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Plasticity of a critical antigenic determinant in the West Nile virus NY99 envelope protein domain III

Jessica A. Plante[a,b], **Maricela Torres**[c], **Claire Y-H Huang**[d], and **David W. C. Beasley**[b,c,e]

[a]Department of Pathology, University of Texas Medical Branch, Galveston, TX 77555, USA

[b]Sealy Center for Vaccine Development, University of Texas Medical Branch, Galveston, TX 77555, USA

[c]Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, TX 77555, USA

[d]Arbovirus Diseases Branch, Division of Vector-Borne Diseases, Centers for Disease Control and Prevention, Fort Collins, CO 80521, USA

[e]Institute for Human Infections and Immunity, University of Texas Medical Branch, Galveston, TX 77555, USA

Abstract

West Nile virus (WNV) is a mosquito-borne flavivirus that causes febrile illness, encephalitis, and occasionally death in humans. The envelope protein is the main component of the WNV virion surface, and domain III of the envelope protein (EIII) is both a putative receptor binding domain and a target of highly specific, potently neutralizing antibodies. Envelope E-332 (E-332) is known to have naturally occurring variation and to be a key determinant of neutralization for anti-EIII antibodies. A panel of viruses containing all possible amino acid substitutions at E-332 was constructed. E-332 was found to be highly tolerant of mutation, and almost all of these changes had large impacts on antigenicity of EIII but only limited effects on growth or virulence phenotypes.

Keywords

West Nile virus; Flavivirus; envelope; domain III

Corresponding author: David W. C. Beasley, University of Texas Medical Branch, 301 University Blvd., Galveston, TX 77555-0609, dwbeasle@utmb.edu, (409)772-1120.
Present contact information for Jessica A. Plante: Department of Epidemiology, University of North Carolina, Chapel Hill, NC 27599, USA, japlante@email.unc.edu

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INTRODUCTION

West Nile virus (WNV) is a member of the Japanese encephalitis serocomplex within the genus *Flavivirus* and family *Flaviviridae*. WNV is distributed worldwide and infection of humans results in outcomes ranging from asymptomatic infections or non-specific fever to neuroinvasive disease (meningitis and/or encephalitis), potentially resulting in polio-like flaccid paralysis and, in some cases, death.(Lindsey et al., 2010; Sejvar, 2007) Up to eight lineages of WNV have been proposed on the basis of molecular phylogenetic analyses, with lineages 1 and 2 being the most geographically widespread.(Vazquez et al., 2010) Lineage 1, which includes the strain introduced to North America in 1999, has traditionally been thought to be the main source of WNV-associated disease. More recently, however, outbreaks involving lineage 2 WNV strains have caused human disease and death in Greece, Italy, Romania, Russia, and, potentially, South Africa.(Barzon et al., 2015; McMullen et al., 2013; Papa et al., 2011; Platonov et al., 2011; Sirbu et al., 2011; Venter and Swanepoel, 2010; Zaayman and Venter, 2012)

The WNV envelope protein (E) is the main surface component of the mature WNV virion. The E ectodomain is composed of three structural domains (Figure 1): domain I (EI) is a central β-barrel connecting domain II (EII) and domain III (EIII) and contains the single glycosylation motif present in some WNV strains; EII contains the flavivirus-conserved fusion loop; and EIII is an Ig-like domain thought to play a significant role in receptor binding.(Kanai et al., 2006; Nybakken et al., 2006) EIII's putative role in receptor binding is based on studies with several flaviviruses that have demonstrated direct attachment of recombinant EIII to target cells and potential receptors (Chu et al., 2005; Hung et al., 2004; Lee et al., 2006),attenuation of *in vitro* and *in vivo* replication associated with single amino acid substitutions (Erb et al., 2010; Hurrelbrink and McMinn, 2001; Zhang et al., 2010), and the potent pre-attachment neutralization of flaviviruses by some antibodies targeting EIII (Crill and Roehrig, 2001).

Although antibodies binding to EIII have been reported to make up only a small fraction of the overall antibody response in human flavivirus infections, they tend to be virus-specific and potently neutralizing.(Crill et al., 2009; Lin et al., 2012a; Throsby et al., 2006; Vratskikh et al., 2013), This, combined with the relative ease of expressing and purifying recombinant EIII protein, has led to several investigations into EIII-based subunit vaccines for WNV and other flaviviruses that have yielded promising results.(Alonso-Padilla et al., 2011; Chu et al., 2007; Dunn et al., 2010; Martina et al., 2008; Spohn et al., 2010) In addition to the EIII-based vaccines, antibody therapies targeting EIII have also been proposed. The monoclonal antibody (mAb) E16 was shown to be protective in mice pre- and post-challenge with WNV (Lai et al., 2010; Morrey et al., 2008; Oliphant et al., 2005; Smeraski et al., 2011). Phase I and II clinical trials (ClinicalTrials.gov - NCT00515385 and NCT00927953, respectively) of a humanized version of that antibody, under the product name MGAWN1, have been performed, although the phase II trial was terminated early due to low enrollment. (MacroGenics, 2009, 2012) and a path forward to licensure of that product is currently unclear.

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Previous research using wild-type (WT) WNV strains or neutralization escape mutants has identified a small number of residues in EIII that can be altered to prevent antibody-mediated neutralization with little or no effect on virus growth in cell cultures or virulence in animal models.(Beasley and Barrett, 2002; Choi et al., 2007; Li et al., 2005; Nybakken et al., 2005; Oliphant et al., 2005; Volk et al., 2004) Residue 332 (E-332), in particular, appears to be a major antigenic determinant. The majority of WNV strains have a threonine atE-332, but naturally occurring variants - including substitutions to alanine, methionine, serine, and lysine - have been found in lineage 1 and 2 strains isolated from humans, equines, bats, and mosquitoes (e.g. GenBank accession nos. AF459403.3, AY688948.1, EU249803.1, GQ502394.1, GQ507480.1, HM051416.1, HM147822.1, HM147823.1, HM488220.1, JX015521.1, and KM052152.1). These sequence variations at E-332 have been shown to reduce neutralization by multiple monoclonal antibodies and by polyclonal antisera raised against EIII.(Li et al., 2005) In particular, 332K variants, including some lineage 2 WNV strains, are entirely resistant to neutralization *in vitro* and/or *in vivo* by MAbs such as 7H2, 5H10, and the candidate therapeutic antibody E16/MGAWN1.(Beasley and Barrett, 2002; Li et al., 2005; Zhang et al., 2010) To define the tolerance of WNV for substitutions at this critical antigenic determinant and the effects on antibody binding and neutralization, a WNV NY99 infectious clone (NY99ic) was used to generate all possible amino acid variants at E-332. Viable variants were recovered and their growth characteristics were assessed in representative mammalian, mosquito and avian cell lines. Mouse virulence was also determined for all recovered variants, with several selected for LD_{50} determination. Finally, *in vitro* antibody binding and neutralization were determined for each variant using several monoclonal antibodies and polyclonal serum raised against EIII.

RESULTS

Recovery and Sequencing of Variants

All 20 possible amino acids at E-332 yielded viable virus. The NY99ic mutants T332E, T332F, T332L, T332N, T332P, T332Q, T332R, T332S, and T332W were recovered via plaque purification from electroporation of Vero cells with mixed *in vitro*-transcribed RNA pools (see Materials and Methods). All other mutants were recovered via electroporation of RNA prepared from single mutant plasmid preparations. Nucleotide sequence analysis revealed no additional mutations in the prM/E region for 18 of the 20 variants. However, both the NY99ic T332P and NY99ic T332W mutants contained an additional mutation from serine to arginine at E residue 66 (E-66). This S66R mutation was present in both the passage 2 plaque purification and passage 3 working stock preparations for each variant despite the fact NY99ic T332P and NY99ic T332W were obtained from separate pools, suggesting that S66R arose relatively rapidly and independently in each mutant during recovery. E-66 is a surface exposed residue in domain II (Figure 1), and it is approximately 80 angstroms from E-332 within the context of a single E monomer. To determine whether the S66R mutation played any compensating role, individual NY99ic T332P, NY99ic T332W, and NY99ic S66R mutants were subsequently recovered via electroporation as passage 0 stocks.

Plaque Morphology and Temperature Sensitivity

Previous studies have compared changes in WNV plaque morphology and apparent titer at 37°C and 41°C (i.e. a "temperature sensitive" phenotype) as possible indicators of attenuation.(Andrade et al., 2011; Davis et al., 2007; Wicker et al., 2012; Wicker et al., 2006) The temperature change had minimal impact on NY99ic WT titer (Table 1), consistent with previous reports, and plaques were approximately 0.5mm in diameter at 37°C and 3mm in diameter at 41°C when measured at 3 days post-infection (Figure 2). Almost all of the NY99ic E-332 mutants produced plaque morphologies comparable to NY99ic WT and had differences of $0.3\log_{10}$ pfu/ml between titers at 37°C and 41°C. Of those, only the slight increases in titer at 41°C for the NY99ic T332I and T332Y mutants were statistically significant. The NY99ic T332P+S66R mutant was the most temperature sensitive, with a $1.5\log_{10}$ pfu/ml decrease in apparent titer at 41°C vs 37°C. Somewhat surprisingly, this relatively large decrease in apparent titer at 41°C was not observed for either the individual NY99ic S66R or the individual NY99ic T332P mutants (Table 1), although the NY99ic T332P mutant did display a small plaque phenotype at 41°C (Figure 2). The NY99ic T332R mutant also exhibited a small plaque phenotype at 41°C but, as with NY99ic T332P, this plaque morphology difference was not accompanied by a titer difference.

Growth in mammalian, avian and mosquito cell lines

NY99ic WT and all of the T332 mutants were replication competent in Vero, duck embryo fibroblast (DEF), and *Aedes albopictus* C6/36 cells. In Vero cells, all viruses were in eclipse at 0.5 days post infection (DPI) and had begun robust amplification by 1 DPI, with peak titers were reached at 3 DPI (Figure 3A). Only the NY99ic T332P+S66R mutant had significantly different titer from NY99ic WT at 3 DPI. Despite similar early growth kinetics observed between 0 and 2 DPI, NY99ic T332P+S66R was $1.7\log_{10}$ PFU/ml lower than NY99ic WT at 3 DPI and maintained that deficit at 4 DPI. No deficiency was noted for NY99ic T332P+S66R in either DEF or C6/36 cells.

Similar to replication in Vero cells, NY99ic WT and the T332 mutants were in eclipse in DEF cells at 0.5 DPI and had begun to amplify by 1 DPI (Figure 3B). Peak titers were observed between 1.5 and 2 DPI. Fifteen of the 19 NY99ic T332 mutants reached peak titers within $\pm 0.5\log_{10}$ PFU/ml of NY99ic WT and were statistically indistinguishable from NY99ic WT by Dunnett's multiple comparisons test. The four NY99ic T332 mutants that did have significantly different peak titers than NY99ic WT were NY99ic T332D, which was $0.6\log_{10}$ PFU/ml lower than NY99ic WT, and NY99ic T332K, T332 M, and T332R, which were $0.6–0.9\log_{10}$ PFU/ml higher than NY99ic WT.

Replication in C6/36 cells resulted in an eclipse phase at 0.5 DPI similar to that observed in Vero and DEF cells, followed by robust amplification from 1 DPI onward (Figure 3C). However, unlike replication in the vertebrate cell lines that reached a peak titer that subsequently decreased, the titers produced by C6/36 cells increased throughout the duration of the experiment to an average peak of 1.4×10^7 PFU/mL at 5 DPI. Only six of the NY99ic T332 mutants were statistically indistinguishable from NY99ic WT at 5 DPI: NY99ic T332A, T332G, T332H, T332L, T332M, and T332V. These mutants were all within $0.2\log_{10}$ PFU/ml of NY99ic WT. Only one mutant, NY99ic T332D, had a higher titer than

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NY99ic WT with an advantage of 0.4$\log_{10}$ PFU/ml at 5 DPI. Of the 12 remaining NY99ic T332 mutants, 10 were 0.2–0.8$\log_{10}$ PFU/ml lower than NY99ic WT at 5 DPI. The two strains with the greatest deficit at 5 DPI were both large aromatic substitutions; NY99ic T332Y had a deficit of 1.0$\log_{10}$ PFU/ml and NY99ic T332W+S66R had a deficit of 1.6$\log_{10}$ PFU/ml.

Virulence in Swiss Webster mice following peripheral inoculation

The impact of the mutations at residue E-332 on virulence was initially assessed via intraperitoneal inoculation of 3–4 week old Swiss Webster mice with a 100PFU dose of each NY99ic mutant or the NY99ic WT parent. Consistent with previous studies, the NY99ic WT virus was highly lethal, with only 5% of mice surviving challenge (Table 1). Of the 22 NY99ic mutants tested, 13 were comparably lethal, with survival rates of ≤10%. Five of the remaining 9 NY99ic mutants (T332I, T332L, T332M, T332R, and T332W+S66R) caused survival rates of 13–20%, and three NY99ic mutants (T332C, T332P, and T332W) had survival rates of 30–47%. The most strongly attenuated mutant was NY99ic T332P+S66R, with a survival rate of 87%. Those subjects that succumbed to infection from NY99ic T332P +S66R also had significantly longer average survival times than was observed for NY99ic WT infection. Interestingly, this mutant appeared to be more attenuated than either the single NY99ic T332P mutant (47% survival) or the NY99ic S66R mutant (0% survival). No clear difference in the apparent level of attenuation was observed in the case of the combined NY99ic T332W+S66R mutant (20% survival) compared to the NY99ic T332W mutant (30% survival).

Following the 100 PFU virulence screen, NY99ic WT and a subset of mutants underwent LD_{50} determination as described elsewhere.(Beasley et al., 2002) The mutants were selected to include those with attenuated or intermediate phenotypes from the virulence screening, plus additional mutants that more closely mirrored the WT parent and reflected different categories of possible amino acid substitutions (acidic, basic, aromatic, etc.) (Table 1). Mutants encoding the S66R mutation were also included. NY99ic WT had a LD_{50} value of 0.4 PFU, similar to previously reported results.(Beasley et al., 2005; Plante et al., 2014) Eight of the 13 NY99ic mutants tested had similar LD_{50} values of <1 PFU. This included T332C, which appeared moderately attenuated during the initial virulence screen but had an LD_{50} value of 0.9 PFU. Three of the NY99ic mutants (T332I, T332R, and T332W+S66R) had slightly increased LD_{50} values between 1–10 PFU. Only the NY99ic T332P mutants, with or without the presence of S66R, had LD_{50} values indicative of strong attenuation. Consistent with the different survival rates observed in the initial virulence screening for those two mutants, the LD_{50} for NY99ic T332P was 452 PFU, and the LD_{50} for NY99ic T332P+S66R was >1000PFU. Therefore, although the S66R mutation was not sufficient to appreciably attenuate NY99ic on its own, its presence did seem to increase attenuation of T332P compared to the individual mutant.

Effects of 332 mutations on EIII antigenicity

The impact of mutations at E-332 on neutralization of virus infection in Vero cells (Table 1; Figure 4) and on binding of selected anti-WNV mAbs and polyclonal anti-EIII serum in Western blots (Figure 5) was assessed. MAbs 5H10 and 7H2 were selected because they

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have overlapping but distinct epitopes on WNV EIII and their binding and neutralization activities are known to be variably affected by previously studied T332A/K/M mutations. (Beasley and Barrett, 2002; Li et al., 2005; Zhang et al., 2010). Following calculation of neutralization indices (NI), hierarchical clustering with k-means analysis defined four categories of neutralization: NI values 2.2 (designated as very strong neutralization); NI values between 1.2 and 1.9 (strong neutralization); NI values between 0.5 and 1.1 (moderate neutralization), and NI values 0.4 (weak neutralization). Almost all mutations caused large reductions in neutralizing activity of 5H10 and 7H2 antibodies compared with the NY99ic WT control (NI values of 2.5±0.0 and 3.3±0.3, respectively). Both of the acidic mutants (NY99ic T332D and T332E), two of the three basic mutants (NY99ic T332H and T332K), and two of the three aromatic mutants (NY99ic T332W+S66R and T332Y) were weakly neutralized by both 5H10 and 7H2. Only NY99ic T332A and T332S retained either a strong or very strong neutralization phenotype by both 5H10 and 7H2. Additionally, many changes to residue T332 resulted in large reductions to neutralization by polyclonal rabbit serum raised against recombinant WNV EIII protein. Only five of the 19 NY99ic T332 mutants (T332A, T332G, T332P+S66R, T332S, and T332V) retained either strong or extremely strong neutralization by the polyclonal serum. The remaining NY99ic T332 mutants were moderately neutralized, except for NY99ic T332H, T332L, T332W+S66R, and T332Y, which were weakly neutralized.

In general, binding by the monoclonal antibodies 5H10 and 7H2 to NY99ic WT and NY99ic T332 mutant cell lysates in Western blots corresponded well with the strength of neutralization (Figure 5). For 5H10, all NY99ic T332 mutants with neutralization indices of 0.9 or less had very faint or non-visible bands in a Western blots except for NY99ic T332E,. For 7H2, NY99ic T332 mutants with neutralization indices between 1.7 and 1.0 (T332A, T332G, T332I, T332L, T332M, T332N, and T332P+S66R) there is variation between strong and weak/non-visible binding, but neutralization indices 1.8 corresponded to strong binding, similar to the NY99ic WT, and neutralization 0.7 corresponded to weak or undetectable binding.

Despite having a strong impact on neutralization by polyclonal anti-EIII serum, mutations at T332 had no impact on binding by the polyclonal anti-EIII serum in a Western blot. This is likely because inoculation with recombinant EIII elicits non-functional antibodies against epitopes that are not accessible in the context of an intact, infectious virion but are accessible in the context of an infected cell lysate.

DISCUSSION

The recovery of viable WNV NY99ic mutants encoding every possible amino acid at E-332 is consistent with related observations regarding the ability of E-332 to tolerate substitution. The extreme plasticity of WNV E-332 has been suggested by both its naturally occurring variation and by the previously reported recovery of neutralization escape mutants that retain a wild-type virulence phenotype.(Armstrong et al., 2011; Beasley et al., 2002; Bernardin, 2010; Li et al., 2005; McMullen et al., 2013; Pybus et al., 2012; Sapkal et al., 2011; Zhang et al., 2010) In addition, a panel of packaged replicon reporters (described by Lee *et* al. as reporter virus particles, or RVPs) was generated that captured the range of possible variation

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at E-332 and a pool of infectious clone-derived viruses with variation at E-332 was used to examine the selective pressure of neutralizing antibodies.(Lee et al., 2013)

In general, changes to NY99ic T332 had only modest impacts on replication kinetics in mammalian, avian, and mosquito cells, and on virulence in mice. The few significant differences that were observed in cell culture were variable between cell types. For instance, only the NY99ic T332P+S66R mutant appeared significantly impaired for growth in Vero cells, but it was more comparable to NY99ic WT in both C6/36 and DEF cells. Similarly, both NY99ic T332W+S66R and T332Y had titers 1log$_{10}$ PFU/ml lower than NY99ic WT in C6/36 cells on 5 DPI, but replicated similarly to WT in Vero an DEF cells. It has been reported that changes to residues in the surface loop residues of EIII in multiple flaviviruses can have differential impacts on replication in mosquito and mammalian cells.(Erb et al., 2010; Huang et al., 2014; Hung et al., 2004) and this also appears to be the case for WNV E-332. It is worth noting that the substitutions which resulted in cell-type-dependent deficits in peak titer all involved the introduction of either large aromatic rings (T332W+S66R and T332Y) or a cyclic structure (T332P+S66R), raising the possibility that the deficit was not a direct consequence of changing E-332 but of the resulting disruption of neighboring residues. Consistent with the extreme plasticity of E-332 in cell culture, the majority of NY99ic T332 mutants retained a highly virulent phenotype in mice. In fact, all but three mutants caused 30% survival following peripheral infection in Swiss Webster mice. Very strong attenuation was only observed with the NY99ic T332P and T332P+S66R mutants. Thus, the detrimental impact of the proline at E-332 observed in Vero cell culture appeared to have a similarly negative effect *in vivo*. While it cannot be entirely ruled out that some of the observed changes in growth and/or virulence might be influenced by suboptimal codon usage associated with the introduced mutations, the especially strong phenotype of the T332P and T332P+S66R mutants is consistent with the disruptive nature of introducing this cyclic amino acid. Furthermore, although codon usage bias can cause demonstrable impacts on flavivirus replication and virulence, those effects have typically been in the context of changes to large segments of the genome, not single codon substitutions.(de Fabritus et al., 2015; Shen et al., 2015)

The independent acquisition of a S66R mutation by both the NY99ic T332P and T332W mutants was an unexpected finding. Similar acquisitions of a positive charge at a surface exposed E residue has previously been associated with tissue culture adaptation in other encephalitic flaviviruses.(Lee et al., 2004; Lee and Lobigs, 2000) A follow-up comparison of NY99ic WT to the individual NY99ic S66R, T332P, T332P+S66R, T332W, and T332W +S66R mutants revealed that S66R had no detectable impact on amplification and peak titer in Vero cells following a low MOI infection (Supplementary Figure S1). However, although the NY99ic S66R mutant was not attenuated in mice, the presence of S66R did appear to exacerbate the attenuation of NY99ic T332P, and also to increase the LD_{50} of NY99ic T332W (Table 1) although neither T332W nor T332W+S66R were strongly attenuated. These observed effects of the S66R mutation are consistent with attenuating effects of other tissue culture associated charge changing mutations. More work is required to characterize the role of the S66R mutation and its direct or indirect interaction with mutations at E-332.

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In contrast to the limited impact of mutations at E-332 on NY99ic replication and virulence, changes to E-332 had a profound impact on antigenicity. None of the nineteen possible NY99ic T332 mutants retained the very strong neutralization phenotype of NY99ic WT with 7H2, and only the NY99ic T332S mutant retained the very strong neutralization phenotype with 5H10. In fact, nine of the 19 possible NY99ic T332 mutants were weakly neutralized by one or both of the EIII mAbs tested. These results are consistent with those previously reported for the anti-WNV EIII mAbs E33 and E16, which largely failed to neutralize a panel of WNV RVPs with basic, acidic, or aromatic substitutions at E-332.(Lee et al., 2013) All nine mutations that resulted in weak neutralization (NI values <0.4) by 5H10 and/or 7H2 also resulted in moderate (NI values 0.5–1.1) or weak neutralization by the polyclonal anti-EIII rabbit serum, confirming that E-332 is a critical antigenic determinant even in the context of a polyclonal population of antibodies against EIII

The ability of NY99ic to tolerate changes at E-332 that ablate or greatly reduce neutralization by monoclonal and polyclonal antibodies while having little to no impact on replication and virulence phenotypes raises an important consideration for the potential use of EIII-based vaccines or therapeutics against WNV. There are six possible amino acid substitutions that require only a single nucleotide change from the predominant WNV 332 codon of ACG: T332A, T332K, T332M, T332P, T332R, and T332S (Figure 4). Of these six possibilities, our data suggest that T332P is significantly impaired and unlikely to persist in nature but four other mutations (T332A, T332K, T332M, and T332S) are already known to exist in naturally occurring WNV strains. These strains with variation at E-332 were collected over a span of decades (1953–2007) from mosquitoes, a bird, a fruit bat, viremic human blood donors, and febrile human patients.(Armstrong et al., 2011; Beasley et al., 2002; Bernardin, 2010; McMullen et al., 2013; Pybus et al., 2012; Sapkal et al., 2011) Of these mutations, only T332K is associated with large reductions or loss of neutralizing activity by anti-EIII MAbs or polyclonal antiserum. However, alternative amino acids with antigenic and virulence characteristics comparable to T332K, particularly T332D, T332E, and T332H are within a single nucleotide substitution of the other known 332 variants. It is worth noting that single residue EIII mutations in naturally occurring WNV strains that strongly reduce neutralization by polyclonal anti-EIII serum or monoclonal antibodies do not significantly impact neutralization by polyclonal sera raised against whole WNV virions. (Li et al., 2005)

Previous work has shown that the proposed therapeutic mAb E16 exerts sufficient selective pressure on a pool of infectious clone-derived E-332 mutants *in vitro* to generate a population composed exclusively of T332K and T332R mutants within a single post-transfection passage(Lin et al., 2012b) .Additionally, *in vivo* experiments have demonstrated that pre-treating mice with the mAb E16 and administering a high but biologically relevant dose of WT WNV can select for neutralization-resistant variants at E-332 which are lethal to the infected animals.(Zhang et al., 2009) Data reported here suggest that WNV could likewise escape from a polyclonal response targeted against EIII. Combined with the previous recovery of 332 variant strains from humans, it seems possible that the administration of an EIII-based subunit vaccine or antibody therapy may potentially select for a resistant population within an infected individual. Such a resistant population would be

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unlikely to take hold in the wild because humans are dead-end hosts, but could complicate treatment and endanger the individual infected.

MATERIALS AND METHODS

Generation of WNV T332 Variants

WNV variants encoding amino acid substitutions were generated via targeted mutagenesis of the pWN-AB/pWN-CG NY99ic using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene, Cedar Creek, TX) as previously described (Beasley et al., 2005). Initially, four variant plasmid pools were generated using degenerate oligonucleotide primers as described previously (Zhang et al 2010). The four pools collectively included all 19 possible amino acid substitutions at residue T332 (primer sequences available upon request). Following electroporation of *in vitro*-transcribed RNA into Vero cells, culture supernatants were harvested at 3–5 days and plaque titrated on Vero cell monolayers in 6 well plates. Individual variants were recovered via plaque purification from those plates and amplified in T-25 flasks of Vero cells. When visible CPE was noted (3 days post infection for most variants), multiple aliquots of each viable variant were frozen down and their identities determined via sequencing. Working stocks of recovered variants representing each of the viable amino acid substitutions were then prepared in Vero cells for subsequent experiments. Each variant recovered via this strategy received three additional Vero cell passages following the passage 0 electroporation. In addition, several variants were recovered directly from individual variant pWN-AB plasmids that were generated using the QuikChange Multi kit with specific mutagenesis primers according to previously described methods.(Zhang et al., 2010)

Nucleotide Sequencing of WNV T332 Variants

Viral RNA was purified from tissue culture supernatants using the QIAamp Viral RNA kit (Qiagen, Germantown, MD) according to the manufacturer's protocol. The prM/E coding region was amplified using the Titan One-Step RT-PCR kit (Roche, Mannheim, Germany) with the WN401 and WN2504A primers.(Beasley et al., 2003) The resulting PCR products were visualized on an agarose gel and the appropriately sized band excised and purified using the QIAquick Gel Extraction kit (Qiagen, Germantown, MD). Traditional Sanger sequencing was performed by the Molecular Genomics Core at the University of Texas Medical Branch using an Applied Biosystems 3130XL instrument using the amplifying primers plus additional internal primers.(Beasley et al., 2003)

Plaque and Temperature Sensitivity Assays

Titers for each virus stock were determined by plaque assays in Vero cells. Briefly, serial 10-fold dilutions of each variant were made in sterile phosphate buffered saline (PBS) and 100μl was used to infect monolayers of Vero cells in 12-well plates. After one hour at room temperature, the plates were washed with PBS and overlaid with a 2ml of a 50:50 mixture of 2XMEM (Gibco, Grand Island, NY) containing 4% bovine growth serum (BGS) (Hyclone, Logan, UT) and 2% agar (Sigma, Portugal). For standard plaque assays, plates were placed at 37°C with 5% CO_2. At 2 days post-infection, 1ml of overlay containing 2% neutral red (Sigma, Irvine, UK) was added to each well, and the plates were wrapped in foil and

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returned to the incubator. Plaques were read with the aid of a light box on days 3 and 4 post-infection. For the temperature sensitivity assays, infected 6-well plates were overlaid with 4ml of media:agar and placed in either a 37°C or a 41°C incubator with 5% CO_2. On day 3 post-infection the plates were fixed with formalin and stained with crystal violet. Differences in titer and plaque morphology at 37°C and 41°C were recorded. Assays were performed in duplicate.

In vitro Growth Kinetics

Vero, C6/36, and duck embryo fibroblast (DEF) cells were infected in duplicate at an MOI of 0.01 for one hour at room temperature, then washed three times with PBS. Cells were maintained in 6ml MEM supplemented with 2% BGS (Vero) or 2%FBS (C6/36 and DEF). Vero and DEF cells were kept at 37°C with 5% CO_2. C6/36 cells were kept at 28°C in an ungassed incubator. For assays in Vero and DEF cells, two 250μL samples were collected at 0, 0.5, 1, 1.5, 2, 3, and 4 days post-infection and stored at −70°C for subsequent titration. C6/36 cell assays were performed similarly, except that an additional sample was collected at 5 days post-infection. Following sample collection fresh media was added to each sample to maintain a constant volume. All samples were titered via plaque assay on Vero cells

Mouse Neuroinvasiveness and LD_{50} Determination

For assessment of virulence, cohorts of 10–20 3–4-week-old female Swiss Webster mice (Harlan Laboratories, Houston, TX) were injected with WNV variants in 100μL volumes via the intraperitoneal (i.p.) route. During the initial screening involving all 20 variants, a single 100 pfu dose of virus was used. During subsequent LD_{50} determination for a subset of viruses, serial 10-fold dilutions ranging from 10^3–10^{-1}pfu were used with cohorts of five mice per dose. Following inoculation, mice were observed for 21 days. Mice exhibiting paralysis or signs of severe illness were humanely euthanized and counted as deceased for that day.

Neutralization by MAbs and polyclonal anti-EIII serum

Neutralization of parental and 332 mutant viruses by commercially-available mAbs and polyclonal anti-EIII sera was assessed by determination of neutralization indices, as described previously.(Beasley and Barrett, 2002; Li et al., 2005) The two MAbs used - anti-WNV 5H10 and 7H2 (Bioreliance Corporation, Rockville, MD) - were previously shown to recognize distinct but overlapping EIII epitopes that include E-332.(Beasley and Barrett, 2002) The rabbit anti-WNV antiserum has been described elsewhere.(Beasley et al., 2004) Briefly, serial 10-fold dilutions of all 20 variants were combined with a constant concentration of monoclonal antibody (2.5ng/μL) or polyclonal antiserum (final concentration of 1:50) diluted in MEM/2% BGS and incubated at room temperature (approximately 22°C) for one hour. A "medium only" control was also included for each virus. The virus:antibody mixtures were then used to infect monolayers of Vero cells in 12-well plates for one hour at room temperature. Cells were overlaid with 2ml/well of 2XMEM/agar and placed in a 37°C incubator with 5% CO_2. After two days, 1ml of a second overlay containing 2% neutral red was added. Plaques were visualized with a light box on days 3 and 4 post-infection. NI values were calculated as the $\log_{10}$ reduction in apparent titer in the

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presence of each antibody/antiserum compared to the no antibody control. Assays were performed in duplicate.

Western blotting

Individual T-25 flasks of Vero cells were infected with viable 332 variants. When early CPE was visible (typically 2 days post-infection), the media was removed and the flask was stored at −70°C overnight. The monolayer was then thawed and lysed in 1ml of 10% SDS for 10 minutes at room temperature. The lysate was then incubated for 30 minutes at 56°C prior to transfer to BSL2. Equal volumes of the resulting cell lysates were then subjected to electrophoresis on 12% SDS polyacrylamide gels under non-reducing conditions. Gels were stained with Coomassie blue for assessment of total protein or transferred to a nitrocellulose membrane for Western blotting. Nitrocellulose membranes were blocked with 3% BSA in TBS, washed, then incubated with primary antibody for one hour at room temperature. Primary antibodies included anti-whole WNV mouse immune ascitic fluid (MIAF) at 1:500, rabbit anti-WNV EIII serum at 1:500, 5H10 at 0.5ng/μL, and 7H2 at 0.25ng/μL. After the primary antibody, the membrane was washed and incubated with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (anti-mouse IgG or anti-rabbit Ig; Sigma, St. Louis, MO) for one hour at room temperature. After additional washing, the membrane was developed with the ECL Western blotting detection reagents (GE Healthcare, Buckinghamshire, UK) and exposed to ECL Hyperfilm (GE Healthcare, Buckinghamshire, UK) for visualization.

Statistical Analysis

Differences in apparent titer at 37°C vs 41°C were compared for NY99ic wild-type (wt) and each T332 mutant using unpaired, two-tailed Student's T-tests. Variation of $\log_{10}$ pfu/ml titer values for each measured time point in the replication curves, survival times, and neutralization indices were assessed by one-way ANOVA. Individual strains that varied from the NY99ic wt control were identified by post-hoc Dunnett's multiple comparisons test. Survival rates were compared using the Kaplan Meier log-rank test. LD_{50} values were calculated using the Spearman Karber method. To assign neutralization indices to a particular group, the appropriate number of clusters was determined using k-means analysis of a pooled dataset of all virus:antibody NI values and NI values were then assigned to a particular cluster using the average method of hierarchical clustering. The significance threshold for all tests was 0.05. Statistical analysis was performed using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla, CA) and R 3.0.2 (R Foundation for Statistical Computing, Vienna, Austria).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- Residue 332 in domain III of West Nile virus NY99 strain envelope protein is highly mutable.
- Mutations at residue 332 had significant effects on antigenicity of WNV domain III but relatively minor effects on growth in cell cultures or mouse virulence.
- Use of monoclonal antibody therapies or subunit vaccines targeted against domain III could potentially select for variants that retain virulence equivalent to the wild-type virus.

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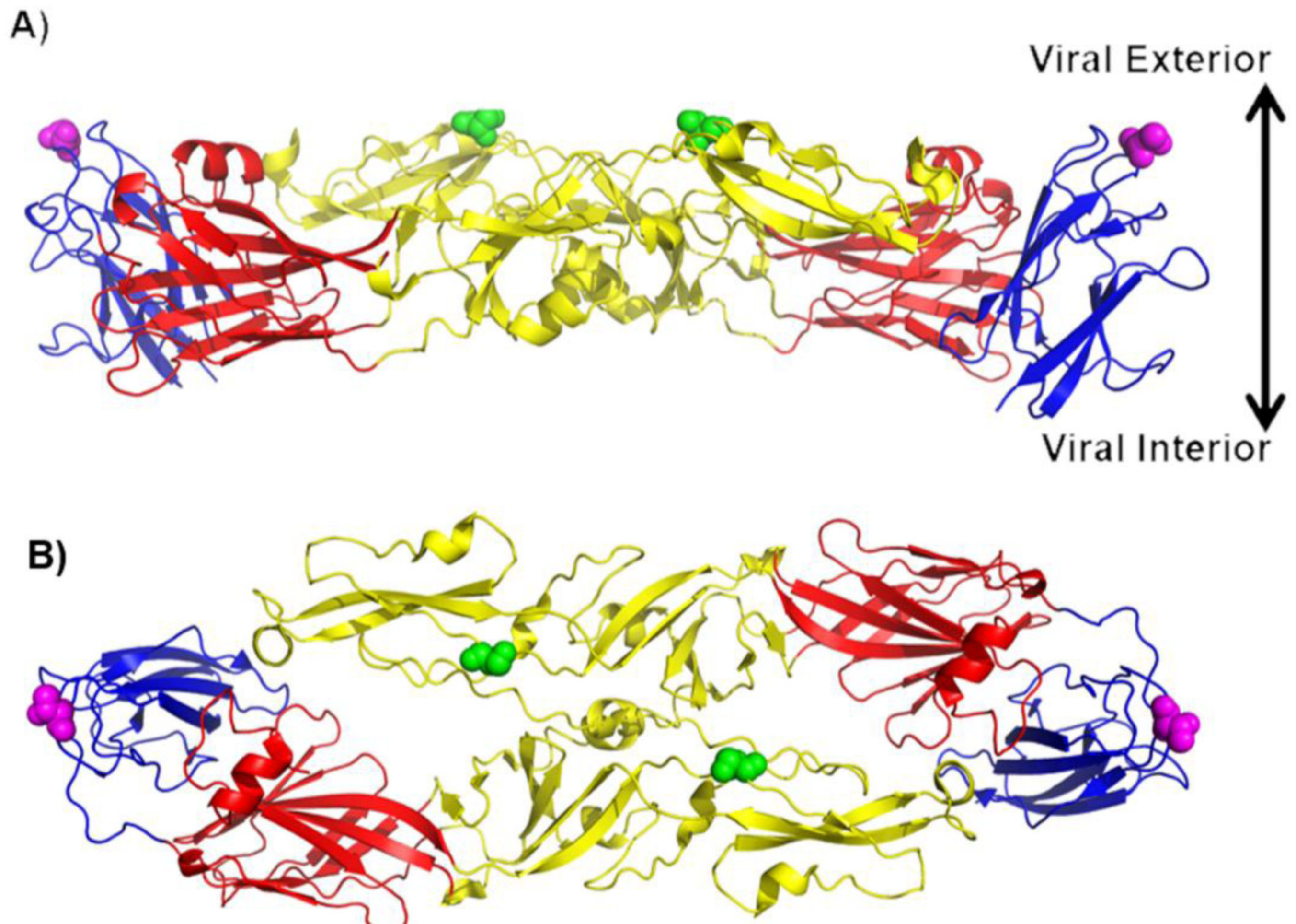

Figure 1.

Location of E-66 and E-332 in the WNV E monomer. EI = red, EII = yellow, and EIII = blue. E-66 is highlighted in green and E-332 is highlighted in magenta. E monomer is shown in both a side (A) and overhead (B) view. Image generated using the 2HG0 crystal structure of the WNV envelope protein aligned to the 3J0B cryo-EM structure of the WNV virion in the PyMol Graphics System, Version 1.7.0.5, Schrödinger, LLC.

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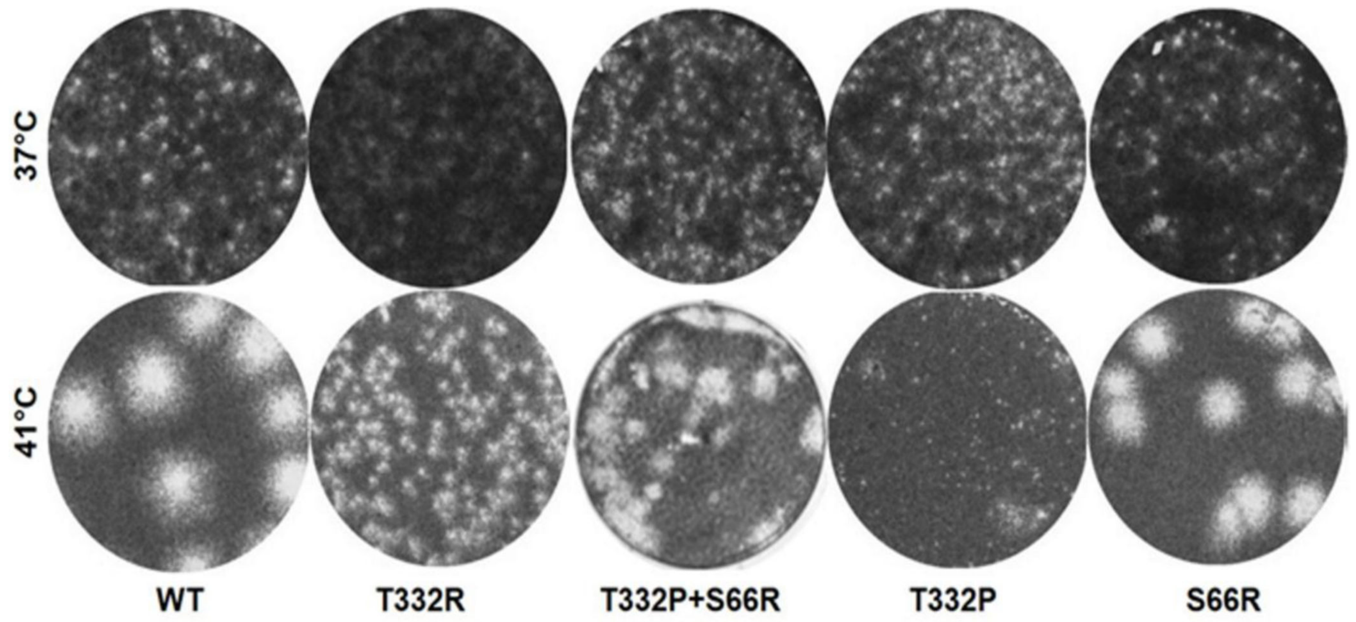

Figure 2.
Plaque morphology of NY99ic WT, T332R, and T332P+S66R at 37°C and 41°C.

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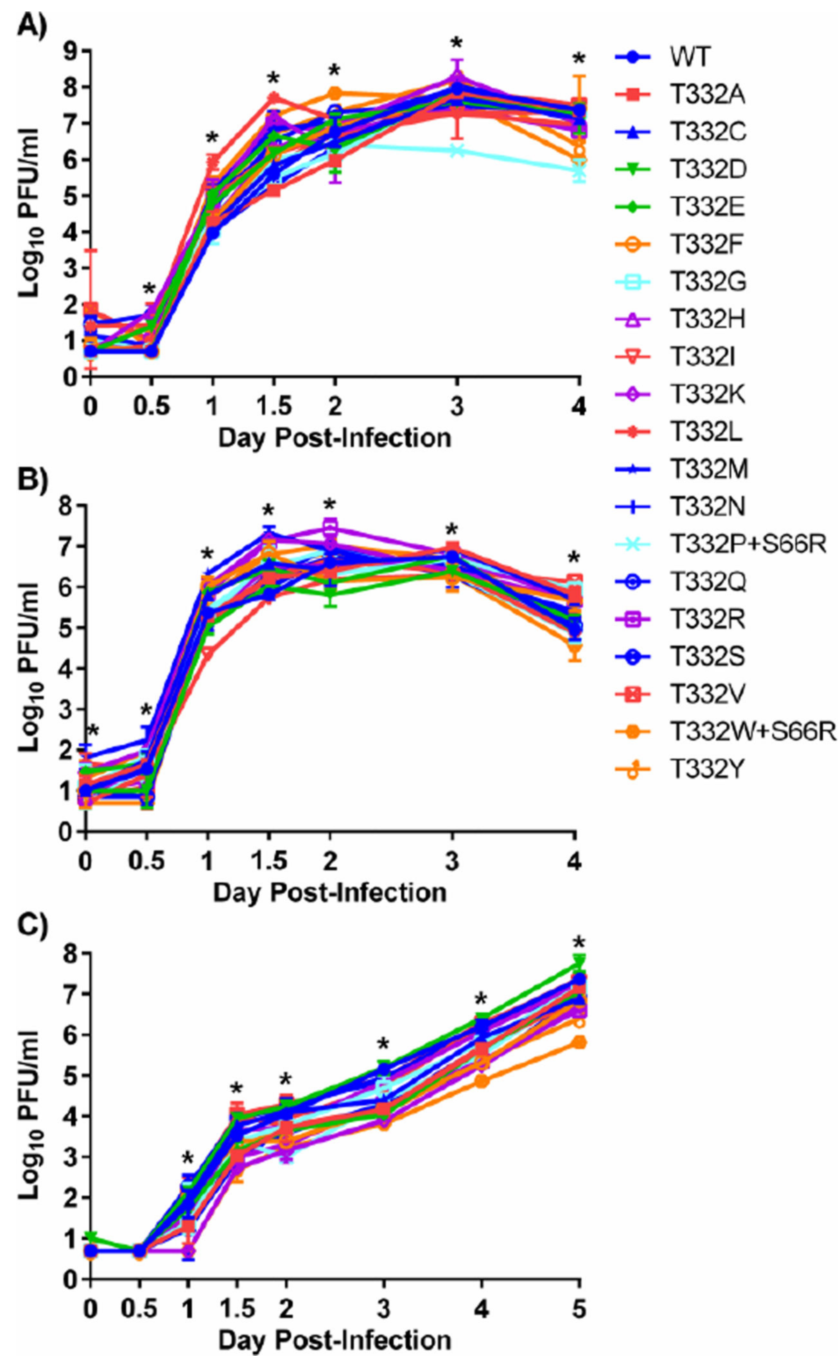

Figure 3.

Replication kinetics of WNV NY99ic WT and T332 mutants in (A) Vero, (B) DEF, and (C) C6/36 cells with an initial multiplicity of infection of 0.01. * = Statistically significant variation between all strains at the indicated timepoint as determined by one-way ANOVA ($p<0.05$)

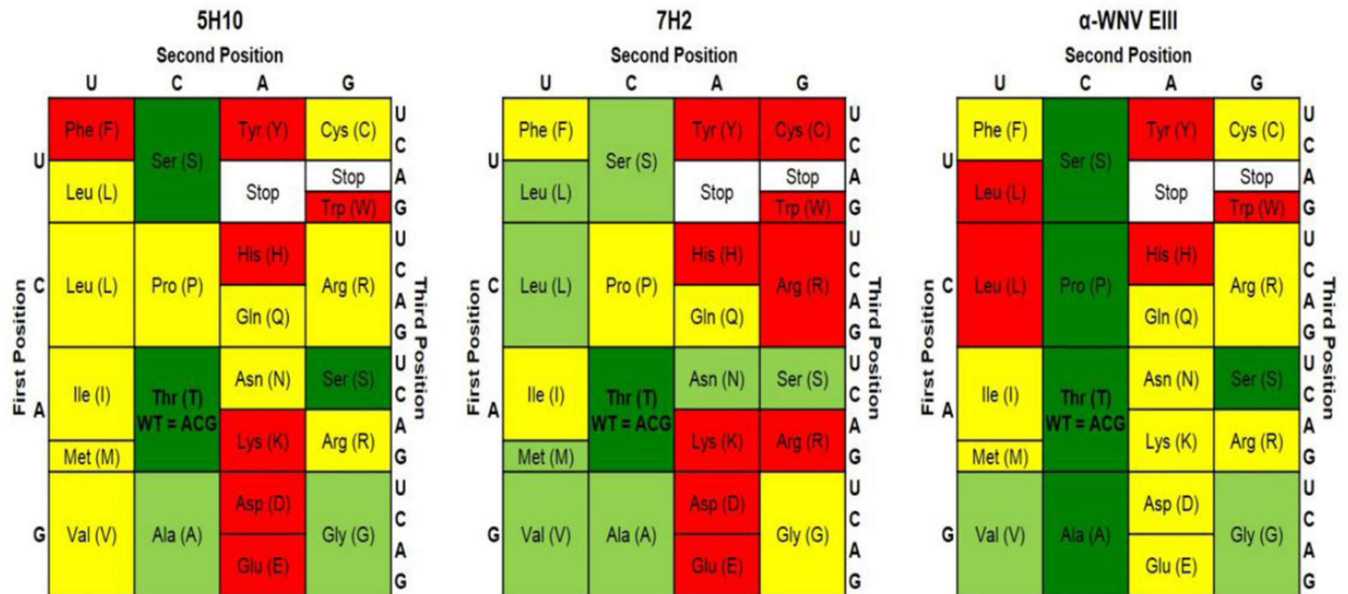

Figure 4.

Heat maps of neutralization phenotypes of NY99ic T332 mutants, arranged by amino acid codon Neutralization indices of NY99ic T332 mutants by 5H10, 7H2, and polyclonal rabbit serum against EIII. Dark green = very strongly neutralized (NI 2.2), light green = strongly neutralized (NI 1.2–1.9), yellow = moderately neutralized (NI 0.5–1.1), red = weakly neutralized (NI 0.4).

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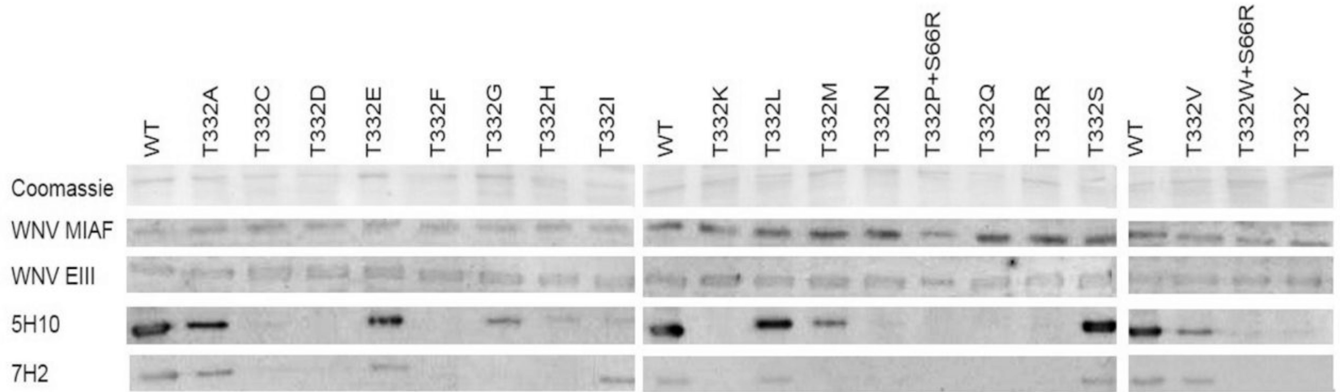

Figure 5.

Binding of monoclonal and polyclonal antibodies to NY99ic WT and T332 mutants

Binding of WNV MIAF, WNV EIII, 5H10, and 7H2 to NY99ic WT and T332 mutants infected cell lysates in Western blots. Bands shown are from the ~50kD region of the gel, corresponding to the size of intact E protein. Coomassie staining and blotting with WNV MIAF are included to confirm the loading of approximately equivalent amounts of protein.

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Table 1

Temperature sensitivity, mouse virulence, and antigenic properties of NY99ic WT and 332 mutant

Strain	Temperature Sensitivity†	Mouse Virulence			Neutralization Indices (Mean±SD)		
		% Survival	MST††±SD	LD_{50}†††	5H10	7H2	α-WNV EIII
NY99ic WT	− 0.29	5	8.7±2.0	0.4	2.5±0.0	3.3±0.3	2.6±0.2
NY99ic T332A	0.06	10	8.3±1.3	ND	1.4±0.1*	1.5±0.0*	2.4±0.0
NY99ic T332C	0.03	47	8.7±1.9	0.9	0.9±0.0*	0.1±0.1*	0.9±0.0*
NY99ic T332D	0.11	0	8.2±1.2	0.8	0.1±0.1*	0.1±0.1*	0.7±0.0*
NY99ic T332E	0.14	0	7.6±1.1	ND	0.0±0.0*	0.1±0.0*	0.8±0.1*
NY99ic T332F	0.23	0	8.0±1.5	ND	0.3±0.1*	0.7±0.0*	0.6±0.2*
NY99ic T332G	0.06	0	8.6±1.6	ND	1.3±0.1*	1.0±0.1*	1.8±0.1*
NY99ic T332H	0.09	10	8.7±2.2	0.4	0.2±0.0*	0.1±0.0*	0.1±0.0*
NY99ic T332I	0.21*	20	10.1±2.3	1.3	0.8±0.1*	1.0±0.1*	0.8±0.3*
NY99ic T332K	0.01	0	8.5±1.0	ND	0.2±0.4*	0.1±0.2*	0.5±0.2*
NY99ic T332L	0.17	13	8.6±0.8	0.7	0.5±0.1*	1.5±0.0*	0.4±0.1*
NY99ic T332M	0.09	20	8.8±2.1	ND	1.0±0.0*	1.7±0.0*	1.0±0.1*
NY99ic T332N	0.00	0	8.3±1.4	ND	0.9±0.3*	1.2±0.1*	0.9±0.0*
NY99ic T332P	−0.12	47	8.8±2.6	452.4	ND	ND	ND
NY99ic T332P+S66R	−1.53*	87*	13.0±7.1*	>1,000	0.7±0.0*	1.1±0.1*	3.1±0.0*
NY99ic T332Q	−0.24	0	9.3±1.5	ND	0.6±0.1*	0.6±0.0*	0.6±0.0*
NY99ic T332R	0.00	20	9.4±2.3	7.9	0.7±0.0*	0.1±0.2*	0.8±0.2*
NY99ic T332S	0.09	7	7.6±1.0	0.2	2.2±0.0*	1.9±0.1*	3.0±0.1
NY99ic T332V	−0.25	0	8.2±0.9	ND	1.0±0.1*	1.8±0.1*	1.6±0.1*
NY99ic T332W	−0.16	30	9.4±1.9	0.3	ND	ND	ND
NY99ic T332W+S66R	0.20	20	8.8±2.1	5.0	−0.1±0.1*	0.3±0.0*	0.3±0.2*
NY99ic T332Y	0.26*	0	9.4±1.3	0.1	0.0±0.1*	0.3±0.1*	0.3±0.0*

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Strain	Temperature Sensitivity†	Mouse Virulence			Neutralization Indices (Mean±SD)		
		% Survival	MST††±SD	LD_{50}†††	5H10	7H2	α-WNV EIII
NY99ic S66R	–0.07	0	9.7±1.9	0.1	ND	ND	ND

†Differences in apparent Log_{10} PFU/ml titer at 41°C compared to 37°C.

††mean survival time (days).

†††50% lethal dose (PFU).

*statistically significant difference ($p<0.05$) as determined by unpaired, two-tailed Student's T-tests of 37°C vs. 41°C titers for each virus (temperature sensitivity), the Kaplan Meier log-rank test comparing each mutant to NY99ic WT (% Survival), or Dunnett's multiple comparison post-hoc test comparing each mutant to NY99ic WT (AST±SD, 5H10 NI, 7H2 NI, and α-WNV EIII NI). ND = not determined.