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The West Nile virus mutant spectrum is host-dependant and a determinant of mortality in mice

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Abstract

To define the impact of mosquitoes and birds on intrahost WNV population dynamics, the mutant spectra that arose as a result of 20 serial *in vivo* passages in *Culex pipiens* and young chickens were examined. Genetically homogeneous WNV was serially passaged 20 times in each host. Genetic diversity was greater in mosquito-passaged WNV compared to chicken-passaged WNV. Changes in the viral consensus sequence occurred in WNV passaged in mosquitoes earlier and more frequently than in chicken-passaged WNV. Analysis of synonymous and nonsynonymous variation suggested that purifying selection was relaxed during passage in mosquitoes. Mortality in mice was significantly negatively correlated with the size of the WNV mutant spectrum. These studies suggest that mosquitoes serve as sources for WNV genetic diversity, that birds are selective sieves, and that both the consensus sequence and the mutant spectrum contribute to WNV phenotype.

Keywords: West Nile virus; Flavivirus; Arbovirus; Evolution; Quasispecies; *Culex*

Introduction

RNA viruses, like other organisms, adapt to available ecological niches or they become extinct. Although RNA viruses posses an inherent potential for high genetic diversity and rapid evolution, arthropod-borne viruses (arboviruses) appear to have slower rates of evolution than other viruses, probably due to their reliance on taxonomically diverse hosts for perpetuation in nature (Weaver et al., 1992). Nonetheless, arboviruses have clearly adapted well to available ecological niches. For example: eastern equine encephalitis virus (Togaviridae:Alphavirus) has diversified into four lineages in the Americas, each perpetuating in a unique transmission cycle (Weaver et al., 1994). Similarly, Powassan virus (Flaviviridae: Flavivirus) has adapted to divergent transmission cycles to produce two known lineages (Ebel et al., 2001). Since its introduction into the New York City area in 1999, West Nile virus (WNV, Flaviviridae:Flavivirus) has undergone a process of adaptation as well, and currently circulating strains are more efficiently transmitted by mosquitoes than the introduced strain (Ebel et al., 2004). The genetic mechanisms that underpin this process of adaptation, however, are not well understood.

RNA viruses exist within hosts as genetically heterogeneous mixtures of variants (i.e. a quasispecies; Eigen, 1971) that differ in varying degrees from a consensus nucleotide sequence. Many factors contribute to the quasispecies structure of RNA viruses. Most important is the lack of error-checking and mismatch-repair mechanisms in virally encoded RNA-dependant RNA polymerases (Holland et al., 1982), which have misincorporation rates of 10^{-3} to 10^{-5} per nucleotide copied (Domingo et al., 1978; Weissmann and Ochoa, 1967). The diversity of viral quasispecies is host and virus dependant (Schneider and Roossinck, 2001), a

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determinant of viral fitness (Martinez et al., 1991), and provides a virus with a molecular "memory" that exists as minority genotypes within the viral mutant spectrum (Ruiz-Jarabo et al., 2002). Recently it was shown that Dengue viruses display quasispecies dynamics in nature with defective genomes maintained within the mutant spectrum by functional complementation (Aaskov et al., 2006). Previously, we demonstrated that WNV is transmitted between hosts in nature as a genetically diverse pool of genomes (Jerzak et al., 2005), supporting the theoretical prediction that quasispecies populations may evolve as a group (Eigen and Schuster, 1977). Thus, a growing body of evidence suggests that RNA viruses, including arboviruses, are structured as quasispecies.

The role of taxonomically divergent hosts in shaping arbovirus quasispecies and the phenotypic consequences of genetic diversity, however, have not been directly examined *in vivo*. Analysis of WNV quasispecies from naturally infected mosquitoes and birds suggested that infections within mosquitoes were more genetically diverse than those in birds (Jerzak et al., 2005). We therefore sought to experimentally determine whether infection of mosquitoes results in greater intra-host genetic diversity than does infection of birds by serially passing WNV derived from an infectious cDNA clone 20 times in either mosquitoes or birds and examining the size and composition of the mutant spectra after 1, 5, 10, 15 and 20 passages. In addition, we determined whether the diversity of the WNV quasispecies influenced the pathogenic potential of serially passed WNV using a C3H mouse model.

Results

Virus passage

The ID50 of WNV was determined to be 0.50 PFU in *Culex pipiens* and 0.66 in young chickens. During sequential passage, infectious titer in the mosquito homogenates at the time of harvest remained fairly constant at approximately 10^6 PFU/0.1 mL clarified homogenate throughout the 20 passages (Fig. 1, Panel A). The infectious titer in chick serum was more variable, ranging from 10^3 to 10^6 PFU/0.1 mL of serum (Fig. 1, Panel B).

Analysis of intrahost genetic diversity

Genetic diversity was determined as both (a) the proportion of nucleotides that differed from the specimen-specific consensus and (b) the proportion of clones that differed from consensus (Fig. 2). Intra-host genetic diversity at passage one was similar to the background error rate, which was determined as described (Jerzak et al., 2005) using a clone-derived WNV population that was expected to be highly genetically homogeneous. WNV passaged in chickens remained genetically homogenous, reaching maximum diversity at passage 10, when a mean of 0.01% of nucleotides and 20% of clones differed from consensus. Thereafter, genetic diversity decreased to approximately the background error rate. In contrast, mosquito passed WNV became more genetically diverse with increasing passage. At passage 20, a mean of 0.03% of nucleotides and 50% of clones differed from consensus. Differences in mean and sequence diversity (i.e. the proportion of mutant nucleotides and clones) was significantly greater (Fisher's exact $P<0.005$) in WNV passed in mosquitoes at passages 15 and 20. WNV passed sequentially in mosquitoes was therefore more genetically diverse than WNV passed in chickens.

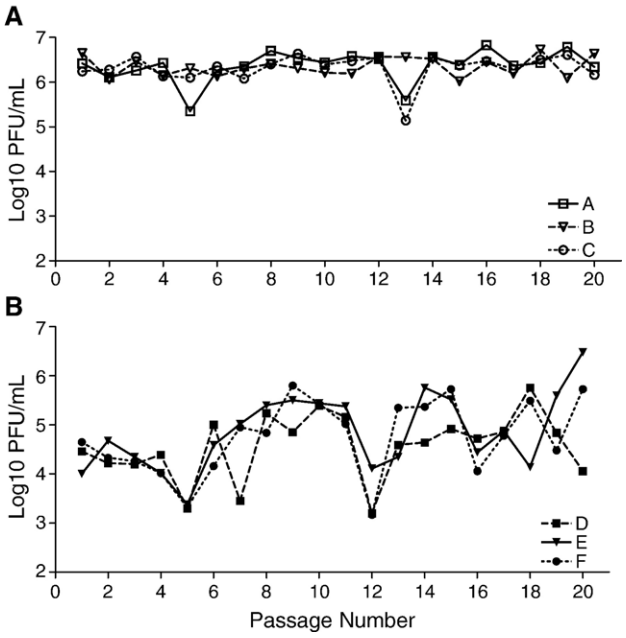

Fig. 1. Mean titer of WNV during sequential passage in mosquitoes (A) and chickens (B). Each of three independent lineages (designated A–F) per host type is shown.

Analysis of complete genome sequences

To determine whether changes occurred to the consensus sequence as a result of serial passage, the complete genome sequence of all six replicates at passage 20 was determined (Table 1). No signature mutations were detected that identified a strain as either mosquito- or chicken-passed. Eleven synonymous and seven nonsynonymous mutations were detected, along with two mutations that occurred in the 3′ noncoding region. Nonsynonymous mutations occurred in both structural and nonstructural coding sequences. The existence of a quasispecies, which was directly examined above, is suggested by the presence of multiple peaks at several positions (for example, a "Y" indicating the presence of both C and T fluorophores at nucleotide position 6662 of mosquito lineage C). These results demonstrate that during passage, changes to the consensus sequence of a WNV population accompany changes to the composition of its quasispecies. Analysis of synonymous and nonsynonymous variation was undertaken for the complete consensus genomes obtained for both mosquito- and chicken passed lineages. d_N/d_S for mosquito-passed WNV was 1.566, and for chick-passed WNV, it was 0.045.

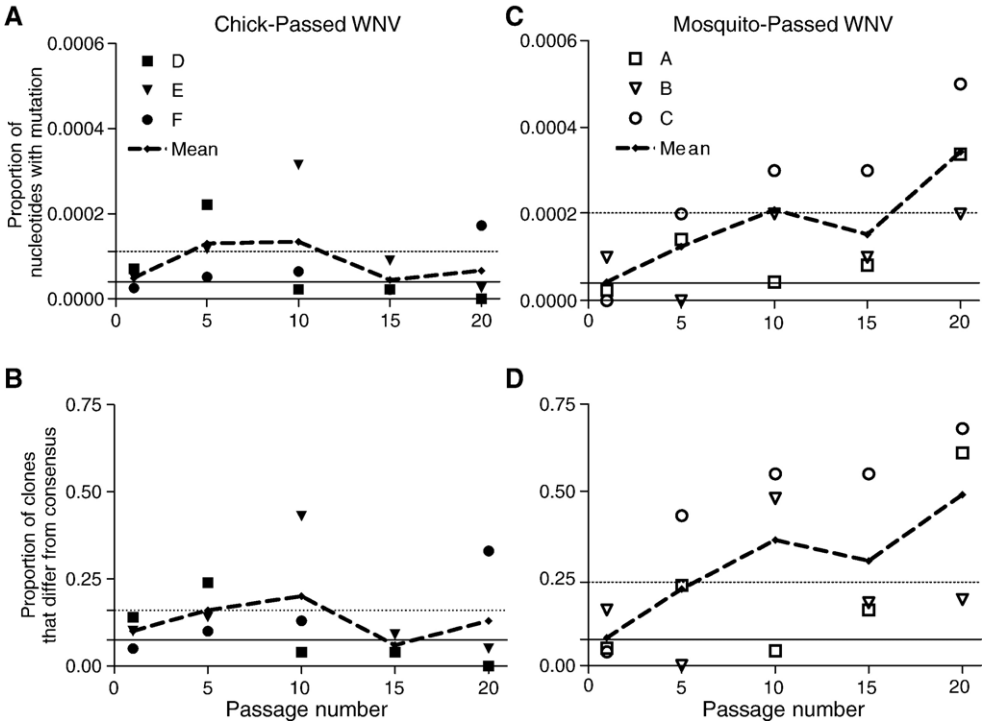

Fig. 2. Host-dependence of the WNV mutant spectrum. WNV derived from an infectious cDNA clone was passaged 20 times in either chickens or mosquitoes, and the mutant spectrum was characterized at passages 1, 5, 10, 15 and 20. Panels A and C: proportion of nucleotides sequenced that differed from the specimen-specific consensus sequence in chicken (A) and mosquito (C) passaged WNV. Symbols indicate values for each of three replicate lineages, designated D, E and F for chickens and A, B and C for mosquitoes. Solid line indicates background error rate as determined by analysis of clone-derived WNV, dotted line indicates homologous field-derived observation (Jerzak et al., 2005). Panels B and D: proportion of clones that differed from the specimen-specific consensus sequence and chicken (B) and mosquito (D) passaged WNV. Open symbols, solid and dotted lines are as above.

Mortality in mice

Mice were peripherally inoculated with genetically characterized WNV strains following passage in chicks and mosquitoes to determine whether the diversity of a virus population is a determinant of mouse mortality. Chicken-passed strains were similar to the parental unpassed WNV in their ability to cause death in mice (Fig. 3A). Mosquito-passed WNV strains were more variable and less pathogenic to mice than the parental strain (Fig. 3B). The survival of mice inoculated with mosquito-passed lineage C was significantly different from those inoculated with the parental virus (Logrank test, $P=0.0038$). The proportion of mice that died as a result of WNV infection was significantly negatively correlated (Fig. 3C) with the proportion of nucleotides in the population that differed from consensus (i.e. the genetic diversity of the infecting population). Parametric (Pearson correlation; $R^2=0.7997$, $P=0.0162$) and nonparametric (Speaman's rank order correlation $P=0.0333$) statistical analyses yielded similar results.

Discussion

Although the role of taxonomically diverse hosts such as mosquitoes and birds in slowing and/or shaping arbovirus evolution has been extensively postulated and studied using *in vitro* systems (Weaver et al., 1999; Novella et al., 1999; Greene et al., 2005; Weaver, 2006), the precise impact of intact hosts has been difficult to determine. In a previous report we showed that, in nature, WNV populations infecting mosquitoes seemed to be more genetically diverse than those infecting birds (Jerzak et al., 2005). This finding, because it suggested a differential evolutionary impact of mosquitoes and birds on WNV, might significantly impact our understanding of how host–virus interactions shape arbovirus population dynamics, including how they persist in complex transmission cycles and emerge as threats to human health. Observational studies of this nature, however, are somewhat difficult to interpret due to the uncertainty introduced through the use of field-collected materials. This is a particular problem with mosquito collections, where it is impossible to rule out the possibility that more than one infected mosquito was present in the infected pool. If this was the case, estimates of genetic diversity could be biased toward the result we observed (i.e. higher genetic diversity). Therefore, we sought to experimentally verify that mosquitoes serve as sources of genetic variation in the WNV transmission cycle. To accomplish this, we used a controlled experimental strategy that relied on serial passage and SC and IT inoculation of chickens and mosquitoes, respectively. Serial passage was used in these studies to amplify the impact of each host on WNV because it was unclear based on our field-derived data whether significantly greater intrahost genetic diversity would be apparent in one or a few passages, or whether it could be detected at all. Artificial modes of infection (SC and IT

Table 1
Consensus changes in WNV genomes following 20 passages

Nt position	Input	Mosquito passage lineage			Chick passage lineage			AA change	AA position[a]
		A	B	C	D	E	F		
297	T					C		–	C
340	C						T	–	C
580	A	G						M to V	prM-39
1697	A			G				E to G	E-244
1889	C			Y				–	E
2200	C					T		L to F	E-412
2291	T		C					V to A	E-442
2573	A	G						Y to C	NS1-35
3033	C						T	–	NS1
3931	C				Y			–	NS2A
4870	C		A					H to N	NS3-87
5229	A					C		–	NS3
5706	T					C		–	NS3
6662	T			Y				M to T	NS4A-65
6696	C						T	–	NS4A
7965	A					G		–	NS5
9123	T	C		C				–	NS5
10266	C		T					–	NS5
10419	A	G						–	3′UTR
10829	T	C						–	3′UTR

[a] Amino acid positions are numbered from the beginning of each protein. For example: prM-39 indicates the 39th amino acid of the premembrane protein.

inoculation) were used in order to isolate the effect of replication in either mosquitoes or chickens on the genetic diversity of WNV by avoiding various barriers to infection and potential population bottlenecks imposed by more realistic transmission systems. Moreover, although sequential infection of a single host type probably occurs in nature only rarely, it was used in this study to amplify the impact of each host on WNV population diversity to readily detectable levels, allowing us to focus on whether infection of mosquitoes might result in higher levels of population diversity than infection of birds.

Because taxonomically diverse species may differ in their inherent susceptibility to WNV, preliminary studies determined the ID50 of WNV in young (1–3 days old) chickens or colonized *Cx. pipiens* mosquitoes. The ID50 values in mosquitoes (0.50 PFU) and chickens (0.66) were remarkably similar, implying that viral infectivity is equivalent in both hosts, and both hosts are highly susceptible to WNV infection. WNV was then passed 20 times at a dose 100 times the ID50 in either mosquitoes or birds, with three replicate lineages per host type. The WNV titers at harvest during mosquito and chicken passage indicate that the virus remained replication-competent during the passage procedure. While titer at harvest remained fairly constant throughout passage in mosquitoes, it was more variable in chickens. Notably, at passage 12, the titer in all three lineages fell 10- to 100-fold. The reasons for this particular sharp decline in titer are unclear, but may be related to the general level of variation in the titer of chick-passed WNV at the time of harvest. These basic data on titer at harvest allow us to conclude that WNV retained its ability to infect and replicate within experimental hosts during the course of this study.

Analysis of intrahost genetic diversity in populations sampled at passages 1, 5, 10, 15 and 20 revealed that while virus passed in chickens remains relatively genetically

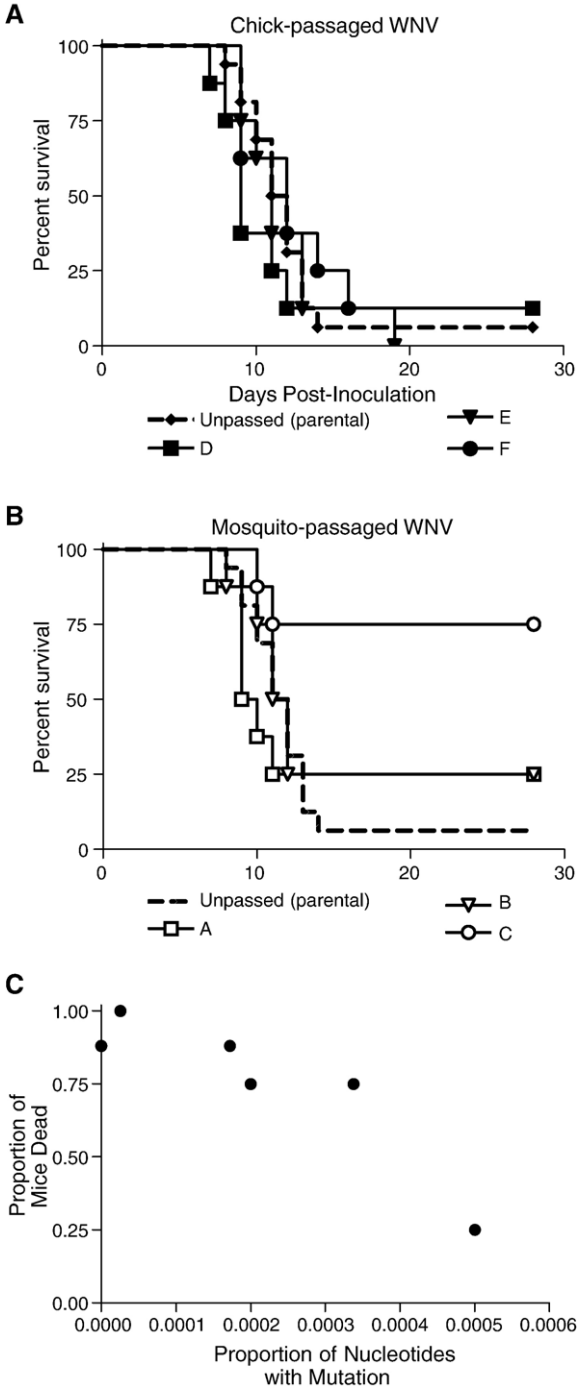

Fig. 3. Survival of mice infected with (A) chicken- and (B) mosquito-passed WNV. Groups of eight 6-week-old female C3H mice were inoculated subcutaneously in the left rear footpad with $10^{2.5}$ PFU of WNV and survival was monitored. Mock-inoculated mice (not shown) remained healthy for the duration of the study. Results were obtained from two independent trials, and the survival curve for mice inoculated with the parental (unpassed) WNV is the combined data from both trials. Lineages are designated D, E and F for chick-passaged and A, B, and C for mosquito-passaged lineages. (C) Percentage mortality at the study conclusion was significantly negatively correlated with overall genetic diversity in the inoculum.

homogeneous, virus passed in *Cx. pipiens* appears to be more genetically diverse. These results are consistent with our field-derived observations that showed virus from mosquitoes to be approximately twice as diverse genetically as virus from birds. Several factors may account for this phenomenon. First, the temperature within mosquitoes is lower than the approximately 39 °C maintained by birds. Our mosquitoes were maintained at a constant temperature of 27 °C. Second, the duration of infection in our studies differed between chickens and mosquitoes. WNV infection in chicks reaches peak viremia titer at 48 h post-inoculation (data not shown). In mosquitoes, WNV inoculated intrathoracically and held at 27 °C tends to reach a plateau at around 7 days postinoculation. We therefore selected 2 and 7 days as harvest points for chicken and mosquito passed WNV, respectively. Other factors acting at the host cell level, such as cell type, intracellular nucleotide availability and host antiviral response, may also contribute to the observed differences in WNV in mosquitoes and birds. Nonetheless, in combination with our field-derived observations, these results establish that WNV reaches greater levels of genetic diversity in mosquitoes compared to birds, and support the hypothesis that in nature, mosquitoes serve as sources of genetic diversity, while birds limit this diversity. In addition, examination of complete genome sequences of WNV after sequential passage in mosquitoes and birds indicated that most nonsynonymous variation occurs in WNV passed in mosquitoes. Indeed, d_N/d_S analysis revealed no evidence of purifying selection in mosquito-passed WNV ($d_N/d_S \sim = 1$), but relatively strong evidence of purifying selection ($d_N/d_S \ll 1$) in chicken-passed WNV. These observations strongly suggest that in mosquito hosts, purifying selection is relaxed, while birds constrain the genetic diversity that accumulates during mosquito infection.

The existence of genetically diverse mutant spectra has been demonstrated for several RNA viruses (Jerzak et al., 2005; Plyusnin et al., 1996; Aaskov et al., 2006; Goodenow et al., 1989; Domingo et al., 1992; Farci and Purcell, 2000; Bonneau et al., 2001). The phenotypic consequences of genetic diversity, however, have been difficult to detect in viruses such as WNV that are typically acute infections in their vertebrate hosts. We therefore used WNV populations of known genetic diversity that were created via *in vivo* passage to evaluate the impact of WNV quasispecies on mortality in mice.

In contrast to human hepatitis C virus infections (Farci et al., 2000) and mumps and poliovirus infections in animals (Sauder et al., 2006; Vignuzzi et al., 2006), more genetically diverse WNV populations caused less mortality in mice. This was somewhat surprising because one might predict that a more diverse population would harbor variants that may replicate more efficiently in mice, leading to increased virulence. Several factors may account for this finding. First, comparison of results across virus genera and model systems may be difficult due to inherent differences in the basic biology of the host–virus interaction. Second, the clone-derived WNV that served as our starting population for passage is highly pathogenic to mice, causing nearly 100% mortality (Fig. 3). The parental virus may therefore represent a peak in pathogenic potential, from which additional gains are significantly more difficult to obtain than losses. Therefore, most mutants within the population would be less pathogenic than the cloned parent. Although we cannot phenotypically characterize all of the components of the mutant spectrum of any of our WNV populations, it seems reasonable that defective and/or poorly replicating viruses may retain some ability to stimulate a host response that limits the range of clinical signs and the associated mortality. Finally, higher genetic diversity was also associated with passage in mosquitoes, raising the possibility that adaptation to mosquitoes during passage resulted in a concomitant loss of pathogenic potential in mice. Very little is known about flavivirus determinants specific to the arthropod–virus interaction. It is therefore not possible to determine from this data whether adaptation to mosquitoes occurred during passage. Future studies should address this issue directly. Importantly, our results do not contradict the several studies that document the impact of the WNV consensus sequence on viral pathogenesis (Beasley et al., 2002; Zhang et al., 2006; Wicker et al., 2006). Rather, in agreement with previous reports on other viruses (Vignuzzi et al., 2006; Sauder et al., 2006; Essajee et al., 2000; Farci et al., 2002), they suggest that the genetic diversity of a virus population is an independent determinant of its phenotype.

Materials and methods

Experimental hosts

Specific pathogen free (SPF) chickens (*Gallus gallus*) were obtained either from Charles River Specific Pathogen Free Avian Services (Franklin, CT) or from Sunrise Farms (Catskill, NY), and maintained in brooders with feed and water constantly available. *Cx. pipiens* mosquitoes were from a colony derived from larvae collected in Pennsylvania and maintained at the Wadsworth Center Arbovirus Laboratories since 2002. Rearing procedures and conditions for experimental mosquitoes are described elsewhere (Ebel et al., 2004).

Virus

WNV was generated from an infectious cDNA clone based on strain 3356, collected from an American Crow (*Corvus brachyrhynchos*) that died on Staten Island in 2000 (AF404756). Methods for clone manipulation and production of infectious WNV are as described previously (Shi et al., 2002). A viral stock was harvested from cells after electroporation without further passage in cell culture, and the titer was determined by plaque assay on African green monkey kidney cells (Vero, ATCC CCL-81). This single viral stock was used for initial passaging.

ID50 determination

The WNV dose that produced infection in 50% of hosts (ID50) was determined for both chickens and *Cx. pipiens* mosquitoes in order to normalize the virus dose against the inherent susceptibility of each host to WNV. Groups of 1–3 day

old chickens were inoculated subcutaneously (SC) in the cervical region, and groups of mosquitoes were inoculated intrathoracically (IT) with serial 10-fold dilutions of WNV stock. At 14 days post-inoculation chicks were bled and infection status was determined by presence of WNV-specific antibodies using an ELISA as described (Ebel et al., 2002). Mosquitoes were harvested 14 days post-inoculation and screened for infectious WNV by plaque assay on Vero cells as follows. Individual mosquitoes were placed into 2 mL safe-lock microcentrifuge tubes containing 1 mL of mosquito diluent (20% heat-inactivated fetal bovine serum [FBS] in Dulbecco's phosphate-buffered saline plus 50 ug/mL penicillin/streptomycin, 50 ug/mL gentamicin, and 2.5 ug/mL fungizone) and one zinc-plated 4.5 mm ball bearing (Daisy Brand, Rogers AR) and homogenized using a Mixer Mill MM300 (Qiagen, Valencia, Calif.) for 30 s at 24 cycles per second. Homogenates were centrifuged at room temperature for 5 min at 13,200 rpm and the clarified supernatants were used to determine infection status. Briefly, confluent Vero cell monolayers in 6 well culture plates were inoculated with 0.1 mL of homogenate. Plates were incubated for 1 h at 37 °C, a primary overlay with 0.6% Oxoid agar in Eagles minimal essential medium containing 10% FBS was applied, and plates were incubated at 37 °C, 5% CO_2. After 2 days, a second overlay containing 0.33% Neutral Red was applied to each well, and plates were read after an additional 24 h. ID50 values were calculated using the method of Reed and Munch.

In vivo virus passage

Twenty passages in chickens and mosquitoes were conducted in three concurrent replicate lineages. One- to three-day-old SPF chickens were initially inoculated SC with 100 times the ID50 (66 PFU) of cDNA clone-derived WNV stock in 0.1 mL animal inoculation diluent (endotoxin-free phosphate-buffered saline supplemented with 1% FBS); two chickens were inoculated for each of three concurrent lineages. Blood was withdrawn by cardiac puncture 48 h post inoculation, and serum was separated, aliquoted, and stored at −80 °C. One aliquot of serum was used to determine the infectious WNV titer by plaque assay on Vero cells as described above, and a second aliquot was diluted to achieve an inoculum of 100 times the ID50 for subsequent passage. *Cx. pipiens* were inoculated IT with 100 ID50 (50 PFU) of cDNA clone-derived WNV stock in 0.1 μl of mosquito diluent. Mosquitoes were held for 7 days at 27 °C and maintained on a 10% sucrose solution. Individual mosquitoes from each lineage were harvested and homogenized as described above. Aliquots of clarified homogenate were stored at −80 °C. One aliquot was used for virus titration, and a second aliquot was diluted and used to inoculate the subsequent passage.

High-fidelity RT-PCR, cloning and sequencing

RNA was extracted from frozen serum and clarified mosquito homogenates using QIAamp Viral RNA spin columns (Qiagen). Reverse transcription (RT) reactions and polymerase chain reactions (PCR) were performed with primers designed to amplify a 1936-bp region encoding the 3′ 1158 nucleotides of the WNV envelope (E) coding region and the 5′ 778 nucleotides of the WNV NS1 coding region [forward primer: WNV1311 (5′-ATGCGCCAAATTTGC-CTGCTCTAC-3′); reverse primer WNV3248 (5′-ATGGGCC-CTGGTTTTGTGTCTTGT-3′)]. To minimize misincorporations introduced during RT-PCR by *Taq* polymerase, we amplified WNV RNA using high-fidelity RT-PCR with *Pfu* polymerase. Reverse transcription (RT) of 5 μl RNA was performed with Sensiscript RT (Qiagen) as specified by the manufacturer at 45 °C for 40 min, followed by heat inactivation at 95 °C for 5 min. The resulting cDNA was used as template for a 'high-fidelity' protocol using *Pfu*Ultra (Stratagene, La Jolla, CA) according to the manufacturer's specifications. Amplification was carried out for 40 cycles at 94 °C for 30 s, 50 °C for 30 s, 72 °C for 4 min, and one cycle at 72 °C for 10 min. PCR products were visualized on a 1.5% agarose gel, and bands of the appropriate size were excised. DNA was recovered using the MinElute Gel Extraction Kit (Qiagen) as specified by the manufacturer. The recovered DNA was ligated into the cloning vector pCR-Script Amp SK (+) and transformed into XL10-Gold Ultracompetent cells (Stratagene) according to the manufacturers protocol. The blue–white color screening method was used to select transformed colonies. White colonies were screened by direct PCR using primers specific for the insert of interest. Plasmid DNA was purified using the QIAprep Spin Miniprep Kit (Qiagen) as specified by the manufacturer. Sequencing was carried out using overlapping primers including the T7 forward and T3 reverse primer. Sequencing was performed at the Wadsworth Center Molecular Genetics Core (WCMGC) using an ABI 373 or 377 automated sequencer according to the manufacturer protocols. Eighteen to twenty-four clones per chick or mosquito per passage analyzed were sequenced.

Consensus sequencing of passage derived WNV

To obtain complete nucleotide sequences of the WNV strains after 20 passages in either mosquitoes or chickens, the genome was amplified by RT-PCR in 16 overlapping fragments using one-step RT-PCR (Qiagen). A complete list of amplification and sequencing primers is available from the authors upon request. RT-PCR products were electrophoretically separated on a 1.5% agarose gel, and bands of the appropriate size were excised. DNA was recovered using the QiaQuick Gel Extraction Kit (Qiagen) as specified by the manufacturer. Full length WNV consensus sequencing was carried out using multiple pairs of overlapping primers and was performed at the WCMGC as previously described (Davis et al., 2005).

Estimates of synonymous and nonsynonymous variation were obtained from the complete coding sequences of all six passed lineages. Ambiguity codes were resolved conservatively (i.e. given the identity of the consensus sequence in the alignment), and d_N/d_S values were calculated using the Jukes–Cantor formulas for d_N and d_S, using Nei–Gojobori estimates

for synonymous and nonsynonymous sites, as described elsewhere (Jerzak et al., 2005).

Sequence analysis

Sequences were compiled and edited using the SeqMan module of the DNAStar software package (DNAStar, Inc., Madison, WI) and a minimum of two-fold redundancy throughout each clone or consensus fragment was required for sequence data to be considered complete. Eighteen to twenty-four clones from each individual chick or mosquito analyzed were aligned using MegAlign within DNAStar. The consensus sequence for each sample was determined, the sequence of each clone was compared to the consensus and mutations were noted. The mean diversity (total number of mutations divided by total number of bases sequenced) and the sequence diversity (percentage of mutated clones) were used as indicators of genetic diversity.

To compare diversity estimates between hosts at each passage, summary measures of mean and sequence diversity were calculated for each host-timepoint analyzed. Numerators consisted of the number of mutations (or number of mutant clones) summed across all three lineages in each host at each timepoint, and denominators were obtained by summing the total number of nucleotides sequenced, or clones examined. Thus, the proportions of mutant nucleotides and clones (i.e. estimates of mean and sequence diversity) were obtained for each host at each passage and compared statistically using Fisher's exact test.

Mortality in mice

To determine whether the amount of quasispecies diversity influences WNV phenotype, the ability of WNV strains that had undergone 20 serial passages to cause disease in mice was evaluated. Five-week-old female C3H/HeN (C3H) mice were obtained from Taconic Farms (Hudson, NY) and allowed to acclimate in the laboratory for 1 week. Viral strains were diluted in animal inoculation diluent. Groups of eight mice were inoculated with $10^{2.5}$ PFU in 10 ul of each passaged WNV strain SC in the left rear footpad. Control groups of mice were inoculated with unpassed, clone-derived WNV or diluent alone. Mice were weighed and clinically assessed daily, and mice that displayed severe disease were euthanized. Serum was harvested from surviving mice at the termination of the study, and infection was confirmed by ELISA, essentially as described (Ebel et al., 2002). All work with animals was conducted in accordance with protocols approved by the Wadsworth Center Institutional Animal Care and Use Committee. Statistical analyses were performed using GraphPad Prism (GraphPad Software, San Diego, CA).

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