Virulence and Evolution of West Nile Virus, Australia, 1960–2012

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Worldwide, West Nile virus (WNV) causes encephalitis in humans, horses, and birds. The Kunjin strain of WNV (WNV_{KUN}) is endemic to northern Australia, but infections are usually asymptomatic. In 2011, an unprecedented outbreak of equine encephalitis occurred in southeastern Australia; most of the ≈900 reported cases were attributed to a newly emerged WNV_{KUN} strain. To investigate the origins of this virus, we performed genetic analysis and in vitro and in vivo studies of 13 WNV_{KUN} isolates collected from different regions of Australia during 1960–2012. Although no disease was recorded for 1984, 2000, or 2012, isolates collected during those years (from Victoria, Queensland, and New South Wales, respectively) exhibited levels of virulence in mice similar to that of the 2011 outbreak strain. Thus, virulent strains of WNV_{KUN} have circulated in Australia for ≥30 years, and the first extensive outbreak of equine disease in Australia probably resulted from a combination of specific ecologic and epidemiologic conditions.

West Nile virus (WNV) is a mosquito-transmitted flavivirus that causes encephalitis. Outbreaks of potentially fatal neurologic syndromes have occurred in Europe and Africa (*1*); recently, however, strains of WNV have caused large outbreaks of encephalitis in humans and horses in the Americas and Australia (*2*,*3*). The Kunjin strain of WNV (WNV_{KUN}) is indigenous to Australia and historically has caused only relatively mild, nonfatal disease in humans and horses. However, in 2011, a large unprecedented outbreak of encephalitis in horses, involving ≈900 reported cases, occurred in southeastern Australia; a high proportion of cases were attributed to the emergence of a virulent strain of WNV_{KUN} (*3*,*4*). WNV_{KUN} has been shown to be enzootic to northern Australia and to have episodic activity in southern regions thought to be associated with periods of heavy rainfall (*5*). However, the epidemiology of WNV_{KUN} seems to have changed over the past decade; virus activity has been detected in the absence of prior flooding and in areas where it was previously not detected (*4*).

Studies comparing the virulence of various WNV strains in mouse models have identified several motifs, residing in both structural and nonstructural genes as well as in the 5′ and 3′ untranslated regions (UTRs). These motifs were associated with enhanced viral invasion of the central nervous system and onset of neurologic disease (*5*–*11*).

To identify potential markers of virulence of WNV_{KUN} in Australia, we investigated evolutionary mechanisms behind the emergence of virulent strain(s) by using established mouse models to compare the neuroinvasive properties of WNV_{KUN} isolates collected from different regions of Australia during 1960–2012. To investigate known markers of WNV virulence, we conducted comparative analyses of viral genome sequences.

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Methods

Cell Culture, Virus Production, and Titration

We used 13 WNV_{KUN} strains isolated during 1960–2012 (Table 1) and African green monkey kidney (Vero) and *Aedes albopictus* mosquito (C6/36) cells, cultured as previously described (*3*). The 1960 prototype WNV_{KUN} strain ($WNV_{KUN1960}$) was used as the attenuated reference virus and was either an unknown passage of the original isolate (WNV_{MRM16}) or derived from an infectious clone of a plaque-purified virus (WNV_{MRM61C}) (*12*,*13*). These isolates were previously shown to be phenotypically identical (*13*,*14*). WNV strain NY99-4132 was obtained from the US Centers for Disease Control and Prevention (Fort Collins, CO, USA) and used as a virulent control. Virus stocks and methods for determination of infectious titers have been described (*3*).

Antigenic Analysis

We compared reactivity of WNV_{KUN} isolates with a panel of monoclonal antibodies (mAbs) with that of reference strains $WNV_{KUN1960}$ and $WNV_{NY99.}$ To do so, we used a fixed-cell ELISA, as previously described (*3*,*5*,*15*).

Virus Replication Kinetics

We performed growth kinetics analysis by infecting Vero and C6/36 cells at a multiplicity of infection of 1 at 37°C (Vero) or 28°C (C6/36) with WNV_{KUN}. Culture supernatants were harvested at 0, 24, 48, and 72 h after infection (Vero) and 0, 24, 48, 72, 96, and 120 h after infection (C6/36) and titrated (*3*). Statistical significance from 3 independent experiments was determined by using 2-way analysis of variance following log transformation (*16*). Mean virus titers were compared between viruses by using the Tukey method for pairwise multiple comparisons (GraphPad Prism, version 6.0; GraphPad Software Inc., San Diego, CA, USA).

Virulence in Mice

Performance of all animal procedures was approved by The University of Queensland Animal Ethics Committee. To determine virus virulence in mice, we intraperitoneally inoculated 20 Swiss white outbred CD-1 mice (weanlings [18–19 days of age] and young adults [28 days of age]) with a range of doses (0.1–10,000 PFU) of each WNV strain (Table 1) (*3*). The significance of clinical differences between groups was calculated by Kaplan-Meier analysis and analyzed by log-rank test where noted (GraphPad Prism, version 6.0). A virus strain was designated as virulent if survival times for mice infected with this strain (both age groups) differed significantly from those of mice infected with the attenuated reference strain $WNV_{KUN1960}$. All virus strains that did not meet this criterion for virulence were designated as attenuated.

Full-length Genome Sequencing

We sequenced 9 WNV_{KUN} genomes (online Technical Appendix Table 1, http://wwwnc.cdc.gov/EID/article/22/8/15-1719-Techapp1.pdf) by using random primer sequencing on extracts of C6/36 cell cultures (*17*). Viral RNA was extracted by using a MagMAX-96 Viral RNA Isolation Kit (Ambion, Waltham, MA, USA) according to the manufacturer's instructions. cDNA synthesis and random PCR amplification were conducted according to previously described methods (*18*), and resultant PCR amplicons were used for sequencing library preparation. DNA libraries were prepared by using a Nextera XT DNA Sample Preparation Kit (Illumina, San Diego, CA, USA) according to the manufacturer's protocols. Paired-end sequencing of 150-bp fragments was performed by using a MiSeq Reagent Kit V2 (300 cycles) and MiSeq Sequencing System (Illumina). Sequencing data were analyzed by using CLC Genomics Workbench version 6.5.0 (http://www.clcbio.com**).** The sequence data were trimmed by

Table 1. WNV_{KUN} strains used during study of virulence and evolution of WNV, Australia, 1960–2012*

Isolate	Year collected	Location	Source	Passage history
MRM16/MRM61C†	1960	Mitchell River Mission, Queensland	Mosquito‡	Unknown
Boort	1984	Victoria	Horse spinal cord	Unknown
K2499	1984	Kimberley region, Western Australia	Mosquito‡	2× C6/36; 1× PSEK
Hu6774	1991	New South Wales	Human	Unknown
K6453	1991	Kimberley region, Western Australia	Mosquito‡	2× C6/36; 1× PSEK
SH183	1991	Victoria	Chicken	Unknown
Gu0631	2000	Gulf of Carpentaria, Queensland	Mosquito‡	3× C6/36
Gu1009	2000	Gulf of Carpentaria, Queensland	Mosquito‡	3× C6/36
K68967	2009	Kimberley region, Western Australia	Mosquito‡	3× C6/36
P9974	2009	Pilbara region, Western Australia	Mosquito‡	3× C6/36
NSW2011	2011	New South Wales	Horse brain	2× C6/36; 1 Vero
K74015	2011	Kimberley region, Western Australia	Mosquito‡	3× C6/36
NSW2012	2012	New South Wales	Mosquito‡	3× C6/36

*C6/36, from *Aedes albopictus* mosquitoes; PSEK, porcine squamous equine kidney cells; WNV, West Nile virus; WNV_{KUN}, Kunjin strain of WNV.
†Prototype strain.
‡*Culex annulirostris.*

using quality scores specified in CLC Genomics Workbench before performing read-mapping analysis. The genome sequences were assembled by read mapping against the reference WNV_{KUN} strain genome (GenBank accession no. JX276662) with use of default parameters in the mapping algorithm. Where gaps in the genome sequence or low coverage (<10 reads/site) were observed, conventional Sanger sequencing was performed to complete or verify the sequence. Oligonucleotide primers sequences designed for these purposes are available upon request to A.A.K or R.A.H.

Bioinformatics Analysis

We used MUSCLE, as implemented in MEGA6 (*19*), to align complete open reading frame (ORF) (10,320 nt) and partial (402 nt) envelope (E) gene nucleotide sequences of the newly sequenced WNV_{KUN} strains, together with those available for 6 other WNV_{KUN} strains and selected WNV isolates, representing different lineages. We estimated maximum-likelihood phylogenetic trees by using PhyML version 3.0 (*20*) and by using substitution models and rates among sites selected with JModelTest version 2.1.5 (*21*). We tested reliability of the inferred trees by using the bootstrap method with 1,000 replicates. All trees were rooted with analogous ORF sequences from Murray Valley encephalitis virus (GenBank accession no. NC000943) and Japanese encephalitis virus (GenBank accession no. EF571853) and visualized by using FigTree version 1.4.0 (http://tree.bio.ed.ac.uk/software/figtree/). Pairwise distances were determined at the nucleotide and amino acid levels by using the p-distance model in MEGA6.

Results

WNV_{KUN} Strains

A panel of previously characterized mAbs (*5*,*15*,*22*–*24*) was used to antigenically type 13 WNV_{KUN} strains. The binding profiles of these mAbs confirmed that all WNV_{KUN} isolates closely resembled the prototype $WNV_{KUN1960}$ strain, including strong recognition by mAb 10A1, known to be specific for WNV_{KUN} strains (Table 2) (*2*,*24*). Only WNV_{KUN} strains isolated before 2000 were recognized by mAb 5H1, which binds a linear epitope in the αA3 motif (residues 39–53) in the methyltransferase domain of nonstructural (NS) protein 5 (*15*). Lack of 5H1 binding was associated with a substitution at residue 49 (Ile-Val) in αA3 (Table 3); this finding was consistent with previous mutagenesis study findings that a substitution of Ile to Ala at this residue was associated with abolition of 5H1 binding (*26*).

Glycosylated E Proteins

All analyzed WNV_{KUN} isolates collected after 1960 contain glycosylated E proteins. The sequence analysis of the E gene of WNV_{KUN} isolates revealed the presence of a conserved potential N-linked glycosylation site at residue 154 in all but the prototype $WNV_{KUN1960}$ isolate (Table 3). To confirm that this site was indeed glycosylated on the viral E protein, each virus was assessed for recognition in ELISA by mAbs 17D7 and 3.101C (Table 2), which specifically recognize glycosylated and unglycosylated WNV_{KUN} E proteins, respectively (*5*,*23*). The results supported the predictions from our sequencing data; all WNV_{KUN} strains except the prototype $WNV_{KUN1960}$ strain were recognized by mAb 17D7 but not by 3.101C.

Table 2. Binding patterns of monoclonal antibodies to WNV strains in ELISA*

		Monoclonal antibodies, by specificity							
				WNV_{KUN}-specific					
Strain	Year of isolation	Panflavivirus, 4G2, anti-env	Pan-WNV, 2B2, anti-env	10A1, anti-env	5D4, anti-NS5	5H1, anti-NS5	Glycosylated E, 17D7	Unglycosylated E, 3.101C	MVEV-specific, 10C6
WNV_{KUN}†									
KUN1960	1960	+	+	+	+	+	–	+	–
Boort	1984	+	+	+	+	+	+	–	–
K2499	1984	+	+	+	+	+	+	–	–
SH183	1991	+	+	+	+	+	+	–	–
K6453	1991	+	+	+	+	+	+	–	–
Hu6774	1991	+	+	+	+	+	+	–	–
Gu0631	2000	+	+	+	+	+	+	–	–
Gu1009	2000	+	+	+	+	+	+	–	–
K68967	2009	+	+	+	+	–	+	–	–
P9974	2009	+	+	+	+	–	+	–	–
NSW2011	2011	+	+	+	+	–	+	–	–
K74015	2011	+	+	+	+	–	+	–	–
NSW2012	2012	+	+	+	+	–	+	–	–
Reference									
WNV_{NY99}	1999	+	+	–	+	–	+	–	–
$MVEV_{1–51}$	1951	+	–	–	–	–	–	–	+

*Binding of a monoclonal antibody was designated as positive if the optical density was at least double the optical density of the negative control (mock-infected C6/36 cells). E, envelope protein; MVEV, Murray Valley encephalitis virus; NS5, nonstructural protein 5; WNV, West Nile virus; WNV_{KUN}, Kunjin strain of WNV; +, positive; –, negative.
†WNV_{KUN} strains collected during 1960–2012, Australia.

Table 3. Amino acid sequences in the West Nile virus genome*

WNV strain	Year of isolation	prM, residue 22/72†	Putative virulence determinant: E protein, residues 154–156‡	NS3, residue 249§	NS5, residue 653¶	NS5, residue 49	3′ UTR residues 64–71
NY99	1999	Val/Ser	Asn-Tyr-Ser (NYS)	Pro	Phe	Val	Present
KUN1960	1960	Ile/Leu	Asn-Tyr-Phe (NYF)	Ala	Ser	Ile	Present
Boort	1984	Ile/Leu	Asn-Tyr-Ser (NYS)	Ala	Phe	Ile	Present
K2499	1984	Ile/Leu	Asn-Tyr-Ser (NYS)	Ala	Phe	Ile	Present
K6453	1991	Ile/Leu	Asn-Tyr-Ser (NYS)	Ala	Phe	Ile	Present
Hu6774	1991	Ile/Leu	Asn-Tyr-Ser (NYS)	Ala	Phe	Ile	Present
Gu0631	2000	Ile/Leu	Asn-Tyr-Ser (NYS)	Ala	Phe	Ile	Present
Gu1009	2000	Ile/Leu	Asn-Tyr-Ser (NYS)	Ala	Phe	Ile	Absent
K68967	2009	Ile/Leu	Asn-Tyr-Ser (NYS)	Ala	Phe	Val	Absent
P9974	2009	Ile/Leu	Asn-Tyr-Ser (NYS)	Ala	Phe	Val	Absent
NSW2011	2011	Ile/Leu	Asn-Tyr-Ser (NYS)	Ala	Phe	Val	Absent
K74015	2011	Ile/Leu	Asn-Tyr-Ser (NYS)	Ala	Phe	Val	Absent
NSW2012	2012	Ile/Leu	Asn-Tyr-Ser (NYS)	Ala	Phe	Val	Absent

*E, envelope; NS, nonstructural; prM, premembrane; UTR, untranslated region.
†(*11*).
‡(*7*).
§(*25*).
¶(*10*).

Growth Kinetics and Plaque Morphology of WNV_{KUN} Strains

Infection of Vero cells at 24 h postinfection demonstrated that $WNV_{KUN1960}$, WNV_{K2499}, WNV_{K6453}, and WNV_{K68967} isolates yielded significantly lower titers than did WNV_{NY99} ($p<0.05$) (Figure 1, panel A). However, by 48 h postinfection, similar titers were reached for all WNV isolates except $WNV_{KUN1960}$.

A similar trend was observed in C6/36 cells, in which viral titers of $WNV_{KUN1960}$, WNV_{K2499}, WNV_{K68967}, and WNV_{GU1009} were significantly lower than those for WNV_{NY99}, $WNV_{NSW2011}$, and WNV_{Boort} at 48 h after infection ($p<0.05$) and titers for WNV_{K6453}, WNV_{K74015}, and WNV_{GU0631} were intermediate. By 96 h after infection, the titers of all WNV isolates except $WNV_{KUN1960}$ were similar.

In terms of plaque morphology of WNV_{KUN} strains, in Vero cells, $WNV_{NSW2011}$ and WNV_{K74015} produced large plaques (average size 4.3 ± 0.63 and 4.3 ± 0.77 mm, respectively), a size similar to those produced by the WNV_{NY99} strain (average size 4.8 ± 0.45 mm). The prototype virus, $WNV_{KUN1960}$, produced very small plaques (average size 2.7 ± 0.47 mm), which differed significantly from those of all other viruses tested during this study ($p<0.0001$). The remaining isolates produced intermediate-sized plaques (average size 3.5–3.9 ± 0.45–0.84 mm) (Figure 2) (*5*). Plaques formed by WNV_{K6453}, WNV_{K74015}, WNV_{Gu1009}, and WNV_{Gu0631} were less well defined than those formed by WNV_{NY99}, $WNV_{NSW2011}$, WNV_{K2499}, and WNV_{Boort}.

Virulence of WNV_{KUN} Strains in Mice

We previously demonstrated that differentiation between virulent and attenuated strains of WNV can be detected in weanling and young adult mice (*3*,*27*). In this study, we found that in addition to $WNV_{NSW2011}$, 3 other WNV_{KUN} isolates (WNV_{Boort}, WNV_{Gu0631}, $WNV_{NSW2012}$) were neuroinvasive in both mouse models (Figure 3; online Technical Appendix Table 2). The WNV_{Boort} strain, obtained from the spinal cord of a symptomatic horse during a small outbreak of equine disease in southeastern Australia in 1984, was neuroinvasive in young adult mice (40% mortality rate at 1,000 PFU); this finding did not statistically differ in this respect from that for $WNV_{NSW2011}$ ($p = 0.3218$)

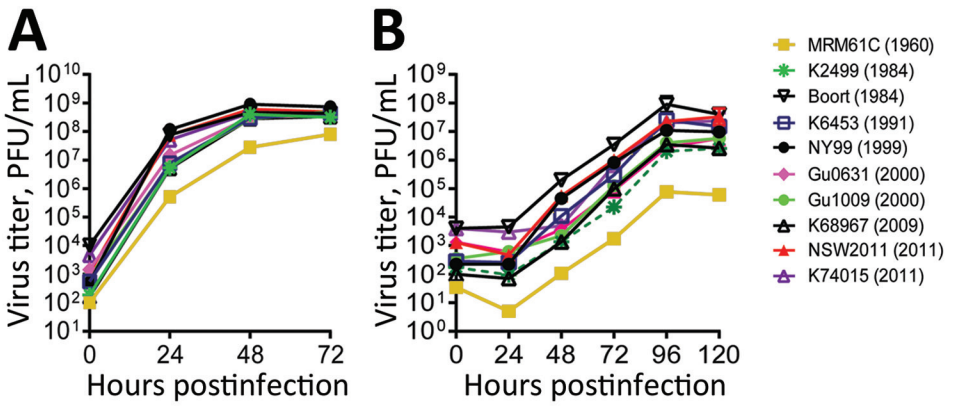

Figure 1. Growth kinetics of West Nile virus strains isolated in Australia, 1960–2012, in Vero (A) and C6/36 (B) cells. Cells were infected with a multiplicity of infection of 1, and the virus titers in the supernatants were determined by plaque assay on Vero cells.

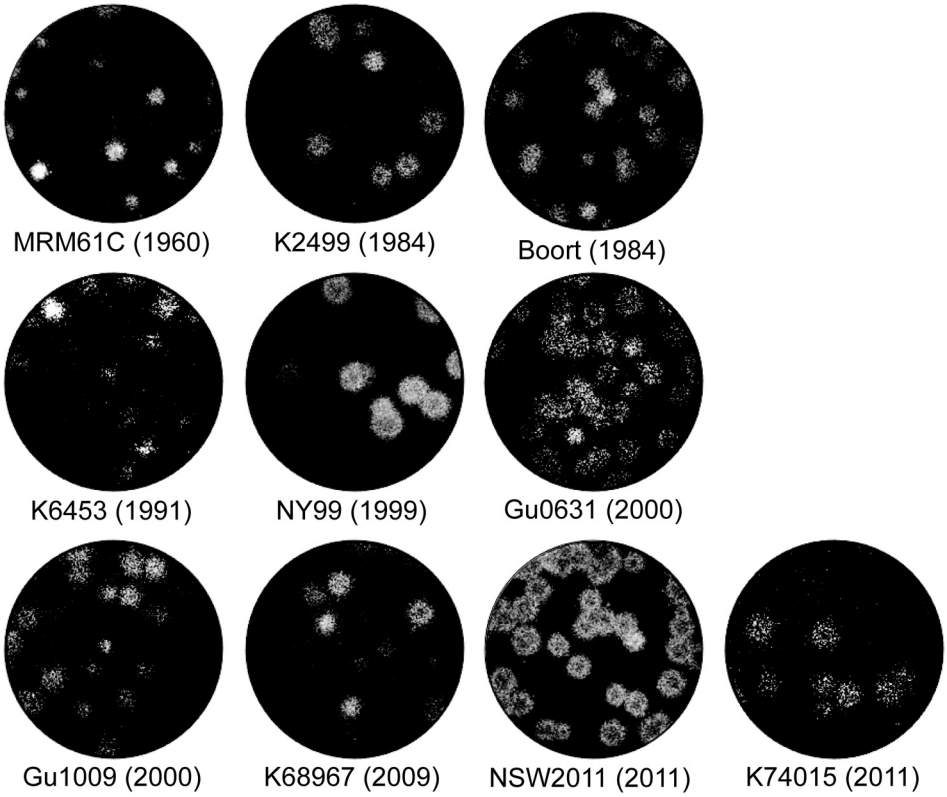

Figure 2. Plaque morphology of representative West Nile virus strains isolated in Australia, 1960–2012. Virus was allowed to adsorb to monolayers of Vero cells for 2 h at 37°C. The cells were then overlaid with Dulbecco Modified Eagle Medium containing 0.5% low melting point agarose and 2% fetal bovine serum. Four days after infection, the cells were fixed with 4% formaldehyde solution and stained with 0.2% crystal violet.

(Figure 3). Two other isolates obtained from mosquitoes, 1 from the Gulf of Carpentaria in 2000 (WNV_{Gu0631}) and 1 from southeastern Australia in 2012 ($WNV_{NSW2012}$), also exhibited levels of neuroinvasive properties in young adult mice similar to those caused by $WNV_{NSW2011}$ (Figure 3). In weanling mice, the virulence of WNV_{Boort}, WNV_{Gu0631}, and $WNV_{NSW2012}$ was also similar to that of $WNV_{NSW2011}$; mortality rates, 50% lethal dose, or time to death did not differ significantly (Table 4). Of note, WNV_{Gu1009} isolated at the same time and from the same region as WNV_{GU0631} was significantly less virulent in young adult mice (Figure 3; online Technical Appendix Table 2). The remaining isolates were relatively attenuated in both young adult (Figure 3) and weanling (online Technical Appendix Table 2) mice and did not differ significantly from the attenuated prototype $WNV_{KUN1960}$ strain ($p>0.05$).

Sequence of Viral Genomes

We sequenced WNV_{KUN} isolates to analyze their relationship to the prototype WNV_{KUN} strains from 1960 (WNV_{MRM16}, WNV_{MRM61C}); the 2011 outbreak strains ($WNV_{NSW2011}$, WNV_{SA2011}, WNV_{V11-03}, and WNV_{V11-07}); and exotic strains of WNV known to be virulent in humans and horses (WNV_{NY99}) or representing different WNV lineages. Phylogenetic analysis of the ORF sequences demonstrated that the WNV_{KUN} strains form a single genetically homogeneous clade within lineage 1 (Figure 4), as previously recognized (*24*); nucleotide and amino acid identities between strains were 96.1%–99.4% and 98.5%–100%, respectively. As expected, the most recent isolates, including the 2011 outbreak strain, were the most divergent, and the early prototype strains (WNV_{MRM61C} and WNV_{MRM16}) occupied the basal lineage of this clade. Recent strains isolated in 2011 and 2012 from southeastern Australian states clustered together and shared high levels of nucleotide (98.6%–100%) and amino acid (99.6%–100%) identities, indicating transmission of a genetically homogeneous virus population during this period. These strains were either virulent for horses or shown in this study to be virulent in mice (Figure 3; online Technical Appendix Table 2). No other association between phylogenetic relationships and virulence was found; other virulent strains clustered closely and interspersed with the attenuated strains identified in this study. An expanded phylogenetic analysis that used 45 partial E gene sequences (402 nt) and a larger range of reference WNV_{KUN} strains showed a similar pattern of relationships (online Technical Appendix Figure).

Of note is the very close relationship (99.9% aa identity) between a virulent 2011 strain isolated from a horse and an isolate obtained from *Culex annulirostris* mosquitoes trapped in New South Wales, Australia, in 2012 ($WNV_{NSW2012}$). Only 3 nonconservative changes were identified between $WNV_{NSW2011}$ and $WNV_{NSW2012}$, located in NS1 (Lys33Arg), NS3 (Phe509Leu), and NS4A

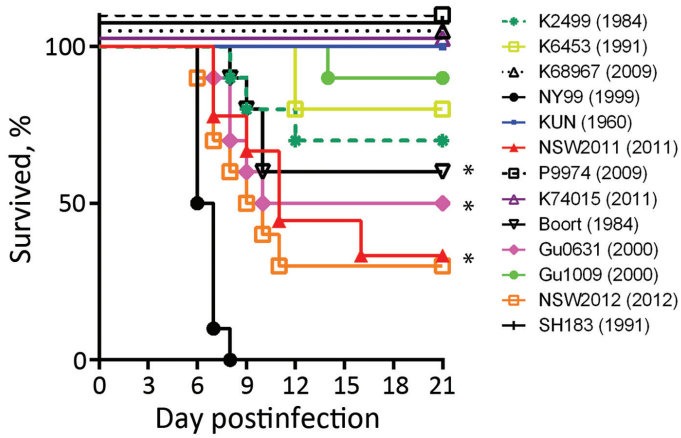

Figure 3. Survival curves for young adult (28-day-old) Swiss outbred mice after intraperitoneal infection with 1,000 PFU of West Nile virus (WNV) strains isolated in Australia, 1960–2012. Groups of 10 mice were infected with each virus. The mice were monitored for 21 days after infection for signs of encephalitis and then euthanized. WNV_{NY99} and $WNV_{NSW2011}$ with previously demonstrated virulence were included as controls. The significance of clinical differences between groups was calculated by Kaplan-Meier analysis and analyzed by log-rank test. Significantly increased virulence over that of $WNV_{KUN1960}$ is indicated by an asterisk (*): WNV_{Boort} ($p = 0.0295$), WNV_{Gu0631} ($p = 0.0115$), and $WNV_{NSW2012}$ ($p = 0.0011$). No significant differences were observed between WNV_{Boort}, WNV_{Gu0631}, and $WNV_{NSW2012}$ compared with $WNV_{NSW2011}$ ($p>0.05$).

(Phe92Leu). These results suggest that the virulent strain either had persisted in New South Wales after the end of the 2011 outbreak or had been reintroduced to the area.

Analyses of predicted gene products from the complete ORF sequence of each WNV_{KUN} isolate revealed that, in addition to the glycosylation site at residues 154–156 in the E protein, all strains isolated after 1960 contained a Phe residue at position 653 in the NS5 protein, which has previously been shown to play a role in resistance to antiviral activity of interferon-α/β (*10*) (Table 3). In contrast, $WNV_{KUN1960}$ contained a Ser residue at position 653 in NS5 (*3*,*5*,*24*,*28*). The Pro residue at position 249 in the NS3 protein, previously shown to be present in WNV strains and associated with increased virulence in birds of some species (*25*), was not present in any of the WNV_{KUN} isolates, which all contained an Ala residue at this position (Table 3).

In addition to an Ile→Val substitution at position 49 in NS5 of WNV_{KUN} isolates collected after 2009, analysis of more contemporary WNV_{KUN} isolates also revealed a consistent 8-nt deletion in the 3′ UTR, just downstream from the ORF stop codon. This deletion was identified in WNV_{Gu1009} and all isolates collected after 2000. In contrast, this deletion was not present in isolates collected before 2000 (Table 3) or in another isolate from Gulf of Carpentaria collected in 2000 (WNV_{Gu0631}). We suggest that these 2 features (Ile→Val 49 residue in NS5 and an 8-nt deletion in the 3′ UTR) can be considered as potential evolutionary markers.

In addition to the genetic variability described above, sequence analysis between virulent and attenuated WNV_{KUN} strains identified other nucleotide differences between isolates, located throughout the viral genome. These differences result in amino acid substitutions (Table 4) and may contribute to observed phenotypic differences.

We also sequenced WNV_{KUN} viral RNA extracted directly from mosquito saliva expectorated onto sugar-soaked nucleic acid preservation cards placed in mosquito traps in Darwin, Northern Territory, in 2012 (WNV_{NT2012}) (*29*,*30*). When partial sequences from E, NS5, and the 3′ UTR from this RNA were aligned, we observed a high level (99.7%) of identity with the $WNV_{KUN1960}$ strains, indicating that viruses genetically homologous to the prototype virus are still circulating in some regions of Australia (online Technical Appendix Figure). Closer analysis revealed a lack of E glycosylation, similar to that found in the prototype strain. However, Phe was identified at position 653 of NS5, similar to that found in recent isolates.

Discussion

Historically, WNV_{KUN} has been associated with only mild disease in humans and rare cases of disease in horses, consistent with data from mouse virulence studies that revealed a relatively attenuated phenotype (*3*,*8*,*27*). Thus, the emergence of an equid-virulent strain of WNV_{KUN}, responsible for ≈900 cases of encephalitis in horses in southeastern Australia, was unprecedented.

Although most WNV_{KUN} isolates examined in this study exhibited an attenuated phenotype, similar to that of the prototype $WNV_{KUN1960}$, we identified an additional 3 strains with neuroinvasive properties in mice similar to those reported for $WNV_{NSW2011}$ (*3*). The first, WNV_{Boort}, was isolated from the spinal cord of a horse with nonsuppurative encephalomyelitis and severe neurologic symptoms in northern Victoria in 1984 (*31*). At that time, 53 animals in the same area were clinically affected. However, a high incidence of Ross River virus–specific antibody in these animals implicated that virus rather than WNV_{KUN} as the primary etiologic agent (*31*). Our results are also supported by another recent study showing virulence of WNV_{Boort} in 18–19-day-old mice (*32*).

The second virulent strain identified in this study, WNV_{Gu0631}, was isolated from *Cx. annulirostris* mosquitoes collected from Normanton, Gulf of Carpentaria, in April 2000. Of note, this virus was isolated in the absence of any reported disease outbreak, as part of a survey for the presence of Japanese encephalitis virus in northern Queensland

Table 4. Comparison of amino acid sequences between virulent and attenuated West Nile virus strains*

Gene and amino acid position in polyprotein (corresponding protein)	Virulent strains		Attenuated Kunjin strains						
	NY99	Boort, NSW2011, NSW2012, Gu0631	KUN1960	K68967	K2499	K6453	K74015	Hu6774	Gu1009
C									
86 (86)	Lys	Lys	Arg	Lys	Lys	Lys	Lys	Lys	Lys
114 (114)	Met	Ile (Boort), Thr (NSW2011, NSW2012, Gu0631)	Met	Thr	Thr	Thr	Met	Thr	Thr
prM									
143 (20)	Thr	Ala	Thr	Ala	Ala	Ala	Ala	Ala	Ala
158 (35)	Ile	Thr	Ile	Thr	Thr	Thr	Thr	Thr	Thr
279 (156)	Val	Thr	Ala	Thr	Thr	Thr	Thr	Thr	Thr
E									
413 (123)	Thr	Thr	Ala	Thr	Thr	Thr	Thr	Thr	Thr
446 (156)	Ser	Ser	Phe	Ser	Ser	Ser	Ser	Ser	Ser
600 (310)	Lys	Arg	Thr	Arg	Arg	Arg	Arg	Arg	Arg
773 (483)	Leu	Phe	Leu	Phe	Phe	Phe	Leu	Phe	Phe
790 (500)	His	His	Tyr	His	His	His	His	His	His
NS1									
1081 (290)	Ser	Asn	Ser	Asn	Asn	Asn	Ser	Asn	Asn
NS2A									
1255 (112)	Val	Val	Ala	Val	Val	Val	Val	Val	Val
1272 (129)	Ile	Ile	Met	Ile	Ile	Ile	Ile	Ile	Ile
1366 (223)	Ile	Ile	Val	Ile	Ile	Ile	Ile	Ile	Ile
NS3									
1520 (146)	Lys	Lys	Arg	Lys	Lys	Lys	Lys	Lys	Lys
1970 (586)	Asn	Ser	Asn	Ser	Ser	Ser	Asn	Ser	Ser
NS4A									
2179 (55)	Ala	Thr	Ala	Thr	Thr	Thr	Thr	Thr	Ala
NS4B									
2296 (23)	Val	Ile	Thr	Ile	Ile	Ile	Ile	Ile	Ile
2324 (51)	Val	Phe	Val	Phe	Phe	Phe	Val	Phe	Phe
2368 (95)	Ala	Ser	Ala	Ser	Ser	Ser	Ser	Ser	Ser
2450 (177)	Met	Ile	Met	Ile	Ile	Ile	Ile	Ile	Ile
2518 (245)	Ile	Ile	Val	Ile	Ile	Ile	Ile	Ile	Ile
NS5									
2629 (101)	Arg	Lys	Arg	Lys	Lys	Lys	Arg	Lys	Lys
3088 (560)	Asp	Asn	Asp	Asn	Asn	Asn	Asp	Asn	Asn

*C, capsid; E, envelope; NS, nonstructural; prM, premembrane.

(*33*). The second Gulf of Carpentaria isolate, WNV_{Gu1009}, was also collected in April 2000, from the town of Karumba, which is ≈30 km from Normanton. However, WNV_{GU1009} is genetically distinct and attenuated to the same degree as the prototype $WNV_{KUN1960}$ in 28-day-old mice (Figure 4). These observations demonstrated that virulent WNV_{KUN} strains might co-circulate with attenuated strains in some regions of Australia. Furthermore, the circulation of neuroinvasive strains may often appear in the absence of disease outbreaks. This suggestion is consistent with our finding that $WNV_{NSW2012}$ was genetically almost identical to the $WNV_{NSW2011}$ and exhibited similar levels of neuroinvasiveness in mice. However, no cases of disease in equids were associated with WNV_{KUN} infection during the 2012 season (*3*,*4*,*34*). This lack of cases suggests that the persistence of virulent strains in southeastern Australia is not the sole determinant for initiating disease outbreaks and that specific climatic and ecologic conditions, perhaps influencing mosquito populations and viral transmission, are also required.

A similar scenario occurred in North America, where an unusually high number of cases in humans (5,387), most in Texas, USA, were reported in 2012. However, sequence analysis of WNV isolates from 2012 revealed that the strains circulating in Texas were virulent and attenuated, and no specific virulence determinants responsible for the increase in cases could be identified (*35*). Instead, other factors, including temperature and changes in mosquito or bird populations, were speculated to have contributed to the magnitude of the 2012 outbreak (*36*).

To identify a phylogenetic association with virulence and to identify potential virulence determinants encoded in the genome of WNV_{KUN} strains, we also performed full-length sequencing of the ORF of several of the viruses studied. Although recent virulent strains were phylogenetically closely related, no other association between phylogenetic grouping and virulence phenotype was found (Figure 4; online Technical Appendix Figure). One notable change in the genome that was clearly associated with the temporal distribution of these viruses was a highly conserved 8-base deletion in the 3′ UTR, just downstream of the ORF stop codon. Isolates from samples collected after 2000, including the virulent $WNV_{NSW2011}$ and attenuated

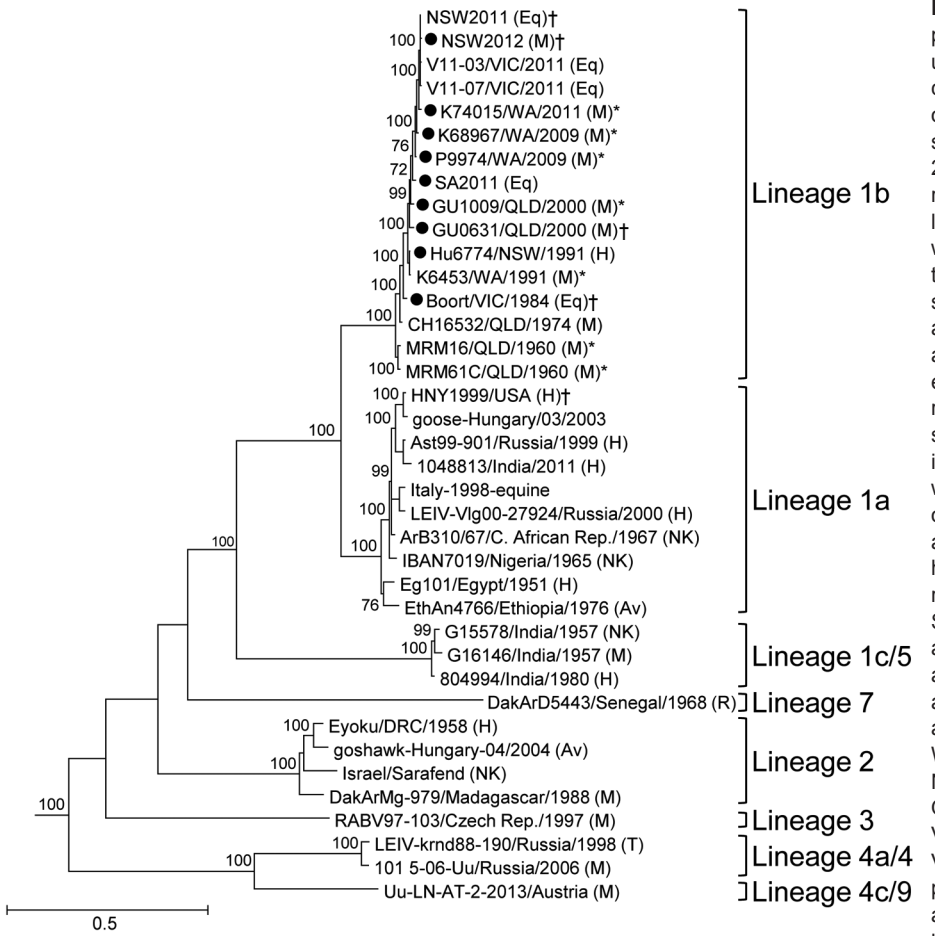

Figure 4. Maximum-likelihood phylogenetic tree estimated by using nucleotide sequences of the complete open reading frame (ORF) of genomes of West Nile virus (WNV) strains isolated in Australia, 1960–2012 (black circles), compared with representative strains from different lineages and clades. The tree was estimated by using a general time-reversible model of nucleotide substitution with a gamma distribution and invariant sites. Bootstrap values are shown on the nodes and are expressed as a percentage of 1,000 replicates; only values >70% are shown. Horizontal branch lengths indicate genetic distance. The tree was rooted with the ORF sequences of Murray Valley encephalitis virus and Japanese encephalitis virus; however, these branches have been removed to improve resolution. Strains that were assessed as having an attenuated virulence phenotype are indicated by a single asterisk (*), and virulent strains are indicated by a dagger (†).The state of origin for WNV_{KUN} strains is shown as follows: NSW, New South Wales; QLD, Queensland; SA, South Australia; VIC, Victoria; WA, Western Australia. Virus sources are indicated in parentheses next to virus identity, as follows: Av, avian; Eq, equine; H, human; M, mosquito; NK, not known; R, rodent; T, tick. Scale bar indicates nucleotide substitutions per site.

strains, invariably contained this deletion. This finding suggests that the deletion is an evolutionary marker but is not directly associated with virulence. This finding is also consistent with our observation that the neuroinvasive 2000 Gulf of Carpentaria isolate, WNV_{Gu0631}, did not have this deletion but that the co-circulating attenuated isolate, WNV_{Gu1009}, collected from the same region at the same time, did have this deletion.

An additional evolutionary change was observed in the a-A3 motif of the methyltransferase domain of the NS5 protein. Isolates obtained before 2009, including the prototype $WNV_{KUN1960}$, contained a conserved Ile residue at position 49. However, all isolates collected after 2009 displayed an Ile→Val substitution at this position. Coincidentally, this substitution abolished the binding of a mAb (5H1) that recognizes a linear epitope comprising the a-A3 peptide (*15*).

Initial comparisons between the virulent isolate $WNV_{NSW2011}$ from a horse and the attenuated prototype $WNV_{KUN1960}$ revealed that several previously identified WNV virulence markers were detected in the former but not in the latter isolate (*3*). These markers included the conserved N-linked glycosylation of the E protein (*7*) and the Phe residue at position 653 in the NS5 protein, associated with resistance to antiviral activity of interferon a/b (*8*). Although these initial observations suggested the involvement of these motifs in the enhanced neuroinvasive properties of the isolate collected from a horse in 2011, our study revealed that, with the exception of $WNV_{KUN1960}$, all strains examined contain both of these markers, regardless of virulence phenotype in mouse models. Thus, it seems that, although these motifs contribute to virulence in mice, they are not likely to be solely responsible for enhancing the neuroinvasive properties of some WNV_{KUN} strains and, hence, not likely to be markers of evolving virulence in recent isolates of WNV_{KUN}.

Additional markers of WNV virulence identified in WNV strains from North America were not detected in any of the WNV_{KUN} isolates. This finding is consistent with our repeated observations that even the equid-virulent

$WNV_{NSW2011}$ is substantially less neuroinvasive than WNV_{NY99} in young adult mice (*3*). These motifs may include Val 22 and Ser 72 residues in the premembrane, which enhance mouse neuroinvasiveness when introduced into the prototype $WNV_{KUN1960}$ (*11*), and the Pro residue at position 249 in NS3, which is associated with enhanced virulence in birds (*25*). The absence of the latter motif in all WNV_{KUN} strains is also consistent with the perceived lack of illness and death among birds in Australia, notably during the 2011 outbreak among equids. Some isolates included in this study (including $WNV_{KUN1960}$, WNV_{SH183}, WNV_{Boort}, and WNV_{Hu6774}) have an unknown passage history. Extensive passage through cells is known to occasionally lead to passage-adapted mutations, and care should be taken when interpreting sequencing data from these virus strains.

WNV_{KUN} is thought to be endemic to the tropical areas of northern Australia, suggesting that virulent viruses emerging in southeastern Australia probably originate from northern Australia. However, WNV_{KUN} recently isolated from mosquitoes in northern Australia, including the 2011 Kimberley isolate WNV_{K74015}, were more attenuated than $WNV_{NSW2011}$. This finding suggests a different explanation for the evolution of virulent WNV_{KUN} viruses, which may be associated with the adaption of WNV_{KUN} to different hosts (avian and terrestrial) or different vector species in temperate regions. In this context, virulence in equids may be just a coincidental outcome of the constraints placed on virus fitness in different geographic locations (*35*–*37*).

Overall, our results show that virulent strains of WNV_{KUN} have been circulating in Australia for ≥30 years and that the first extensive outbreak of disease among horses in Australia in 2011 probably resulted from a combination of ecologic and epidemiologic conditions rather than the emergence of a novel, more virulent strain. Further studies evaluating viral fitness of West Nile virus quasispecies in terms of population-dependent host–virus interactions, are warranted.

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Dr. Prow is a postdoctoral research fellow at the QIMR Berghofer Medical Research Institute and is engaged in research to characterize the pathogenesis and virulence of WNV strains in murine models. She also has a special interest in understanding the neuroinvasive properties of neurotrophic flaviviruses.

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Virulence and Evolution of West Nile Virus, Australia

Technical Appendix

Technical Appendix Table 1. GenBank accession numbers and details of WNV strains used for phylogenetic analysis in study of virulence and evoloution of WNV, Australia

Isolate	Location	Year collected	Source	GenBank accession no.
MRM16	Mitchell River Mission, QLD	1960	*Cx. annulirostris*	GQ851602
MRM61C	Mitchell River Mission, QLD	1960	*Cx. annulirostris*	D00246
MRM5373	Mitchell River Mission, QLD	1966	*Cx. annulirostris*	AF196509
OR134	Kimberley region, WA	1973	*Cx. annulirostris*	AF196506
OR4	Kimberley region, WA	1973	*Cx. annulirostris*	AF196523
OR205	Kimberley region, WA	1973	*Cx. annulirostris*	AF196515
OR130	Kimberley region, WA	1973	*Cx. annulirostris*	AF196492
OR166	Kimberley region, WA	1973	*Cx. annulirostris*	AF196499
CH16532C	Charleville, QLD	1974	*Cx. annulirostris*	JX276662
CH16549E	Charleville, QLD	1974	*Cx. annulirostris*	AF196520
CH16514C	Charleville, QLD	1974	*Cx. annulirostris*	AF196501
CH16465C	Charleville, QLD	1974	*Cx. annulirostris*	AF196504
OR393	Kimberley region, WA	1974	*Cx. annulirostris*	AF196503
OR354	Kimberley region, WA	1974	*Cx. annulirostris*	AF196518
WK436	Kimberley region, WA	1979	*Cx. annulirostris*	AF196507
CX238	Kimberley region, WA	1982	*Cx. annulirostris*	AF196502
M695	VIC	1982	*Cx. annulirostris*	AF196496
M1465	VIC	1983	*Cx. annulirostris*	AF196522
V407	Jabiru, NT	1983	*Cx. annulirostris*	AF196508
Boort*	Boort, VIC	1984	Horse spinal cord	KT934796
K2499	Kimberley region, WA	1984	*Cx. annulirostris*	AF196498
FC15	Kimberley region, WA	1986	*Cx. annulirostris*	AF196510
K5374	Kimberley region, WA	1989	*Cx. annulirostris*	AF196517
K1738	Kimberley region, WA	1989	*Cx. annulirostris*	AF196494
Hu6774*	Southern NSW	1991	Human	KT934797
K6453	Kimberley region, WA	1991	*Cx. annulirostris*	GQ851603
K6590	Kimberley region, WA	1991	*Cx. annulirostris*	AF196500
K6547	Kimberley region, WA	1991	*Cx. annulirostris*	AF196521
SH183	VIC	1991	Chicken	AF196491
P1553	Kimberley region, WA	1994	*Cx. species*	AF196495
CY3404	Cape York, QLD	2000	*Cx. sitiens* subgroup	AY251614
Gu0631*	Gulf of Carpentaria, QLD	2000	*Cx. annulirostris*	KT934798
Gu1009*	Gulf of Carpentaria, QLD	2000	*Cx. annulirostris*	KT934799
TS5656	Torres Strait, QLD	2000	*Cx. sitiens* subgroup	AY251611
TS5677	Torres Strait, QLD	2000	*Cx. sitiens* subgroup	AY251612
TS5750	Torres Strait, QLD	2000	*Cx. sitiens* subgroup	AY251613
TS5926	Torres Strait, QLD	2000	*Cx. sitiens* subgroup	AY251610
K68967*	Kimberley region, WA	2009	*Cx. annulirostris*	KT934802
P9974*	Pilbara region, WA	2009	*Cx. annulirostris*	KT934800
NSW2011	Eastern NSW	2011	Horse brain	JN887352
SA2011*	Hallett, SA	2011	Horse brain	KT934803
K74015*	Kimberley region, WA	2011	*Cx. annulirostris*	KT934801
V11-03	VIC	2011	Horse CNS tissue	JX123030
V11-07	Euroa, VIC	2011	Horse CNS tissue	JX123031
NSW2012*	Eastern NSW	2012	*Cx. annulirostris*	Submitted (1863956)
NT S3083	Leanyer swamp, NT	2012	Mosquito (FTA card)	KC492441
Eg101	Cairo, Egypt	1950	Human	AF404756
EthAn4766	Ethiopia	1976	Bird	AY603654
Italy-1998-equine	Tuscany, Italy	1998	Horse	AF404757
LEIV-Vlg00-27924	Volgograd, Russia	2000	Human	AY278442
Ast99-901	Volga delta, Russia	1999	Human	AY603654

Isolate	Location	Year collected	Source	GenBank accession no.
1048813	Kerala, India	2011	Human	KC601756
HNY1999	New York, USA	1999	Human	AF202541
goose-Hungary/03	Hungary	2003	Goose	DQ118127
IBAN7019	Nigeria	1965	Unknown	GQ851607
ArB310/67	Central African Republic	1967	Unknown	GQ851608
Sarafend	Israel	Unknown	Unknown	AY688948
Eyoku	Democratic Republic of Congo	1958	Human	HM147824
DakArMg-979	Madagaskar	1988	*Cx. quinquefasciatus*	HM147823
goshawk-Hungary-04	Hungary	2004	Goshawk	DQ116961
Rabensburg (97-103)	South Moravia, Czech Republic	1997	*Cx. pipiens*	AY765264
LEIV-krnd88	NW Caucasus, Russia	1998	*Dermacentor marginatus*	AY277251
101_5-06-Uu	Volgograd, Russia	2006	*Uranotaenia unguiculata*	FJ159129
G16146	India	1957	Mosquito	KJ831223
G15578	India	1957	Not known	GQ851604
804994	Bangalore, India	1980	Human	DQ256376
DakArD5443	Senegal	1968	*Tatera kempi* (rodent)	EU082200
WNV-Uu-LN-AT-2013	Burgenland State, Austria	2013	*Ur. unguiculata*	KJ831223

*WNV_{KUN} strains sequenced as part of this study.

Technical Appendix Table 2. Virulence of West Nile virus strains in 18–19-d–old mice after intraperitoneal infection

Virus strain and dose, pfu	No. mice died/no. mice injected	Average survival time, d	LD_{50}
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	
KUN (1960)[#*]			
1000	9/10	8.4	
100	4/10	8	
10	6/10	10.2	13.4 pfu
1	3/10	12	
NSW2011*			
1000	10/10	7.1	
100	10/10	7.4	
10	10/10	7.7	
1	7/10	8.3	0.5 pfu
0.1	1/10	10	
NSW2012			
10	10/10	7.4	
1	7/10	8.3	0.5 pfu
0.1	1/10	10	
K2499			
1000	10/10	7.4	
100	10/10	7.8	
10	9/10	9.5	2.0 pfu
1	3/10	17.1	
0.1	0/12	21	
K6453			
1000	6/10	12.4	
100	5/10	15.4	
10	4/10	17.1	
1	1/10	20.1	100 pfu
0.1	0/10	21	
K68967			
1000	10/10	7.4	
100	10/10	7.8	
10	5/10	14.3	10 pfu
1	4/10	15.7	
0.1	0/10	21	
K74015			
10	5/10	9	10 pfu
P9974			
1000	9/10	8.5	
100	9/10	10.3	

Virus strain and dose, pfu	No. mice died/no. mice injected	Average survival time, d	LD_{50}
10	5/10	15.5	10 pfu
1	1/10	19.9	
0.1	0/10	21	
SH183			
100	10/10	7	
10	8/10	10.1	2.5 pfu
1	3/10	17.7	
Boort			
100	10/10	6.9	
10	10/10	7.2	
1	9/10	9.4	
0.1	5/10	15.1	0.1 pfu
Gu1009			
100	9/10	9.3	
10	9/10	10.1	
1	9/10	10	0.4 pfu
0.1	2/10	18.9	
Gu0631			
100	10/10	7.2	
10	10/10	7.6	
1	10/10	7.8	0.2 pfu
0.1	2/10	18.6	

*Reference virus, data from (*1*).

Technical Appendix Figure. Maximum-likelihood phylogenetic tree estimated using partial envelope gene sequences (402 nt) of WNV_{KUN} strains and reference WNV strains. The tree was estimated using a general time-reversible model of nucleotide substitution with a gamma distribution and invariant sites. Bootstrap values are shown on the nodes and are expressed as a percentage of 1,000 replicates; values >70% only are shown. Horizontal branch lengths indicate genetic distance proportional to the scale bar. The tree generated using the same reference sequences as for the open reading frame phylogeny and was rooted with Murray Valley encephalitis and Japanese encephalitis sequences. To improve resolution, all branches of reference sequences have been removed except for lineage 1, clade a. Strains sequenced as part of this study are indicated by a closed circle. Those that were assessed as having an attenuated virulence phenotype are indicated by a single asterisk, while virulent strains are indicated by a double asterisk. The state of origin for WNV_{KUN} strains is shown with the following abbreviations: NSW, New South Wales; QLD, Queensland; SA, South Australia; VIC, Victoria; WA, Western Australia. Virus source is indicated in parentheses next to its identity, as follows: Av, avian; Eq, equine; H, human; M, mosquito. WNV, West Nile virus.

Reference

1. Frost MJ, Zhang J, Edmonds JH, Prow NA, Gu X, Davis R, et al. Characterization of virulent West Nile virus Kunjin strain, Australia, 2011. Emerg Infect Dis. 2012;18:792–800. PubMed http://dx.doi.org/10.3201/eid1805.111720