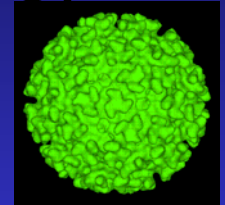




Epiornitic determinants of West Nile virus in North America: a murder of crows

CDC WNV annual meeting
February 23, 2006, San Francisco, CA

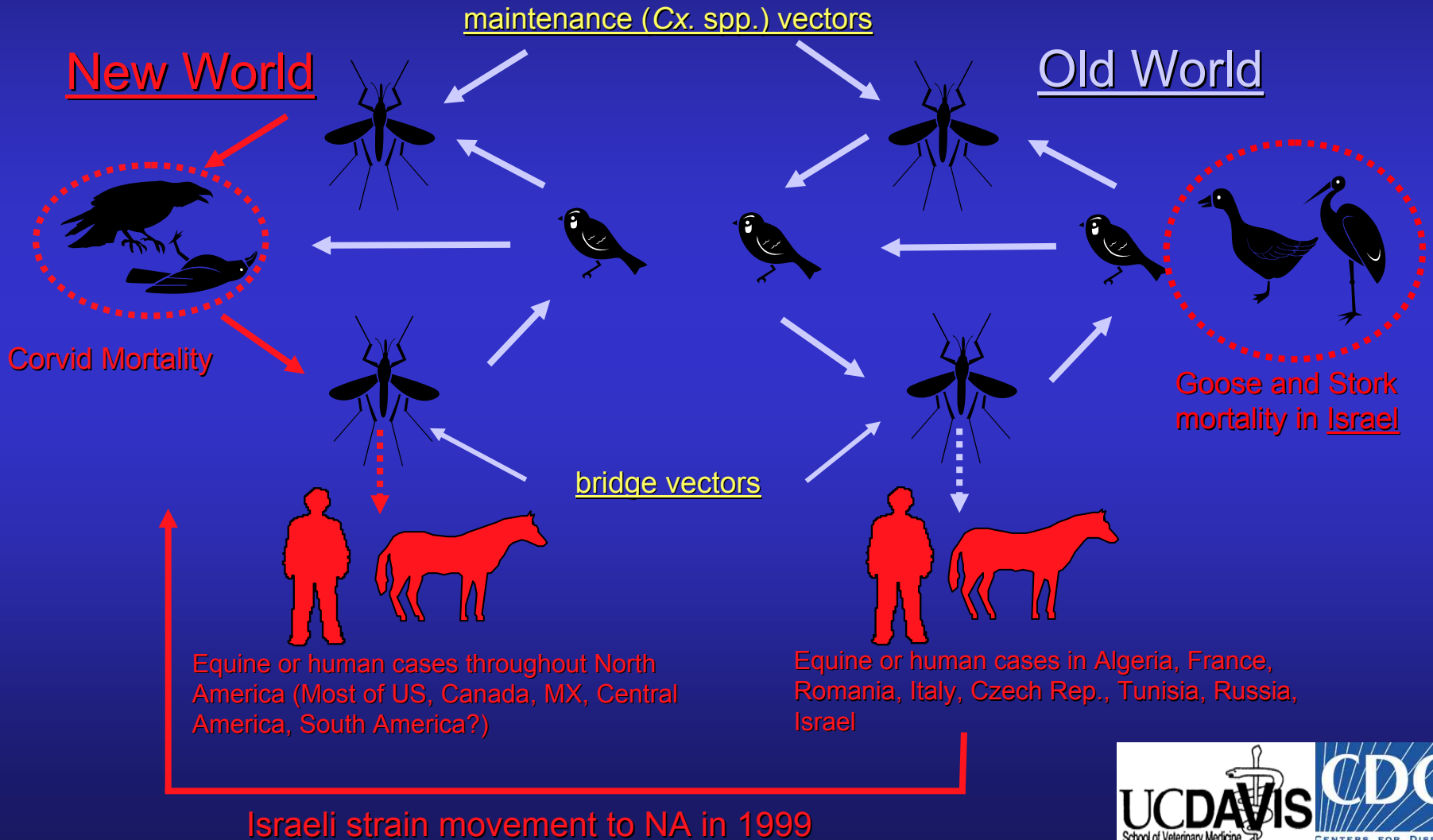


Aaron C. Brault^{1,2,3}, Stanley A. Langevin^{1,3}, Richard A. Bowen⁴,
Leslie Woods^{2,5}, Nicholas A. Panella³, Claire Y.-H. Huang³,
Nicholas Komar³, Ann M. Powers³, Barry R. Miller³, Richard M.
Kinney³, David W.C. Beasley⁶ and Alan D.T. Barrett⁶

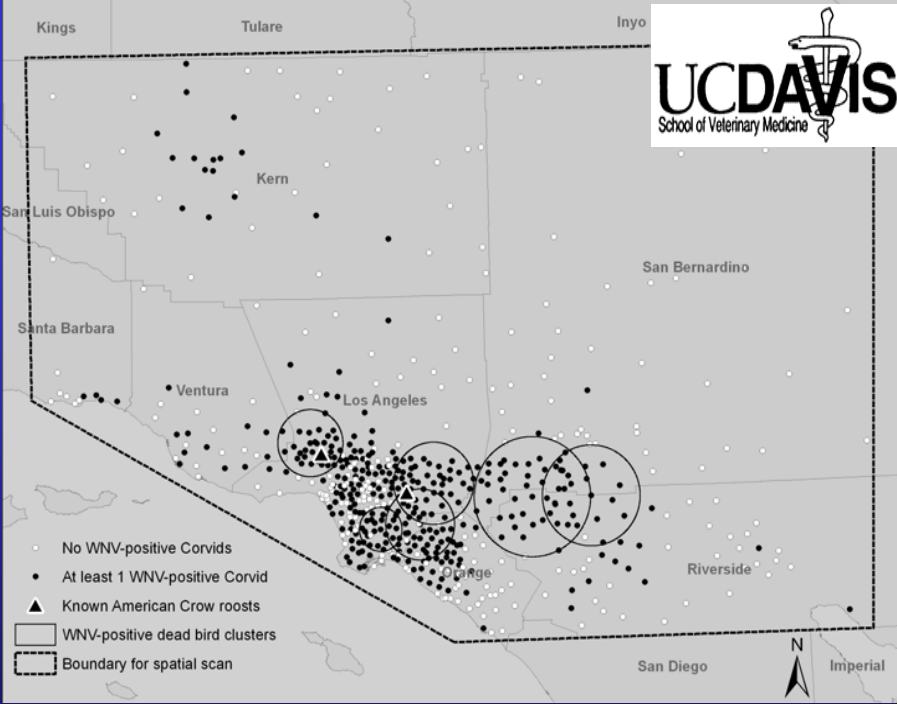
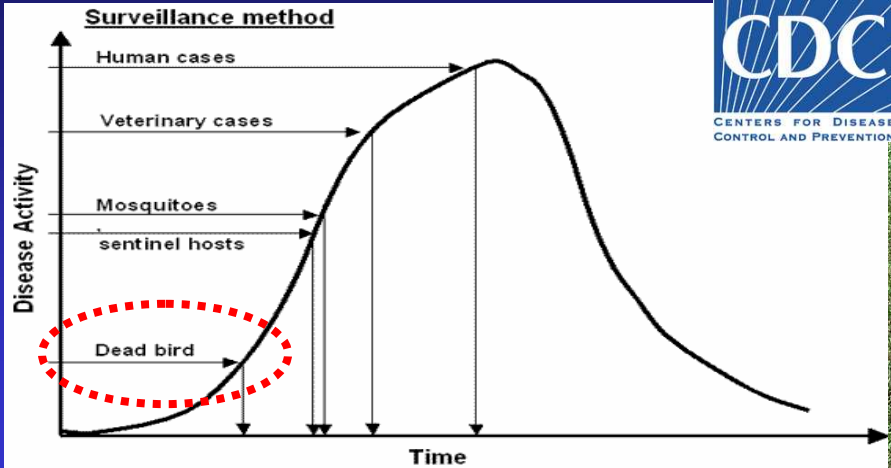
¹Center for Vector-borne Diseases, ²Department of Pathology, Microbiology and Immunology, School of Veterinary Medicine, University of California, Davis, CA; ³Division of Vector-Borne Infectious Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, U.S. Department of Health and Human Services, Fort Collins, CO; ⁴Department of Biomedical Sciences, Colorado State University, Fort Collins, CO; ⁵California Animal Health and Food Safety Laboratory, Davis, CA.; Center for Tropical Diseases and Department of Pathology, University of Texas Medical Branch, Galveston, TX



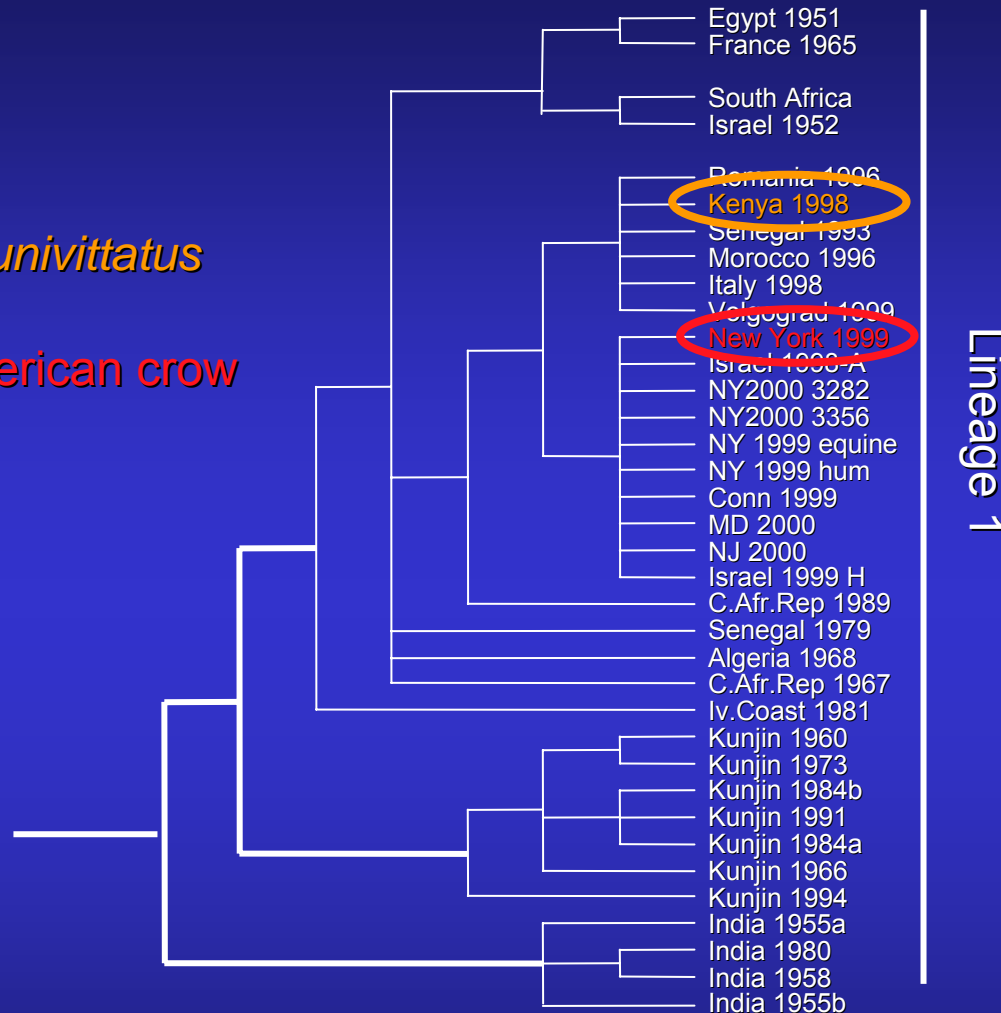
West Nile viral transmission cycles



American crow mortality as a sentinel for WNV viral activity



Phylogenetic Relationships among West Nile Viruses

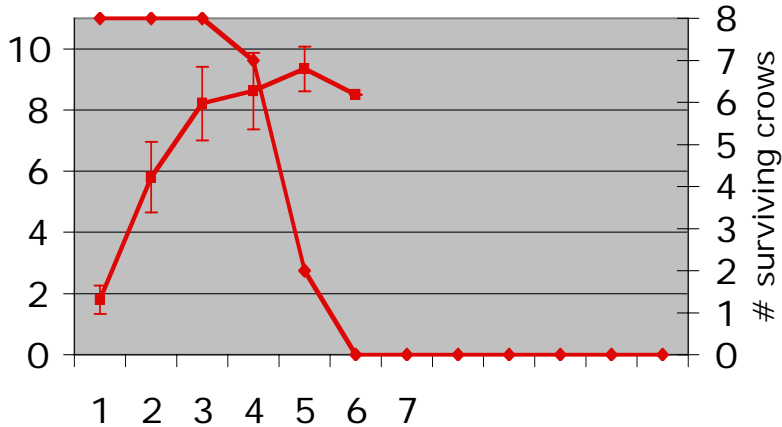


Kenya-98- *Culex univittatus*
(V1, BHK1)
New York-99- American crow
(V1)

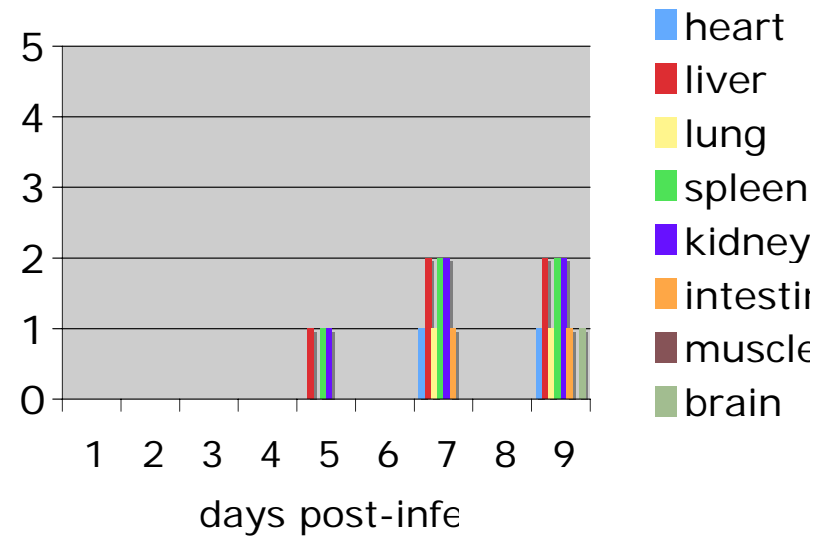
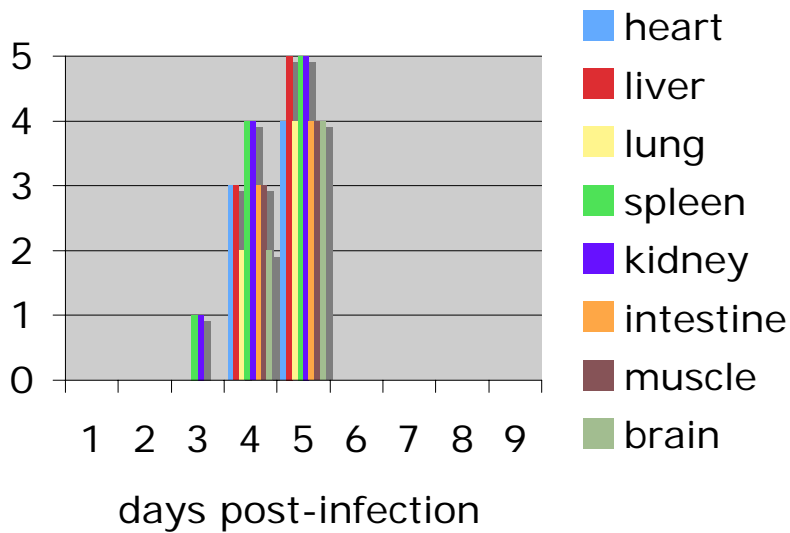
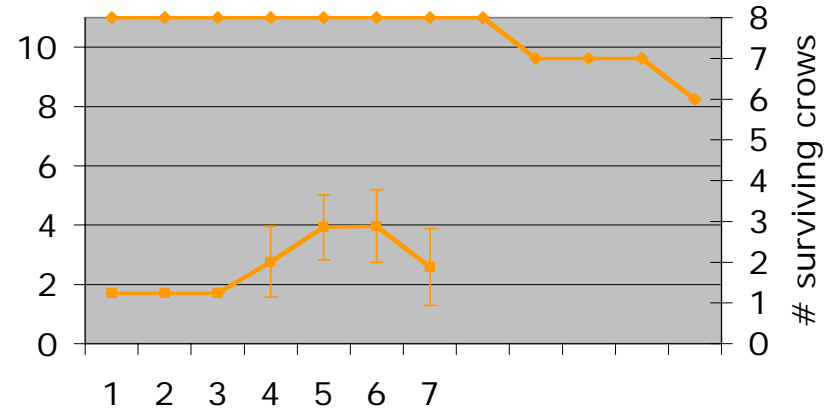
Lanciotti, R. S., J. T. Roehrig, V. Deubel, J. Smith, M. Parker, K. Steele, B. Crise, K. E. Volpe, M. B. Crabtree, J. H. Scherret, R. A. Hall, J. S. MacKenzie, C. B. Cropp, B. Panigrahy, E. Ostlund, B. Schmitt, M. Malkinson, C. Banet, J. Weissman, N. Komar, H. M. Savage, W. Stone, T. McNamara, and D. J. Gubler. 1999. Origin of the West Nile virus responsible for an outbreak of encephalitis in the northeastern United States. *Science* 286:2333-7.

Viremia and survival data for crows inoculated with WNVs

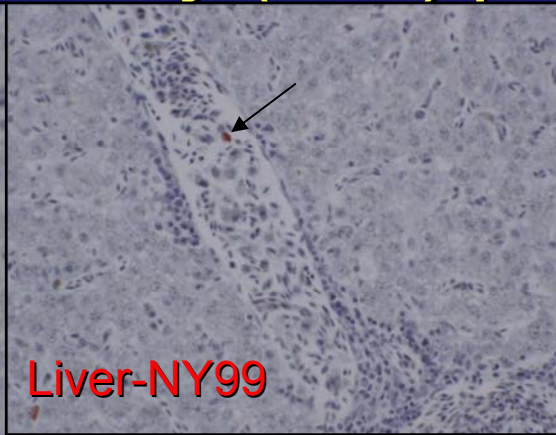
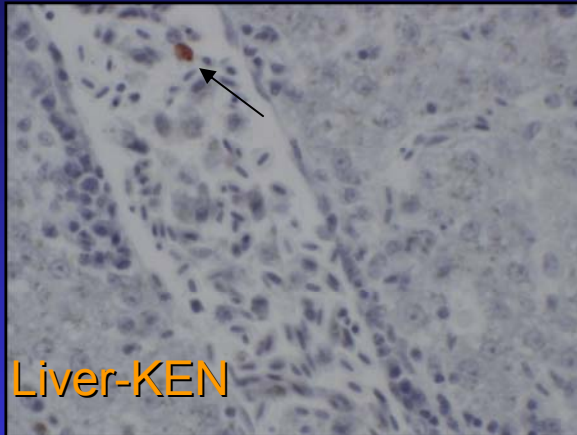
NY-99 survival vs viral product



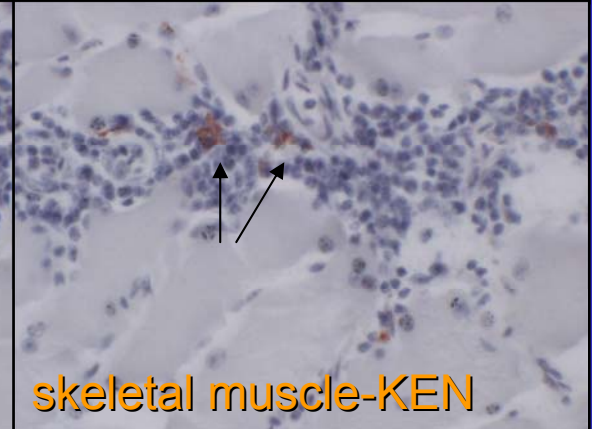
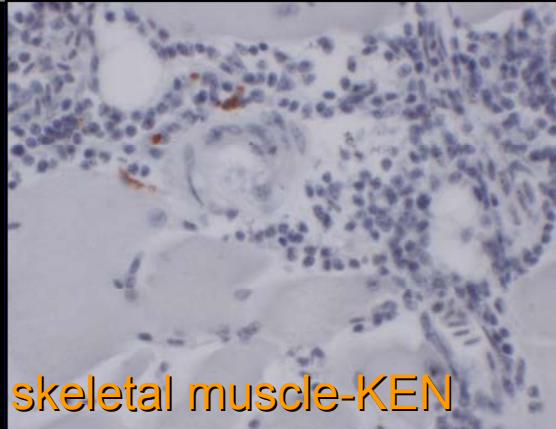
KEN survival vs. viremia



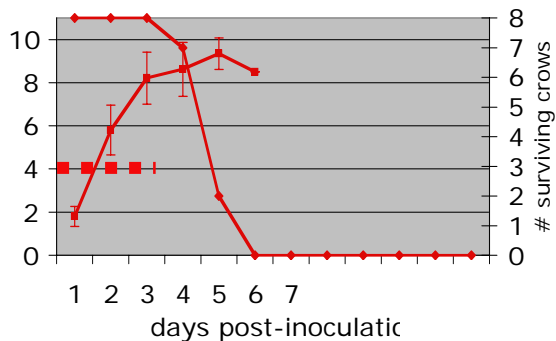
Immunohistochemistry (IHC) positive leukocytes



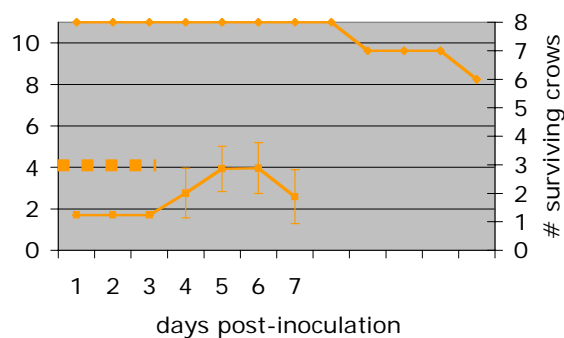
Crows were sacrificed at dpi 1-5. Birds were necropsied and tissues stored in 10% buffered formalin, paraffin embedded, sectioned and IHC was performed using WNV polyclonal antiserum.



NY-99 survival vs viral product



KEN survival vs. viremia



Amino acid differences between NY99 and KEN genomes

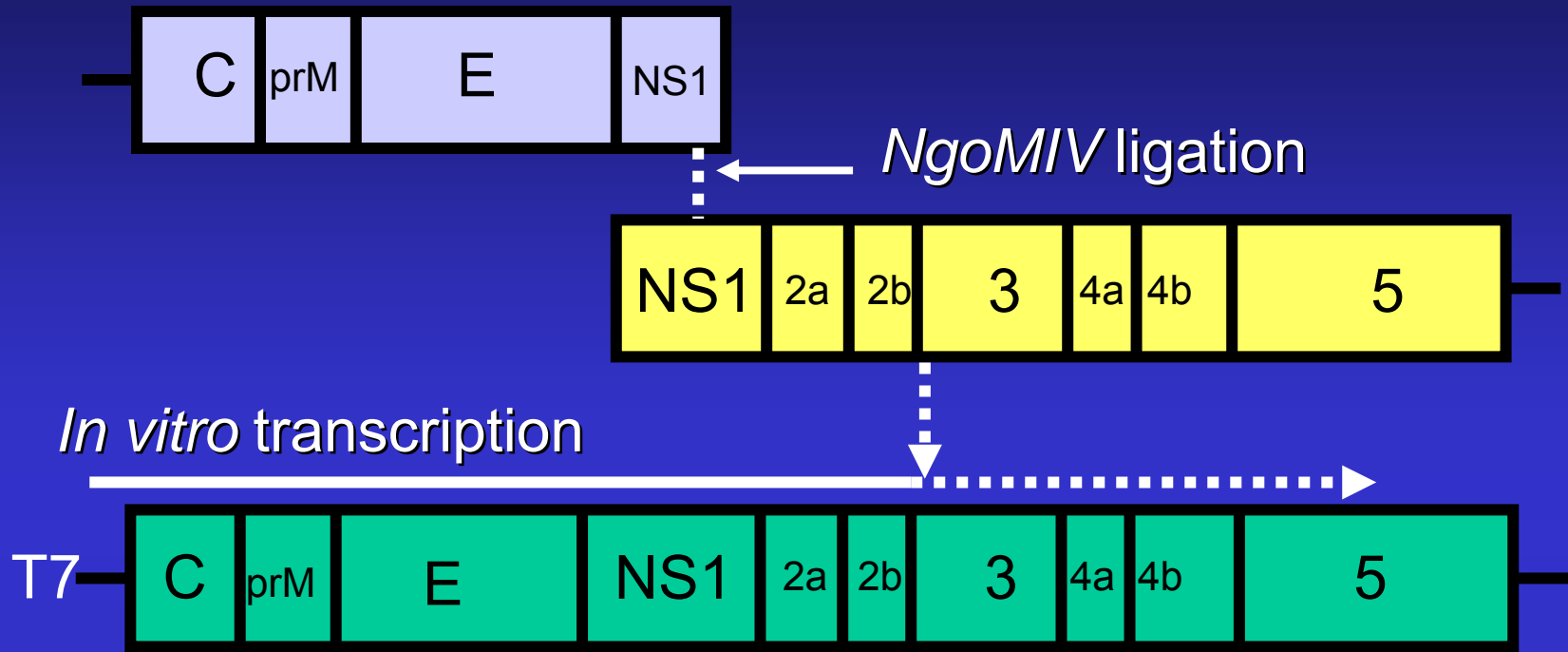
Gene region	aa position	NY99	KN-3829
Capsid	108	Lys	Asn
Capsid	113	Val	Ala
Envelope	126	Val	Ala
Envelope	159	Val	Ile
.....			
NS1	70	Ala	Ser
NS2a	52	Thr	Ala
NS2b	103	Val	Ala
NS3	249	Pro	Thr
NS3	356	Thr	Ile
NS4a	85	Ala	Val
NS4b	249	Glu	Asp

Charrel, R. N., A. C. Brault, P. Gallian, J. J. Lemasson, B. Murgue, S. Murri, B. Pastorino, H. Zeller, R. de Chesse, P. de Micco, and X. de Lamballerie. 2003. Evolutionary relationship between Old World West Nile virus strains. Evidence for viral gene flow between Africa, the Middle East, and Europe. *Virology* 315:381-8.

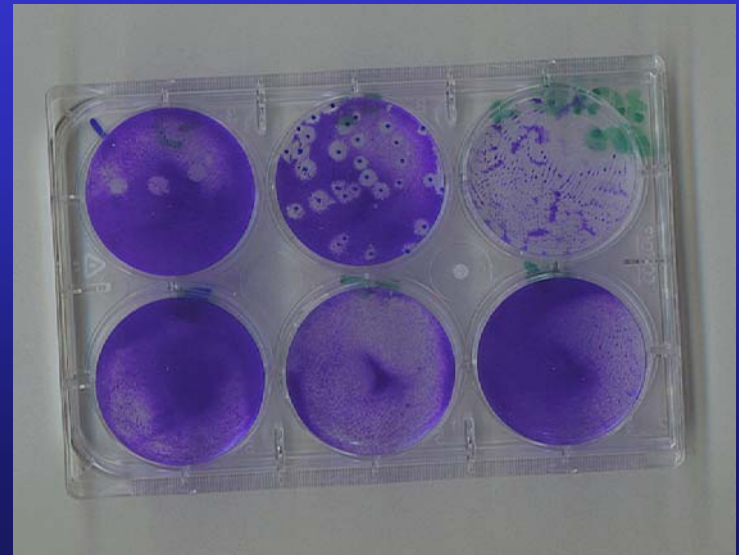
Non-conservative substitution within the helicase domain of NS3.

In addition to the coding amino acid differences, 22 nucleotide differences were identified in the 3'NCR. The 5'NCR regions from both genomes were identical.

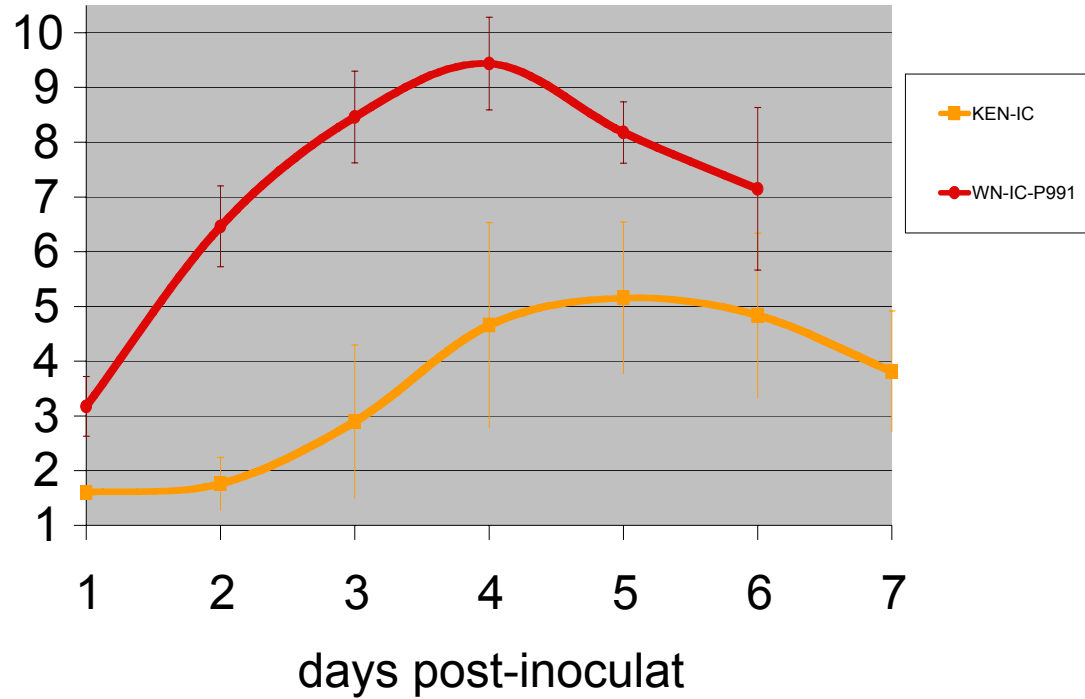
Generation of recombinant WNV



Plasmid DNAs were ligated at a common *NgoMIV* site and ligation product was used as template for *in vitro* transcription



Gene region	aa position	P991	KN-IC
Capsid	108	Lys	Asn
Capsid	113	Val	Ala
Envelope	126	Val	Ala
Envelope	159	Val	Ile
NS1	70	Ala	Ser
NS2a	52	Thr	Ala
NS2b	103	Val	Ala
NS3	249	Pro	Thr
NS3	356	Thr	Ile
NS4a	85	Ala	Val
NS4b	249	Glu	Asp



E-159 (I→V)

NS2b-103 (A→V)

NS3-249 (T→P)

NS3-356 (I→T)

E-126(A→V)

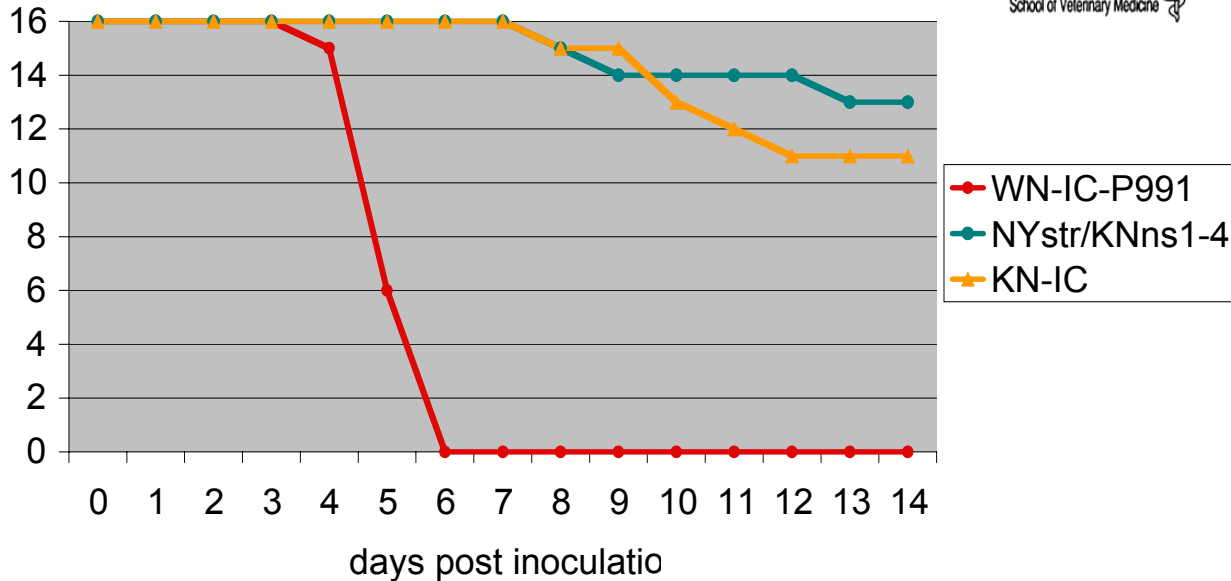
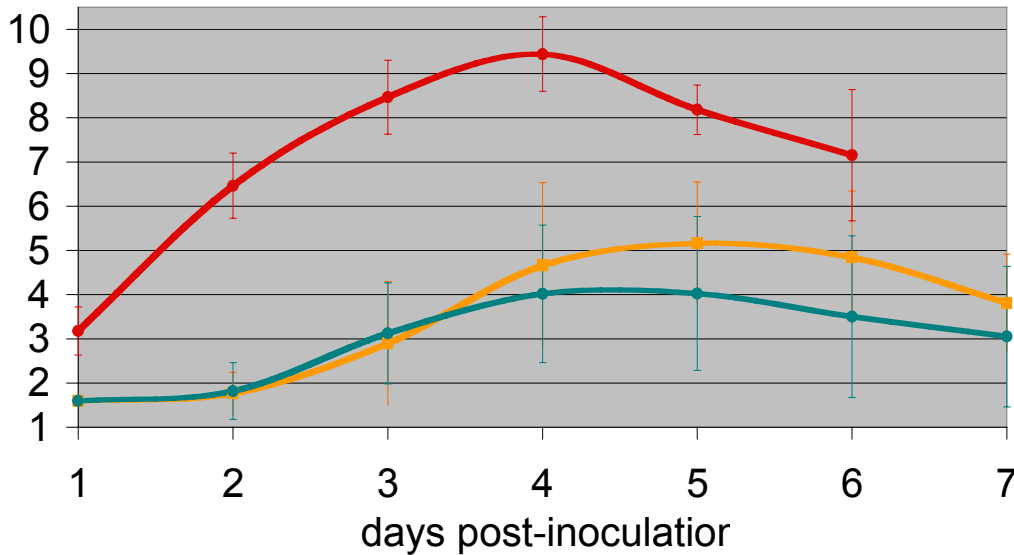
NS1-70(S→A)

NS4a-85(V→A)

NS4b-249(D→E)



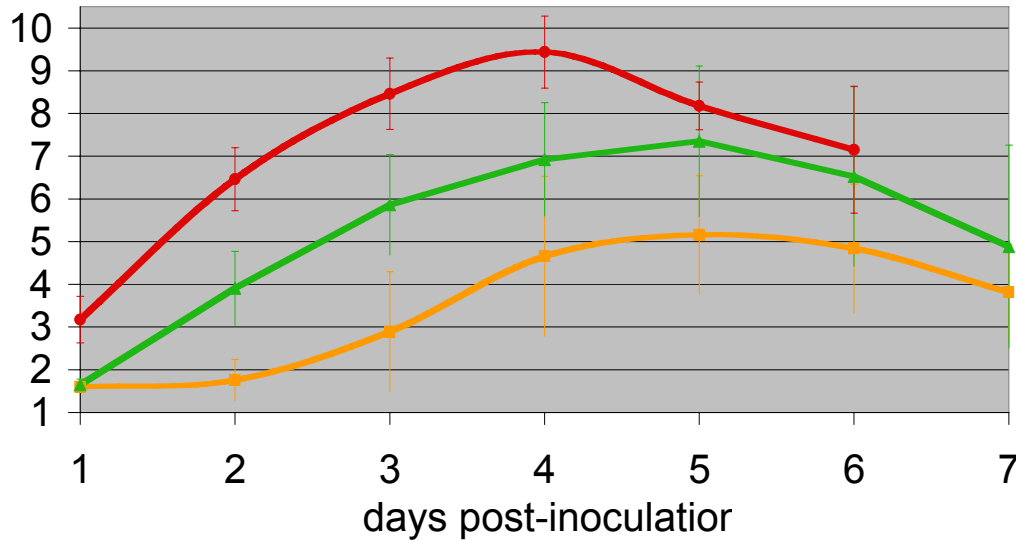
Role of structural amino acid substitutions



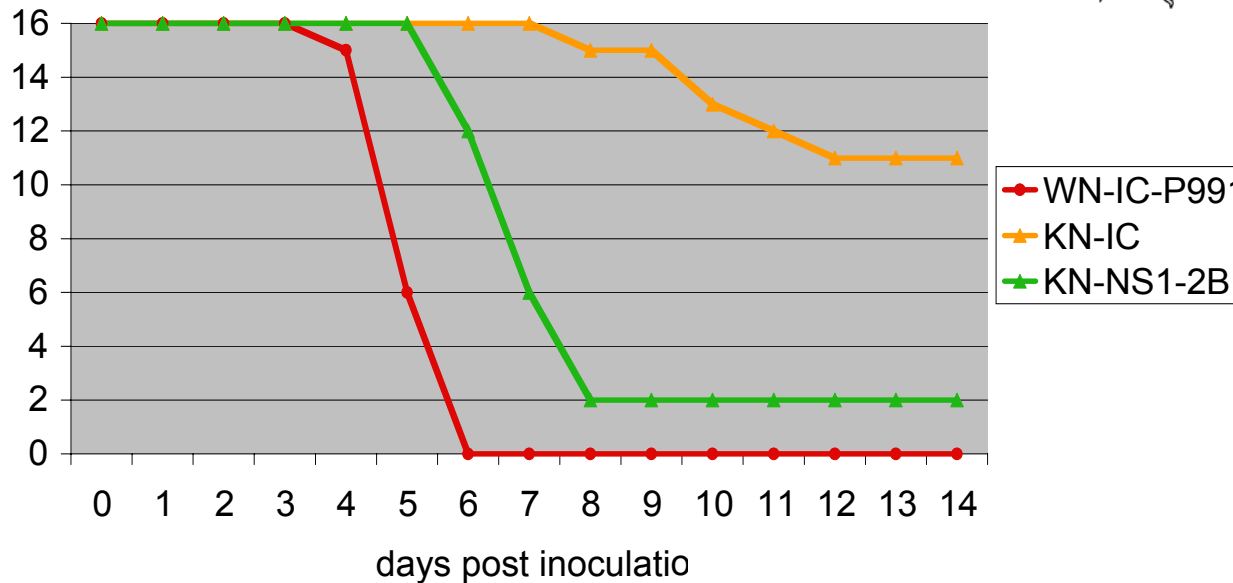
Gene	aa position	P991	KN-IC
Capsid	108	Lys	Asn
Capsid	113	Val	Ala
Envelope	126	Val	Ala
Envelope	159	Val	Ile
NS1	70	Ala	Ser
NS2a	52	Thr	Ala
NS2b	103	Val	Ala
NS3	249	Pro	Thr
NS3	356	Thr	Ile
NS4a	85	Ala	Val
NS4b	249	Glu	Asp

Generation of chimera with the structural aa of NY99 does not increase virulence or viremia in the KEN genetic backbone.

Role of NS3-4b substitutions

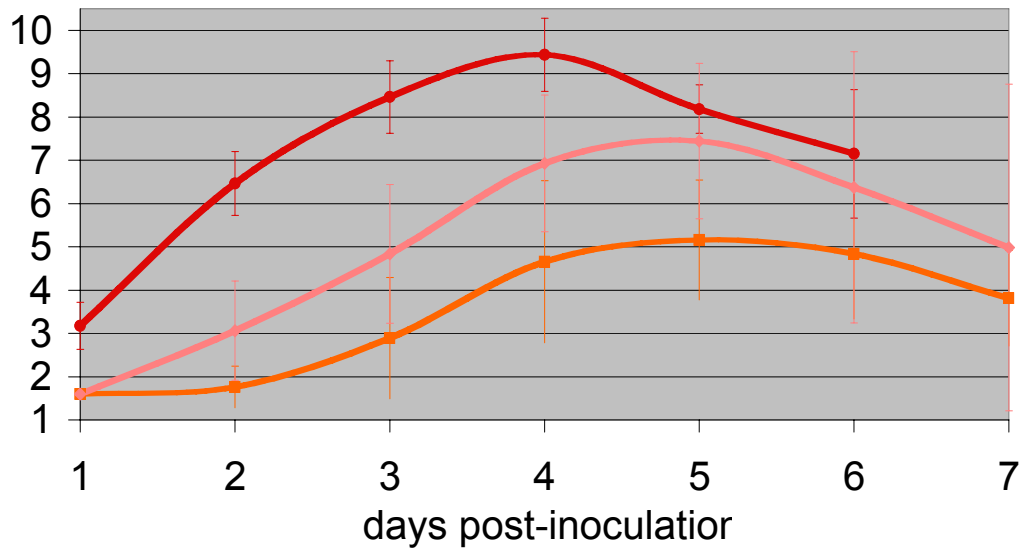


Gene	aa position	P991	KN-IC
NS1	70	Ala	Ser
NS2a	52	Thr	Ala
NS2b	103	Val	Ala
NS3	249	Pro	Thr
NS3	356	Thr	Ile
NS4a	85	Ala	Val
NS4b	249	Glu	Asp

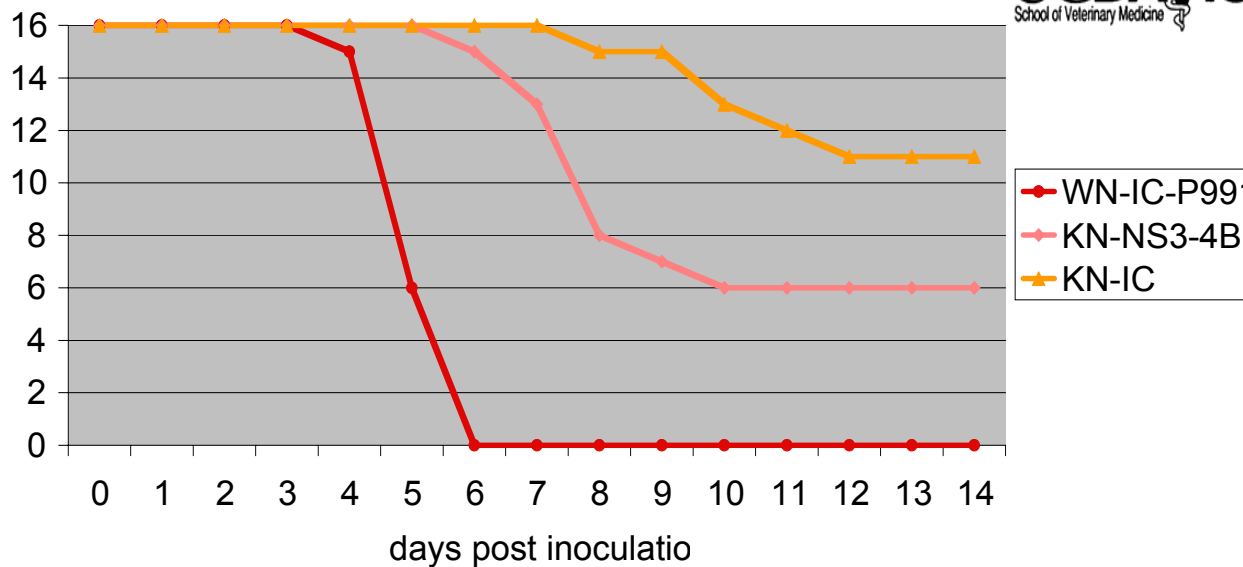


Generation of chimera with the NS3-4b of NY99 increases virulence from 30% to 90% with an increase in mean viremia of >300-fold.

Role of NS1-2b substitutions

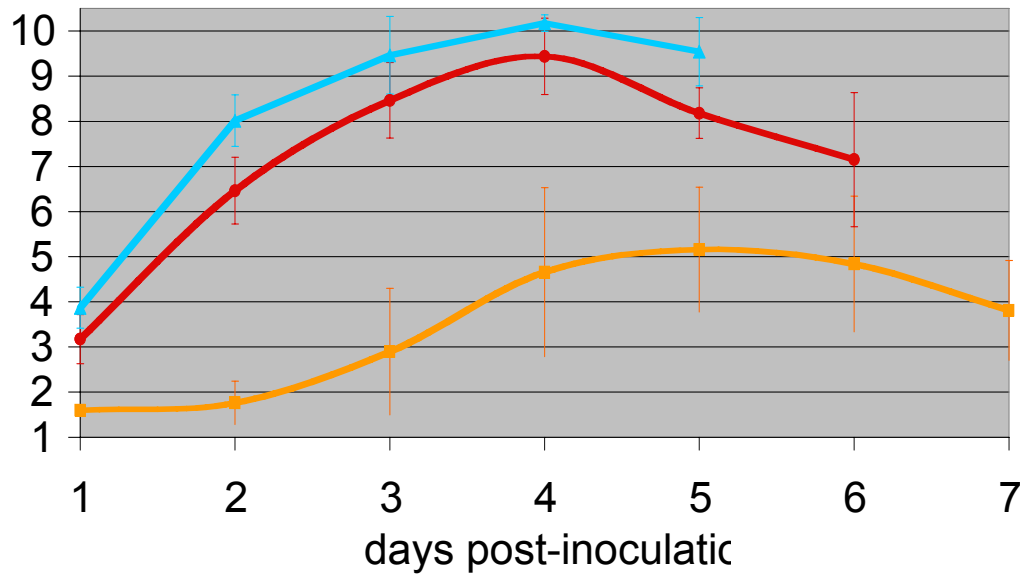


Gene	aa position	P991	KN-IC
NS1	70	Ala	Ser
NS2a	52	Thr	Ala
NS2b	103	Val	Ala
NS3	249	Pro	Thr
NS3	356	Thr	Ile
NS4a	85	Ala	Val
NS4b	249	Glu	Asp

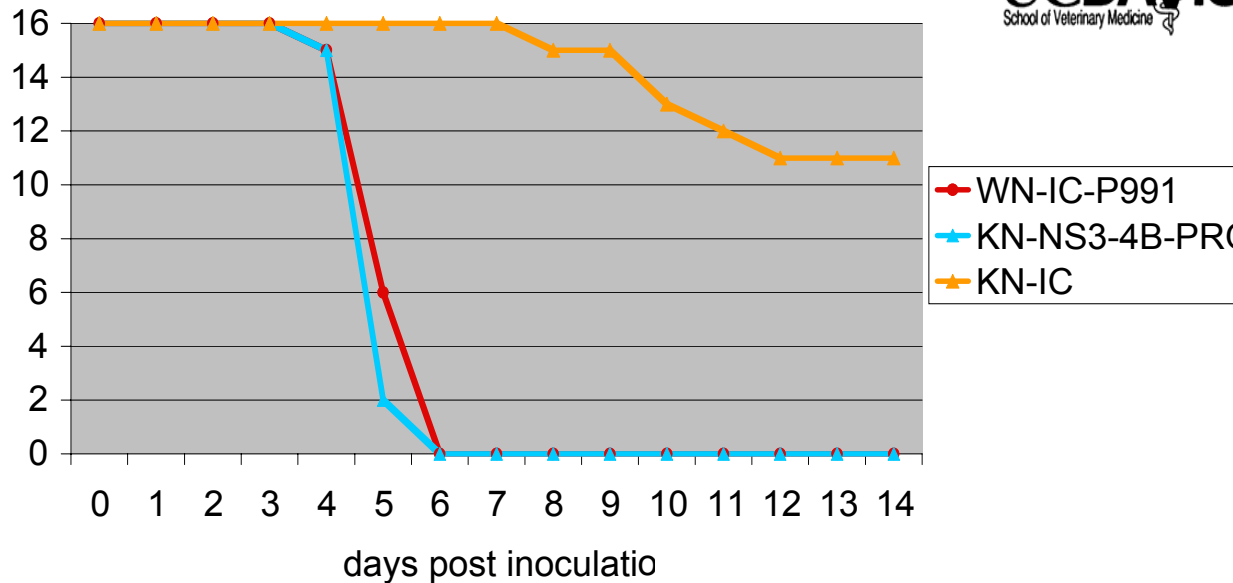


Generation of chimera with the NS1-2b of NY99 increases virulence from 30% to 60% with an increase in mean viremia of >300-fold.

Role of NS1-2b and NS3-249^{Pro}

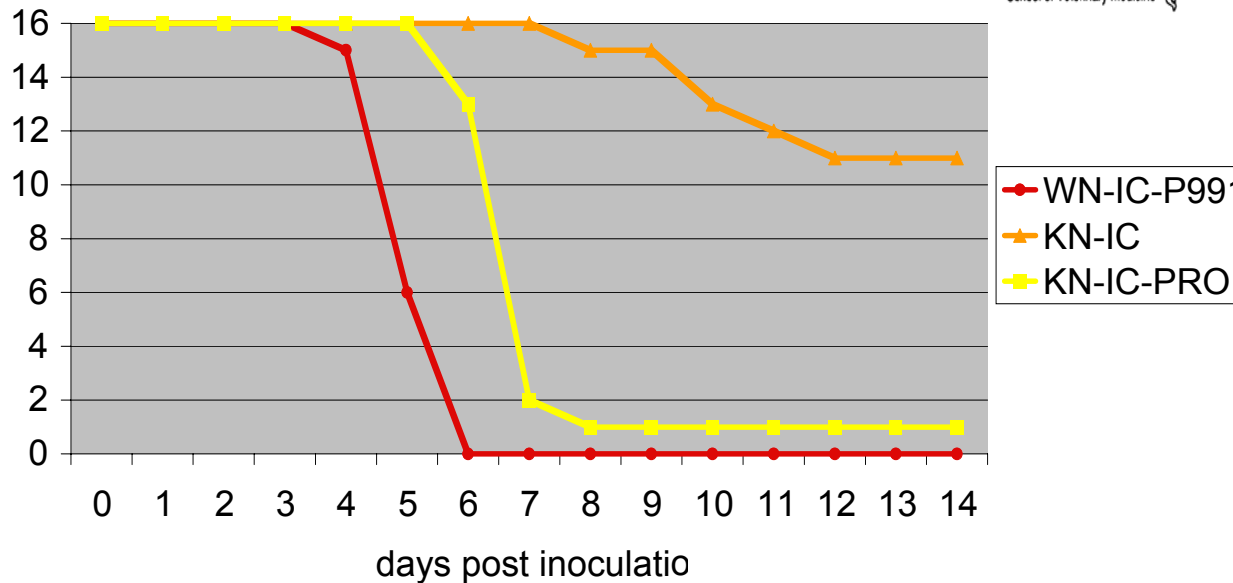
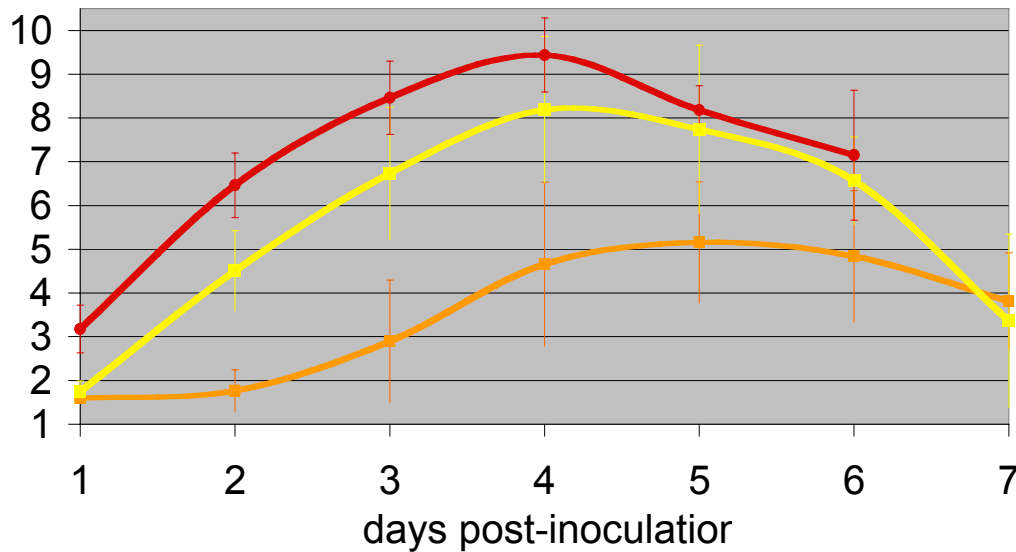


Gene	aa position	P991	KN-IC
NS1	70	Ala	Ser
NS2a	52	Thr	Ala
NS2b	103	Val	Ala
NS3	249	Pro	Thr
NS3	356	Thr	Ile
NS4a	85	Ala	Val
NS4b	249	Glu	Asp



Reconstitution of the NS3-249^{Pro} in the NS1-2b backbone generates a wild-type virulence and viremia phenotype

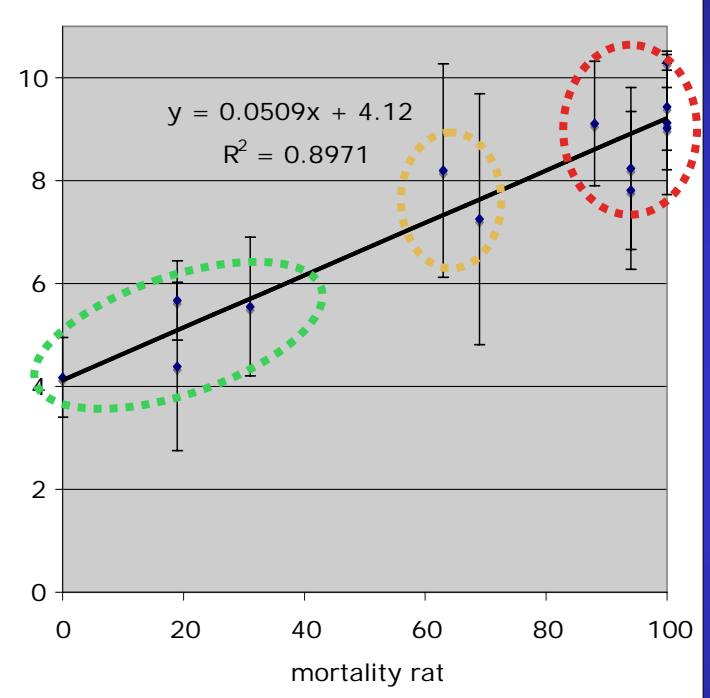
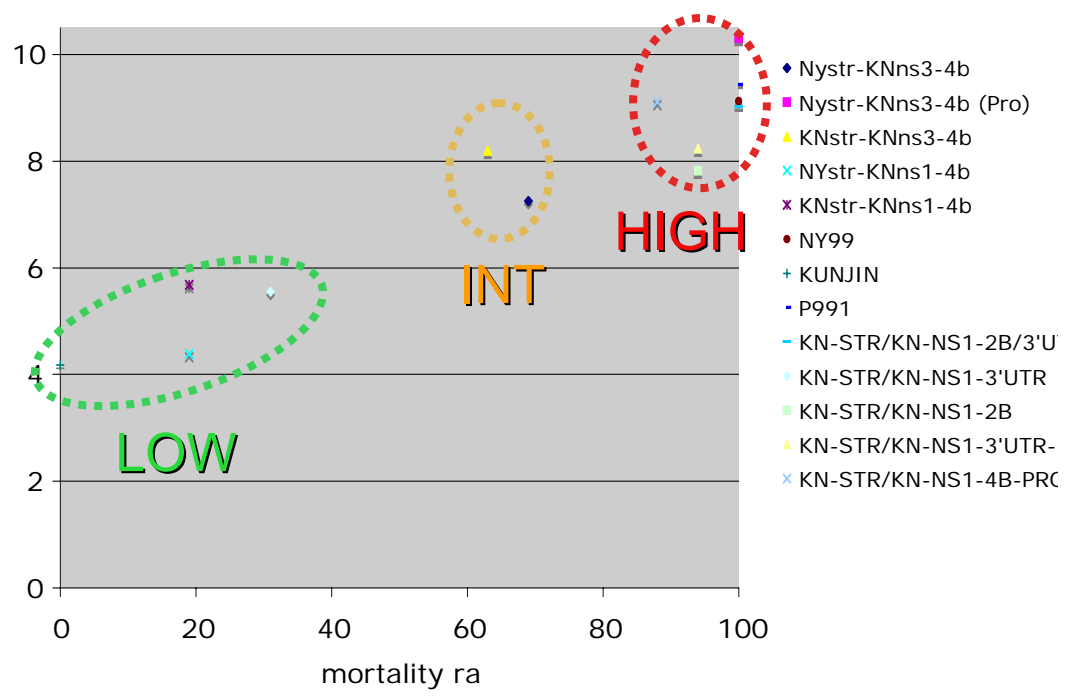
Role of NS3-249-Pro



Gene	aa position	P991	KN-IC
NS1	70	Ala	Ser
NS2a	52	Thr	Ala
NS2b	103	Val	Ala
NS3	249	Pro	Thr
NS3	356	Thr	Ile
NS4a	85	Ala	Val
NS4b	249	Glu	Asp

Insertion of the 249-Pro increases viremia >1,000-fold and increases virulence from 30% to 94%.

Positive correlation between viremia and mortality

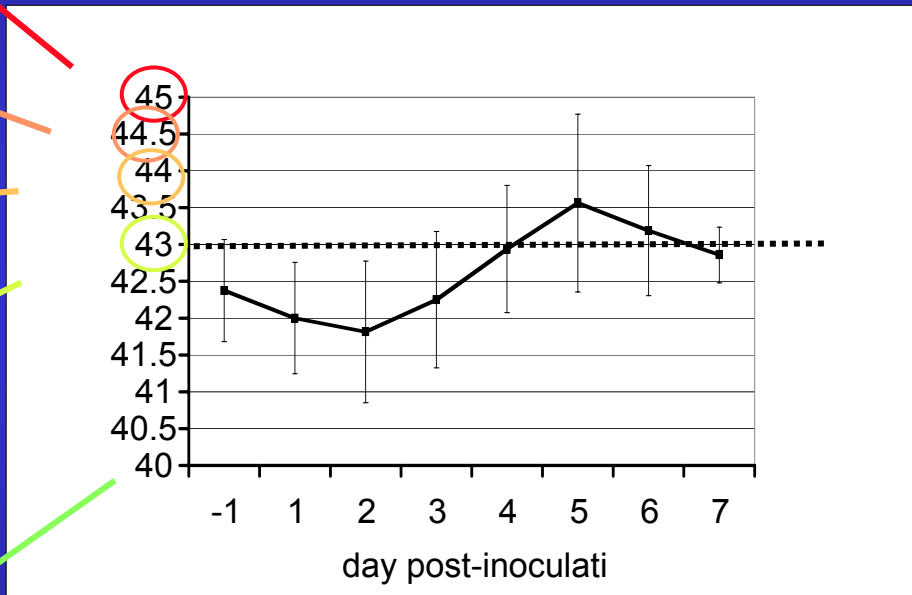


Lack both the NY99 NS1-2B as well as the NS3-249^{Pro}
 Contain the NY99 NS1-2B; Lack the NS3-249^{Pro}
 Contain the NS3-249^{Pro} with or without the NY99 NS1-2B



Differential *in vitro* temperature replication

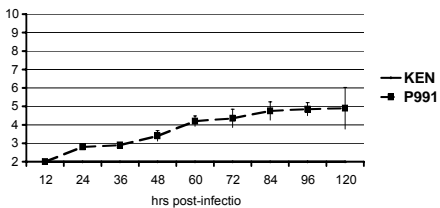
Mean body temperatures in infected AMCRs



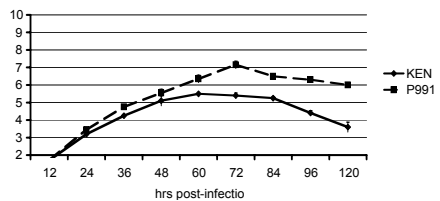
Replication in duck embryonic fibroblast cells

(DEF). Monolayers of DEF cells were infected at an moi of 0.01 of P991 and KEN-IC viruses. Culture supernatants were sampled at 12 hr time-points (12 hpi-120 hpi). Titers were determined by plaque assay on Vero cells.

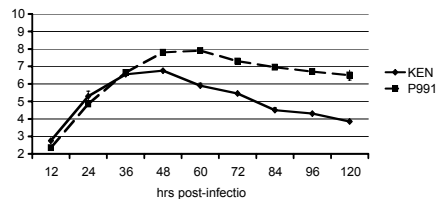
DEF-45°C



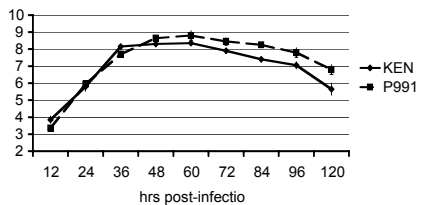
DEF-44.5°C



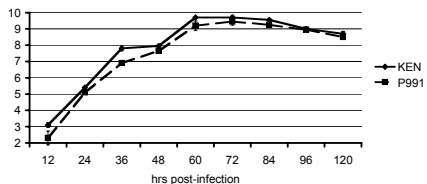
DEF-44°C



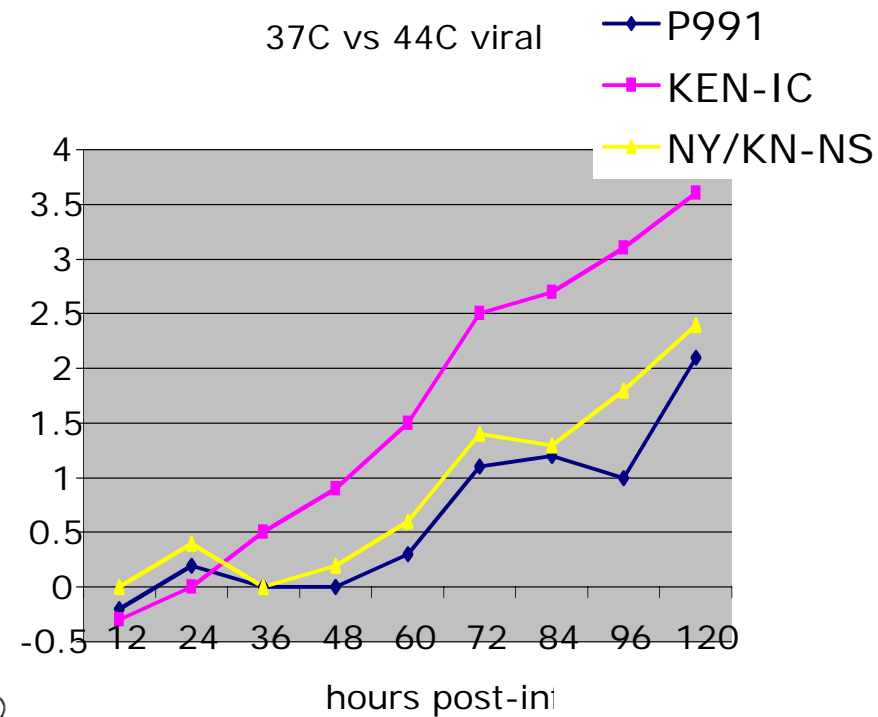
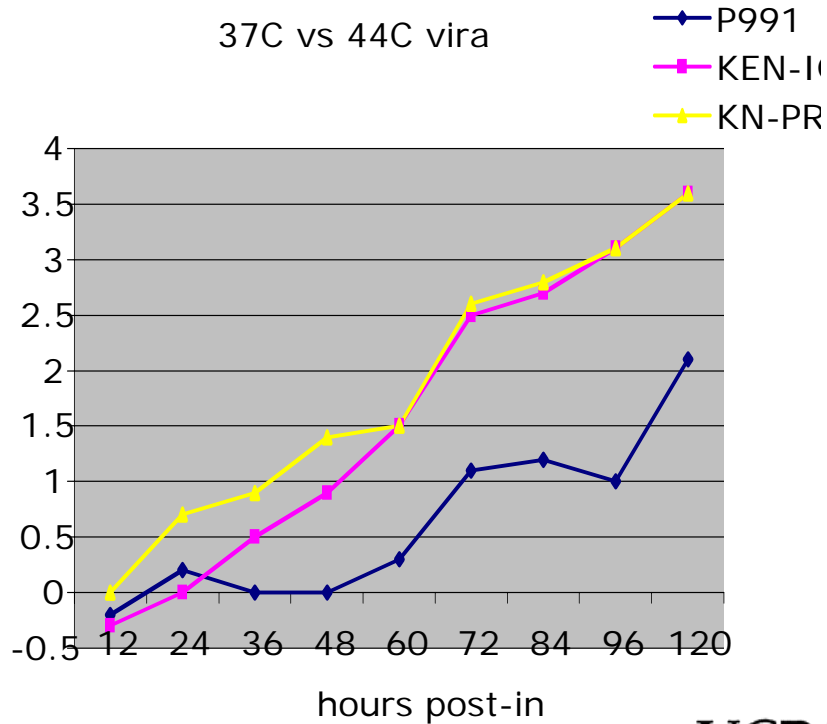
DEF-43°C



DEF-37°C



NS3-249^{Pro} not temperature sensitivity determinant



*DEN-2 (PDK-53) vaccine candidate indicate that an NS3-250 substitution involved in a *ts* phenotype

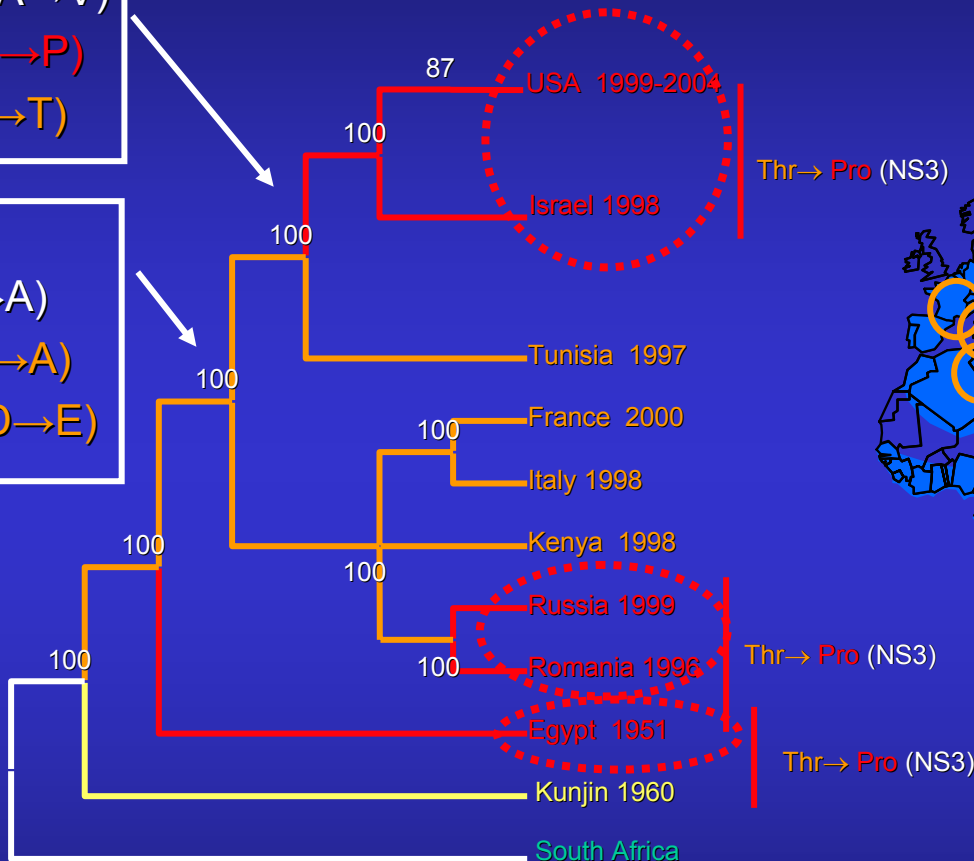
Butrapet, S., C. Y. Huang, D. J. Pierro, N. Bhamarapavati, D. J. Gubler, and R. M. Kinney. 2000. Attenuation markers of a candidate dengue type 2 vaccine virus, strain 16681 (PDK-53), are defined by mutations in the 5' noncoding region and nonstructural proteins 1 and 3. *J Virol* 74:3011-9.

Monolayers of DEF cells were infected at an moi of 0.01 of P991, KEN-IC and recombinant WNVs. Culture supernatants were sampled at 12 hr time-points (12 hpi-120 hpi). Titers were determined by plaque assay on Vero cells. Viral titers calculated between culture supernatants drawn from 37°C were compared to those from 44°C cultures and are represented as GMT log differentials.

Distribution of West Nile viral NS3 genotypes

- ⊗ E-159 (I→V)
- ? NS2b-103 (A→V)
- + NS3-249 (T→P)
- ⊗ NS3-356 (I→T)

- ⊗ E-126(T→I)
- ? NS1-70(S→A)
- ⊗ NS4a-85(V→A)
- ⊗ NS4b-249(D→E)



- His- lineage II
- Ala- Kunjin
- Thr
- Pro



NS3-249^{Pro} substitution is involved in the avian virulence phenotype
 Synergistic effect of NS1-2B mutations? Mechanism? Selection for Virulence?

Genomic sequence of TM-171 Mex03 isolate



Isolate from dead raven at wildlife reserve in Villahermosa, Tabasco.

RNA and, subsequently, Vero cell passaged virus sent to UTMB.

46 nucleotide differences (0.42%) from NY99; 4 amino acid differences:

prM/M-141

Ile \boxtimes Thr

E-156

Ser \boxtimes Pro* (loss of glycosylation motif)

NS4B-245

Ile \boxtimes Val*

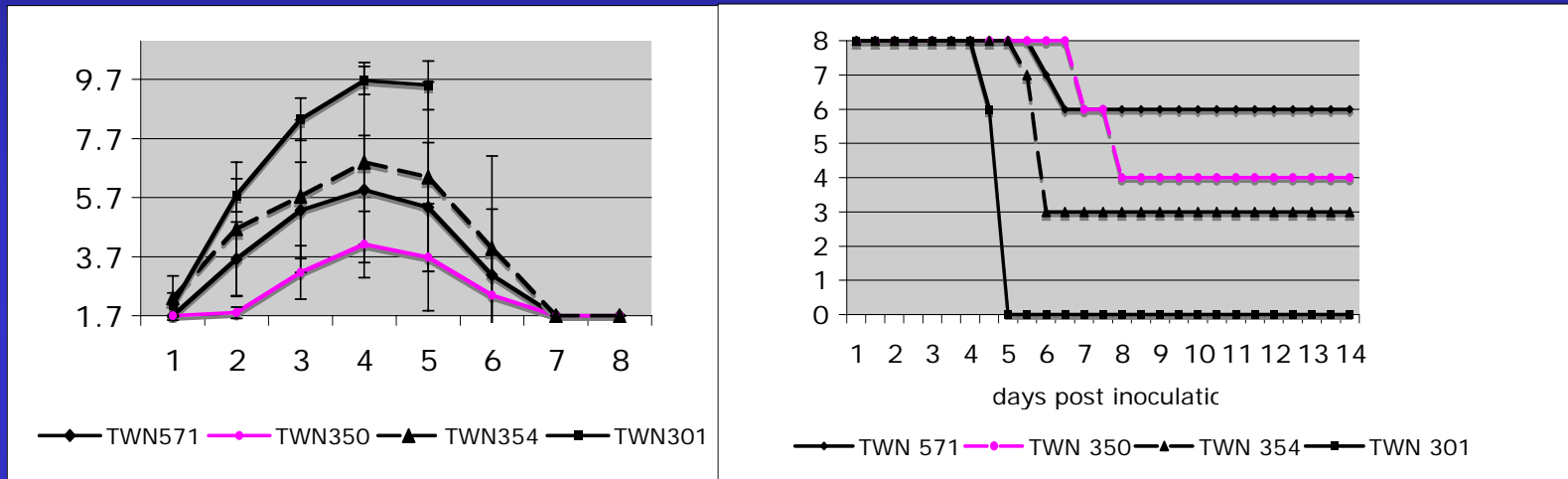
NS5-898

Thr \boxtimes Ile*



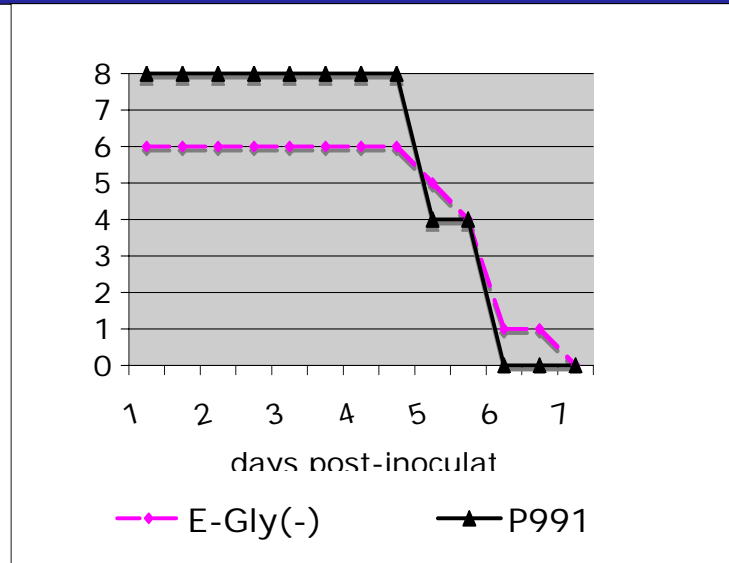
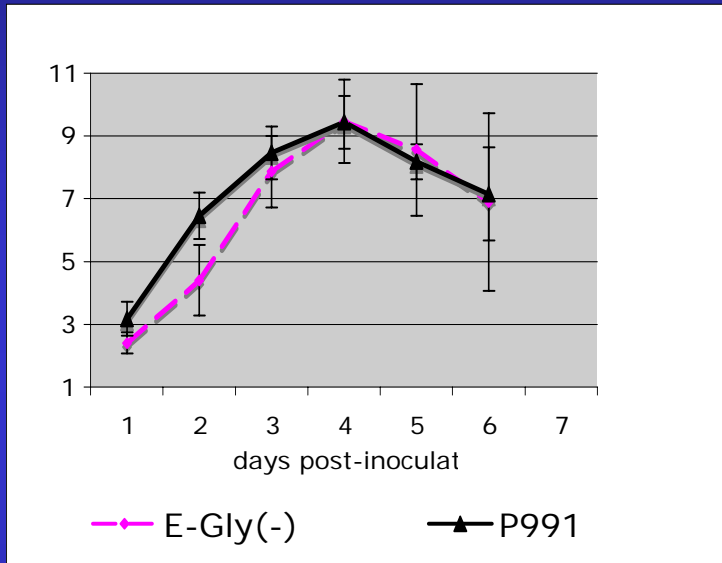
Virus Strain	Sequence	Glycosylation	Source	Location	Accession number
TWN 301	N-Y-S	Yes	Bird 1461	Texas	AY712947
TWN 571	N-Y-S	Yes	Pigeon 1153	Texas	AY712945
TWN 350	N-Y-P	No	TM171-03/Raven-small plaque variant	Mexico	AY660002
TWN 354	N-Y-S	Yes	TM171-03/Raven-large plaque variant	Mexico	AY660002

Infection of AMCRs with WNV strains with Differential Mouse neurovirulence phenotypes



Eight AMCRs were inoculated subcutaneously with 1500 PFU of the respective WNV strains. TWN 350, 354 (MX) and TWN 301, 571 (TX).

Role of E glycosylation for virulence modulation in AMCR?



NYS
↓
NYP



Site-directed Mutagenesis was utilized for the single site ablation of the E glycosylation motif; the resulting virus [E-gly(-)] was utilized for the inoculation of 6 AMCRs.

Conclusions

- Differences in replication of KEN vs NY99 WNVs appears to be the result of alternative viral production in circulating leukocytes
- The NS3-249 helicase mutation is the dominant virulence determinant which is modulated by NS1-2B determinants
- AMCR virulence is modulated by peak viremia; the NS3-249 substitution has been associated with WNV emergence over the past 50 years.
- Some mouse attenuated strains of WNV have reduced virulence potential in AMCRs as well (not a complete correlation-KEN and gly(-) viruses)

Acknowledgements



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University of QLSD

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- Nick Panella
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- Mike Bunning (USAF)
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- Claire Huang
- Ann M. Powers
- Janae Raetz
- Tiffany Whitehurst

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- Paul Gordy
- Max Teehee
- Laura Austgen

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- Alan D.T. Barrett
- David W.C. Beasley



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CDC UO1 CI000235