Characterization of Virulent West Nile Virus Kunjin Strain, Australia, 2011

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To determine the cause of an unprecedented outbreak of encephalitis among horses in New South Wales, Australia, in 2011, we performed genomic sequencing of viruses isolated from affected horses and mosquitoes. Results showed that most of the cases were caused by a variant West Nile virus (WNV) strain, $WNV_{NSW2011}$, that is most closely related to WNV Kunjin (WNV_{KUN}), the indigenous WNV strain in Australia. Studies in mouse models for WNV pathogenesis showed that $WNV_{NSW2011}$ is substantially more neuroinvasive than the prototype WNV_{KUN} strain. In $WNV_{NSW2011}$, this apparent increase in virulence over that of the prototype strain correlated with at least 2 known markers of WNV virulence that are not found in WNV_{KUN}. Additional studies are needed to determine the relationship of the $WNV_{NSW2011}$ strain to currently and previously circulating WNV_{KUN} strains and to confirm the cause of the increased virulence of this emerging WNV strain.

In Australia, Murray Valley encephalitis virus (MVEV) and West Nile virus (WNV) Kunjin (KUN) strain are the main etiologic agents of arboviral encephalitis in humans, which usually occurs as isolated sporadic cases or occasional small outbreaks, mainly in northwestern Australia and rarely in southern regions (Figure 1, panel A) (*1*). MVEV is the more virulent pathogen and the only proven cause of fatal arboviral encephalitis in humans in Australia (*2*). WNV_{KUN} infections are infrequent and less severe (*3*). Horses are also susceptible to these viruses and have been involved in WNV outbreaks elsewhere, most notably in the United States in an outbreak that began in 1999. In Australia, infection with WNV_{KUN} has been detected intermittently in horses in the Southeast, but reports of encephalitis caused by this virus are rare (*3*). In New South Wales (NSW), Australia, the seroprevalence of WNV_{KUN} in horses is <5% (P.D. Kirkland and A. Read, unpub. data); infection is confined to inland areas where flooding supports large mosquito populations and water birds are a reservoir and amplifying host. Even in years when WNV_{KUN} has caused disease in humans, disease has rarely been observed or confirmed in horses (*3*,*4*).

In 2011, an outbreak of encephalitis occurred among horses in NSW. To analyze this strain of WNV_{KUN}, we conducted genomic sequencing, antigenic profiling, in vitro growth kinetics, and mouse virulence studies on virus isolates from diseased animals and mosquitoes.

Materials and Methods

Disease Outbreak

In late February 2011, neurologic disease was reported in several horses in northwestern and southwestern NSW. The number of cases and geographic distribution gradually increased. By mid-June 2011, specimens from ≈300 horses were submitted to the virology laboratory at Elizabeth Macarthur Agriculture Institute (Menangle, NSW, Australia). Many more horses probably were affected.

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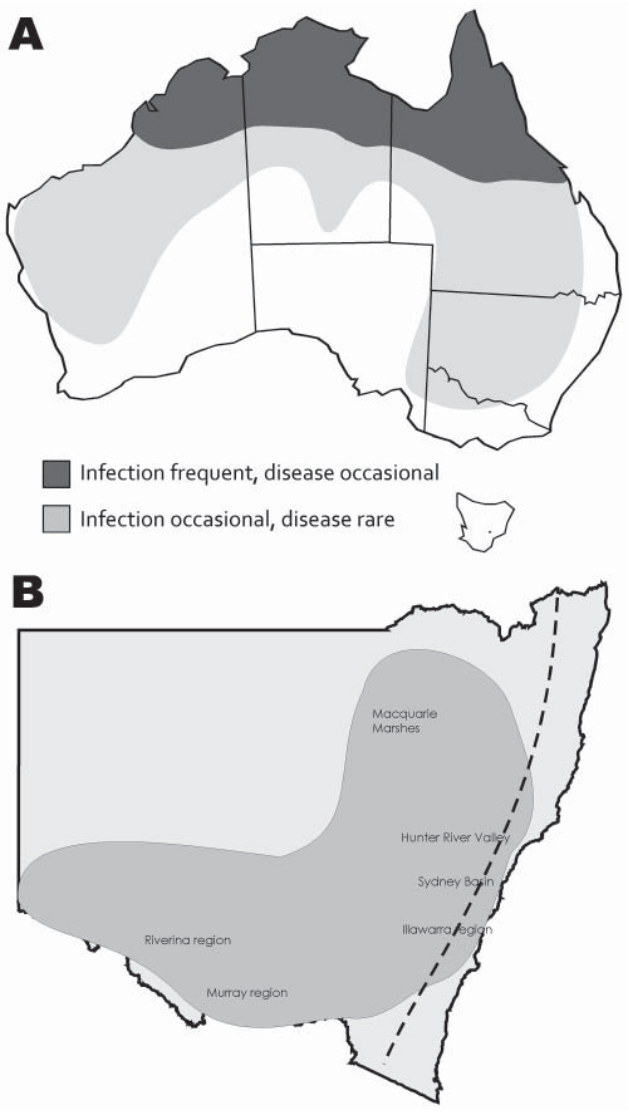

Figure 1. Known distribution of West Nile virus infection and disease caused by Kunjin strain (A) and distribution of encephalitis cases among equids (B), New South Wales, Australia, 2011. Dashed line indicates the Great Dividing Range.

Diseased horses were located throughout most of NSW, west of the Great Dividing Range, but also extending through the Hunter River Valley region, Sydney Basin, and Illawarra coastal region immediately south of Sydney (Figure 1, panel B). Cases also occurred in other southern Australia states. Clinical signs were generally consistent with those described in horses infected with WNV in the United States (*5*). A detailed report of the clinical signs, virology, and pathology of equine cases will be published elsewhere.

Specimen Collection

Whole brains were removed from 12 horses at postmortem examination. Half of each brain was fixed in 10% neutral buffered formalin; the other half was held fresh at 4°C. Upon receipt, we collected small pieces of fresh and formalin-fixed tissue from several locations in the cerebrum and cerebellum and along the brain stem and cervical spinal cord. If virus isolation could not be performed on fresh samples within 24 h after receipt, we held the samples at –80°C until tested. Before testing, we prepared 10% tissue homogenates in RPMI medium (Life Technologies, Carlsbad, CA, USA) containing antimicrobial drugs.

Mosquitoes were collected throughout NSW, as part of the NSW Arbovirus Surveillance and Mosquito Monitoring Program, by using dry ice–baited light traps. The mosquitoes were submitted live to the Medical Entomology Laboratory at Westmead Hospital (Westmead, NSW, Australia) for species identification, arbovirus isolation, and virus identification (*6*).

Cells and Viruses

We propagated Vero 76 cells in Dulbecco modified minimum essential medium (DMEM; Life Technologies) supplemented with 10% fetal bovine serum (FBS). C6/36 *Aedes albopictus* mosquito cells were maintained in RPMI medium supplemented with 10% FBS, and BHK21 cells were maintained in DMEM containing 5% FBS. All cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). We used prototype WNV_{KUN} MRM61C (*7*) and WNV New York 99 (WNV_{NY99}) 4132 strains (*8*) for comparison with $WNV_{NSW2011}$. Stocks of WNV_{KUN} MRM61C and WNV_{NY99} 4132 that most closely resembled the low-passage level $WNV_{NSW2011}$ were prepared by electroporation of BHK21 cells with WNV_{KUN} or WNV_{NY99} RNA (prepared from corresponding infectious cDNA clones) and passaging them 1× in C6/36 cells. Viral supernatants were harvested 5 days later. Viral titers for each viral stock were determined by plaque assay on Vero 76 cells.

Virus Neutralization Tests

We conducted microneutralization tests (*9*) in Vero cells by using 25–100 infectious units (measured as 50% tissue culture infective doses) of $WNV_{NSW2011}$, WNV_{KUN}, and WNV_{NY99}; we used 2-fold dilutions of serum from an initial dilution of 1:20. Results were scored as 80% reduction in virus growth or 100% inhibition of virus growth. Reduction in virus growth was determined by assessing the extent of cytopathic effect in each well. Inhibition of virus growth was determined by the absence of viral antigen in the cells of each well when tested with a WNV-reactive monoclonal antibody (mAb) in ELISA.

Nucleic Acid Purification

We used the MagMax-96 Viral RNA Isolation Kit (Ambion, Austin, TX, USA) on a magnetic particle handling system (Kingfisher 96; Thermo Electron Corporation, Vantaa, Finland) to extract total nucleic acid from clarified 10% brain homogenate (50 μL) or tissue culture fluid. Purified nucleic acids were eluted in 50 μL of kit elution buffer and used immediately for PCR amplification or stored frozen at ≈–20°C

Real-Time Reverse Transcription PCR

We used a published WNV real-time reverse transcription PCR (rRT-PCR) (*10* [assay 2]) with the following variations: Black Hole Quencher (Biosearch Technologies, Novato, CA, USA) was used instead of TAMRA on the probe, the internal control system was not used, and 5 μL of RNA was used as template. The AgPath-ID One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA, USA) was used for the rRT-PCR on a 7500HT Fast Real-Time PCR System (Applied Biosystems). We ran rRT-PCR reactions in standard mode, according to conditions recommended by the mastermix manufacturer.

Virus Isolation

Supernatant from the 10% brain homogenate was placed on monolayers of *A. albopictus* C6/36 mosquito cells in cell culture tubes. The cultures, which were maintained in RPMI medium containing antimicrobial drugs at standard concentrations and supplemented with 2% FBS, were incubated at 28°C for 5–7 days. Culture supernatants were then passaged up to 3× on BHK21 cells maintained in Basal Medium Eagle (MP Biomedicals, Sydney, Australia) containing antimicrobial drugs and 2% FBS. We regularly examined cultures by light microscopy for cytopathic effects. We used rRT-PCR to confirm the identity of virus isolates in culture supernatants or to confirm that there was no virus replication in the absence of cytopathology. Viral RNA recovered from culture fluid at the first or second passage in BHK21 cells was sequenced as described below. A virus isolate obtained from the first brain examined was designated $WNV_{NSW2011}$. Virus isolation was conducted on homogenates of mosquitoes by similar methods but with passage onto BHK and PSEK cells after initially being placed on C6/36 cells. We identified virus isolates by using immunoassays with generic and specific mAbs.

$WNV_{NSW2011}$ virus harvested from the first passage in C6/36 cells was used to examine plaque morphology and virulence in mice. The virus was passaged 1× in Vero76 cells for 4 d and 1× in C6/36 cells for 5 d. Virus supernatant was centrifuged at 500 × *g* at 4°C for 5 min before being stored at −80°C

Reactivity with mAbs

Reactivity of the new isolate with a panel of mAbs was compared with that of WNV_{KUN} and WNV_{NY99} by using a fixed-cell ELISA (*11*). The mAbs and their characteristics follow: mAb 10C6, anti–nonstructural protein (NS) 1 (reactive with MVEV); mAb 10A1, anti-envelope glycoprotein (anti-E; specific for WNV_{KUN}); mAb 2B2, anti-E (reactive with WNV); mAb 3.1112G, anti-NS1 (reactive with WNV); mAb 5H1, anti-NS5 (strong reaction with WNV_{KUN} strains, weak for WNV_{NY99}, nonreactive with WNV strains from other lineages); mAb 17D7, anti-E (specific for strains of WNV with glycosylated E); mAb 3.101C, anti-E (specific for strains of WNV with unglycosylated E); and the pan–flavivirus-reactive mAbs (i.e., 4G4, anti-NS1; and 4G2, anti-E) (*12–15*; J. Hobson-Peters et al., unpub. data).

Nucleic Acid Sequencing

For sequencing of the whole genome, we used total nucleic acid purified from virus-infected cell culture supernatant as template in 5 RT-PCRs with primers designed to cover the coding regions of any WNV genome (Table 1). RT and amplification were performed by using the SuperScript III One-Step RT-PCR System (Invitrogen, Carlsbad, CA, USA) with primers at 20 μM. RT was performed at 50°C for 30 min, followed by denaturation at 94°C for 2 min. PCR amplification involved 40 cycles (95°C for 30 s, 55°C for 1 min, 68°C for 4.5 min) followed

Table 1. Primers used for viral RNA amplification and genomic sequencing of WNV isolates from horses and mosquitoes, Australia, 2011*

RT-PCR region	Forward primer, 5′ → 3′ (relative genome position†)	Reverse primer, 5′ → 3′ (relative genome position†)
Amplification and sequencing of whole genome		
5′ NTR capsid	TAGTTCGCCTGTGTGAGCTG (5′ NTR-2)	TTGAAAATTCCACAGGAATGG (capsid-1772)
Capsid-NS2A	GTGATAGCATTGGGCTCWCA (capsid-1720)	ATCTTGAAGGYYGCCATGAG (NS2A-1760)
NS2A-NS3	CACTGATGTGTTACGCTATGTCA (NS2A-3678)	CAAAGTCCCAATCATCGTTCT (NS3-5807)
NS3-NS5	CGGTTTGGTTTGTGCCTAGT (NS3-5687)	CCAACTTCACGCAGGATGTA (NS5-9235)
NS5–3′ NTR	GACCACTGGCTTGGAAGAAA (NS5-9169)	CTGGTTGTGCAGAGCAGAAG (3′ NTR-10955)
Partial sequencing of key regions of genome		
NS3	GTGCTGGTAAAACAAGGAGG (NS3-5201)	TGTATCCTCTAGCCGCGATG (NS3-5493)
NS5	TCGGCCCAGATGATGTG (NS5-9575)	CGGCATGGAACCACCAGTGTTC (NS5-9860)

*Primers were designed from available sequences in GenBank to cover the coding regions of any WNV genome. WNV, West Nile virus; RT, reverse transcription; NTR, nontranslated region; NS, nonstructural protein.
†WNV_{NY99} GenBank accession no. NC_009942.1.

by a final extension at 68°C for 10 min. Reaction products were visualized after electrophoresis on a ethidium bromide–stained 1% agarose gel. Reaction products were purified directly (MinElute PCR Purification Kit; QIAGEN, Valencia, CA, USA) or excised from the gel and cleaned (Gel MinElute PCR Purification Kit; QIAGEN). Purified nucleic acid was sequenced at the Australian Genome Research Facility (Sydney) by using the primers used to generate the PCR product. Each RT-PCR was run 3× and sequenced in both forward and reverse orientation. Sequence data were assembled by using Sequencher software (Gene Codes Corp., Ann Arbor, MI, USA). For subsequent isolates from horses and mosquitos, we used primers designed from the sequence of the $WNV_{NSW2011}$ genome (Table 1) to amplify and sequence the NS3 and NS5 regions, in which changes had been identified. The same RT-PCR and sequencing methods were used, except that the annealing temperature was 50°C and extension time was 1 min. The nucleic acid sequences were translated and then aligned with $WNV_{NSW2011}$ and WNV_{KUN} (GenBank accession nos. JN887352 and D00246.1, respectively) by using ClustalW (www.clustal.org).

Bioinformatics Analysis

Complete coding regions of selected WNV isolates, representing all lineages and clades and including all complete KUN sequences, were aligned with the $WNV_{NSW2011}$ sequence as described (*16*). This alignment was transferred to BioEdit (www.mbio.ncsu.edu/BioEdit/bioedit.html) for manual editing before construction of phylogenetic trees. Maximum-likelihood trees were constructed by using PhyML (*17*). Trees were rooted by using the Japanese encephalitis virus Nakayama sequence (GenBank accession no. EF571853), which was removed from the final tree for clarity.

Endoglycosidase Digestion

To examine glycosylation of the E protein, viral proteins from cultures of infected C6/36 cells were digested as described (*18*). Proteins were separated and analyzed by Western blot. Samples were loaded with reducing sodium dodecyl sulfate–polyacrylamide gel electrophoresis buffer (NuPAGE LDS Sample Buffer; Invitrogen) on a 4%–12% NuPAGE Gel (Invitrogen). Electrophoresed proteins were electroblotted onto nitrocellulose paper (Hybond C; GE Healthcare, Little Chalfont, UK) and immunostained with anti-E mAb (*11*).

Plaque Morphology

We allowed the virus to adsorb to monolayers of Vero 76 cells in 6-well plates for 2 h at 37°C. The cells were overlaid with DMEM containing 0.75% low melting point agarose and 2% FBS. Four days after infection, the cells were fixed with 4% formaldehyde solution and stained with 0.2% crystal violet.

Virulence in Mice

Groups of 10 weanling (18–19 days old) or young adult (4 weeks old) Swiss outbred CD1 mice were injected intraperitoneally with 10-fold dilutions of virus. The mice were monitored for 21 days after injection and euthanized when signs of encephalitis were evident. All animal procedures had received prior approval from The University of Queensland Animal Ethics Committee.

Results

Virus Isolation and Initial Characterization

Viral RNA was detected by WNV-specific rRT-PCR in fresh brain tissue from 6 of 12 horses showing signs of encephalitis. Viruses were isolated from 4 of these samples; each showed distinct cytopathology in BHK21 and Vero cells. rRT-PCR of the culture fluids and immunoperoxidase staining of the cells with pan–flavivirus-reactive and WNV-specific mAbs confirmed the isolation of a West Nile–like virus. The first isolate was named NSW2011 and designated $WNV_{NSW2011}$. Eight isolates of $WNV_{NSW2011}$ were isolated from *Culex annulirostris* Skuse mosquitoes during the 2011 vector season. Of the 8 isolates, 5 were from mosquitoes collected in the Riverina region of southwestern NSW (Hanwood, 4 isolates; Barren Box, 1 isolate); 2 were from the Murray region in southern NSW (Mathoura, 1 isolate; Moama, 1 isolate); and 1 was collected at Lower Portland in the outer western Sydney region of NSW. No other isolates of WNV were obtained.

Antigenic Analysis of $WNV_{NSW2011}$

To antigenically type $WNV_{NSW2011}$ in a fixed-cell ELISA (*11*), we used a panel of mAbs previously shown to differentiate between strains of WNV_{KUN} and other WNVs (*11*,*13*–*15*,*19*,*20*). The recognition patterns showed that the $WNV_{NSW2011}$ isolate most closely resembled Australian WNV_{KUN} strains; the WNV_{KUN}-specific mAb 10A1 reacted strongly with prototype WNV_{KUN} and $WNV_{NSW2011}$ but not with WNV_{NY99} (Table 2). However, the anti-NS5 mAb 5H1, which is also specific for WNV_{KUN} isolates from Australia (*15*), failed to react with $WNV_{NSW2011}$ and WNV_{NY99}, but it bound strongly to WNV_{KUN}. The reaction patterns of mAbs 17D7 and 3.101C, which react specifically with glycosylated and unglycosylated WNV E antigens, respectively (*11*,*14*; J. Hobson-Peters et al., unpub. data), indicated that, unlike the E protein of WNV_{KUN}, the E protein of $WNV_{NSW2011}$ is glycosylated.

To assess the level of antigenic crossreactivity between $WNV_{NSW2011}$, WNV_{KUN}, and WNV_{NY99}, we assessed neutralization titers for homologous and heterologous

Table 2. Binding pattern of monoclonal antibodies to the viral antigens of 3 WNV strains in fixed-cell ELISA, Australia, 2011*

	Monoclonal antibody, by specificity								
	Pan-flavivirus		WNV group		WNV_{KUN}		Unglycosylated WNV E protein	Glycosylated WNV E protein	MVEV
Virus	4G4, anti-NS1	4G2, anti-E	2B2, anti-E	3.91D, anti-E	10A1, anti-E	5H1, anti-NS5	3.101C	17D7	10C6, anti-NS1
$WNV_{NSW2011}$	+	+	+	+	+	–	–	+	–
WNV_{KUN}†	+	+	+	+	+	+	+	–	–
WNV_{NY99}‡	+	+	+	+	–	–	–	+	–

*WNV, West Nile virus; KUN, Kunjin; E, envelope; MVEV, Murray Valley encephalitis virus; NS, nonstructural protein; NS, nonstructural protein; NSW, New South Wales; + positive; –, negative; NY, New York.
†Prototype WNV_{KUN} strain MRM-61C.
‡North American WNV strain.

viruses in immune serum samples from the following sources: horses infected during the 2011 outbreak, horses infected with WNV_{KUN} in the Northern Territory of Australia several years earlier, and horses infected with WNV in the United States. Convalescent-phase serum samples from $WNV_{NSW2011}$-immune horses had neutralizing titers similar to those of the homologous virus ($WNV_{NSW2011}$) and of WNV_{KUN} and WNV_{NY99} (Table 3). Serum samples from WNV_{KUN}-immune horses from Northern Territory and from WNV-immune animals from the United States showed a similar pattern of cross-neutralization. However, serum samples from horses from Northern Territory and the United States showed slightly less neutralizing efficiency of $WNV_{NSW2011}$; this was likely due to a higher dose (≈4-fold) of virus in the assay (Table 3). Overall, these results are consistent with those in our previous reports showing a high level of cross-neutralization between WNV_{KUN} and WNV_{NY99} strains (*21*,*22*).

Nucleotide and Amino Acid Sequence Analysis of $WNV_{NSW2011}$

A comparison of the nucleotide sequence of the complete coding region of the first isolate of $WNV_{NSW2011}$ with sequences available in GenBank confirmed that $WNV_{NSW2011}$ was genetically most closely related to Australian WNV_{KUN} isolates (Figure 2). A detailed

Table 3. Neutralizing titers of serum samples from WNV–infected horses against 3 WNV strains, Australia, 2011*

	% Inhibition of CPE/growth†					
	$WNV_{NSW2011}$, 100 infectious units		WNV_{KUN}, 26 infectious units		WNV_{NY99}, 32 infectious units	
Horse serum samples	80‡	100§	80	100	80	100
Control¶						
1	<20	<20	<20	<20	<20	<20
2	<20	<20	<20	<20	<20	<20
3	<20	<20	<20	<20	<20	<20
4	<20	<20	<20	<20	<20	<20
5	<20	<20	<20	<20	<20	<20
NSW#						
04	640	320	1,280	1,280	640	320
06	320	160	640	640	1,280	160
08	320	320	1,280	1,280	640	640
28	320	320	640	640	320	320
36	320	160	640	640	320	640
NT**						
111473	**80**	**20**	**640**	**320**	**160**	**160**
104714	**320**	**80**	**640**	**640**	**640**	**640**
110910	80	40	160	160	160	160
98727	40	40	160	160	80	80
WNV††						
1	**160**	**40**	**640**	**40**	**320**	**320**
2	**320**	**160**	**1,280**	**640**	**160**	**160**
3	**80**	**160**	**320**	**320**	**640**	**640**
4	**40**	**20**	**160**	**160**	**320**	**320**
5	**320**	**80**	**1,280**	**320**	**640**	**640**
mAb 3.91D‡‡	>2,560	>2,560	>2,560	>2,560	>2,560	>2,560

*Determined, as described (*14*), by microneutralization assay in Vero cells. WNV, West Nile virus; CPE, cytopathic effect; NSW, New South Wales; KUN, Kunjin; NY, New York; NT, Northern Territory; mAb, monoclonal antibody.
†**Boldface** indicates serum samples with >4-fold difference in titer between virus strains.
‡Determined by using a microscope to assess the level of CPE in each well compared with that in control wells.
§Determined by the absence of viral antigen in the cell monolayer of each well when tested with a WNV-reactive mAb in ELISA.
¶Samples from uninfected horses.
#Samples from horses infected with WNV during the 2011 outbreak in New South Wales, Australia.
**Samples from horses infected with WNV_{KUN} in Northern Territory, Australia.
††Samples from horses infected with WNV in the United States.
‡‡This mAb has potent WNV-neutralizing activity (*11*).

comparison of deduced amino acid sequences for the entire coding region of $WNV_{NSW2011}$, WNV_{KUN}, and WNV_{NY99} further confirmed a closer relationship between $WNV_{NSW2011}$ and WNV_{KUN} than between $WNV_{NSW2011}$ and WNV_{NY99}. There was a 42-aa difference (18 nonconserved changes) between $WNV_{NSW2011}$ and WNV_{KUN} and an 89-aa difference (38 nonconserved changes) between $WNV_{NSW2011}$ and WNV_{NY99} (online Appendix Table, wwwnc.cdc.gov/EID/article/18/5/11-1720-TA1.htm). At least 2 of the known WNV virulence markers present in WNV_{NY99} but not in WNV_{KUN} were found in $WNV_{NSW2011}$ (online Appendix Table). The glycosylation tripeptide (N-Y-S) at residues $E_{154–156}$, which allows N-linked glycosylation at a conserved site on the E protein, domain I, and is associated with virulence in most WNV strains (*24*), was present in $WNV_{NSW2011}$; its presence is consistent with the mAb recognition profile. A phenylalanine residue at aa 653 in NS5, which also is associated with enhanced virulence of WNV strains (*24*), was present in $WNV_{NSW2011}$. Because the WNV_{KUN} strain–specific mAb 5H1 did not react with $WNV_{NSW2011}$, we also examined the predicted amino acid sequence of the NS5 protein that corresponded to the linear epitope previously mapped for 5H1 to residues 41–53 in the methyltransferase domain (*15*). $WNV_{NSW2011}$ and WNV_{NY99} contain a substitution (I—V) at residue 49 that is not contained in WNV_{KUN}, confirming the critical role of this residue for 5H1 binding. Together these data suggest that $WNV_{NSW2011}$ represents a virulent WNV strain that has emerged in Australia. However, the amino acid substitution in NS3, which is believed to be associated with increased virulence of WNV_{NY99} in birds in North America (*25*), was not present in isolate $WNV_{NSW2011}$. Sequencing of the key regions of the other WNV isolates obtained during this outbreak (3 from horses and 8 from mosquitoes) showed that each was indistinguishable from $WNV_{NSW2011}$.

In vitro Growth Properties and E Protein Glycosylation Status of $WNV_{NSW2011}$

The average plaque size of $WNV_{NSW2011}$ (Figure 3, panel A) was 4.2 mm ± 0.5 mm, much closer to WNV_{NY99} (4.7 mm ± 0.8 mm) than to the prototype WNV_{KUN} (2.8 mm ± 0.4 mm). To confirm the presence of an N-linked glycan on the E protein of $WNV_{NSW2011}$, we used Western blot to analyze endoglycosidase-digested viral protein. Analysis showed that the E protein of $WNV_{NSW2011}$ and WNV_{NY99} migrated slightly faster than the undigested control protein. This result is consistent with N-linked glycosylation (Figure 3, panel B). However, consistent with the lack of a potential glycosylation site on the E protein of most WNV_{KUN} isolates, we found no evidence of N-linked glycosylation for WNV_{KUN} (*11,20,26*).

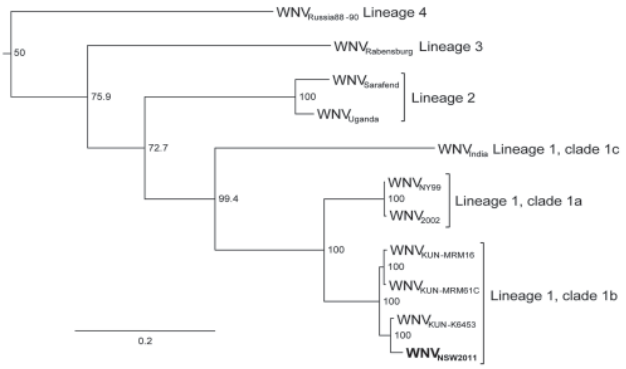

Figure 2. Maximum-likelihood tree based on nucleotide sequences of the complete open reading frame of genomes of West Nile virus (WNV) NSW2011 (**boldface**) and representative strains of WNV from the different lineages and clades. All published complete Kunjin (KUN) virus sequences are included. Bootstrap values are shown on the nodes and are expressed as a percentage of 1,000 replicates. Sequences downloaded from GenBank were $WNV_{Russia88–90}$, AY277251; $WNV_{Rabensburg}$, AY765264; $WNV_{Sarafend}$, AY688948; WNV_{Uganda}, AY532665; WNV_{India}, DQ256376; WNV_{NY99}, AF196835; WNV_{2002}, GU827998; $WNV_{KUNV-MRM16}$, GQ851602; $WNV_{KUNV-MRM61C}$, AY274504; and $WNV_{KUNV-K6453}$, GQ851603. NY, New York; NSW, New South Wales. Horizontal branch lengths indicate genetic distance proportional to the scale bar.

Neuroinvasive Properties of $WNV_{NSW2011}$ in mice

Injection of 18- to 19-day-old (weanling) mice with 10-fold dilutions of virus showed that substantially lower doses of $WNV_{NSW2011}$ (50% lethal dose [LD_{50}] 0.5 PFU), compared with WNV_{KUN} (LD_{50} 13.4 PFU), induced neurologic signs (Table 4); and the time to disease onset was substantially shorter (Table 4; Figure 3, panel C). In contrast, the LD_{50} for WNV_{NY99} (0.1 PFU) was lower than that for $WNV_{NSW2011}$, and neurologic signs developed more rapidly (Table 4; Figure 3, panel C). Only WNV_{NY99} and $WNV_{NSW2011}$ caused a substantial number of deaths among 4-week-old (young adult) mice. Compared with $WNV_{NSW2011}$, WNV_{NY99} exhibited a lower LD_{50} (240 PFU vs. 0.7 PFU, respectively) and a shorter time to death (10.7 days vs. 6.6 days, respectively, at 1,000 PFU) (Table 5; Figure 3, panel C).

Discussion

It is estimated that at least 1,000 horses were affected during an unprecedented outbreak of encephalitis in southeastern Australia during 2011. The case-fatality rate was 10%–15%, and diseased animals had clinical signs consistent with those observed during a WNV outbreak in the United States. Not only was the Australian outbreak unique and unprecedented in size and disease severity, but its epidemiologic features also differed from those

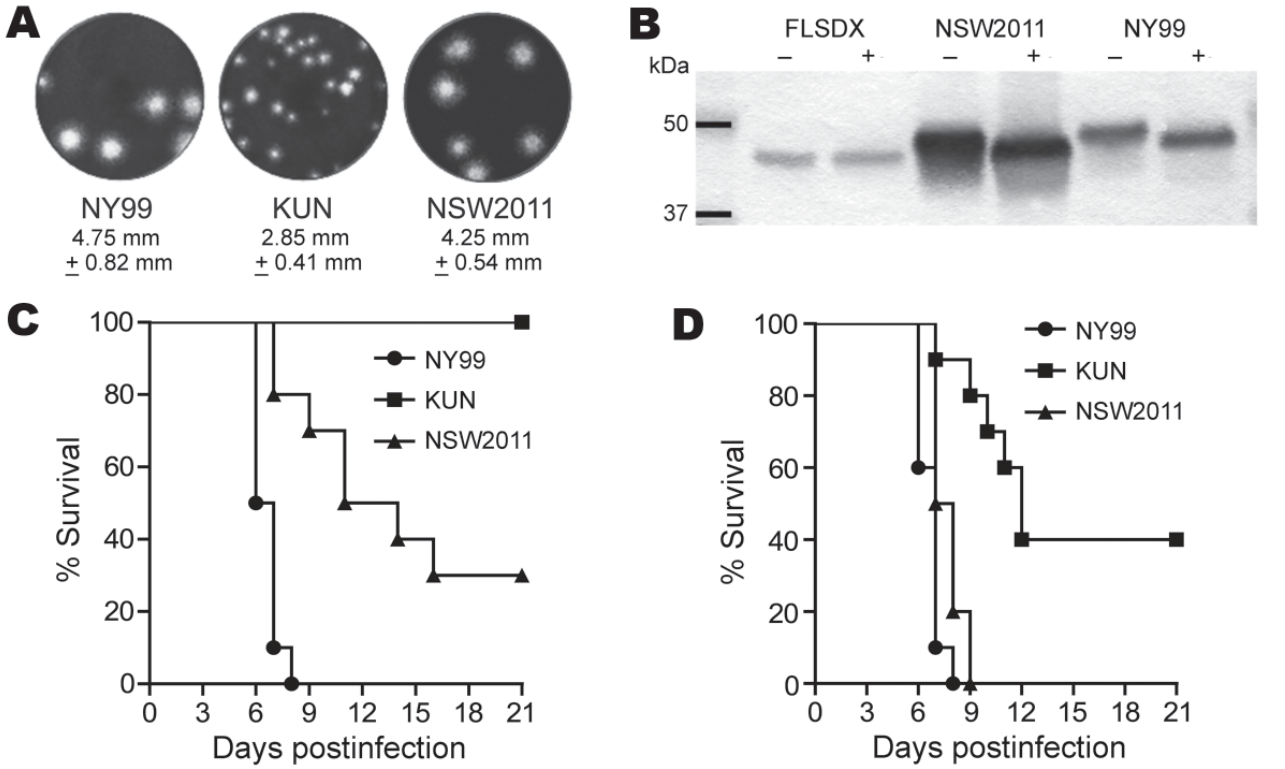

Figure 3. Studies of West Nile virus (WNV) properties in cell cultures and mice. A) Plaque morphology of WNV_{NY99}, prototype WNV_{KUN}, and $WNV_{NSW2011}$ in Vero cells. Cells in 6-well plates were infected with specified virus and overlaid with 0.75% low melting point agarose in Dulbecco modified minimum essential medium (Life Technologies, Carlsbad, CA, USA) containing 2% fetal bovine serum. Four days after infection, the cells were fixed with 4% formaldehyde and stained with 0.2% crystal violet. B) Assessment of envelope (E) protein glycosylation of $WNV_{NSW2011}$, WNV_{KUN} and WNV_{NY99} by endoglycosidase digestion (PNGase F; Roche Diagnostics, Basel, Switzerland). Viral proteins in culture supernatant were digested by PNGase F (+) or undigested (–) and then resolved on sodium dodecyl sulfate–polyacrylamide gel electrophoresis. The migration rate of the E protein in each sample was determined by Western blot with E glycoprotein–specific monoclonal antibodies. C) Young adult (4 weeks old) or D) weanling (18–19 days old) Swiss outbred mice survival after intraperitoneal injection with 1,000 PFU (adult) or 10 PFU (weanling) of WNV_{NY99}, WNV_{KUN}, or $WNV_{NSW2011}$. The mice were monitored for 21 days after injection for signs of encephalitis and then euthanized. The differences in virulence in weanling and adult mice between different pairs of viruses were all highly significant, as calculated by log rank Mantel-Cox algorithm with exact p values: for adult mice, WNV_{NY99} vs. WNV_{KUN} $p<0.0001$, WNV_{NY99} vs. $WNV_{NSW2011}$ $p = 0.0001$, and WNV_{KUN} vs. $WNV_{NSW2011}$ $p = 0.0012$; and for weanling mice, WNV_{NY99} vs. WNV_{KUN} $p<0.0001$, WNV_{NY99} vs. $WNV_{NSW2011}$ $p =0.0004$, and WNV_{KUN} vs. $WNV_{NSW2011}$ $p = 0.0006$. NY, New York; KUN, Kunjin; NSW, New South Wales.

observed previously in Australia. In particular, WNV_{KUN} now has been detected on the eastern seaboard of NSW, close to major urban areas, including the largest 3 cities (Sydney, Newcastle, and Wollongong). This detection occurred despite relatively small mosquito populations in many of these areas, suggesting that the virus is more virulent and probably transmitted more efficiently than other strains between mosquito vectors and mammalian hosts. Characterization of virus isolated from the brain of an animal that died showed a variant strain of WNV most closely related to WNV_{KUN}. Typing of $WNV_{NSW2011}$ by reactivity with a panel of mAbs indicated the virus was antigenically more similar to the native Australian WNV_{KUN} strains than to exotic WNV strains. However, for $WNV_{NSW2011}$, the reaction profile of mAbs 17D7 and 3.101C differed from that of the prototype WNV_{KUN}. Similar to WNV_{NY99} and other virulent strains of WNV, $WNV_{NSW2011}$ E protein was glycosylated at residue 154. This finding was further confirmed by gene sequencing and endo-glycosidase F digestion analysis. Glycosylation of WNV E protein at this site is thought to enhance virus dissemination in the infected host by increasing the efficiency of assembly and release of virus particles from infected cells (*27*). Previous studies showed that a phenylalanine residue at aa 653 in NS5, observed in $WNV_{NSW2011}$ and WNV_{NY99} but not in WNV_{KUN}, is associated with increased resistance to interferon, which may also enhance virulence in the host (*24*). Virulence studies in weanling and young adult mice clearly demonstrate that $WNV_{NSW2011}$ is substantially more neuroinvasive than the

Table 4. Virulence of 3 WNVs in 18- to19-day-old mice after intraperitoneal injection, Australia, 2011*

Virus and dose, PFU	No. mice/no. died	Average survival time, d	LD_{50}
WNV_{NY99}			
100	10/10	6.1	
10	10/10	6.7	
1	10/10	6.9	0.1 PFU
0.1	5/10	7.8	
WNV_{KUN}			
1,000	9/10	8.4	
100	4/10	8	13.4 PFU
10	6/10	10.2	
1	3/10	12	
$WNV_{NSW2011}$			
1,000	10/10	7.1	
100	10/10	7.4	
10	10/10	7.7	0.5 PFU
1	7/10	8.3	
0.1	1/10	10	

*WNV, West Nile virus; LD_{50}, dose at which 50% of the mice died; NY, New York; KUN, Kunjin; NSW, New South Wales.

prototype strain of WNV_{KUN}, which might explain the severity of the 2011 outbreak. However, the association between the identified and perhaps other amino acid changes and increased virulence of $WNV_{NSW2011}$ in horses and mice will require further confirmation by using site-directed mutagenesis of an infectious cDNA clone.

Another unusual aspect of the 2011 outbreak was the absence of encephalitis caused by WNV_{KUN} in humans. In contrast, several confirmed cases of Murray Valley encephalitis in humans were recorded in southeastern Australia during this time. This absence of disease in humans suggests that ecologic and/or epidemiologic features of the virus transmission cycle, such as small mosquito populations and timely alerts, probably resulted in less exposure of the human population to $WNV_{NSW2011}$.

The US outbreak of WNV was associated with high mortality among several bird species, particularly American crows (*Corvus brachyrhynchos*). In contrast, increased mortality among birds of any species was not reported during the 2011 outbreak in southeastern Australia. The lack of disease in birds in Australia supports the hypothesis that the amino acid substitution observed in WNV_{NY99} (Ala—Pro at aa 249 in NS3) (*25*) is associated with increased virulence in birds because this change was not present in the $WNV_{NSW2011}$ isolate. However, this observation should be viewed with some caution because of the species differences between birds in Australia and the United States and because disease was limited or absent when a species of Australian crow (Little Raven [*Corvus mellori*]) was experimentally infected with WNV_{NY99} (*28*).

Table 5. Virulence of 3 WNVs in 4-week-old mice after intraperitoneal injection, Australia, 2011*

Virus and dose, PFU	No. mice/no. died	Average survival time, d	LD_{50}
WNV_{NY99}			
100	10/10	8.3	
10	10/10	8.2	0.7 PFU
1	5/10	8.6	
0.1	2/10	10	
WNV_{KUN}			
1,000	0/10	21	>1,000 PFU
$WNV_{NSW2011}$			
1,000	7/10	10.7	
100	2/10	11	240 PFU
10	3/10	10.3	

*WNV, West Nile virus; LD_{50}, dose at which 50% of the mice died; NY, New York; KUN, Kunjin; NSW, New South Wales.

Taken together, our results show that the $WNV_{NSW2011}$ isolate is closely related to Australian WNV_{KUN} strains. However, in contrast to the prototype WNV_{KUN} strain (MRM-61C), the new virus has several amino acid substitutions that are likely to be the reason for enhanced virulence in horses. More extensive epidemiologic studies in the field and experimental studies in the laboratory are required to determine the relation of $WNV_{NSW2011}$ to other currently and previously circulating WNV_{KUN} strains and to confirm which viral proteins and amino acid residues are associated with increased virulence of $WNV_{NSW2011}$ in horses.

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Dr Frost is a scientist engaged in research to characterize new and emerging viral pathogens among animals. She also has a special interest in the application of molecular methods for the diagnosis of viral diseases.

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