	N	E	
NS2A			
1177 (34)	M	V	
1181 (38)	L	M	
1182 (39)	I	V	
1186 (43)	V	T	
1197 (54)	V	L	
1198 (55)	L	V	
1201 (58)	V	I	
1211 (68)	S	A	
1239 (96)	K	R	
1240 (97)	A	S	
1255 (112)	V	A	
1262 (119)	H	Q	
1264 (121)	A	C	
1265 (122)	R	H	
1266 (123)	Q	H	
1267 (124)	I	L	
1268 (125)	L	I	
1269 (126)	L	T	
1290 (147)	T	S	

TABLE 5 (Continued)

Gene and aa position in polyprotein (corresponding protein position)	NY99 4132 virus ^a amino acid	Koutango Dak ArD 5443 virus ^b amino acid	No. of substitutions in each gene (no. of nonconservative changes), % total changes
1301 (158)	L	M	9 (2), 22
1310 (167)	R	K	
1311 (168)	R	C	
1324 (181)	V	I	
1326 (183)	I	V	
1330 (187)	I	L	
1333 (190)	K	R	
1335 (192)	S	N	
1336 (193)	A	S	
1342 (199)	G	S	
1355 (212)	L	A	
1359 (216)	M	L	
1366 (223)	I	L	
NS2B			
1400 (26)	I	V	
1433 (59)	D	N	
1435 (61)	S	T	
1438 (64)	S	N	48 (19), 40
1449 (75)	V	I	
1459 (85)	F	Y	
1473 (99)	M	I	
1481 (107)	I	V	
1494 (120)	V	I	
NS3			
1538 (33)	S	N	
1592 (87)	H	Q	
1597 (92)	Q	V	
1614 (109)	V	I	
1615 (110)	R	Q	
1620 (115)	V	I	
1660 (155)	I	V	
1667 (162)	I	V	
1677 (172)	D	E	
1680 (175)	I	A	
1682 (177)	A	V	
1708 (203)	R	K	
1720 (215)	R	K	
1725 (220)	A	V	
1754 (249)	P	T	
1758 (253)	N	S	
1772 (267)	T	H	
1778 (273)	P	S	
1811 (306)	E	S	
1827 (322)	T	S	
1829 (324)	D	E	
1836 (331)	S	A	
1841 (336)	L	I	
1852 (347)	S	T	
1861 (356)	T	V	
1863 (358)	K	R	
1873 (368)	M	L	
1887 (382)	K	R	
1889 (384)	V	I	
1940 (435)	I	L	
1951 (446)	E	D	

(Continued on following page)

TABLE 5 (Continued)

Gene and aa position in polypeptide (corresponding protein position)	NY99 4132 virus ^a amino acid	Koutango Dak ArD 5443 virus ^b amino acid	No. of substitutions in each gene (no. of nonconservative changes), % total changes
1954 (449)	A	S	22 (11), 50
1955 (450)	V	I	
1972 (467)	S	A	
1991 (486)	F	S	
2011 (506)	I	V	
2014 (509)	F	L	
2016 (511)	Q	E	
2023 (518)	Y	F	
2024 (519)	T	S	
2045 (540)	T	A	
2053 (548)	A	S	
2061 (556)	V	I	
2067 (562)	R	K	
2074 (569)	R	K	
2075 (570)	T	V	
2078 (573)	I	V	
2102 (597)	I	T	
NS4A			28 (13), 46
2127 (3)	I	V	
2129 (5)	L	V	
2135 (11)	K	R	
2142 (18)	G	T	
2145 (21)	W	M	
2148 (24)	L	F	
2178 (54)	I	V	
2179 (55)	A	V	
2186 (62)	V	I	
2188 (64)	T	S	
2189 (65)	M	L	
2193 (69)	F	L	
2209 (85)	A	V	
2213 (89)	V	T	
2221 (97)	A	S	
2222 (98)	E	D	
2224 (100)	P	S	
2234 (110)	L	M	
2259 (135)	V	I	
2264 (140)	V	I	
2265 (141)	M	L	
2266 (142)	T	A	
NS4B			28 (13), 46
2276 (3)	M	L	
2281 (8)	K	R	
2284 (11)	S	N	
2288 (15)	S	N	
2291 (18)	G	A	
2293 (20)	R	G	
2294 (21)	I	V	
2296 (23)	V	K	
2300 (27)	F	L	
2301 (28)	S	N	
2302 (29)	M	I	
2314 (41)	A	S	
2321 (48)	T	S	
2369 (96)	L	F	
2373 (100)	A	V	

TABLE 5 (Continued)

Gene and aa position in polypeptide (corresponding protein position)	NY99 4132 virus ^a amino acid	Koutango Dak ArD 5443 virus ^b amino acid	No. of substitutions in each gene (no. of nonconservative changes), % total changes
2387 (114)	A	T	86 (39), 45
2427 (154)	I	M	
2449 (176)	I	V	
2450 (177)	M	I	
2456 (183)	L	I	
2459 (186)	V	L	
2460 (187)	V	I	
2466 (193)	K	R	
2476 (203)	T	S	
2477 (204)	A	S	
2513 (240)	I	V	
2518 (245)	I	V	
2521 (248)	M	L	
NS5			
2539 (11)	V	F	
2543 (15)	R	K	
2548 (21)	K	R	
2553 (25)	T	N	
2561 (33)	I	V	
2565 (38)	S	L	
2573 (45)	K	R	
2602 (74)	V	I	
2657 (129)	G	K	
2663 (135)	R	K	
2667 (139)	C	S	
2682 (154)	A	P	
2684 (156)	V	I	
2690 (162)	I	L	
2691 (163)	R	K	
2705 (177)	R	K	
2709 (181)	V	I	
2722 (194)	K	R	
2725 (197)	L	V	
2757 (229)	V	I	
2760 (232)	S	A	
2762 (234)	N	S	
2775 (247)	R	K	
2802 (274)	S	N	
2805 (277)	S	G	
2809 (281)	N	R	
2815 (287)	R	K	
2816 (288)	R	Q	
2824 (296)	H	L	
2826 (298)	E	P	
2840 (312)	D	E	
2901 (373)	K	R	
2902 (374)	Y	K	
2905 (377)	N	D	
2909 (381)	N	D	
2913 (385)	A	N	
2916 (388)	A	K	
2920 (392)	R	K	
2925 (397)	S	T	
2931 (403)	R	S	
2950 (422)	R	S	
2966 (438)	D	E	

(Continued on following page)

TABLE 5 (Continued)

Gene and aa position in polypeptide (corresponding protein position)	NY99 4132 virus ^a amino acid	Koutango Dak ArD 5443 virus ^b amino acid	No. of substitutions in each gene (no. of nonconservative changes), % total changes
2967 (439)	E	Q	
3048 (520)	I	V	
3054 (526)	T	G	
3055 (527)	R	K	
3074 (546)	R	K	
3081 (553)	A	G	
3085 (557)	E	R	
3086 (558)	L	F	
3088 (560)	D	E	
3093 (565)	R	L	
3116 (588)	D	N	
3118 (590)	R	K	
3160 (632)	I	V	
3163 (635)	D	E	
3169 (641)	T	G	
3173 (645)	G	E	
3174 (646)	P	A	
3175 (647)	K	R	
3177 (649)	R	K	
3178 (650)	T	S	
3181 (653)	F	S	
3231 (703)	T	V	
3234 (706)	Y	H	
3238 (710)	Q	Y	
3247 (719)	T	A	
3268 (740)	V	I	
3282 (754)	R	K	
3283 (755)	D	E	
3300 (772)	F	L	
3318 (790)	V	M	
3333 (805)	G	K	
3344 (816)	E	A	
3353 (825)	E	D	
3365 (837)	K	R	
3388 (860)	A	P	
3397 (869)	Q	H	
3398 (870)	V	A	
3413 (885)	V	I	
3418 (890)	S	T	
3420 (892)	K	R	
3425 (897)	T	A	
3426 (898)	T	S	
3427 (899)	L	V	
3441 (903)	T	S	
Total			371 (173), 47

^a Accession number [HQ596519](#).^b Accession number [EU082200](#).

WNV_{KOU} have been from gerbils, which are not commonly associated with WNV strains from other lineages, the transmission cycle of WNV_{KOU} may be ecologically distinct. Despite being included in a number of phylogenetic studies of WNV, little is known about this virus, including its ability to cause infection in humans or animals. While a single symptomatic human infection has been reported in a laboratory worker in Senegal, there have been no natural human cases attributed to WNV_{KOU} (48). How-

ever, an unusual outbreak of neurological disease occurred in Sudan in 2002 in areas relatively close to the region where the Koutango virus was first isolated. During this outbreak, the causative agent was deemed to be a West Nile-like virus, but no viral isolates were obtained, precluding further genetic analysis (49). This outbreak was atypical in that it occurred solely in children and also was associated with severe neurological involvement, including seizures, a symptom prominent in adult mice infected with WNV_{KOU} in this study.

WNV_{Sarawak} was isolated in 1966 from *Culex pseudovishnui* mosquitoes collected in Sarawak, Borneo, and originally was classified as a strain of Kunjin virus (50). Further characterization of this virus led to its reclassification into lineage VI (51, 52). WNV_{Sarawak} is an example of the sporadic activity of WNV in southeast Asia and has not been associated specifically with disease in humans or animals. Results from this study suggest that this virus is attenuated in established murine models; thus, it is less likely to pose a risk to humans. WNV_{Sarawak} contains an unglycosylated envelope protein at residues 154 to 156 that has previously been reported to be an important determinant of virulence for lineage I strains (19, 22, 24, 53). Glycosylation of the envelope protein also has been associated with increased particle secretion and infectivity, promoting more efficient packaging and replication (25). Therefore, based on previous studies using lineage I strains, it was expected that WNV_{Sarawak} would have replicated at the same efficiency as other unglycosylated strains. However, WNV_{Sarawak} replicated as efficiently as other glycosylated WNV strains. Collectively, the *in vitro* and *in vivo* data for WNV_{Sarawak} question the contribution of the glycosylated envelope protein alone in terms of virulence for viruses branching outside the major lineages.

The argument is further strengthened with survival and virulence data for the unglycosylated lineage VII virus, WNV_{KOU}. While in cell culture this virus was not able to replicate to the same efficiency as its glycosylated counterparts, *in vivo* data suggest that WNV_{KOU} is more virulent than the North American NY99 strain, both in terms of time to death and overall mortality. A closer investigation of the mechanism behind this enhanced virulence revealed that replication in CNS tissues was similar for both WNV_{NY99}- and WNV_{KOU}-infected animals, indicating that both viruses are able to efficiently enter the CNS and replicate in neurons. However, a significant difference was observed in the ability of these two viruses to replicate and subsequently be cleared from the periphery of the mouse. Consistent with published works (54), WNV_{NY99} was able to mount significant viremia and viral load in the spleen, which peaked by day 3 and then was cleared subsequently or significantly reduced by the animal's immune response. However, this pattern was not shared by mice infected with WNV_{KOU}, which had a delayed but more prominent viremia that was not cleared before the mice died. Furthermore, no reduction in the viral load in the spleen was observed for the duration of the experiment. The poor viral clearance in WNV_{KOU}-infected animals most likely is due to the reduced induction of neutralizing antibodies, a critical control measure in severe WNV infection (44). In peripheral tissues, WNV infection is thought to be restricted or controlled by innate and adaptive immune responses, including serum IgM (44), IFN- α/β (55), IFN- γ (56), cytolytic CD8⁺ T cells (54), and cell-intrinsic IRF-3-dependent antiviral responses (57). Further investigation into these aspects of the innate and adaptive immune response comparing WNV_{NY99}- and

WNV_{KOU}-infected mice will shed light on their contribution to differences observed in pathogenicity.

While overall the total amount of infectious virus detected in both WNV_{NY99}- and WNV_{KOU}-infected mice was similar in both the brain and spinal cord, there were notable differences in the levels of viral antigen in brain sections, where more widespread viral antigen was detected in WNV_{KOU}-infected animals on day 7 postinfection. Overall, more neuropathology was observed in WNV_{KOU}-infected animals than in WNV_{NY99}-infected animals; however, the mechanism behind neuron loss appears to be caspase 3 independent. A very marked IL-6 response was detected in brain and periphery of WNV_{KOU}-infected mice, and this may have contributed to increased cellular stress. Notably, Cusick et al. showed that IL-6, produced by microglia and infiltrating monocytes, causes seizures following virus infection (58). Seizures were a prominent feature of the clinical presentation of the WNV_{KOU}-infected mice in the terminal phase. Aberrant cellular stress responses, including cyclic AMP (cAMP) response element-binding transcription factor homologous protein (CHOP), may be responsible for the enhanced pathology of WNV_{KOU}-induced neuronal damage, as this protein has been shown to be activated during WNV infection, leading to apoptosis (59). Alternatively, WNV infection is known to trigger apoptosis by activating noncaspase proteases, such as calpains and cathepsins (60, 61). Levels of these enzymes during infection may provide some insight into the differences in pathology seen between WNV_{KOU}- and WNV_{NY99}-infected animals. In addition, further studies into the postneuroinvasion events in disease progression may provide insight into additional virus and host determinants leading to the enhanced virulence of WNV_{KOU}.

Sequence analysis between WNV_{NY99} and WNV_{KOU} revealed a high level of nonconservative amino acid changes, with nearly 50% of all amino acid changes within each gene segment representing a nonconservative change. In addition, WNV_{KOU} did not contain any known published virulence motifs, including residue 72 of prM and E protein glycosylation, associated with enhanced viral assembly and/or secretion of virus (19, 21), 249 in NS3, which has been associated with enhanced virulence in birds (62), or 653 of NS5, associated with increased resistance to interferon (20), suggesting that additional virulence determinants within the genome are responsible for the enhanced virulence phenotype of WNV_{KOU}. Of particular interest may be the accumulated nonconservative changes in E domain III, which is known to contain potent antibody neutralizing epitopes (29, 43). Amino acid changes in the WNV_{KOU} genome in E domain III may explain the induction of a poor neutralizing antibody response seen in WNV_{KOU}-infected mice. Previous studies have identified key contact residues (amino acids 306, 307, 330, and 332) in E domain III that form the neutralizing epitope of the potent humanized neutralizing monoclonal antibody E16 (63). While amino acid changes were not identified at these particular residues for WNV_{KOU}, nonconservative changes close to these residues at 310, 312, 314, and 328 were identified which may affect neutralizing epitopes for WNV_{KOU}. Furthermore, nonconservative amino acid changes also were identified in close proximity to the furin cleavage site at residues 86 and 87, which may influence virus maturation. This in turn could influence the neutralizing antibody response to WNV_{KOU} infection, as a recent study has identified that the maturation status of WNV virions affects the efficiency of neutralization by antibody (64).

Additional West Nile-like viruses have been isolated from south Asia, the Czech Republic, and Russia and are proposed to form lineages III to V (13, 14, 65). Since cases of WNV encephalitis and febrile disease (66), as well as multifocal retinitis (67), have been reported for some of these new viruses, further analysis for similarities and differences in disease development compared to lineage I and II strains is warranted. Collectively, data generated during this project have identified a virulent West Nile-like virus that branches outside the major lineages and has the potential to cause disease in humans and animals.

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