Genetic and phenotypic characteristics of West Nile virus in North America

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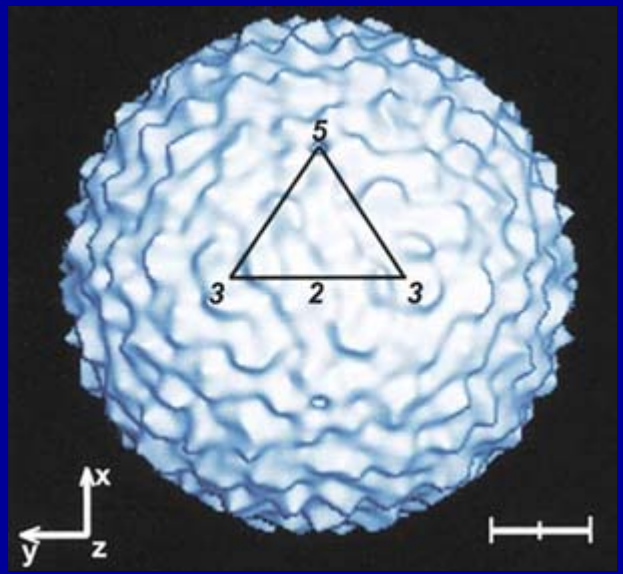
Virus Proteins

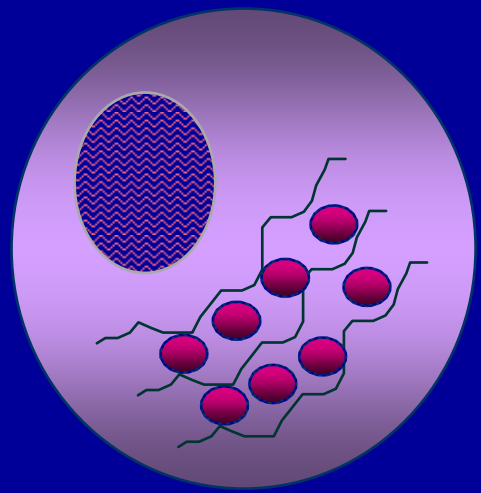
Structural proteins

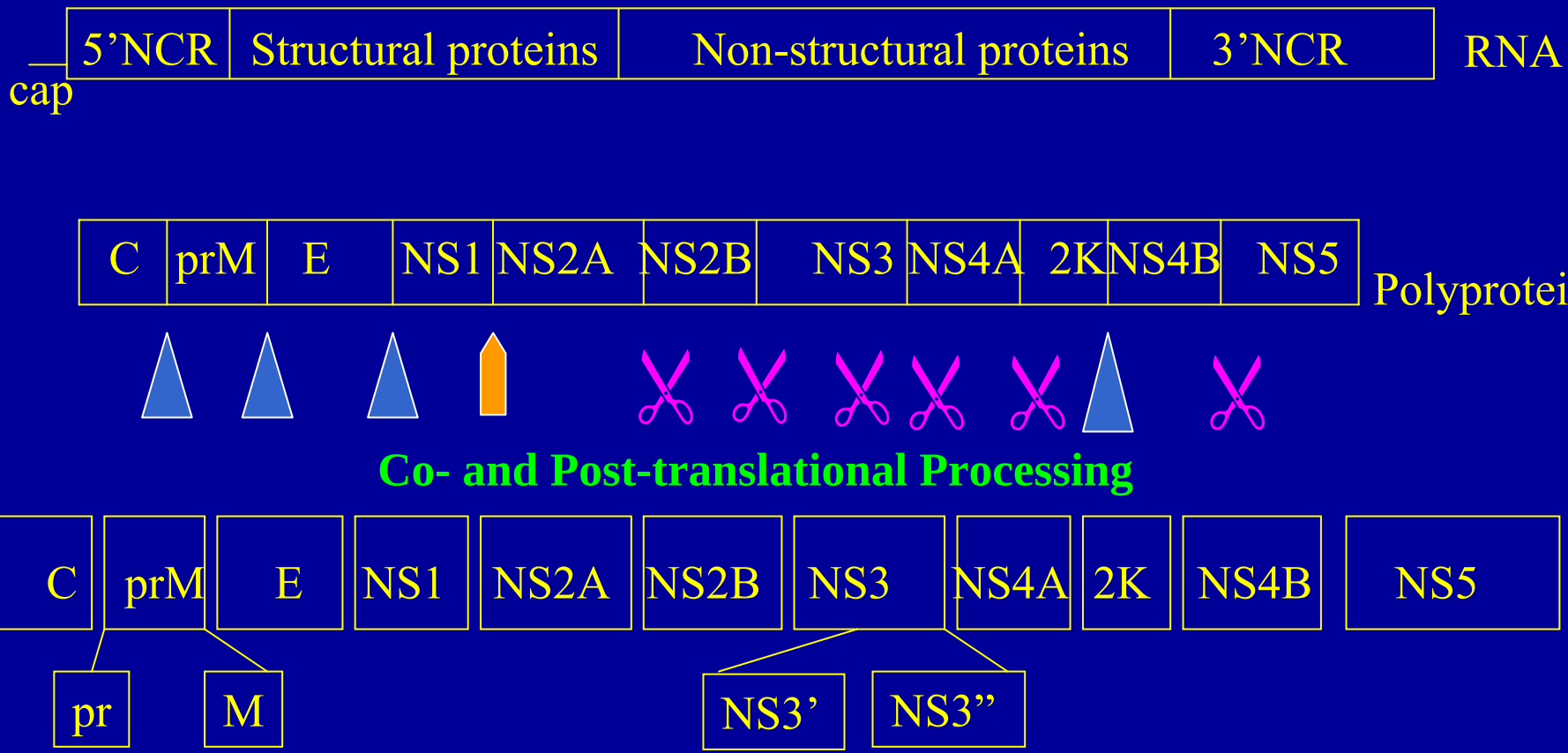
- C, M, E

"Nonstructural" proteins

- **NS1, NS2A, NS2B, NS3, NS4A, NS4B, NS5**







Functions of the E protein

- Major virus immunogen and target of neutralizing antibodies
- Receptor binding (tissue tropism).
- Chu and Ng (*J Biol Chem 279:54533-41, 2004*): $\alpha_v\beta_3$ integrin is cell receptor.
- Davis et al (*J Virol 80:1290-1301, 2006*): DC-SIGNR (L-SIGN; CD209R) [dendritic cell-specific intercellular adhesion molecule-grabbing nonintegrin] is an ancillary receptor.

E-protein

- Approximately 54 kDa
- Glycosylated at 0 or 1 site for West Nile virus
- Most West Nile virus strains have one site
  - E154-E156 : NYS
- Non-glycosylated viruses generated by three routes
  - SYS
  - NYP/NYA
  - Delete E156-E159

Table 1. Properties of West Nile and Kunjin virus strains used in this study

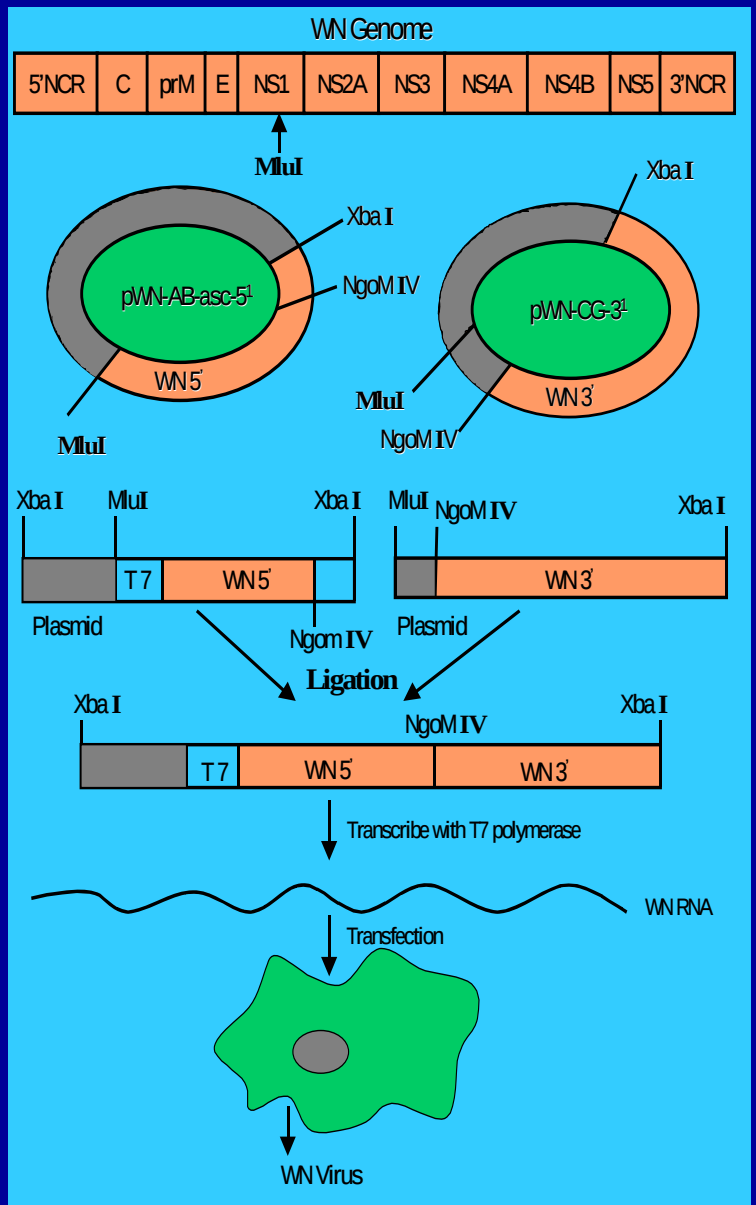
Designation	Strain	Origin	Year	Lineage †	CHO ‡	i.p.LD_{50} (pfu)
CAR67	ArB-310/67	Central	1967	I	-S	12.6
NIG65	IbAn7019	Nigeria	1965	I	+	3.2
SEN79	ArD-27875	Senegal	1979	I	+	0.2
KEN98	KEN3829	Kenya	1998	I	+	<0.1
USA99a	31A	United States	1999	I	+	0.5
USA99b	**385-99**	**U.S**	**1999**	**I**	**+**	**3.2**
IND68	68856	India	1968	I	+	3.2
EGY50	Egypt101	Egypt	1950	I	-S	50*
EGY52	Ar248	Egypt	1952	I	-N	n.d.
ISR52	TL443	Israel	1952	I	-S	8000*
ISR53	Goldblum	Israel	1953	I	-S	≥100000
ETH76a	**EthAn4766**	**Ethiopia**	**1976**	**I**	**-N**	**2000***
ETH76b	EthAn4767	Ethiopia	1976	I	-N	≥100000
AUS60	MRM16	Australia	1960	KUN	-S	≥100000
AUS91	K6453	Australia	1991	KUN	+	≥100000
IND57	IG-15578	India	1957	IND	-S	8000*
IND80	804994	India	1980	IND	-S	50*
SEN90	ArD-76104	Senegal	1990	II	-Δ	50*
CAR82	ArB3573/8	Central	1982	II	+	0.8
SA58a	SAH-442	South Africa	1958	II	+	3.2
CON58	Evoku	Congo	1958	II	-S	0.8
SA89	SPU116-89	South Africa	1989	II	+	5.0
UGA37	B956	Uganda	1937	II	-Δ	7.9
MAD88	ArMg-979	Madagascar	1988	II	+	500*
SA58b	SAAn2842	South Africa	1958	II	+	125*
CYP68	Q3574-5	Cyprus	1968	II	-N	≥100000
MAD78	DakAnMg7	Madagascar	1978	II	-S	≥100000

* LD_{50} values could not be reliably calculated for these strains

† based on sequencing of a 3'NCR fragment; see Fig. 1

‡ indicates presence or absence of potential N-linked glycosylation site in the E protein sequence (E154-156 NYS): +, present; -. Absent; N, asparagines mutant (N→S); S, serine mutant (S→P/A); Δ, deletion mutant.

Flow chart for generation of virus from infectious clone



Rich Kinney, CDC
Fort Collins

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Comparisons of glycosylated and non-glycosylated variants *(Beasley et al. J Virol 79:8339-47, 2005)*

Wild-type strains

NY99	wild-type strain 382-99 (NY99)	gly +
ETH76a	wild-type strain EthAn4766 (ETH76a)	gly –

Infectious clone-derived variants

NY99ic	infectious clone of 382-99	gly +
NY99/ETH	prM and E of ETH76a in NY99ic	gly –
NY99/E154	Asn→Ser mutation at 154 of NY99ic	gly –
NY99/ETHgly	NY99/ETH S → N mutation at E-154	gly +

Mouse virulence phenotypes of gly +/- strains

Strain	Neuroinvasion (i.p.)		Neurovirulence (i.c.)	
	LD_{50} (pfu)	AST (days)	LD_{50} (pfu)	AST (days)
NY99	1.3	8.3±1.2	0.5	6.8±1.1
NY99ic	1.3	8.1±1.0	0.3	6.7±0.7
ETH76a	126	9.6±1.0	1.3	5.8±1.9
NY99/ETH	200	11.0±1.9	1.1	5.3±0.4
NY99/E154	126	10.2±2.9	1.1	5.8±0.8

(using 3-4 week old female NIH Swiss mice)

Mouse virulence phenotypes of gly +/- strains

(Beasley et al J Virol 79: 8339-47, 2005)

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NY99	1.3	8.3±1.2	0.5	6.8±1.1
NY99ic	1.3	8.1±1.0	0.3	6.7±0.7
ETH76a	126	9.6±1.0	1.3	5.8±1.9
NY99/ETH	200	11.0±1.9	1.1	5.3±0.4
NY99/E154	126	10.2±2.9	1.1	5.8±0.8
NY99/ETHgly	**2.0**	**8.8±1.7**	**0.8**	**6.7±1.1**

(using 3-4 week old female NIH Swiss mice)

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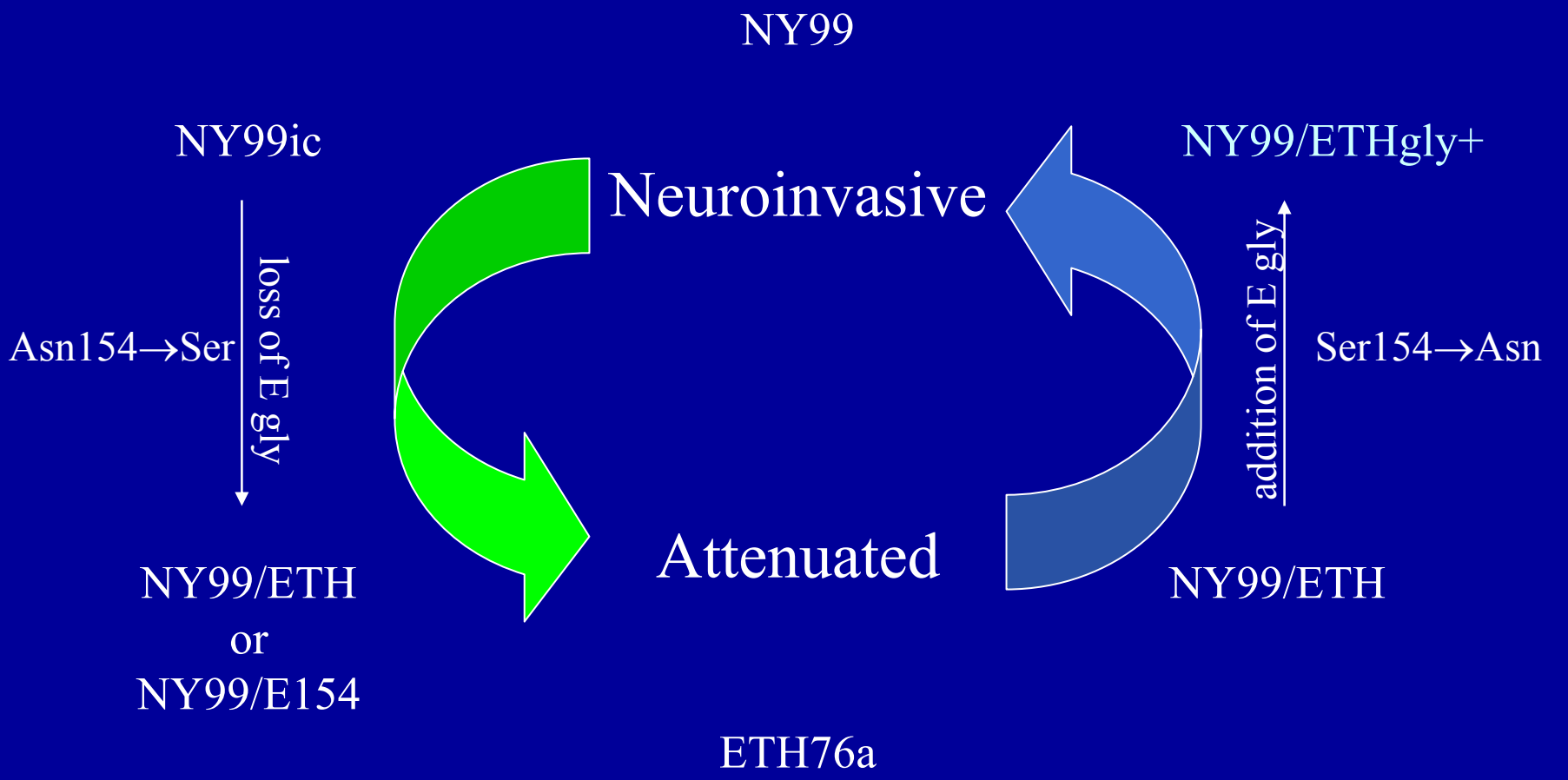
Confirmation of importance of E glycosylation

Infectious clone	Parental strains	Infectious clone



Courtesy of David Beasley

Titers of NY99ic and NY99/E154 infectious clone-derived WNV variants in the serum and brains of NIH Swiss mice determined at daily intervals following intraperitoneal inoculation

Day	Animal #	NY99ic		NY99/E154	
		Serum titer (pfu/mL)	Brain titer (pfu/brain)	Serum titer (pfu/mL)	Brain titer (pfu/brain)
1	1	2.0×10^2	--*	--	--
	2	7.5×10^2	--	--	--
	3	4.0×10^3	--	--	--
2	1	1.0×10^4	--	--	--
	2	2.0×10^3	--	--	--
	3	6.5×10^3	--	--	--
3	1	1.3×10^3	--	--	--
	2	9.0×10^2	--	--	--
	3	6.5×10^3	5.0×10^1	--	--
4	1	--	5.0×10^2	--	--
	2	--	5.5×10^4	--	--
	3	--	--	--	--
5	1	--	3.5×10^6	--	--
	2	--	2.5×10^2	--	--
	3	--	3.3×10^4	--	--
6	1	--	8.8×10^5	--	--
	2	--	--	--	--
	3	--	1.8×10^7	--	--
7	1	--	2.8×10^7	--	--
	2	--	6.3×10^3	--	--
	3	--	$>1.3 \times 10^8$	--	2.8×10^7

Genomic sequence of TM-171 Mex03 isolate

(Beasley et al. EID 10: 2221-4, 2004)

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 → Thr
E-156	Ser → Pro* (loss of glycosylation motif)
NS4B-245	Ile → Val*
NS5-898	Thr → Ile*

Mouse virulence of Mex03 glycosylation variants.

Virus	E154-156	i.p. LD_{50} (pfu)	A.S.T. ± s.d. (days)
Mex03	mixed	0.5	8.2 ± 1.8
Mex03-pp1	NYP	>1000	--
Mex03-pp2	NYP	794	10.3 ± 0.6
Mex03-pp5	NYS	3.2	8.5 ± 0.8
Mex03-pp6	NYS	2.0	9.2 ± 1.0

n.d. – not determined

All strains contained prM-141 I→T; NS4B-245 I→V; NS5-898 T→I

Summary – E protein glycosylation

- Loss of E protein glycosylation results in partial attenuation mouse virulence of NY99 virus.
- Degree of attenuation similar to non-glycosylated lineage 1 strain ETH76a.
- Non-glycosylated strains retain neurovirulence comparable to NY99.

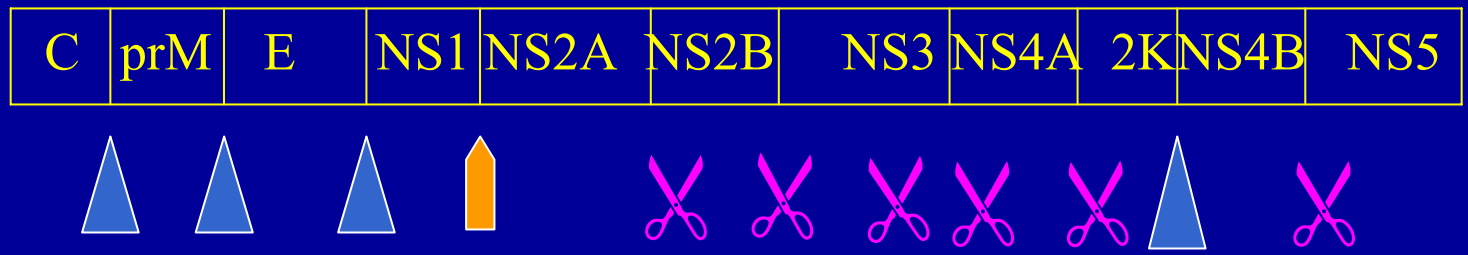
Virus persistence in golden hamster model

Tonry et al. (*Am J Trop Med Hyg 72: 320-4, 2004*)
Tesh et al. (*J Inf Dis 192: 287-95, 2005*)
Ding et al.(*Am J Trop Med Hyg 73: 803-7, 2005*)

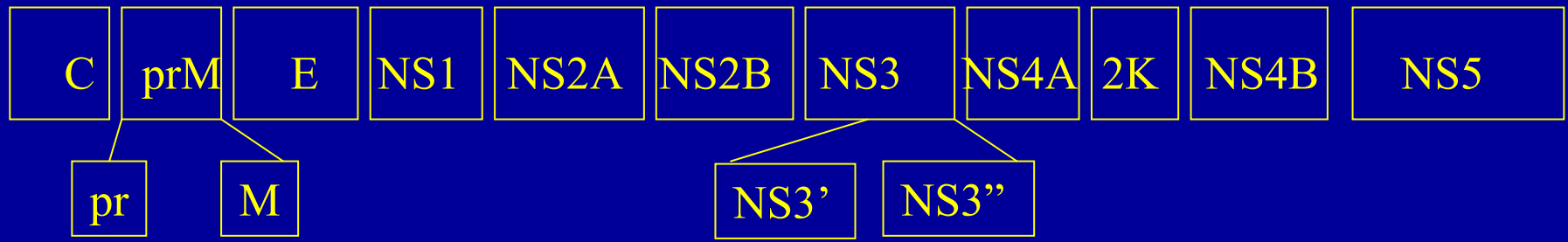
- Hamsters can get persistent renal infections and viruria.
- Persistent virus has become attenuated for neuroinvasive disease but causes renal disease.
- 0.04-0.09% nucleotide changes in genome.
- Amino acid substitutions in E (2), NS1 (1), NS2B (1) and NS5 (2) + 3'UTR (3).
- Viral RNA detected in urine up to 8 months post infection.

Molecular epidemiology of West Nile virus

Flavivirus genome



Co- and Post-translational Processing



Signal peptidase site
Unique site
NS2B-NS3 protease site

NS3 Protease, helicase, NTPase

NS5 Methyltransferase, RNA polymerase

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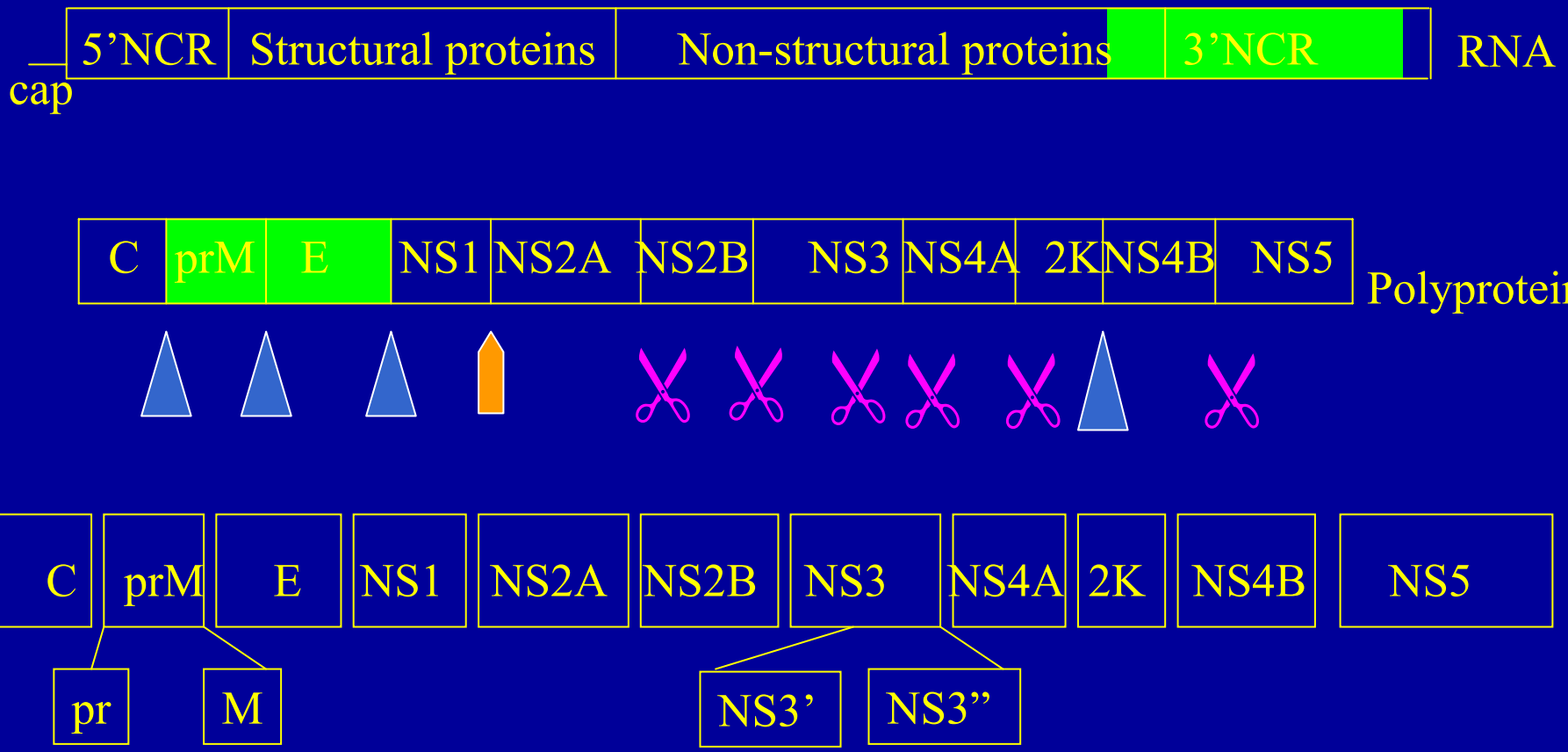
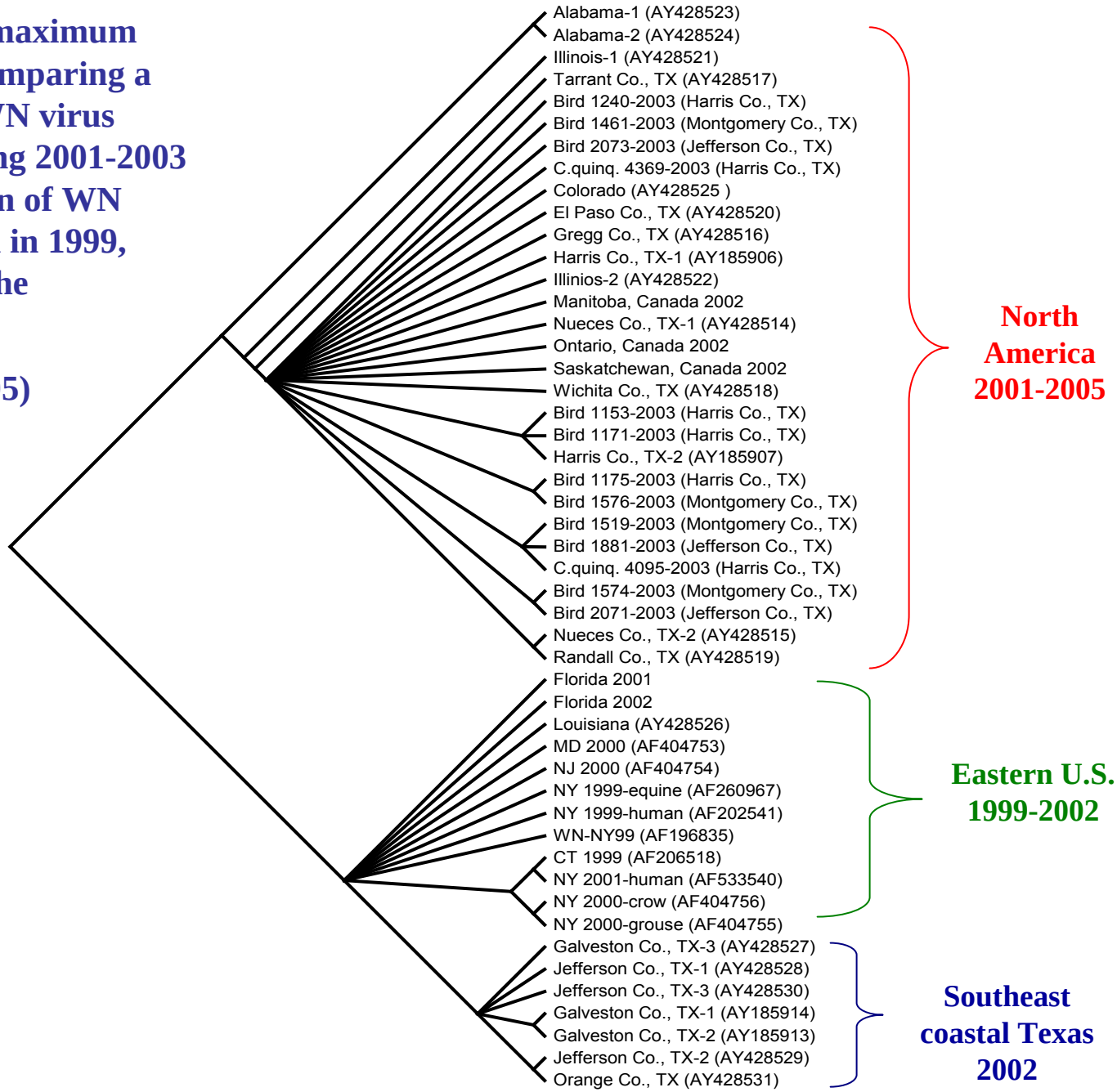
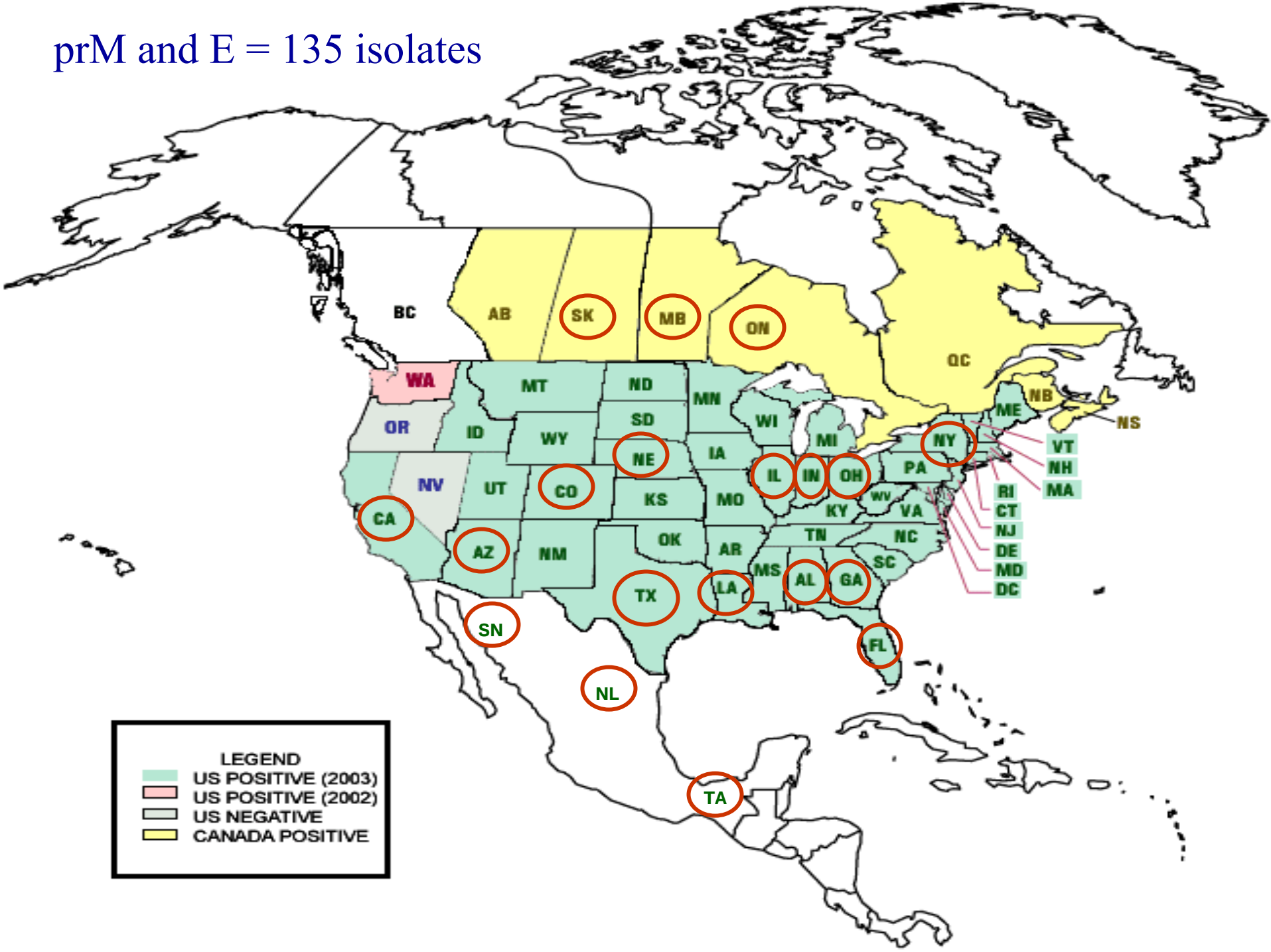


Cladogram based on maximum parsimony analysis comparing a 2004-nt sequence of WN virus isolates collected during 2001-2003 to a homologous region of WN virus isolates collected in 1999, 2000, and 2001 from the northeastern U.S.

(Davis et al., 2003, 2005)



prM and E = 135 isolates



Bayesian analysis of prM and E genes of 115 North American WNV isolates. The following criterion were used in analysis:

N gen= 150,000

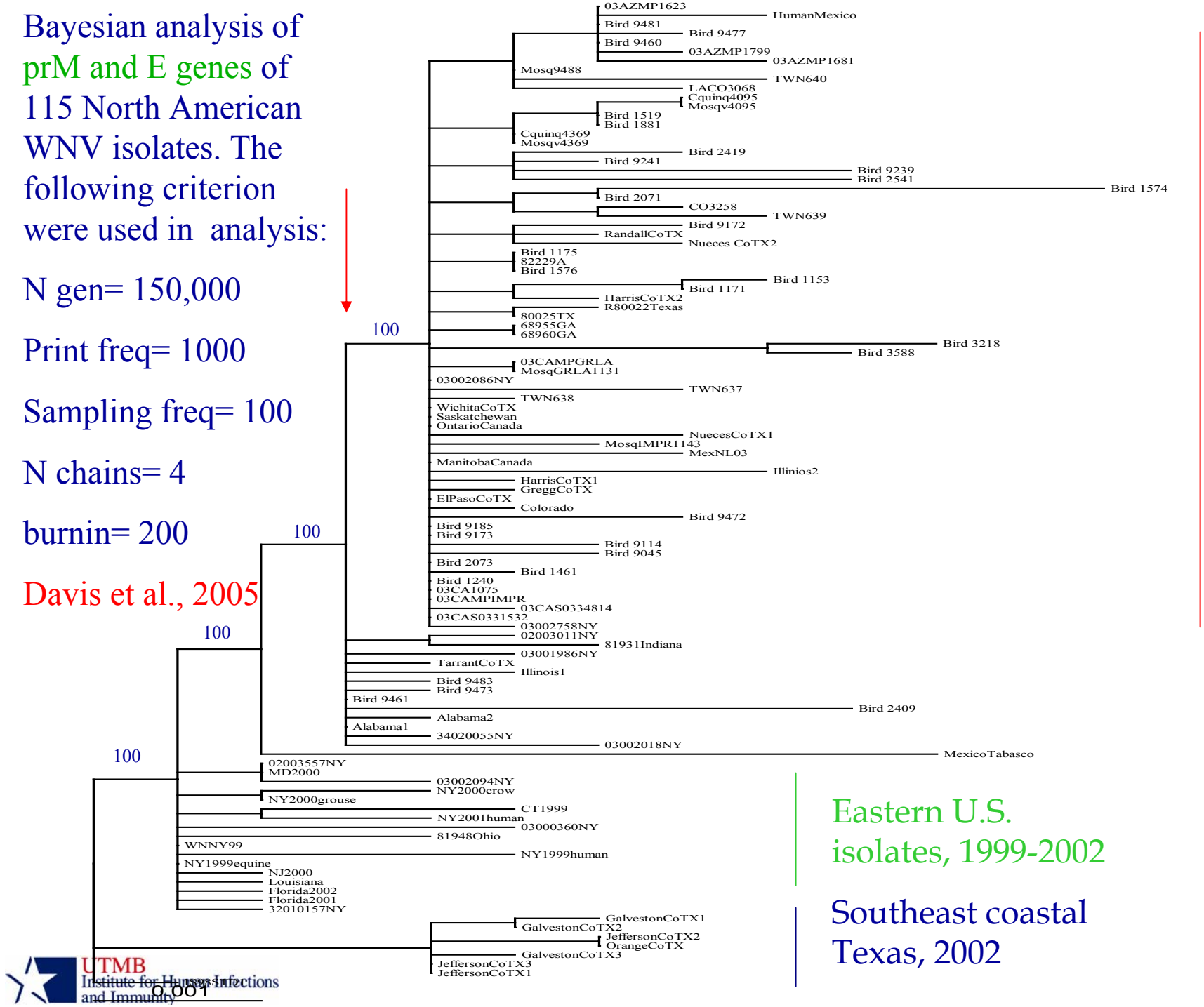
Print freq= 1000

Sampling freq= 100

N chains= 4

burnin= 200

Davis et al., 2005



North America genotype

Eastern U.S. isolates, 1999-2002

Southeast coastal Texas, 2002

Nucleotide/amino acid substitutions conserved in all isolates of North American clade 2002-2004 relative to WN-NY99

Davis et al., Virology 342:252-65, 2005

5'UTR	Capsid	prM	E	NS1	NS2A	NS2B	NS3	NS4A	NS4B	NS5	3'UTR
None	None	660 (C to U)	1442 (U to C)*	None	3774 (U to C)	None	4803 (C to U)	None	6996 (C to U)	7938 (U to C)	10851
			2466 (C to U)		4146 (A to G)		6138 (C to U)			9352 (C to U)	(A to G)
							6238 (C to U)				
							6426 (C to U)				

Nucleotide positions correspond to WN-NY99.

*Encodes E159 (Val to Ala) amino acid substitution.

Poster # 51, Grinev et al., has amino acid substitutions in C, NS1 and NS4A

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SE coastal Texas strain

(Granwehr et al., J Clin Micro 42: 5375-7, 2004)

Residue	SE coastal Texas	Lineage I	Lineage II
E-76	Ala	Thr	Thr
NS1-94	Gly	Glu	Asn
NS2A-90	Met	Met	Lys
NS2A-138	Ile	Val	Val
NS4B-173	Ile	Val	Val
NS5-526	Ile	Thr	Thr

Conclusions

- Evidence suggests limited, but continuing, divergence from isolates collected in 1999 and 2000
- Emergence of genetically distinct variants
    - Dominant North America variant since at least 2001
    - Coastal SE Texas variant in 2002 only → become extinct?
- Dominant variant has emerged throughout the majority of North America with >90% of isolates collected in 2002 and after belonging to the dominant clade
  - Emergence of novel genotype corresponds with displacement or extinction of earlier genotypes
- Subclades are readily illustrated depending on year of isolation and geographic location
  - Higher degree of nucleotide identity within individual states and during the same transmission season
- Majority of nucleotide changes are transitions (U ←→ C).
- No strong selection → genetic drift?
- Genetic mutations could eventually lead to phenotypic changes in viral virulence?

Phenotypic variants – Texas 2003

Davis et al., Virology 330: 342-50, 2004

Nucleotide sequencing and phenotypic comparisons of 29 WNV isolates collected by Harris Co. Mosquito Control Division in and around Houston, TX between 9 May – 8 Sept. 2003:

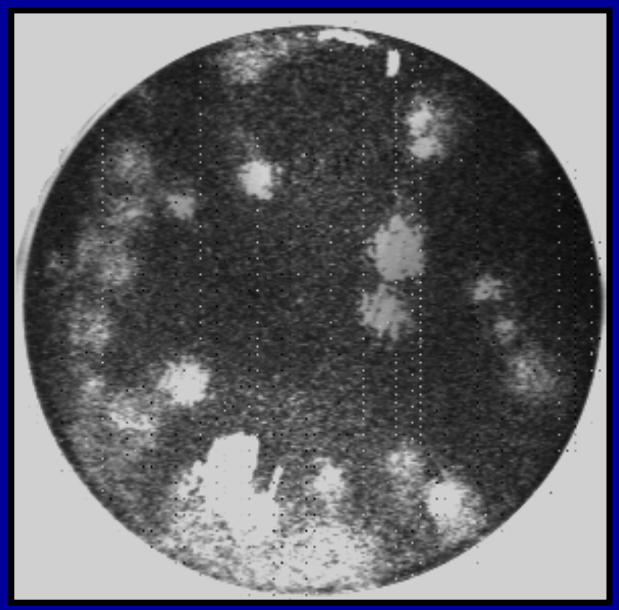
- 17 from dead birds
- 12 from mosquito pools (*Culex spp.*)

Viruses isolated in Vero cells, amplified by one additional passage and plaque titrated in Vero cells.

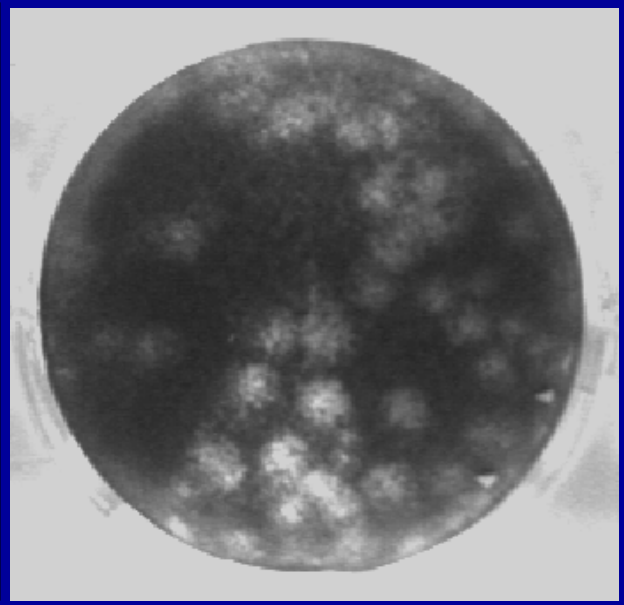
6 strains (5 bird, 1 mosq.) with small plaque (SP) morphology

Plaque morphology of WNV isolates

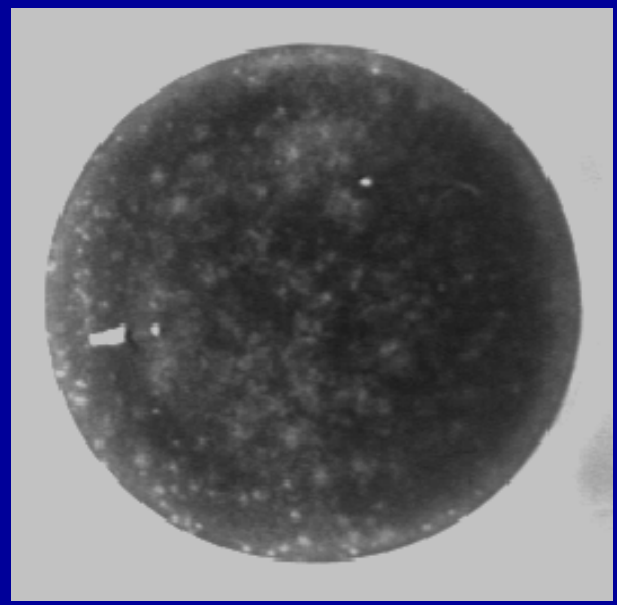
WN-NY99 (382-99)



WNV 2002



WNV 2003 sp



Mean plaque size ≥ 1.5mm

Mean plaque size < 1.0mm

In Vero cells, 72 hours post-infection.

Stained with crystal violet.

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Phenotypic comparisons of LP/SP WNV strains

Small plaque phenotype often a marker of attenuation of flaviviruses.

Other phenotypic comparisons:

- Temperature sensitivity at 39.5°C
- Multiplication in cell culture and in vivo
- Mouse neuroinvasion

  - i.p. inoculation of 3-4 week old Swiss Webster mice

Phenotypic comparisons of LP/SP WNV strains

Strain	Source	Plaque	TS index*	ip LD_{50} (pfu)	AST ± sd	ic LD_{50} (pfu)
382-99	Flamingo	L	-0.5	0.8	7.2±0.6	0.1
TWN93 (02)	Bird	L	0.3	0.5	8.0±1.0	0.1
TWN301 (03)	Bird	L	-0.2	0.6	7.0±1.0	0.2
TWN305 (03)	Bird	S	0.3	51,000	9.0±4.0	0.1
TWN274 (03)	Bird	S	-2.7	23,000	9.5±1.0	0.3
TWN382 (03)	Mosq.	S	-1.8	645,000	8.3±3.0	0.1
TWN269 (03)	Bird	S	-2.7	2,000	9.7±3.3	nd

*TS index is $\log_{10}$ difference in virus titer at 39.5°C compared with 37°C; negative values indicate decreased titers.

nd – not determined; L = large, S = small.

Serum and brain viremia in mice following ip inoculation of 10^3 pfu of WN-NY99 (neuroinvasive) vs. Bird 1153 (non-neuroinvasive)

Days post inoculation	Animal	WNNY99 (382-99)		Bird 1153 sp, ts, att	
		Serum titer (pfu/ml)	Brain titer (pfu/brain)	Serum titer (pfu/ml)	Brain titer (pfu/brain)
1	1	2,000	-	3,000	-
	2	4,000	-	1,500	-
	3	1,400	-	4,000	-
2	1	4,000	-	1,650	-
	2	11,500	-	250	-
	3	1,650	-	750	-
3	1	17,000	-	100	-
	2	15,000	-	100	-
	3	6,000	-	250	-
4	1	-	2,000	-	-
	2	-	500	-	-
	3	-	4,000	-	-
5	1	-	3,000	-	-
	2	-	1,000	-	-
	3	-	750	-	-
6	1	-	135,000	-	-
	2	-	300,000	-	-
	3	-	750,000	-	-
7	1	-	1,150,000	n.d.	n.d.
8	1	-	1,350,000	n.d.	n.d.

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* - indicates no virus detected

WNV isolate	Nucleotide position	Gene/region	Nucleotide change	Amino acid change
Harris Co., TX 2002	1442	E	U to C	V159A
lp, non-ts, non-att	7699	NS5	A to C	T6P
	10408	3' UTR	C to U	n/a
	10851	3' UTR	A to G	n/a
Bolivar P., TX 2002*	1192	E	A to G	T76A
lp, non-ts, non-att	2749	NS1	A to G	E94G
ΔAY289214	3937	NS2A	G to A	V138I
	7432	NS4B	G to A	V173I
	9256	NS5	C to U	T526I
	10494	3' UTR	U to C	n/a
	10768	3' UTR	U to A	n/a
	10851	3' UTR	A to G	n/a
Bird 1461*	1442	E	U to C	V159A
lp, non-ts, non-att	5151	NS3	A to U	E180D
ΔXXXXXX	5593	NS3	G to A	E327K
	6871	NS4A	G to A	V134M
	9535	NS5	G to U	A618S
	10408	3' UTR	C to T	n/a
	10851	3' UTR	A to G	n/a
Bird 1153*	931	prM	G to A	V156I
sp, ts, att	1442	E	U to C	V159A
ΔXXXXXX	7661	NS4B	A to G	E249G
	10091	NS5	C to U	A804V
	10596	3' UTR	A to G	n/a
	10774	3' UTR	C to U	n/a
	10799	3' UTR	A to G	n/a
	10851	3' UTR	A to G	n/a
Bird 1171*	931	prM	G to A	V156I
sp, ts, att	1442	E	U to C	V159A
ΔXXXXXX	7661	NS4B	A to G	E249G
	8279	NS5	G to U	R199L
	9743	NS5	C to A	A687D
	10091	NS5	C to U	A804V
	10596	3' UTR	A to G	n/a
	10774	3' UTR	C to U	n/a
	10799	3' UTR	A to G	n/a
	10851	3' UTR	A to G	n/a
	11000	3' UTR	G to U	n/a
Bird 1175	1442	E	U to C	V159A
sp, ts, att	7661	NS4B	A to G	E249G
	10408	3'UTR	C to U	n/a
	10851	3' UTR	A to G	n/a
Bird 1519	478	prM	A to G	N4D
sp, att	1442	E	U to C	V159A
	10851	3' UTR	A to G	n/a
Mosq. V4369*	478	prM	A to G	N4D
sp, att	1442	E	U to C	V159A
ΔXXXXXX	7636	NS4B	A to G	T240A
	8566	NS5	C to U	H295Y

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Summary of mutations in 5 attenuated isolates

- prM-4 N→ D
- prM-156 V→I
- E-159 A→V
- NS4B-240 T→A
- NS4B-249 E→G
- NS5-199 R→L
- NS5-295 H→Y
- NS5-687 A→D
- NS5-804 A→V
- 3'UTR: 10408, 10596, 10774, 10799, 10851, 11000

Conclusions

- First evidence of phenotypic variation in North American West Nile virus
- Attenuation of mouse neuroinvasiveness may be indicative of attenuation in birds? horses? humans?
- No indication that attenuated viruses persist in nature
  - No more SP/mouse attenuated isolates identified
  → another extinct lineage?
- Sequencing and reverse genetics studies can be used to identify molecular determinants of phenotypic variation

Genetic and phenotypic variation in New York, 2002

Ebel et al. 2004. Am J Trop Med Hyg 71: 493-500

- North America genotype emerged in New York in 2002 and replaced the Eastern US genotype.
- In vivo mosquito transmission studies (*Culex pipiens*)
  - Significantly higher proportion of mosquitoes
    - became infected (infectious virus in bodies) and
    - developed disseminated infections (infectious virus in legs) following feeding with North America genotype compared to WN-NY99
  - Significantly higher proportion of mosquitoes were able to transmit (infectious virus in salivary secretions) at days 5 and 7 post-feed with dominant genotype
- Displacement of WN-NY99 genotype by dominant genotype may be due to differences in mosquito transmission efficiency (reduction of EIP with dominant genotype)?

Genetic variation in naturally infected mosquitoes and birds

Jerzak et al (*J Gen Virol 86: 2175-83, 2005*)

- WNV RNA populations are quasispecies.
- RNA sequences in mosquitoes had greater genetic diversity than birds.
- Indicates strong purifying selection taking place in nature.

Al Dupuis, Greta Jerzak, Yongqing Jia & Laura Kramer, unpublished

- Small plaque morphology.
- Non-neuroinvasive in mice.
- Low viremia in chicks.
- Reduced dissemination in mosquitoes.
- Amino acid changes in prM and NS2A.

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