Published in final edited form as:
Virology. 2008 May 10; 374(2): 256–260. doi:10.1016/j.virol.2008.02.032.

Genetic diversity and purifying selection in West Nile virus populations are maintained during host switching

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Abstract

To investigate differential evolutionary rates and selective forces of WNV in hosts and vectors, we measured the genetic diversity that arose during alternating passage in mosquitoes and birds. Within-host genetic diversity was monitored in each of three experimentally passed replicates, and the complete genome sequence of each WNV strain was determined after passage. The intra-host genetic diversity that arose during alternating passage was significantly greater than the diversity generated during chicken-only passage and similar to mosquito-only passage. d_N/d_S ratios suggested purifying selection similar chick-passed virus, but not mosquito-passed virus. Thus, the abundant genetic variation contributed to WNV populations through infection of mosquitoes and the strong purifying selection contributed by infection of birds may be maintained despite frequent host switching.

Keywords

West Nile virus; Flavivirus; Arbovirus; Host Switching; Quasispecies; Natural Selection

Introduction

West Nile virus (WNV; Flaviviridae, Flavivirus) was introduced into North America in 1999 (Lanciotti et al., 1999) where it rapidly adapted to local transmission cycles (Davis et al., 2005) and spread throughout the contiguous United States (Hayes & Gubler, 2006). Studies aimed at understanding the basic evolutionary dynamics and population structure of WNV have quantified its mutation rate, determined that it has reached peak prevalence in North America, and found it to be an essentially “panmictic” population subject to strong purifying selection (Jerzak et al., 2005; Bertolotti et al., 2007; Snapinn et al., 2007). Further, it was found that WNV, like other RNA viruses, is genetically diverse within hosts, suggesting the possibility that WNV populations may be structured as a quasispecies (Jerzak et al., 2005). WNV populations within mosquitoes are more genetically diverse than those within birds in (a) naturally infected specimens and (b) when an initially genetically homogeneous population was sequentially passed exclusively in either mosquitoes or birds. The greater genetic diversity

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in mosquitoes was attributed to relaxed purifying selection in these hosts (Jerzak et al., 2006; Jerzak et al., 2005).

In nature WNV perpetuates by alternately replicating in mosquitoes and birds. *In vitro* studies with WNV demonstrate that, following sequential passage in mosquito cell culture, a single alternate passage in avian cell culture can significantly decrease genetic diversity (Ciota et al., 2007). Although the impact of alternating replication has been suggested through observational and experimental studies of several arboviruses to result in tight evolutionary constraints on the genome and consequent slowed evolution (Weaver et al., 1992; Holmes, 2003; Jenkins et al., 2002), the mechanism(s) through which this constraint may be imposed has not systematically been addressed *in vivo*.

Accordingly, we examined the hypothesis that host switching *per se* results in the evolutionary constraint typical of natural arbovirus populations using WNV as a model system. Specifically, we examined both inter- and intrahost population dynamics of WNV to determine whether populations that underwent alternating replication in mosquitoes and birds would adopt levels of genetic diversity, selective signatures and nucleotide substitution rates similar to WNV that replicated exclusively in either of these hosts, or whether alternating replication would balance the differential effects of mosquitoes and birds on WNV populations. WNV derived from an infectious cDNA clone was passed alternately in mosquitoes and birds for a total of 20 passes. Three experimental replicates were conducted, and genetic diversity was assessed at various timepoints during passage. The complete genomes of all three replicates were sequenced at the termination of the study to determine the cumulative selective pressures acting on WNV during alternating passage and to quantify the rate at which mutations became fixed. Finally, results obtained for WNV that underwent alternating replication in mosquitoes and birds was compared to those obtained from WNV that underwent replication solely in mosquitoes or birds.

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. *Culex 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 BHK-21 cells after RNA 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

To normalize the virus dose for alternating passage against the inherent susceptibility of mosquitoes and chickens to WNV, the virus dose that produced infection in 50% of hosts (ID50) was determined in preliminary experiments described elsewhere (Jerzak et al., 2006). Briefly, 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. Confluent Vero cell monolayers in 6 well culture plates were inoculated with 0.1 ml of mosquito homogenate. Plates were incubated for 1 hr at 37ºC, a primary overlay with 0.6% Oxoid agar in Eagles minimal essential medium containing 10% FBS was applied, and plates were incubated at 37ºC, 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 hours. ID50 values were calculated using the method of Reed and Munch.

In vivo virus passage

Alternating passage was conducted in three concurrent replicate lineages. Female *Cx. pipiens* were inoculated IT with 100 ID50 (50 PFU) of clone-derived WNV stock in 0.1 μl of diluent. Mosquitoes were held for 7 days at 27ºC and provided with a 10% sucrose solution. Individual mosquitoes from each lineage were harvested and homogenized. Aliquots of clarified homogenate were stored at −80ºC. One aliquot was used for virus titration by plaque assay on Vero cells, and a second aliquot was diluted and used to inoculate the subsequent passage in chickens. Chicks, similarly, were inoculated SC with 100 times the ID50 (66 PFU) of titered WNV from the previous mosquito passage in 0.1ml 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 hours post inoculation, and serum was separated, aliquoted, and stored at −80ºC. Virus from chick passage was titered, diluted to 100 times the mosquito ID50 and inoculated into mosquitoes, as above. Alternating passage was repeated a total of ten times for each host, or 20 total passages for each lineage.

High-Fidelity RT-PCR, Cloning and Sequencing

Intrahost genetic diversity was assessed at six timepoints during alternating passage: Mosquito pass 1, chick pass 1 (cumulative passage 2), mosquito pass 5 and chick pass 5 (cumulative passes 9 and 10) and mosquito and chick pass 10 (cumulative passes 19 and 20, respectively). RNA was extracted from clarified mosquito homogenates and chick serum using QiaAmp Viral RNA spin columns (Qiagen). Reverse transcription (RT) reactions and polymerase chain reactions (PCR) were performed using *Pfu*Ultra (Stratagene, La Jolla, CA) and products were cloned as described elsewhere (Jerzak et al., 2005). Clones were sequenced by the University of New Mexico Health Sciences Center DNA Research Services and the Wadsworth Center Molecular Genetics Core (WCMGC) using ABI 373, 377 and 3130XL automated sequencers according to the manufacturer protocols. Eighteen to twenty-two clones per chick or mosquito per passage analyzed were sequenced.

The background error rate of the methods described above was determined experimentally as described elsewhere (Jerzak et al., 2005). Briefly, WNV derived from an infectious cDNA clone (Shi et al., 2002), which is expected to be genetically homogeneous, was processed according to protocols used for experimentally passed WNV specimens and mutation rates determined. These control observations indicated that our methods introduce errors in 0.004 percent of nucleotides examined. All mutations observed were C to A tranversions and all resulted in changes to the predicted amino acid sequence. Based on the advertised error rate of *Pfu*Ultra (4.3×10^{-7} errors/base copied), the length of the amplicon (1937 nt) and number of PCR cycles (40) used in these studies, we would have expected to observe two mutations in our control observations. Three were observed, confirming the genetic homogeneity of our WNV prior to passage.

Consensus Sequencing of passage derived WNV

To obtain complete nucleotide sequences of the WNV strains after ten rounds of mosquito-chick passage, the genome was amplified by RT-PCR in 16 overlapping fragments using one-step RT-PCR (Qiagen) and sequenced at the UNM HSC DNA Research Services and the WCMGC as described elsewhere (Davis et al., 2005).

Sequence and Phylogenetic 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. Clones from each WNV passage 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 within-host genetic diversity 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 mosquitoes and birds 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 WNV that underwent alternating passage, and compared to WNV that was passed exclusively in either mosquitoes or birds statistically using Analysis of Variance (ANOVA) and Tukey's HSD test.

Several methods were used to determine whether the sequence of WNV that underwent alternating *in vivo* replication was under different selective pressures, and evolved at a slower rate compared with single-host passed WNV. Estimates of synonymous and nonsynonymous variation were obtained from the complete coding sequences of all six passed lineages. Ambiguity codes in consensus sequences were resolved by visual inspection of raw trace data and assigning the base to the nucleotide with the highest corresponding fluorescent peak. 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). To assess evolutionary rates, rates of nucleotide substitution were calculated by dividing the total number of mutations in the complete genome sequences by the total number of nucleotides sequenced, and then dividing by the total number of passages (i.e. 20).

Results

Virus Passage

Infectious titer was monitored during the alternating passage regime to evaluate the possibility that by inoculating only 100 times the WNV ID50 into mosquitoes and chicks, we would impose population bottlenecks that would fix deleterious mutations in the population leading to decreases in replication efficiency. WNV titers fluctuated between approximately 10^5 and 10^7 PFU/0.1 mL of mosquito homogenate or chick serum during passage without consistent increases or decreases in titer at harvest in either mosquitoes or birds (Figure 1).

Intrahost Genetic Diversity

Intrahost genetic diversity during passage was measured to determine whether alternating replication would balance genetic diversity between values obtained through single-host passage in mosquitoes and birds. Diversity was measured as both the proportion of nucleotides

that differed from the consensus sequence and the proportion of clones that differed from consensus (Figure 2, Panels A and B). After 20 alternating passages, mean genetic diversity was 0.035 percent, and sequence diversity was 51%. Genetic diversity remained low through the fifth set of passages (cumulative passages 9 and 10) with genetic diversity of most passed lineages at or below the error rate of the methods used in this study. A single lineage was more diverse, particularly at passages 9 and 10, than the other replicate lineages. At passage set 10 (cumulative passages 19 and 20) all lineages were more genetically diverse than the input, clone-derived WNV that was used to initiate passage. At these timepoints, alternately passed WNV populations were as diverse genetically as WNV that had been passed exclusively in mosquitoes, and more diverse than WNV that had been passed exclusively in chickens (Figure 2, Panel C).

Complete Genome Sequences, Selective Pressures and Substitution rates

Complete genome sequences were determined for all lineages at the termination of the study, and d_N/d_S values were computed to assess selective pressures acting on WNV alternately passed in mosquitoes and birds. The number of nucleotide changes to the consensus sequences compared to the input sequence was low. Two mutations were noted in each passed strain, with two of these resulting in changes to the predicted amino acid sequence (Table 1). One, an I to T substitution at position 126 of the E protein is located on the exposed face of domain II and is not obviously associated with known host binding or membrane fusion domains. The T to I substitution in the NS4b protein is located within the carboxy-terminal ectodomain of this protein. The functional significance of this mutation is also unclear at present. The d_N/d_S ratio for alternately passed WNV was 0.313.

Substitution rates were calculated in order to determine whether alternating replication in mosquitoes and birds resulted in slower rates of evolution compared to single host-passed WNV. Virus passed alternately in mosquitoes and chickens accumulated a total of six nucleotide substitutions in three complete genomes. The substitution rate of WNV that underwent alternating passage in mosquitoes and birds was thus 1.0×10^{-5} substitutions per site per passage. This rate was similar to WNV passed in either chickens or mosquitoes [1.5×10^{-5} and 1.7×10^{-5}, respectively (chi squared P=0.5946)].

Discussion

Arthropod-borne RNA viruses perpetuate in nature by alternately replicating in vertebrate and invertebrate hosts. Alternating replication is thought to fundamentally impact the evolution of these agents: convention holds that arboviruses make an evolutionary “trade-off” that results in moderately efficient infection and replication in both hosts at the cost of extremely high efficiency in either. A corollary of this trade-off is that arboviruses are particularly evolutionarily constrained because they must recognize host receptor molecules that may be quite divergent, replicate in a variety of cellular environments, evade both arthropod and vertebrate antivirus responses, etc.. Arbovirus populations, therefore, seem to be characterized by relative evolutionary stasis due to the selective constraints imposed by reliance on taxonomically divergent hosts for perpetuation in nature (Weaver et al., 1999; Weaver, 2006).

The observation of slowed evolution among arboviruses, paradoxically, stands in contrast to two well-accepted aspects of their biology. The first is their extraordinary ability to generate genetic diversity. The error-prone replication characteristic of most RNA viruses (Holland et al., 1982), along with their rapid replication rates and large population sizes, results in a high level of genetic diversity within infections that has been termed a quasispecies (Eigen, 1971). Secondly, arboviruses clearly adapt well to available ecological niches. Chikungunya virus (Togaviridae:Alphavirus), for example, has recently evolved strains that more efficiently infect

an important mosquito vector, *Aedes albopictus* (Tsetsarkin et al., 2007). Similarly, since its introduction into North America, WNV has evolved strains that are more efficiently transmitted by local mosquitoes (Ebel et al., 2004; Moudy et al., 2007; Snapinn et al., 2007). Arbovirus populations may therefore be more dynamic than is generally acknowledged.

Previously, we examined the impact of replication in mosquitoes and birds on WNV quasispecies by sequentially passing WNV derived from an infectious cDNA clone exclusively in either host (Jerzak et al., 2006). These studies demonstrated that mosquitoes and birds differentially impact WNV populations: WNV passed sequentially in mosquitoes reached extremely high levels of genetic diversity, while virus passed sequentially in chickens did not. In addition, a signature of strong purifying selection was detected only in chicken-passed WNV. Thus, in the WNV-mosquito-bird system, mosquitoes apparently provide a source for viral genetic diversity because purifying selection is relaxed in these hosts. In contrast, infection in birds seemed to limit genetic diversity through strong purifying selection. These results suggested that evolutionary constraint in WNV may be due to infection of birds where purifying selection is particularly strong, and not to alternating replication *per se*. Therefore, we examined intrahost genetic diversity, evolutionary rates and natural selective forces in WNV that underwent alternating infection of mosquitoes and birds.

To normalize the infecting dose during the passage regime we titered infectious virus at each passage and inoculated 100 times the ID50 (either 50 or 66 PFU depending on the host) for the next passage. We observed infectious titers that fluctuated between approximately 10^7 PFU and 10^5 PFU, with no marked trend toward increased or decreased titer during the course of the study, suggesting that population bottlenecks and strong founder effects did not negatively impact virus population during this study. These data on titer at harvest during passage indicate, not surprisingly, that the WNV populations alternately passed in mosquitoes and chickens remained replication competent during passage.

WNV replication in mosquitoes leads to high levels of within-host genetic diversity, while replication in birds results in greatly reduced genetic diversity (Jerzak et al., 2006). It was therefore expected that WNV that underwent alternating replication would balance the distinct impacts of these hosts. Specifically, we expected that the requirement for replication in vertebrates would constrain mosquito-generated genetic diversity, resulting in a population that would be somewhat less genetically diverse compared to mosquito-only passed WNV. Surprisingly, analysis of intrahost genetic diversity in three WNV lineages subjected to alternating replication demonstrated high levels of population genetic diversity. After 20 passes, the proportion of nucleotides sequenced and the proportion of clones that differed from consensus (0.03469 and 0.5100, respectively) were similar to values obtained when WNV was passed 20 times in mosquitoes without replication in birds. This was a surprising result because Ciota et al. showed that a single passage in vertebrate cells was sufficient to remove most genetic diversity that had accumulated during sequential *in vitro* passage in C6/36 cells (Ciota et al., 2007). The contrasting results we report highlight the importance of *in vivo* studies using intact hosts and suggest that alternating host replication does not significantly constrain within-host population genetic diversity in WNV. In particular, WNV that replicates alternately in mosquitoes and birds adopts within-host population dynamics similar to WNV that replicates only in mosquitoes. Analysis of synonymous and nonsynonymous variation in alternately passed WNV demonstrated a d_N/d_S ratio of 0.313. This result is consistent with purifying selection and similar to, but weaker than, d_N/d_S of WNV passed exclusively in chickens [0.039, (Jerzak et al., 2006)]. d_N/d_S of WNV passed exclusively in mosquitoes was 2.139, showing an absence of purifying selection. Therefore, the distinct contributions of mosquitoes and birds to WNV populations are maintained during host switching, suggesting that the virus does not suffer a cost of alternating replication, at least from the perspective of virus population genetics. Rather, our findings suggest a potential population-level advantage of alternating replication.

Specifically, genetic plasticity is contributed through infection of mosquitoes and high fitness is maintained via infection of birds.

Analysis of substitution rates obtained from complete genome sequences of passed WNV demonstrated that alternating passage resulted in slightly (but not statistically significantly) fewer substitutions than did passage in a single host. These findings were based on only a small number of substitutions, limiting our statistical power to draw firm conclusions. Nonetheless, they suggest that the slower evolutionary rates observed among the arboviruses may be only partially attributable to the requirement for replication in divergent hosts. It is interesting that the substitution rates are similar despite differing levels of purifying selection in the various passed WNV lineages. Different replication temperatures in mosquitoes (~27ºC) compared to birds (~39ºC) or differences in host-derived replicase factors, for example, could lead to decreased replicase fidelity and concomitant higher mutation rates in birds compared to mosquitoes (Ackermann & Padmanabhan, 2001; Kushner et al., 2003).

We observed measures of within-host genetic diversity and natural selection that were higher than those observed in naturally occurring WNV populations. Specifically, intrahost genetic diversity and d_N/d_S were higher than has been reported for enzootically transmitted WNV (Jerzak et al., 2005). These results suggest that additional stochastic and/or specific mechanisms influence WNV populations in nature. This is not particularly surprising since the model system used in this study relied on IT inoculation of mosquitoes and SC inoculation of chickens. This system bypasses several phases of infection that might impose either selective or stochastic limits on viral genetic diversity. For example, initial infection of the mosquito midgut might randomly limit the number of genomes that initiate mosquito infection, as has been shown for the alphavirus eastern equine encephalitis virus (Smith et al., 2007). On the other hand, oral delivery by mosquitoes might specifically reduce genetic diversity by selecting for more rapidly replicating and/or disseminating variants. Based on the observed levels of within-host genetic diversity and purifying selection reported here and those observed in naturally circulating WNV (Jerzak et al., 2005) we expect that natural transmission (i.e. transmission that does not bypass initial midgut infection, etc.) would remove approximately 50% of the genetic diversity observed in our model system, and strengthen purifying selection by approximately tenfold. Experiments currently in progress will address this issue directly.

Acknowledgements

The authors thank Pam Chin for mosquito rearing, and David Young, Kelly Fitzpatrick and Robert Nofchissey for technical assistance. Edward C. Holmes provided helpful comments on the manuscript. Sequencing was performed at the UNM DNA research facility and the Wadsworth Center Molecular Genetics Core facility. The work was supported in part by funds from the National Institutes of Allergy and Infectious Disease, National Institutes of Health, under grants AI055609 and AI067380. The BSL-3 animal facility at the Wadsworth Center was used, which is funded in part by the Northeast Biodefense Center's animal core on the NIH/NIAID award U54A17158. Ivy Brown is supported by University of New Mexico Initiative to Maximize Student Diversity, which is funded by the National Institute of General Medical Sciences, National Institutes of Health, under grant GM060201.

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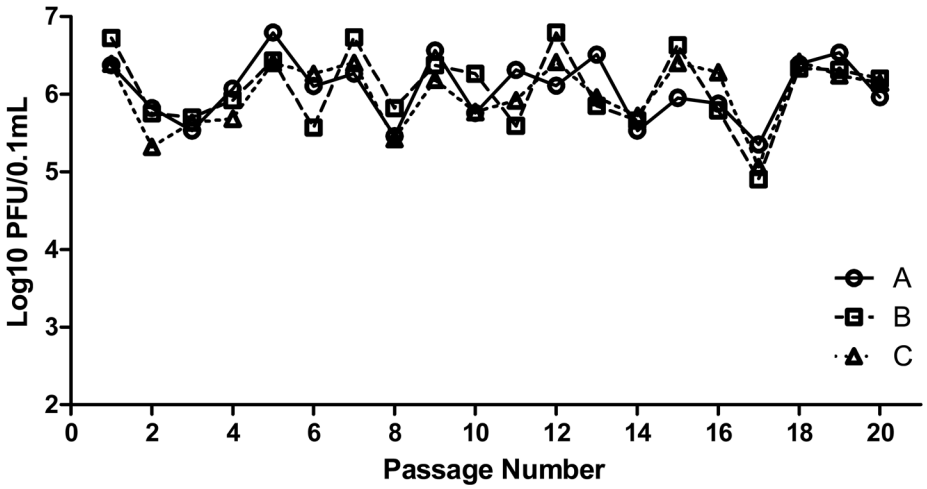

Figure 1.
Titer of WNV during alternating passage in mosquitoes and birds. Each of three replicate lineages, designated A–C, is shown.

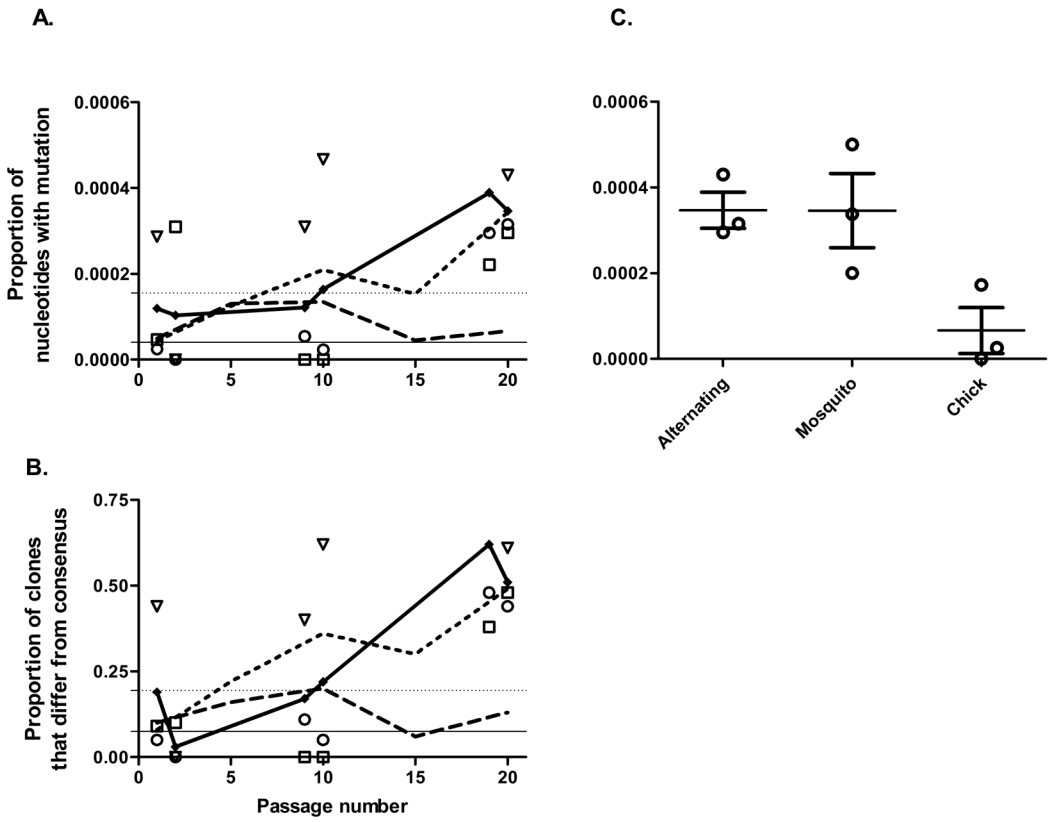

Figure 2.

Intrahost genetic diversity in WNV subjected to alternating host passage is similar to WNV passed solely in mosquitoes. WNV derived from a cDNA clone was passed in mosquitoes and chickens as described in the text, and genetic diversity was characterized during passage, and expressed as either the proportion of nucleotides (Panel A) or clones (Panel B) that differed from consensus. Open symbols indicate individual observations from three replicate lineages. Heavy solid line indicates mean of three alternately passed lineages, and dotted and dashed lines indicate diversity measures for mosquito- and chicken- passed WNV, respectively [taken from Jerzak et al (Jerzak et al., 2006)]. The light horizontal solid line indicates the background error rate, which was determined experimentally, and the light dotted line indicates genetic diversity obtained from field-collected WNV-infected mosquitoes and birds (Jerzak et al., 2005). After 20 alternating passes (Panel C), intrahost genetic diversity from WNV subjected to alternating passage was similar to mosquito-only passed WNV, and different from chick-only passed WNV (ANOVA, $P=0.0320$, Tukey's HSD post test, Alternating vs. Mosquito, $q=0.016$, $P>0.05$; Alternating vs. Chick, $q=4.407$ $P<0.05$; Mosqito vs. Chick $q=4.391$, $P<0.05$).

Table

Nucleotide substitutions in WNV strains following alternating replication in mosquitoes and chickens.

Nt Position:	Input base	Passed Lineage:			AA change	AA Position*
		A	B	C		
1343	T			C	I to T	E-126
1795	A		G		-	E
2320	T		C		-	E
4014	A	C			-	NS2A
7637	C			T	T to I	NS4B-241
7845	C	T			-	NS5

*Amino Acids are numbered from the beginning of the protein