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Preexisting inter-serotype immunity drives antigenic evolution of dengue virus serotype 2

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Abstract

Dengue virus (DENV) infects roughly 400 million people annually, causing febrile and hemorrhagic disease. While preexisting inter-serotype immunity (PISI) provides transient protection, it may drive severe disease over time. PISI's impact on virus evolution, however, is less understood. Retrospective epidemiological analyses suggest that PISI may drive DENV evolution. Using *in vitro* directed evolution, we explored how DENV2 evolves in the presence of DENV3/4 convalescent serum. Two post-passaging mutations (E-I6M and E-N203D) were then studied for fitness effects in mammalian and insect hosts and immune escape. E-I6M resisted neutralization, altered fitness in mammalian cell culture models, and had no effect in *Aedes albopictus* mosquitoes. E-N203D showed no change in neutralization sensitivity, reduced fitness in a DENV-naïve epithelial model, and no effects in the other models. These results align with surveillance data, where E-I6M emerged and disappeared, while E-203D and E-203N cocirculate, thus suggesting that PISI can drive DENV evolution.

Keywords

Evolution; Flaviviruses; Cross-reactive Immunity; dengue virus; Inter-serotype Immunity

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[7] Author Contributions

Conceptualization, J.W.L; methodology, J.M.M, and J.W.L; cloning, J.M.M; characterization, J.M.M; formal analysis, J.M.M and J.W.L; validation, J.M.M; writing—original draft preparation, J.M.M; writing—review and editing, J.M.M and J.W.L; visualization, J.M.M, and J.W.L. All authors contributed to the article and approved the submitted version.

[6] Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

1 Introduction

Dengue virus serotypes 1–4 (DENV1–4; genus *Flavivirus;* family *Flaviviridae)* infect roughly five percent of the global population annually (Bhatt et al., 2013). While $82% of all DENV infections are subclinical (Bhatt et al., 2013), the clinical manifestations can be severe, including vascular leakage, pleural effusion, thrombocytopenia, and death (Simmons et al., 2012). Disease outcomes following DENV infection are heavily influenced by preexisting inter-serotype immunity (PISI) (Burke et al., 1988; Guzmán et al., 2002). The relative risk of developing hemorrhagic disease is 5–500 fold higher in people with a prior DENV infection (Endy et al., 2004). A proposed driver of this phenomenon is antibody-dependent enhancement (ADE) (Guzmán et al., 2002; Halstead et al., 2002; Katzelnick et al., 2017; Katzelnick et al., 2020). ADE occurs when previously protective anti-DENV antibodies begin to wane, resulting in non-neutralizing antibodies binding to the virus, enabling leukocyte uptake via the Fcγ receptor (Dejnirattisai et al., 2016). This can lead to enhanced viral replication and more severe pathologies (Flipse et al., 2016; Katzelnick et al., 2017). While PISI's role in disease is well understood, its impact on DENV evolution is not.

Immune-driven evolution has been characterized for several flaviviruses, such as West Nile virus (Sapkal et al., 2011) and Zika virus (Marano and Weger-Lucarelli, 2023). Functionally, this process involves partial neutralization of a viral population by preexisting host antibodies (Marchi et al., 2021), which selects for a sub-population of neutralization-resistant viruses to become the founders for subsequent generations (Coffey et al., 2013; Morris et al., 2020). It has been theorized that PISI plays a role in DENV evolution based on retrospective data (Katzelnick et al., 2021). However, there has not been a comprehensive laboratory analysis of PISI-driven evolution. It is critical to understand PISI-driven evolution, as mutations derived from this process could have implications on pathogenesis and transmission (Chen et al., 2022).

To address this gap, we employed a previously characterized *in vitro* directed evolution workflow (Marano and Weger-Lucarelli, 2023) to assess the effects of neutralizing intrapatient PISI-driven evolution by passaging an Asian DENV2 isolate in serum from convalescent or DENV naïve patients from the Americas. After passaging, we sequenced the viral populations using next-generation sequencing (NGS) and identified enriched amino acid changes reported in natural isolates. Using a DENV2 reverse genetics platform, we engineered two mutations unique to anti-DENV passaging within the envelope, E-I6M and E-N203D. We assessed the impacts of the mutations on fitness in various mammalian cell lines and *Aedes albopictus* mosquitoes and their sensitivity to neutralization. We observed that the mutations had altered fitness in a variety of mammalian environments, but no effects in *Aedes albopictus* mosquitoes. Further, we observed that E-I6M became more resistant to anti-DENV neutralization, while E-N203D did not affect neutralization sensitivity. These results align with surveillance data of these variants emerging and either disappearing (E-I6M) or being maintained in circulation (E-N203D). By reproducing naturally occurring mutants using an in vitro directed evolution system, we provide additional evidence that PISI plays a significant role in DENV evolution

2 Methods

2.1 Cells, Viruses, and Serum Sources

We obtained Vero cells (CCL-81) and U937-DC-SIGN cells (CRL-3253) from the American Type Culture Collection (ATCC). A549 cells and HEK293A cells were kindly provided by Drs. Nisha Duggal and Jamie Smyth of Virginia Tech, respectively. All cells were maintained at 37°C with 5% CO_2. We cultured Vero, A549, and HEK293A cells using Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 1% nonessential amino acids, and 0.1% gentamicin. U937-DC-SIGN cells were cultured in Roswell Park Memorial Institute medium (RPMI-1640) supplemented with 2 mM L-glutamine, 5% FBS, 1% nonessential amino acids, 0.1% gentamicin, and 0.05 mM 2-mercaptoethanol. We obtained DENV1 strain R99142 and DENV2 strain PUO-218 from the Arbovirus Reference Collection at the Centers for Disease Control and Prevention in Fort Collins, Colorado, USA. DENV3 strain BC188/97 (NR-3801) and strain DENV4 703–4 (NR-48801) were obtained from the Biodefense and Emerging Infections Research Resources Repository (BEI Resources). Previously characterized, de-identified human serum was used for passaging (Marano and Weger-Lucarelli, 2023). Convalescent serum from DENV patients with no history of ZIKV infection was acquired from Boca Biolistics, LLC. We obtained our control serum from blood donors from Kentucky, USA, via Biomedical Products and Services, INC.

2.2 Plaque Reduction Neutralization Test (PRNT)

Serum samples were serially diluted in RPMI-1640 with 10 mM HEPES and 2% FetalPure bovine serum (Genesee Scientific 25–525H), hereafter referred to as viral diluent. We then mixed a solution containing 800 plaque-forming units per mL (PFU/mL) of virus with the diluted serum at equal volumes. This resulted in an average of 40 plaques based on our control well where we mixed virus with viral diluent instead of serum. After a one-hour incubation at 37°C, we transferred the virus-serum mixture to a confluent 24-well plate of Vero cells. Following a one hour adsorption period, overlay media containing 1.5% methylcellulose (Spectrum Chemical catalog ME136–100GM), 4% FetalPure FBS (GenClone 25–525), and 0.2 mg/ml of gentamicin (Gibco™ catalog 11811023) was added to each well (Marano and Weger-Lucarelli, 2023). Plates were fixed six (DENV3 and DENV4) or seven (DENV1 and DENV2) days later. We defined the $PRNT_{50}$ as the highest reciprocal dilution that neutralized the virus by at least 50% (Timiryasova et al., 2013).

2.3 Viral Passaging

We mixed DENV2 stocks with an equal volume of anti-DENV or control serum diluted 1:80 and incubated the virus-serum mixture for one-hour at 37°C. We used this mixture to inoculate a 24-well plate of 80–90% confluent Vero cells at a multiplicity of infection (MOI) of 1 in triplicate. After a one-hour adsorption period, the cells were washed with phosphate-buffered saline (PBS), and fresh media supplemented with the appropriate human serum was added. We monitored cells in 12-hour intervals for cytopathic effects (CPE). When the cells demonstrated >75% CPE, assessed by visual inspection, we harvested the supernatant and stored it at −80°C until titration by plaque assay. This process was repeated for a total of five passages, each time in triplicate, where the MOI for each passage was maximized based on the available titer (0.01–1) to provide the greatest viral diversity prior to challenge.

2.4 Library Preparation, Next-Generation Sequencing (NGS) Analysis, and Phylogenetic Analysis

We prepared libraries from RNA isolated from the unpassaged virus, virus passaged in anti-DENV serum, and virus passaged in control serum. To remove host nucleic acids, viral supernatant was mixed with 250 units/mL of Benzonase (Millipore Sigma E1014–5KU) diluted in 10x Benzonase Buffer (200 mM Tris-Cl [pH 7.5], 100 mM NaCl, 20 mM MgCl2) (Berg et al., 2016) and 250 units/mL of RNase A (Millipore Sigma 10109142001)(Aryani and Denecke, 2015), and incubated for three hours at 37°C. We performed RNA extraction using the Zymo Quick-RNA Viral Kit (R1035). To remove smaller RNA molecules, we purified the samples using a 0.8x bead selection with sparQ PureMag magnetic beads (95196–005). We performed first-strand cDNA synthesis using random nonamers and the Maxima H-Reverse Transcriptase kit (EP0751) and second-strand synthesis using Q5 High-Fidelity 2X Master Mix (M0492S) (Bates et al., 2021). Library fragmentation and adapter/index ligation was performed using the sparQ DNA Frag & Library Prep Kit from Quantabio (Cat. 95194–024). We sequenced the libraries using 150 bp paired-end reads on the Illumina Novaseq 6000 (Novogene Co., Ltd.). NGS data were analyzed using a previously described pipeline (Marano and Weger-Lucarelli, 2023). Briefly, BBduk v39.01 was used to trim adapters and remove low-quality bases (Bushnell, 2014), Burrows-Wheeler Aligner v0.7.17 (BWA) (Li and Durbin, 2009) was used to map reads to the DENV2 reference genome (Accession number ON398847.1), variants were called with LoFreq v2.1.5 (Wilm et al., 2012), and new consensus sequences for the unpassaged viruses were produced using the Genome Analysis Toolkit v 4.2.6.1 (GATK) (McKenna et al., 2010). To generate phylogenetic trees, To generate phylogenetic trees, we used Nextstrain (Auspice v2.49.0) for DENV2 envelope positions 6 and 203 (Hadfield et al., 2018). Nexus files associated with each trial were directly exported and visualized using FigTree v1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/). Individual genotypes were then highlighted using FigTree to aid in visualization and differentiation.

2.5 Generation and Rescue of Single Mutant Viruses by Bacteria-Free Cloning

Mutagenic PCR primers were designed using SnapGene 6.0.2 software (GSL Biotech). We performed mutagenic PCRs using SuperFi II Master Mix (Thermo Fisher 12368010) and purified the products using the Macherey-Nagel NucleoSpin Gel and PCR Clean-up kit (740609). The PCR products were assembled using the NEB Builder HiFi DNA Assembly Master Mix (E2621L). We digested the assemblies with DpnI (R0176S), Lambda exonuclease (M0262S), and Exonuclease I (M0293S) to remove non-circularized products. The assembly product was amplified using EquiPhi29™ DNA Polymerase (Thermo Scientific A39390). RCA products were transfected into HEK293A cells (Marano and Weger-Lucarelli, 2023) to produce a p0 stock. We sequenced the p0 stocks of the mutants derived from the anti-DENV passaged population by Sanger and NGS methods to confirm that the mutation was introduced with no additional unwanted mutations. For the mutant derived from the control population, we sequenced the p0 stock by Sanger sequencing.

2.6 *In vitro* Competition Assays

Mutant and wild-type viruses were mixed at a 1:1 PFU ratio, hereafter referred to as the competition mix. We validated the composition of each competition mix by RT-PCR (qScript XLT One-Step RT-PCR Kit 95143–200) followed by Sanger sequencing to assess the proportions of the viruses and plaque assay to confirm the expected titer. The proportion of each virus was calculated using QSVanalyzer (Carr et al., 2009). Vero and A549 cells were infected with the competition mix at an MOI of 0.01. For the Vero cells with serum supplementation experiments, the competition mix was mixed with equal volumes of the 1:80 diluted anti-DENV serum and incubated for 1 hour. For the U937-DC-SIGN cells, we used an MOI of 1 (Fowler et al., 2018). For all infections, after an adsorption period (1 hour for Vero and A549 cells and 2 hours for U937-DC-SIGN cells), we washed the cells with PBS to remove residual competition mix before adding growth media. Virus was harvested at 2 (U937-DC-SIGN), 3 (Vero and A549), or 4 (Vero with anti-DENV serum) days post-infection (dpi). We performed extraction-free RT-PCR, Sanger sequencing, and analysis identically to our competition mix validation. Relative fitness, W(t), was calculated as $W(t) = F(t)/F(0)$, where F(t) is defined as the ratio of the mutant virus following the competition and F(0) is defined as the ratio of the mutant virus in the competition mix (Marano and Weger-Lucarelli, 2023). We considered the mutant to have no effect on fitness in an environment if $W=0$. If $W>1$ or $W<1$, we would consider the mutant virus to have increased or decreased fitness, respectively.

2.7 Mosquito Rearing and *In vivo* Competition Assays

We reared *Aedes albopictus* Gainesville mosquitoes based on previously published methods (Zheng et al., 2015). Mosquitoes were maintained under controlled environmental conditions, with a temperature of 28°C, a relative humidity of 75%, and a 12:12 light/dark photoperiod. During their larval stages, mosquitoes were raised on ground Nishikoi fish food. Adult mosquitoes had access to a 10% sucrose solution via cotton balls. Approximately 6–8 days after emerging as adults, they were separated into containers at a female-to-male ratio of 5:1. Before their infectious blood meal, mosquitoes were subjected to a 24-hour period of sugar and water deprivation. The “naïve” blood meal included defibrinated sheep’s blood, a competition mix with 106 PFU/mL, and 0.5 μM ATP. For the “immune” blood meal, the virus was pre-treated by incubating it with a 1:80 diluted anti-DENV serum for one hour, with all other conditions remaining consistent. Mosquitoes were allowed to feed for one hour using the Hemotek membrane feeder system (SP4W1–3). Following the feeding, female mosquitoes were anesthetized at 4°C and those that had fed to repletion were isolated into new containers and maintained for ten days. After this period, mosquitoes were terminally anesthetized using triethylamine. Whole mosquito bodies were collected in viral diluent, which was supplemented with 50 μg/mL gentamicin and 2.5 μg/mL amphotericin B. Mosquitoes were processed by adding a single sterile metal bead per tube and homogenized at 30 Hz for 2 minutes using the Qiagen TissueLyser II (85300). Subsequently, samples were diluted 1:5 in viral diluent and underwent processing in a manner consistent with the in vitro competition assays mentioned earlier.

2.8 Statistical Analysis

Statistical analysis was performed in Prism 9 (GraphPad, San Diego, CA, USA). For the *in vitro* competition assays, a Shapiro-Wilk test for normality was performed to ensure normality, and then a one-sample two-tailed t-test was performed. For *in vivo* competition assays, a Fisher's exact test was used to determine which species (mutant or WT) became dominant. A two-way ANOVA with Dunnett's correction for multiple comparisons was used to compare the neutralization of the mutant viruses to the wild type and the growth kinetics of the passaged populations.

3 Results

3.1 Donor Serum Characterization and Passaging Virus

Previously, we characterized donor serum from the Dominican Republic and the United States by plaque reduction neutralization tests (PRNTs) against all four DENV serotypes (Marano and Weger-Lucarelli, 2023). Serum from the United States was used as a DENV naïve control as nearly all adults in the Dominican Republic are seropositive for anti-DENV antibodies (Yamashiro et al., 2004). We re-examined the $PRNT_{50}$ data to identify patients without prior exposure to DENV2. To do this, we used a preexisting framework used in DENV serosurveillance (Sasmono et al., 2018; Sharp et al., 2019). Patients were defined as having monotypic immunity if they had a $PRNT_{50} \geq 10$ for a single serotype or a $PRNT_{50} \geq 10$ for multiple serotypes with a single predominant serotype (4-fold higher than the other serotypes). Multitypic immunity was defined as having a $PRNT_{50} \geq 10$ for multiple serotypes without a single predominant serotype. Based on these parameters, we concluded that anti-DENV Donor C had been previously infected with DENV3 and DENV4 but not DENV2 (Table 1). Therefore, we used serum from anti-DENV Donor C for passaging DENV2 (hereafter called anti-DENV serum).

We passaged DENV2 strain PUO-218, an Asian isolate, in Vero cells in triplicate in the presence of anti-DENV serum at the $PRNT_{50}$ concentration (1:80 dilution) or control serum at the same dilution using our previously published methods (Marano and Weger-Lucarelli, 2023). We monitored the cells and harvested the supernatant when we observed >75% cytopathic effect (CPE), as determined by visual inspection. We then titered the harvested virus (Figure 1A) and used it for a subsequent passage. By the fifth passage, we observed that the time to produce CPE in the anti-DENV passaged virus was consistently 1.5 days longer than the virus passaged in the control serum at the same passage (Figure 1B).

3.2 Identification of enriched mutations following passaging

To determine if replication in the presence of anti-DENV serum resulted in viral genetic changes, we sequenced viral RNA from the unpassaged virus and passaged populations. We focused on the premembrane (prM), envelope (E), and non-structural protein 1 (NS1), as these regions are the major targets for neutralizing antibodies (Vaughan et al., 2010). Using an allelic frequency cut-off of 0.05, we detected 56 total mutations in the protein-coding regions of the unpassaged and passaged populations (Supplemental Tables 1–3). Mutations that were present in the unpassaged and passaged populations with no change in allelic frequency were excluded, as they did not appear to be under selection. Mutations that occurred in both the control and anti-DENV passaged population but not in the unpassaged stock, specifically the variants at position 3154 (NS1-K245E), 6977 (NS4b-M51T), and 7148 (NS4b-T108I), are possible cell culture adaptations, and we excluded them from further analysis. We identified five nonsynonymous mutations in the prM-E-NS1 coding region unique to the anti-DENV serum passaged virus that increased in frequency post-passaging (Table 2). These five mutations were examined for their occurrence in nature using Nextstrain by generating phylogenetic trees based on the genotypes at each position (Hadfield et al., 2018). All but E-D154G were detected in nature; however, only E-N203D is currently circulating (Table 2). Furthermore, E-I6M was the only mutation detected in multiple replicates. Therefore, we selected E-I6M and E-N203D (Supplemental Figure 1, Supplemental Figure 2, and Figure 2A) for further analysis.

3.3 Mutants identified during passage in convalescent dengue serum have neutral to negative fitness in mammalian cell culture.

Using a DENV2 reverse genetics system, we generated the E-I6M and E-N203D mutants. To assess the fitness effects of the mutations in mammalian cells, we performed competition assays comparing the mutants to WT DENV2 by mixing the viruses at 1:1 PFU ratios. Relative fitness was calculated by comparing the proportion of the mutant virus before and after infection (Liu et al., 2021; Marano and Weger-Lucarelli, 2023). We tested four mammalian cellular environments: Vero cells without supplementation of anti-DENV serum, representing an immune naïve model (Immune Naïve); Vero cells supplemented with the anti-DENV pool, representing a dengue immune model (Immune); A549 cells representing a human epithelial cell model (Human Epithelial) (Chiu et al., 2014); U937-DC-SIGN cells, representing a human monocyte model (Human Monocyte) (Wong et al., 2012). E-I6M had significantly reduced fitness in Vero cells, regardless of immune status, and human epithelial cells, but had neutral to weakly increased fitness in human monocytes (Figure 2B). In contrast, E-N203D had reduced fitness only in the Vero immune naïve environment and neutral fitness in all other mammalian cellular environments (Figure 2C).

3.4 Mutants identified during passage in convalescent dengue serum have neutral fitness in mosquitoes.

As DENV is a two-host virus, assessing the impacts of the PISI-selected mutants in mosquitoes is essential to understand transmission potential. To accomplish this, we fed *Aedes albopictus* an artificial blood meal containing the competition mixes used in the *in vitro* studies. To model patients with PISI, we also pretreated the virus with the anti-DENV serum before feeding. We collected the mosquitoes ten days post-feeding and tested the whole-body homogenate to determine the proportion of the mutant virus compared to the starting competition mix. We observed that regardless of the host’s immune status, roughly half of mosquitoes became dominated by WT DENV2 and half with the mutant virus. This indicates a stochastic and not a deterministic effect, likely due to the natural bottlenecks in the mosquitoes (Fitzmeyer et al., 2023). We, therefore, concluded that the mutations had no effect on the fitness of DENV2 in the mosquitoes (Figure 2D).

3.5 Neutralization escape is not correlated to enhanced fitness in an immune environment after heterologous immune selection.

To understand the impact of the mutations on neutralization sensitivity, we performed PRNTs using the anti-DENV serum (Figure 3). The anti-DENV serum neutralized recombinant WT DENV2 similarly to the parental virus ($PRNT_{50} = 80$) (Table 1). While E-I6M had reduced fitness in the presence of anti-DENV serum during competition assays (Figure 2B), it was more resistant to neutralization by anti-DENV serum ($PRNT_{50} = 20$, $p < .0001$). In contrast, E-N203D showed no significant change in neutralization sensitivity ($PRNT_{50} = 80$, $p = 0.6139$) nor fitness in an immune environment (Figure 2C). When we examined the population from which the mutants were derived, we did not observe any changes in neutralization sensitivity (Figure 4A). To further contextualize the results, we generated a mutant from the control serum passaged population (Supplemental Table 3), E-L277S, and tested its neutralization sensitivity. E-L277S had significantly increased sensitivity to neutralization ($PRNT_{50} > 640$, $p < .0001$) (Figure 3), which was mirrored at the population level (Figure 4A). When we assessed the replication of the passaged populations in Vero cells, we observed that the control serum passaged virus had increased replicative fitness (Figure 4B). In contrast, when we tested the control populations in Vero cells supplemented with anti-DENV serum, the control population lost that fitness advantage (Figure 4C). The fitness loss of the PISI mutants in immune naïve Vero cells was not reflected at the population level (Figure 4C).

4. Discussion

DENV remains one of the most significant threats to human health globally. While the relationship between PISI and severe disease is well characterized (Guzmán et al., 2002; Halstead et al., 2002; Katzelnick et al., 2017; Katzelnick et al., 2020), there still exists a significant gap regarding the role of PISI on DENV evolution. Immune-driven evolution has been characterized for many pathogens (Sui et al., 2008; Sapkal et al., 2011; Doud et al., 2018; Mishra et al., 2020; Haslwanter et al., 2021; Marano and Weger-Lucarelli, 2023), and has been theorized to be a driver of DENV evolution (Katzelnick et al., 2021); however, experimental investigations have been limited. To address this gap, we passaged DENV in serum from DENV convalescent or DENV naïve patients to model intrapatient selection and then studied the evolutionary and fitness consequences. Within the anti-DENV serum passaged population, we identified two previously reported, naturally occurring, unique mutations within the envelope protein, E-I6M and E-N203D, which we engineered using a reverse genetics platform. We then assessed their impacts on replication in various host environments and immune escape.

The envelope protein of DENV, like all flaviviruses, contains three non-continuous domains (EDI, EDII, and EDIII) (Modis et al., 2004). When translated, envelope protein monomers form a polymer (dimer or trimer) that mediates cell attachment and entry, with each domain playing a distinct role. EDI has been hypothesized to be responsible for the conformational changes of the envelope protein during cell entry and egress (Zhang et al., 2004). EDII is considered critical for cellular fusion (Allison et al., 2001), while EDIII contains the purported receptor-binding domain (Chin et al., 2007). Interestingly, monoclonal antibodies that target EDI and EDII, the regions where E-I6M and E-N203D occur, are more broadly neutralizing across all four DENV serotypes (Beltramello et al., 2010).

Previously, we reported on the impacts of preexisting cross-reactive DENV immunity on ZIKV evolution (Marano and Weger-Lucarelli, 2023). In this previous study, we found that passaging ZIKV in anti-DENV serum resulted in increased fitness in mammalian cells and reduced fitness in mosquitoes (Marano and Weger-Lucarelli, 2023). Therefore, we hypothesized that passaging DENV in anti-DENV serum would yield similar findings, aligning with the trade-off hypothesis (Wilson and Yoshimura, 1994; Kassen, 2002). However, E-I6M demonstrated significantly reduced fitness in mammalian epithelial cells, regardless of immune status, and had neutral to weakly increased fitness in human monocytes (Figure 2B). In contrast, E-N203D only demonstrated reduced fitness in immune naïve mammalian cells and had no effect in all other mammalian cellular environments (Figure 2C). Both mutants had no fitness effect in *Aedes albopictus* mosquitoes, regardless of host immune status (Figure 2D). These results indicated that while the trade-off hypothesis can explain some instances of immune-driven viral evolution, it does not apply to all cases.

We also examined the neutralization sensitivity of the mutants. Our previous result with ZIKV demonstrated that passaging in anti-DENV serum resulted in mutants with varying neutralization phenotypes, ranging from escape to sensitization. We hypothesized that we would observe similar behaviors with DENV. Our neutralization sensitivity assays supported this hypothesis (Figure 3), with E-I6M become more resistant neutralization and E-N203D having no change in neutralization sensitivity. Interestingly, similar to results with ZIKV, the mutant derived from the control serum passaged virus was significantly more sensitive to neutralization. These results indicate that PISI protects DENV from adopting neutralization-sensitive phenotypes. Interestingly, similar to our previous results with ZIKV, the mutation derived from the control passaged population, E-L277S, was more sensitive to neutralization. These data indicate that pressure from anti-DENV neutralizing immunity protects DENV, like ZIKV, from adopting a hypersensitive neutralization phenotype.

Taken together, these results align with epidemiological findings. While E-I6M became resistant to neutralization, as demonstrated with a PRNT, it cannot outcompete WT DENV2 in most mammalian cells, even in the presence of anti-DENV serum. These behaviors likely explain how E-I6M emerged briefly in the 1960s and 1980s and then quickly disappeared (Supplemental Figure 1) (Hadfield et al., 2018). A follow-up study should be performed to assess if similar behaviors would be observed in the two naturally occurring but currently non-circulating mutations within prM that we detected. In contrast to E-I6M, because E-N203D did not affect fitness in DENV immune environments, human cells, or mosquitoes, it suggests that E-203D should be able to cocirculate alongside E-203N, the WT. This is observed in nature, with E-203D and E-203N being the two dominant genotypes globally (Supplemental Figure 2) (Hadfield et al., 2018). Interestingly, there is a geographic and genotype biasing at this site, with E-203N being more prevalent in Asia and the Asian I genotype, while E-203D is more prevalent in the Americas and the Asian-American genotypes. How E-203D became dominant in the Americas is unclear, given that we observed no positive fitness effects with the mutation. One hypothesis is that it became dominant due to a genetic bottleneck (Sittisombut et al., 1997), and thus E-203D became a founder for DENV2 in the Americas. Alternatively, there may be epistatic effects within the genotypes that reveal fitness effects, a phenomenon observed with chikungunya virus (Tsetsarkin et al., 2011). Because we inserted E-203D into PUO-218, which is from the Asian I genotype, necessary epistatic interactions may not have been present to produce a fitness effect.

Limitations of the study:

We used only a single DENV2 isolate and convalescent serum from the Dominican Republic for this study. We may have observed different results if we used a different DENV2 isolate or serum from different sources. Also, our focus in this study was to characterize the impacts of humoral immunity; however, cross-reactive T-cells can be protective in secondary DENV infections (Zompi et al., 2012) and may drive virus evolution. By passaging in only mammalian cells, our model represented multiple rounds of replication and antibody selection within a single host. Use of an alternating passaging system between mosquitoes and mammalian cells could have been used to mimic natural host cycling (Deardorff et al., 2011; Talavera-Aguilar et al., 2021; Regla-Nava et al., 2022). Moreover, our choices of cell lines may have limited our sensitivity. Had we used an animal model to assess replication, fitness differences may have been observed. However, because animal models, specifically mouse models for DENV, often fail to replicate human infection (Chen and Diamond, 2020; Kayesh and Tsukiyama-Kohara, 2022), the current animal models have also lacked sensitivity and/or translatability. Regarding our selection of mutants for analysis, while Nextstrain acquires data from several databases, including NCBI, GISAID, and ViPR (Hadfield et al., 2018), it is possible that variants within our study have emergent in recent years, but were not reported. Finally, while we generated only single mutants, we could have engineered mutant viruses containing multiple mutations to determine if epistasis was occurring within the viral populations (Ferreira et al., 2021); this may have led to more drastic phenotypes in competition assays.

Conclusions:

We demonstrated that PISI selection alters the evolutionary trajectory of DENV. Specifically, we found that by passaging DENV in serum from DENV convalescent patients, we could replicate the emergence of genotypes found in nature, E-I6M and E-N203D. These two mutations demonstrated changes in replicative fitness and neutralization sensitivity; however, these results did not align with the trade-off hypothesis, which we had previously reported for ZIKV passaged under similar conditions. We also observed that passaging DENV in the presence of anti-DENV serum constrains the population from adopting highly susceptible neutralization phenotypes. These results substantiate the findings of Katzelnick et al., demonstrating that PISI drives DENV evolution (Katzelnick et al., 2021). These results improve our understanding of PISI as a driver of DENV evolution and provide a framework to dissect other inter-flavivirus interactions, such as other DENV serotype combinations and those within the Japanese encephalitis complex (Rathore and St. John, 2020).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 2A was generated using BioRender. The Virginia Tech Genomic Sequencing Center performed Sanger sequencing. The Center for Biostatistics and Health Data Science provided statistical support. We thank Dr. Christina Chuong and Kelsey Marano for editing the manuscript.

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5 Data Availability Statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the Supplementary Material.

9 References

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Highlights

- Pre-existing inter-serotype immunity is critical in DENV pathology but is under-studied in evolution.
- We replicated the emergence of natural DENV mutations using directed evolution in the presence of cross-reactive neutralizing antibodies.
- Neutralization escape did not correlate with increased fitness in immune environments.
- DENV passaged without cross-reactive neutralizing antibodies gained fitness in a DENV-naive environment but became more sensitive to neutralization.
- These findings highlight the importance of PISI on DENV evolution.

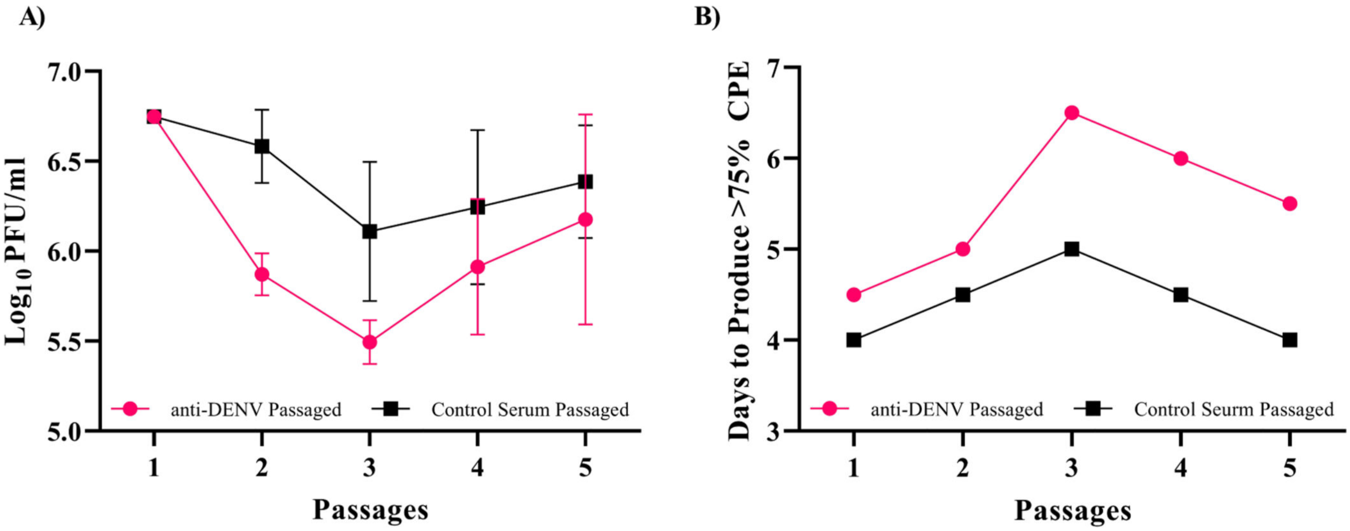

Figure 1: Dengue virus serotype 2 (DENV2) Passaging Benchmarks.

DENV2 stocks were used to infected Vero cells at a multiplicity of infection (MOI) for each passage was maximized based on the available titer (0.01–1). The supernatant was harvested when >75% of cells demonstrated cytopathic effects (CPE), by visual inspection, and then titered for subsequent passages. Data represent the three independent lineages within each passaging condition (anti-dengue virus (DENV) or Control Serum). A) The post-passage titers of the anti-DENV serum and control serum passaged virus, as measured by plaque assay B) The days post-infection where this occurred when cells reached >75% CPE were recorded for each passage. The error bars represent the standard deviation from the mean titer (A) or time to reach 75% CPE (B).

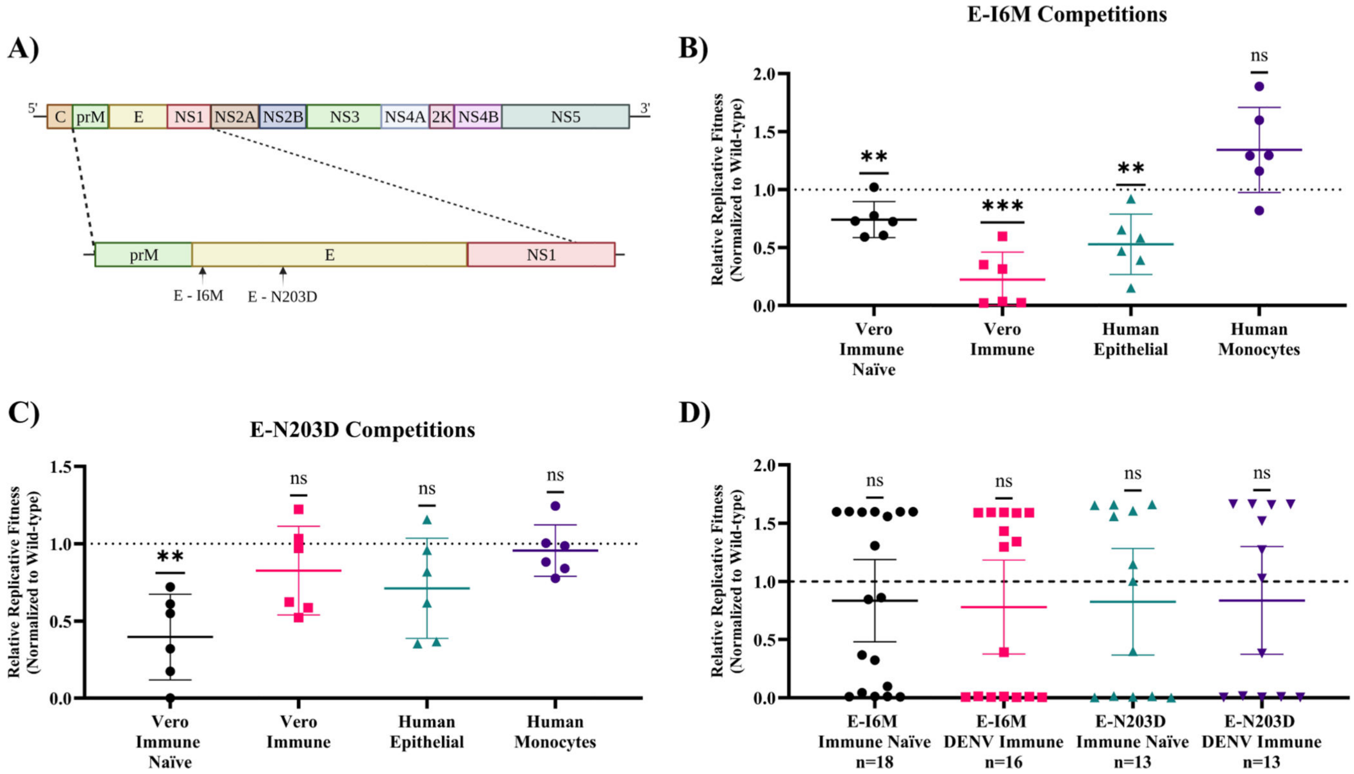

Figure 2: Dengue virus (DENV) mutants enriched following passage in the presence of anti-DENV convalescent serum have neutral to negative fitness effects in mammalian cells and no effects in mosquitoes.

A) Two mutations were selected (E-I6M and E-N203D) to assess replicative fitness. WT virus was mixed with each mutant virus at a 1:1 PFU ratio and used to infect cell lines (MOI=0.01 or MOI = 1) and mosquitoes (10^6pfu/ml). Post-infection, virus was harvested and sequenced. The relative fitness was calculated by comparing the proportion of the mutant virus before and after infection. (B-C) In vitro competition assays. B) E-I6M and C) E-N203D represent results on cell lines (t-test represented by ** p< .0021, *** p = .0002, ns- nonsignificant). The error bars represent the standard deviation from the mean relative replicative fitness. D) Represent results in mosquitoes. Each data point represents the whole-body homogenate of a single mosquito. The error bars represent the standard deviation from the mean of pooled fitness across all processed mosquitoes (n represents the number of mosquitoes) from two independent experiments, and the data was analyzed using a Fisher’s exact test.

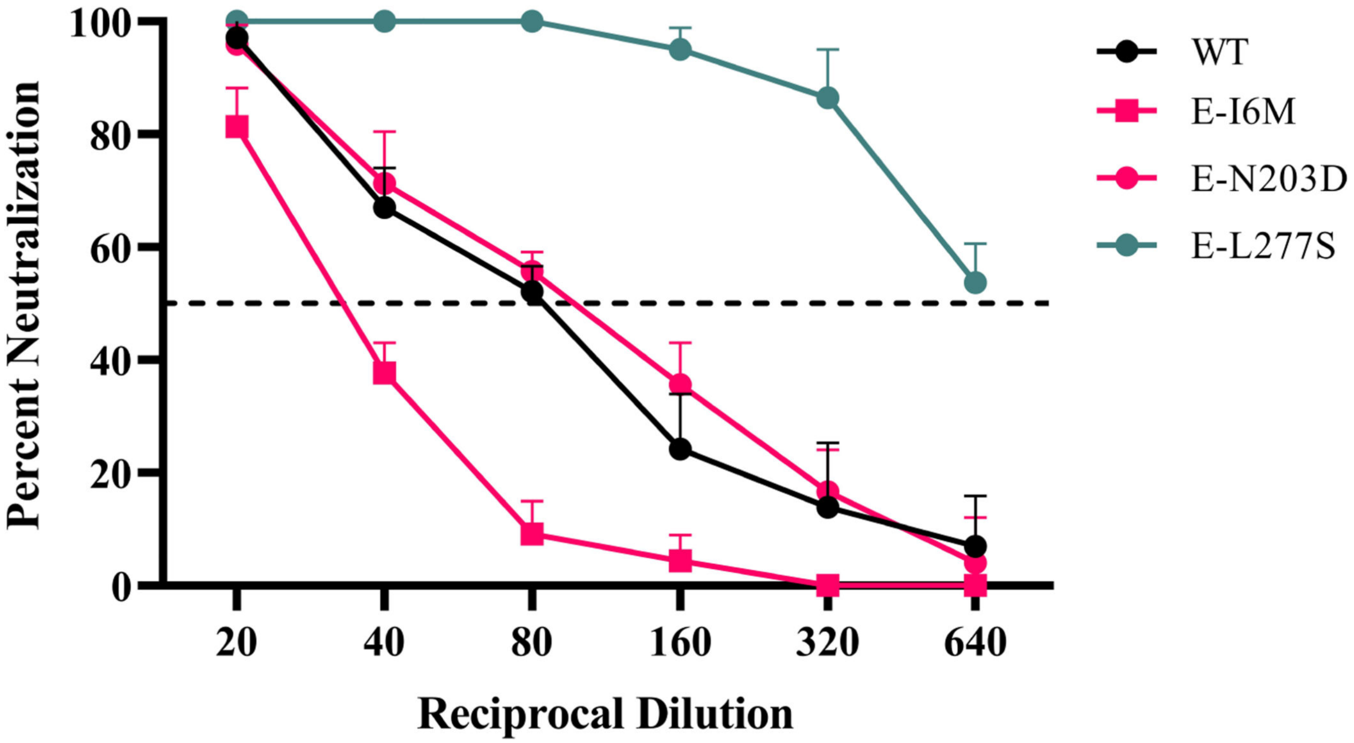

Figure 3: DENV mutants enriched following passage in the presence of anti-DENV convalescent serum demonstrate escape or no change in neutralization sensitivity.

anti-DENV serum were serially diluted and mixed with 800 PFU/ml of WT DENV (black line), mutants from the anti-DENV population (red lines), those identified from the Control Serum-passaged population (green lines). Data are from two biological replicates, each with three technical replicates, and the error bars represent the standard deviation from the mean. The dotted line represents the limit of detection.

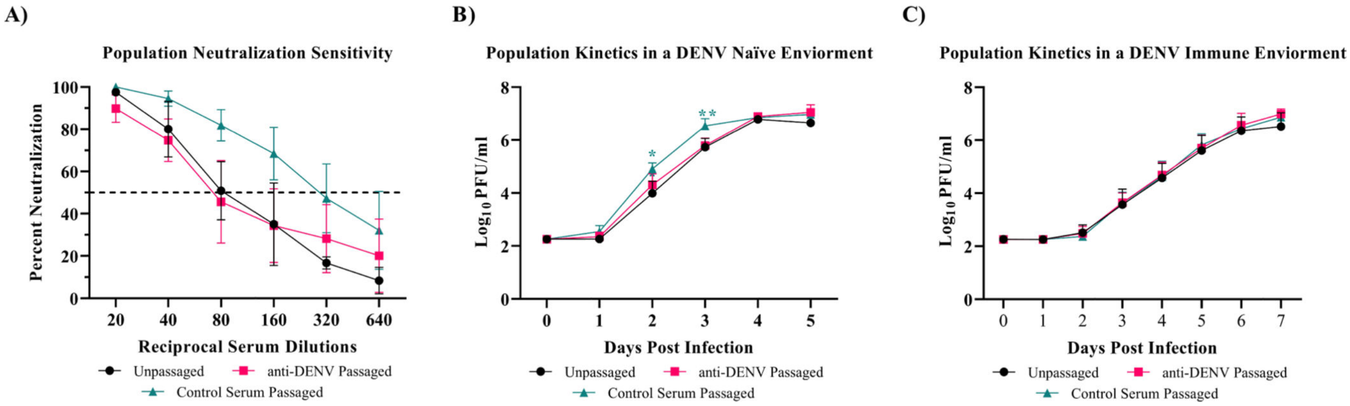

Figure 4: Neutralization Dynamics and Replicative Fitness of Passaged Populations.

WT DENV (black line), passage 5 of the anti-DENV passaged population (red lines), or passage 5 of the Control Serum-passaged population (green lines) were tested for neutralization sensitivity and replication. Data are from two biological replicates, each with three technical replicates, and the error bars represent the standard deviation from the mean. A) The anti-DENV serum was serially diluted and mixed with 800 PFU/mL of each virus, and percent neutralization was recorded at each dilution. (B-C) Vero cells were either infected at an MOI of 0.01 with untreated virus (B) or virus incubated with the anti-DENV serum for 1 hour. Statistics were performed using a two-way ANOVA with a Dunnett's correction for multiple comparisons comparing the passaged viruses to the unpassaged virus (ns - nonsignificant, * - p = 0.0139, ** - p = 0.001, *** - p = 0.005, **** - p < 0.0001).

Table 1:
Retrospective Diagnosis of Blood Donors.

Donor blood was serially diluted and mixed with dengue virus (DENV) serotypes 1–4. The reciprocal of the highest serum dilution that neutralized 50% of the challenge virus is reported as the $PRNT_{50}$ value. These data represent two biological replicates, each with three technical replicates. We defined the infecting serotype as PRNT50 ≥ 4-fold higher than the other DENV serotypes. A multi-typic infection is defined as a patient having >2 DENV infections and cannot be differentiated by $PRNT_{50}$. The sample we selected for our passaging, anti-DENV Patient C, is presented in bold.

Virus	anti-DENV Patient A	anti-DENV Patient B	anti-DENV Patient C	anti-DENV Patient D	Control Serum
DENV1 R99142	640	1280	**160**	10240	<20
DENV2 PUO218	640	10240	**80**	2560	<20
DENV3 BC188/97	1280	320	**640**	5120	<20
DENV4 703–4	640	640	**360**	1280	<20
Retrospective Diagnosis	Multi-typic Infection	DENV2	**DENV3/4**	Multi-typic Infection	Naïve

Table 2:
Variants Identified in anti-DENV Passaged Dengue Virus (DENV) Populations.

Non-synonymous mutations within the prM-E-NS1 coding region. If a mutation was not found in a replicate at the limit of detection, allelic frequency (AF) = 0.05, they are identified as N/A. The mutations that were selected for downstream use are bolded. No mutations meeting our criteria were found in NS1. Nextstrain was used to identify the year of the most recent isolate containing the identified mutation.

nt Position	Protein	Reference	Alternative	AA Position	AA Change	Allelic Freq. Rep. 1	Depth Rep.1	Allelic Freq. Rep. 2	Depth Rep.2	Allelic Freq. Rep. 3	Depth Rep.3	Year of Last Isolate
601	Premembrane	C	T	prM −55	[L/F]	N/A	N/A	0.691398	48953	N/A	N/A	2019
614	Premembrane	A	G	prM - 59	[N/S]	0.352588	34831	N/A	N/A	N/A	N/A	1996
954	**Envelope (Domain 1)**	**A**	**G**	**E - 6**	**[I/M]**	**0.256574**	**23463**	**N/A**	**N/A**	**0.088496**	**120503**	**1981**
1397	Envelope (Domain 1)	A	G	E - 154	[D/G]	0.071492	54873	N/A	N/A	N/A	N/A	N/a
1543	**Envelope (Domain 2)**	**A**	**G**	**E – 203**	**[N/D]**	**0.347833**	**45450**	**N/A**	**N/A**	**N/A**	**N/A**	**2023**