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Evolution of resistance to fluoroquinolones by dengue virus serotype 4 provides insight into mechanism of action and consequences for viral fitness

Stacey L.P. Scroggs [a,1,*], Jordan T. Gass [a], Ramesh Chinnasamy [b], Steven G. Widen [c], Sasha R. Azar [d], Shannan L. Rossi [d,g], Jeffrey B. Arterburn [b], Nikos Vasilakis [d,e,f,g], Kathryn A. Hanley [a]

[a] Department of Biology, New Mexico State University, Las Cruces, NM, USA
[b] Department of Chemistry and Biochemistry, New Mexico State University, Las Cruces, NM, USA
[c] Department of Biochemistry & Molecular Biology, The University of Texas Medical Branch, Galveston, TX, USA
[d] Department of Pathology, The University of University of Texas Medical Branch, Galveston, TX, USA
[e] Center for Biodefense and Emerging Infectious Diseases, The University of University of Texas Medical Branch, Galveston, TX, USA
[f] Center for Tropical Diseases, The University of University of Texas Medical Branch, Galveston, TX, USA
[g] Institute for Human Infection and Immunity, The University of University of Texas Medical Branch, Galveston, TX, USA

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ABSTRACT

Drugs against flaviviruses such as dengue (DENV) and Zika (ZIKV) virus are urgently needed. We previously demonstrated that three fluoroquinolones, ciprofloxacin, enoxacin, and difloxacin, suppress replication of six flaviviruses. To investigate the barrier to resistance and mechanism(s) of action of these drugs, DENV-4 was passaged in triplicate in HEK-293 cells in the presence or absence of each drug. Resistance to ciprofloxacin was detected by the seventh passage and to difloxacin by the tenth, whereas resistance to enoxacin did not occur within ten passages. Two putative resistance-conferring mutations were detected in the envelope gene of ciprofloxacin and difloxacin-resistant DENV-4. In the absence of ciprofloxacin, ciprofloxacin-resistant viruses sustained a significantly higher viral titer than control viruses in HEK-293 and HuH-7 cells and resistant viruses were more stable than control viruses at 37 °C. These results suggest that the mechanism of action of ciprofloxacin and difloxacin involves interference with virus binding or entry.

1. Introduction

Members of the genus *Flavivirus*, notably DENV and ZIKV, pose a great and growing threat to public health, but at present no approved antiviral therapies are available to treat any flaviviral infection (Bhatt et al., 2013; Boldescu et al., 2017; Aliota et al., 2017). Drug repurposing offers the fastest route to move anti-flaviviral drugs into the clinic (Barrows et al., 2016), and to this end we have recently demonstrated that three FDA-approved fluoroquinolones, enoxacin, difloxacin, and ciprofloxacin, suppress replication of six flaviviruses, including DENV and ZIKV, in cultured HEK-293 (human embryonic kidney) cells (Scroggs et al., 2020). Additionally, we found that treatment with enoxacin suppressed replication of ZIKV in the testes of interferon-deficient A129 mice *in vivo*, although it did not impact ZIKV titer in the serum, brain, or liver in these mice (Scroggs et al., 2020). Further, Xu et al. (2019) recently reported that enoxacin also suppresses ZIKV replication in human neural progenitor cells (hNPCs) and brain organoids (Xu et al., 2019). Thus, fluoroquinolones offer a promising candidate for a repurposed therapy for flavivirus infections. However, several key aspects of the fluoroquinolone-flavivirus interaction must be elucidated before these drugs can move forward on the path to the clinic, particularly: (i) the barrier to resistance to these drugs, (ii) patterns of cross-resistance among fluoroquinolones, (iii) the fitness consequences of evolution of drug resistance, (iv) the mechanism(s)-of-action of

* Corresponding author.
E-mail address: stacey.scroggs@nih.gov (S.L.P. Scroggs).
[1] Present address: Stacey L. P. Scroggs, Biology of Vector-Borne Viruses Section, Laboratory of Virology, Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Hamilton, MT USA.

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fluoroquinolones against flaviviruses.

Resistance evolution has been a serious obstacle to successful treatment of other RNA viruses, particularly HIV, hepatitis C virus (HCV), and influenza (Asahchop et al., 2012; Iyidogan and Anderson, 2014; Bloom et al., 2010). Moreover, cross-resistance to structurally related drugs can occur and must be investigated as cross-resistance can render an entire class of antivirals ineffective (Halfon and Locarnini, 2011; Hussain et al., 2017). The utility of an antiviral also depends on the barriers to resistance (Hussain et al., 2017; Robinson et al., 2011a; Wensing et al., 2010; Poveda et al., 2014). A drug that imposes a high genetic barrier for the evolution of resistance is more useful than a drug that allows for evolution of resistance to occur within a few passages or within a single host infection (Wensing et al., 2010; Tong et al., 2014). RNA viruses have been shown to evolve drug resistance via one of two mechanisms: (i) specific antagonism of drug action, which often results in a decrease in fitness in the absence of the drug (Bloom et al., 2010; Nijhuis et al., 1999; Back et al., 1996; Coffin, 1995; Eyer et al., 2017; Deng et al., 2014; Jensen et al., 2019) or (ii) a general increase in fitness, which can sometimes be sustained following withdrawal of the drug (Jensen et al., 2019; Quinones-Mateu et al., 2002). A decrease in fitness of resistant viruses could negatively impact viral transmission and alleviate disease burden, while a gain in fitness could exacerbate disease and could increase transmission, although these two phenotypes may also be uncoupled by evolution of drug resistance.

Previous studies have revealed multiple impacts of fluoroquinolones on both cellular and viral processes that may confer antiviral activity and shape the genetic pathway to resistance. First, multiple fluoroquinolones enhance RNA interference (Melo et al., 2011; Shan et al., 2008; Zhang et al., 2008). Xu et al. (2019) recently reported that enoxacin suppresses ZIKV in human neural progenitor cells (hNPC) and suggested the efficacy was due to enhancement of RNAi. Second, multiple fluoroquinolones have been shown to suppress HCV, possibly by interfering with the viral helicase (Khan et al., 2012; Anwar et al., 2020). Third, ciprofloxacin inhibits cellular helicases (Simon et al., 2013), which are targeted and bound by the flavivirus capsid protein (Xu et al., 2011) and the untranslated regions (Ward et al., 2011; Li et al., 2014) to regulate viral replication and aid in virion assembly. Fourth, ciprofloxacin and trovafloxacin prevent cellular apoptosis (Fukumoto et al., 2013; Poon et al., 2014), a process that flaviviruses suppress early in infection by activating the phosphatidylinositol 3-kinase pathway (Lee et al., 2005), but activate later in infection with the capsid, NS2A, and NS3 protease proteins (Melian et al., 2013; Urbanowski and Hobman, 2013; Prikhod'ko et al., 2002). Fifth, ciprofloxacin suppresses autophagy (Fukumoto et al., 2013). The loss of autophagy results in non-infectious virions and a decrease in viral RNA production during DENV infection (Mateo et al., 2013). Lastly, the fluoroquinolone levofloxacin inhibits rhinovirus replication by reducing expression of the cellular receptor required for viral entry (Yamaya et al., 2012). Any one of these mechanisms, or combinations thereof, may be responsible for the efficacy of fluoroquinolones to suppress flaviviruses. There may also be functions of fluoroquinolones that have not been described in the literature that contribute to their anti-flaviviral efficacy. The location of mutations specific to fluoroquinolone-resistant viruses can suggest possible mechanisms-of-actions.

In the current study, we harnessed the power of experimental evolution to investigate fluoroquinolone-flavivirus interactions. We hypothesized that: (i) the barrier to resistance against fluoroquinolones would be low and that, therefore, resistance would evolve within ten passages in cultured cells, (ii) resistance to one fluoroquinolone would confer cross-resistance to other fluoroquinolones due to the high structural similarity of these drugs, and (iii) resistance would evolve via specific evasion of the drug, resulting in a decrease in fitness in the absence of the drug. To test these hypotheses, we serially passaged DENV-4 in triplicate in the presence or absence of fluoroquinolones, in human embryonic kidney cells (HEK-293). While DENV-4 evolved resistance to ciprofloxacin in 7 passages (passage 7 lineages termed DENV-Cipro A,B,C) and difloxacin in 10 passages (DENV-Diflox A, B, C), it did not evolve resistance to enoxacin in 10 passages (DENV-Enox A,B, C). We predicted that DENV-Cipro and control lineages of DENV-4 passaged 7 times in media (DENV-DMEM A,B,C) would share common mutations relative to the parent DENV-4 (DENV-P) that confer adaptation to HEK-293 cells (Blaney et al., 2001), but that DENV-Cipro lineages would also possess a unique constellation of mutations, some conferring fluoroquinolone-resistance, and that these latter mutations would provide insight into the fluoroquinolone mechanism-of-action (Zou et al., 2009; Yang et al., 2011, 2014; Kato et al., 2016; Qing et al., 2010; Xie et al., 2011; Byrd et al., 2013a, 2013b; Griesemer et al., 2017; van Cleef et al., 2013). We also used the DENV-Cipro and DENV-Diflox lineages to test the hypothesis that evolution of resistance to one fluoroquinolone will confer cross-resistance to other members of this class. Finally, we quantified the fitness and stability of resistant, control and parent virus in the absence of drug in mammalian and mosquito cultured cells and mosquitoes *in vivo* to draw inference about the mechanisms underlying resistance. Evolutionary studies suggest that gains in flavivirus fitness in the mammalian host come at the cost of a loss in fitness in the mosquito vector (a trade-off) (Deardorff et al., 2011; Vasilakis et al., 2009; Ciota et al., 2013). As both DENV-Cipro and DENV-DMEM had been passaged in HEK-293 cells, we predicted that these lineages would both have lower fitness in mosquitoes than DENV-P, and that DENV-Cipro would be more disadvantaged than DENV-DMEM. In sum, these studies seek to move the evaluation of FDA-approved drugs for antiviral repurposing beyond the demonstration of efficacy to consider the consequences of resistance.

2. Materials and methods

2.1. Viruses and cells

Dengue virus serotype 4 (rDEN-4 Dominica p4) was derived from full-length clone p4 (Durbin et al., 2001a) and passaged four times in Vero cells. Replicate vials of a working stock were collected in 1X SPG (2.18 mM sucrose, 38 mM potassium phosphate [monobasic], 72 mM potassium phosphate [dibasic], 60 mM L-glutamic acid), clarified by centrifugation, and stored at −80 °C. HEK-293 cells were obtained from ATCC (CRL-1573); Vero, HuH-7, and C6/36 cells were obtained from the lab of Dr. Stephen Whitehead (NIAID, NIH). HEK-293 cells were maintained at 37 °C with 5% CO_2 in Dulbecco's minimum essential medium (DMEM/F12; Gibco, Life Technologies, Grand Island, NY) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), 2 mM L-glutamine (Gibco), and 0.5% antibiotic-antimycotic (penicillin, streptomycin, and amphotericin B; Gibco). Vero cells were maintained at 37 °C with 5% CO_2 in minimum essential medium (MEM, Gibco) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 0.05 mg/mL gentamycin (Gibco). HuH-7 cells were maintained at 37 °C with 5% CO_2 in DMEM/F12 supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, and 0.05 mg/mL gentamycin. C6/36 cells were maintained at 32 °C with 5% CO_2 in MEM supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 2 mM nonessential amino acids (Gibco), and 0.05 mg/mL gentamycin. Viral titers were determined via serial dilution onto HEK-293, Vero, HuH-7 or C6/36 cells followed by immunostaining using previously described methods (Scroggs et al., 2020; Durbin et al., 2001b; Hanley et al., 2008). Briefly, each virus was serially diluted ten-fold and inoculated onto confluent cells in 24-well plates. After 2 h of incubation at 37 °C with occasional rocking, infected cells were overlaid with 1% methylcellulose in OptiMEM (Gibco) that had been supplemented with 2% FBS, 2 mM L-glutamine, and 0.05 mg/mL gentamycin. Plates were incubated for five days under maintenance conditions, after which cells were fixed with ice cold methanol: acetone (1:1) for HEK-293 and 90% methanol for Vero, HuH-7, and C6/36 cells for 30 min. Viral plaques were immunostained using DENV-4-specific hyperimmune mouse ascitic fluid and peroxidase-labeled goat anti-mouse secondary antibody (KPL,

Gaithersburg, MD) then developed with KPL True Blue Peroxidase Substrate (SeraCare, Milford, MA) and counted to calculate viral titer.

2.2. Antiviral compounds

For each experiment, a fresh working stock of enoxacin (Sigma-Aldrich, E3764, St. Louis, MO), difloxacin (Sigma-Aldrich, D2819, St. Louis, MO), or ciprofloxacin (Corning, 86393-32-0, Manassas, VA) at a concentration of 1.5 mM was sonicated in nanopore water with 3 mM lactic acid (Sigma-Aldrich, L1750, St. Louis, MO) and sterilized via passage through a 0.2 μm filter as described in (Scroggs et al., 2020). The compounds were diluted to their final concentrations in HEK-293 cell culture media.

2.3. Selection of drug-resistant viruses

Fluoroquinolone-resistant DENV-4 was generated by passaging the virus in triplicate in the presence of enoxacin, difloxacin, or ciprofloxacin (three independent lineages per drug) until resistance was detected (Fig. 1a, Fig. 1c and e) or for a total of ten passages. This endpoint was chosen based on selection for flavivirus resistance to other candidate antivirals in cell culture, in which a range of 7–21 passages was required to detect resistance (Eyer et al., 2017; Zou et al., 2009; Kato et al., 2016; Qing et al., 2010; Xie et al., 2011; Byrd et al., 2013a, 2013b; Deas et al., 2007), though we recognize that studies that did not detect resistance may not be in the published literature due to negative publication bias (Easterbrook et al., 1991). Additionally, one set of triplicate control lineages were passaged in cell culture media. For each passage, T25 flasks of HEK-293 cells at 80% confluency were infected at a multiplicity of infection (MOI) of 0.1. After 2 h incubation at 37 °C with 5% CO_2, infected cells were washed with 1x PBS and treated with 5 mL of each drug at the specified concentrations or media for each passage (Fig. 1a, c, and 1e). The effective concentration 50 (EC_{50}) values for difloxacin and ciprofloxacin, 10.1 μM, and 19.6 μM respectively as reported in (Scroggs et al., 2020) were used to initiate passage, however, the concentration of ciprofloxacin and difloxacin was increased 2-fold after 3 or 4 passages, respectively, to accelerate evolution of drug resistance. Based on preliminary experiments, passaging with enoxacin was

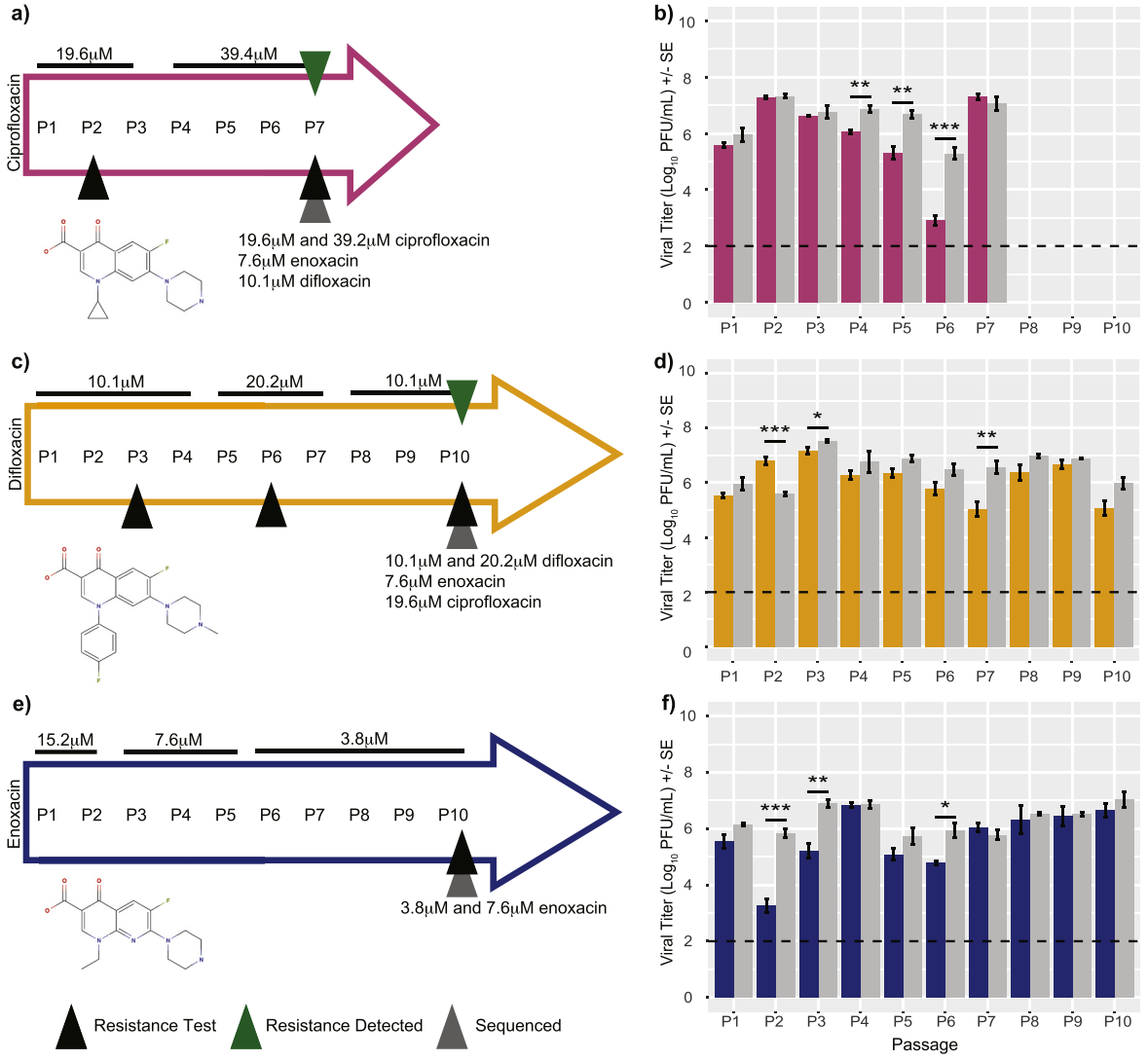

Fig. 1. DENV-4 was passaged in the presence of **(a,b)** ciprofloxacin, **(c,d)** difloxacin, and **(e,f)** enoxacin, at indicated concentrations for 10 total passages or until resistance was detected. Black triangles indicate the passage at which formal resistance tests were conducted at specified concentrations, green triangles indicate when resistance was confirmed, and grey triangles indicate when the genomes (for the ciprofloxacin treatment) or the structural genes (for the difloxacin and enoxacin treatments) were sequenced. Chemical structures of designated fluoroquinolones are depicted. Mean viral titers±SE by passage for DENV-4 passaged with DMEM media control (grey bars, b,d,f) or ciprofloxacin (b, pink bars), difloxacin (d, yellow bars), and enoxacin (f, blue bars). Dashed line indicates the limit of detection. *P < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

initiated at 2x the EC_{50} or 15.2 μM. When viral titers following drug treatment were not high enough to initiate the next passage (enoxacin passages 3 and 6 and difloxacin passage 8), the passage was repeated at half the previous drug concentration. All drug concentrations remained below the cytotoxic concentration 50 (CC_{50}) of 1504.0 μM, 763.0 μM, 537.6 μM for ciprofloxacin, difloxacin, and enoxacin in HEK-293 based on (Scroggs et al., 2020). After 5 days of incubation, viral supernatants were collected in 1x SPG, clarified by centrifugation, and stored at −80 °C. Viral titers were determined by plaque assay in HEK-293 cells.

2.4. Antiviral resistance assays

Resistance was suspected when the mean viral titers of fluoroquinolone-passaged viruses were equal to or greater than media-control viruses. When resistance was suspected, a formal resistance test was conducted by infecting duplicate T25 flasks of HEK-293 cells with each of the three lines of virus passaged in that fluoroquinolone, the three control lines, or the parent virus (N = 7 virus lines) at MOI: 1.0. Two hours after infection, one flask of infected cells per virus was treated with the specific fluoroquinolone at a specified concentration (Fig. 1) in 5 mL media and the other flask was treated with 5 mL media. After five days, the viral supernatants were collected, clarified, frozen, and viral titers determined via plaque assay. As shown in Fig. 1, resistance of the ciprofloxacin lineage to ciprofloxacin was suspected and formally tested as described above at passage 2 (P2) but resistance was not detected; resistance was suspected and confirmed at P7. Resistance of difloxacin-passaged lineages to difloxacin was suspected at P3 and P6, but resistance was not detected; resistance was suspected and confirmed at P10. Resistance of enoxacin-passaged lineages to enoxacin was never suspected and was only evaluated at P10.

Ciprofloxacin-resistant lineages (hereafter termed DENV-Cipro A,B or C) were tested for cross-resistance to enoxacin and difloxacin at 7.6 μM and 10.1 μM, the EC_{50} of each drug, respectively, and difloxacin-resistant viruses (DENV-Diflox A,B,C) were tested for cross-resistance to enoxacin and ciprofloxacin at 7.6 μM and 19.6 μM, the EC_{50} of ciprofloxacin, respectively. To test for cross-resistance, triplicate T25 flasks of HEK-293 cells were infected with each of the three lines of viruses resistant to the specified fluoroquinolone, the three control lines, or the parent virus (N = 7 virus lines) at MOI: 1.0. Two hours after infection, one flask of infected cells per virus was treated with the first target drug in 5 mL media, the next infected flask was treated with the second target drug in 5 mL media, and the last infected flask was treated with 5 mL media. After five days, the viral supernatants were collected, clarified, frozen, and viral titers determined via plaque assay.

2.5. Library preparation and high-throughput sequencing of DENV-Cipro, DENV-DMEM, DENV-P

Whole genomes from all DENV-Cipro and DENV-DMEM lineages from passage 7 and the parental virus (DENV-P) were deep sequenced as follows: Libraries for sequencing were prepared with the NEBNext Ultra II RNA Prep Kit (New England BioLabs, Inc.) following the manufacturer's protocol. Briefly, ~70–100 ng of RNA was fragmented for 15 min, followed by cDNA synthesis, end repair and adapter ligation. After 8 rounds of PCR the libraries were analyzed on an Agilent Bioanalyzer and quantified by qPCR. Samples were pooled and sequenced with a paired-end 75 base protocol on an Illumina (Illumina, Inc) NextSeq 550 using the High-Output kit.

Reads were processed with Trimmomatic v0.36 (Bolger et al., 2014) to remove low quality base calls and any adapter sequences. The *de novo* assembly program ABySS v1.3.7 (Simpson et al., 2009) was used to assemble the reads into contigs, using several different sets of reads, and kmer values from 20 to 40. Contigs greater than 400 bases long were compared against the NCBI nucleotide collection using BLAST. A nearly full length DENV-4 viral contig was obtained in each sample. All the remaining contigs mapped to either host cell ribosomal RNA or mitochondria. The trimmed reads from each sample were mapped to the sample consensus sequence with BWA v0.7.17 (Li and Durbin, 2009) and visualized with the Integrated Genomics Viewer v2.3.26 (Robinson et al., 2011b) to confirm a correct assembly.

For single nucleotide variant and insertion/deletion calling the trimmed reads from each sample were mapped to the parental reference sequence (Durbin et al., 2001a) with BWA. The LoFreq v2.1.3.1 (Wilm et al., 2012) call and call-indels commands were used for variant calling, after the mapped reads were preprocessed with the LoFreq viterbi and indelqual commands to fix alignments at the read ends and insert indel quality scores, respectively. Variant calls were made relative to the parent sequence and were filtered at a level of 0.5%. Intrahost genetic diversity was estimated by calculating the mean variant frequency for each replicate (Grubaugh et al., 2015, 2016). Shannon entropy was calculated for each replicate (Grubaugh et al., 2015).

2.6. RNA isolation, RT-PCR, and Sanger sequencing of DENV-Diflox, DENV-Enox, and DENV-P

To determine whether mutations detected in the envelope of DENV-Cipro were also present in virus lineages passaged in the presence of other fluorquinolones, the capsid, pre-membrane, and envelope genes of the DENV-Diflox, DENV-Enox, DENV-DMEM passaged 10 times, and DENV-P were sequenced (n = 10 viruses). Viral RNA was isolated using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) per the manufacturer's protocol. RNA was reverse transcribed and amplified using previously validated (Durbin et al., 2001b) forward (17F: 5′ GGACCGACAAGGACAGTTCCAAAT 3′) and reverse (2546R: 5′ TCTCGCTGGGGACTCTGGTTGAAAT 3′) primers (Eurofins, Louisville, KY) and SuperScript™ III One-Step RT-PCR kit with Platinum™ Taq DNA Polymerase (Invitrogen, Carlsbad, CA) using the following thermocycler conditions: 50 °C for 30 min; 94 °C for 3 min; 9 cycles at 94 °C for 10 s, 60 °C for 30 s, and 68 °C for 4 m; 29 cycles at 94 °C for 10 s, 60 °C for 30 s, and 68 °C for 4 m with 5 s added at each cycle; and a final extension at 68 °C for 7 min. Amplicons were sequenced via Sanger sequencing at the Genomic Analysis Core Facility at the University of Texas at El Paso using the amplification primers listed above as well as four validated internal primers (996R: 5′ ATGCTCCACCTGAGACTCCTTCC 3′, 860F: 5′ GGCTTATATGATTGGGCAAACAGG 3′, 1683R: 5′ CCTGTCTCTTGGCATGAGGAACC 3′, 1551F: 5′ ACATGGCTCGTGCATAAGCAATGG 3′) (Durbin et al., 2001b). The resulting forward and reverse sequences were clipped for quality and aligned in Geneious (version 9.0.5 (Kearse et al., 2012)) with the reference genome (GenBank AY648301.1). Mutations were identified in the passaged viruses compared to DENV-P.

2.7. Replication kinetics

To evaluate the impacts of ciprofloxacin resistance on DENV-4 replication dynamics in human and mosquito cells, 7 T25 flasks of 80% confluent HEK-293, HuH-7, Vero, or C6/36 cells were infected at MOI 0.1, based on the viral titer determined in HEK-293, with each of the DENV-Cipro or DENV-DMEM lineages (both from passage seven) or DENV-P. After 2 h of incubation with occasional rocking, the flasks were washed with 1X PBS and 5 mL of cell-specific media was added to each flask. Viral supernatants were collected on the day of infection as well as 1, 2, 3, 4, 6, and 8 days post-infection (dpi). For replication curves with HuH-7 and Vero cells, the time points for collection were extended to include 9, 10, 11, and 12 dpi as the plateau viral titer was not detected by 8 dpi. At each time point, 1 mL of the media was removed from each flask and 1 mL of fresh media was added back to each flask. The viral supernatants were clarified by centrifugation at 1200 rpm for 10 min at 4 °C, stored at −80 °C in 1X SPG, and viral titers were determined using the same cell line as that used for the replication dynamics (HEK-293, HuH-7, Vero, or C6/36).

2.8. Stability assay

To detect differences in virion stability between DENV-Cipro and DENV-DMEM, 7 T25 flasks of 80% confluent HEK-293 cells were infected at MOI: 0.1 with each of the DENV-Cipro or DENV-DMEM lineages (both from passage seven) or DENV-P. After 2 h of incubation with occasional rocking, the flasks were washed with 1X PBS and 5 mL of cell-specific media was added to each flask. On the expected day of peak viral titer, day 3, the viral supernatants were collected on ice then clarified by centrifugation at 1200 rpm for 10 min at 4 °C. Immediately after centrifugation, 45 μL of each clarified supernatant was added to cryotubes with 405 μL Leibovitz L15 media (Gibco, Life Technologies, Grand Island, NY) and placed into a 37 °C incubator, one tube for each virus and for each time point (0 h post-incubation, 2, 4, 6, 8, 12, 24, and 30). At each time point, the cryotube was removed from the incubator and 50 μL of 10X SPG was added before the tube was snap frozen on a dry ice-methanol bath. Frozen samples were stored at −80 °C and viral titers were determined using HEK-293 cells as described above.

2.9. Mosquito infection

Eggs from *Aedes aegypti* (Rockefeller strain) were hatched and reared as described in (Andrade et al., 2016). Infectious bloodmeals were prepared and mosquito feeding was conducted as described in (Hanley et al., 2008). Briefly, 1 mL of virus was mixed with 2 mL of washed red blood cells from defibrinated rabbit blood (Hemostat, Dixon, CA, USA) in 10% sucrose; 75 μl of 120 mM ATP was added to each meal immediately prior to feeding. Sealed cartons containing ~30 mosquitoes were starved for 24 h, placed under parafilm-covered feeders warmed to 37 °C, and allowed to feed for 20 min. Engorged mosquitoes were separated into new cartons and incubated at 27 °C with 80% relative humidity on a 12-h light and 12-h dark cycle. The mosquitoes were provided 10% sucrose in cotton pledgets *ad libitum*. After 10 days, mosquitoes were cold-killed and stored at −80 °C, after which whole mosquitoes were homogenized in Hank's balanced salt solution (Gibco) supplemented with 10% FBS, 250 μg/ml amphotericin B (Gibco), 1% ciprofloxacin, and 150 μg/mL clindamycin and viral titer was determined as described above in C6/36 cells with the addition of 5 μg/ml amphotericin B to the methylcellulose overlay media.

2.10. Statistical analyses

Viral titer data were log transformed, assessed for normality and then compared using t-tests or two-way ANOVAs as appropriate. Repeated measures ANOVA was used to detect differences in replication kinetics and percent change in viral titer from the stability assay between the ciprofloxacin-resistant and media control viruses. DENV-P was not included in the repeated measures ANOVA as there was only one parental replicate. Differences in variant frequency, Shannon entropy, and mosquito mortality (%) were detected by ANOVA. Differences in variant frequency and Shannon entropy among genes and between treatments were identified using a two-way ANOVA. Differences in the counts of mosquito bodies positive for DENV-4 infection was detected using contingency table analyses. Tukey-Kramer or pairwise t-tests, as specified, were used to ascertain *post hoc* pairwise differences. Statistics were conducted in R using packages: tidyverse, plyr, dplyr, lme4, car, emmeans, lsmeans, and pbkrtest.

3. Results

3.1. Evolution of resistance to each of three fluoroquinolones by DENV

In this study, we passaged DENV in the presence and absence of ciprofloxacin, difloxacin, and enoxacin until resistance was detected or 10 total passages were completed. Drug resistant viruses provide the opportunity to further assess the mechanism-of-action for each drug by identifying mutations associated with resistance and evaluate the consequences of resistance. Resistance was defined as a lack of a difference between the mean viral titers of the media-passaged control viruses cultured in media and viruses passaged in a specific fluoroquinolone cultured in the presence of that fluoroquinolone.

Resistance of the three DENV-4 Cipro lineages to 19.6 μM and 39.2 μM ciprofloxacin, which represent the EC_{50} and twice the EC_{50} of this drug, was assessed after two passages but not detected (Figs. S1a–b). After seven passages with increasing concentration of ciprofloxacin (Fig. 1a and b), DENV-Cipro resistance to ciprofloxacin was detected (Fig. 2a, Fig. S1c). A two-way ANOVA revealed that DENV-DMEM and DENV-Cipro responded significantly differently to the treatment conditions (i.e. treatment with media or ciprofloxacin), indicating that the DENV-Cipro lineages had gained drug resistance ($F(1,8) = 40.7$, $P = 0.0002$). Treatment of both DENV-DMEM and DENV-Cipro with 39.4 μM ciprofloxacin (2x the EC_{50}) resulted in a significant decrease in titer for both virus lineages relative to treatment with media, but the decrease in viral titer of DENV-DMEM after ciprofloxacin treatment was greater than that of DENV-Cipro (4.2 log decrease vs 1.9 log decrease). There was no significant difference in mean viral titers between DENV-DMEM treated with media and DENV-Cipro treated with ciprofloxacin. In the absence of ciprofloxacin, replication of DENV-Cipro was not different than DENV-DMEM, and in the presence of ciprofloxacin, DENV-Cipro replicated to significantly higher levels (2.8 log increase) than DENV-DMEM.

Resistance to difloxacin was evaluated after three passages (Figs. S2a and b) and six passages (Fig. S2c) but was not confirmed. After ten passages (Fig. 1c and d), DENV-DMEM and DENV-Diflox responded significantly differently to the treatment conditions (i.e. treatment with media or difloxacin) at 10.1 μM difloxacin (the EC_{50} of this drug, Fig. 2b) but not 20.2 μM difloxacin (Fig. S2d), indicating that DENV-Diflox had gained resistance to lower concentrations of difloxacin. While treatment with 10.1 μM difloxacin significantly reduced the titer of DENV-DMEM compared to the media treatment, the same concentration of drug did not cause a similar reduction of DENV-Diflox (Fig. 2b). DENV-DMEM and DENV-Diflox were both suppressed by 20.2 μM (2x the EC_{50}) difloxacin to at or below the level of detection and there was not a significant difference between the mean viral titers of the DENV-DMEM treated with media and the DENV-Diflox treated with difloxacin (Fig. 2b).

After DENV-4 was passaged ten times in the presence of enoxacin (Fig. 1e and f), DENV-DMEM and DENV-Enox responded to treatment with media or enoxacin (3.8 μM or 7.6 μM) similarly (Fig. 2c), indicating that DENV-Enox had not gained drug resistance. The mean viral titer for DENV-DMEM treated with media was significantly different from DENV-Enox treated with the EC_{50} for enoxacin (7.6 μM, the EC_{50} of this drug)) (Fig. 2c), although it was not significantly different from the mean viral titer for DENV-Enox treated with half the EC_{50}. These data indicate a trend toward enoxacin resistance but did not satisfy our *a priori* criteria for resistance.

3.2. Mutations associated with fluoroquinolone resistance

To identify mutations associated with fluoroquinolone resistance, the entire open reading frame of all DENV-Cipro, DENV-DMEM, and DENV-P replicates were sequenced, as well as the structural genes of the DENV-Diflox and DENV-Enox replicates. Only two coding mutations became predominant (>50% of all reads) in the DENV-Cipro but not in the DENV-DMEM viruses; these included substitution V15L in domain I of the envelope glycoprotein (E) in two replicates of DENV-Cipro and E417A in domain III of E in one replicate of DENV-Cipro (Table 1). One of these substitutions, E417A, was also found in one replicate of DENV-Diflox (Table 2). Other coding mutations in the E, NS2B and NS4B genes and non-coding mutations in the untranslated regions (UTRs), many of which had been previously identified in the literature as HEK-adapting mutations, were found in at least a subset of both drug-resistant and

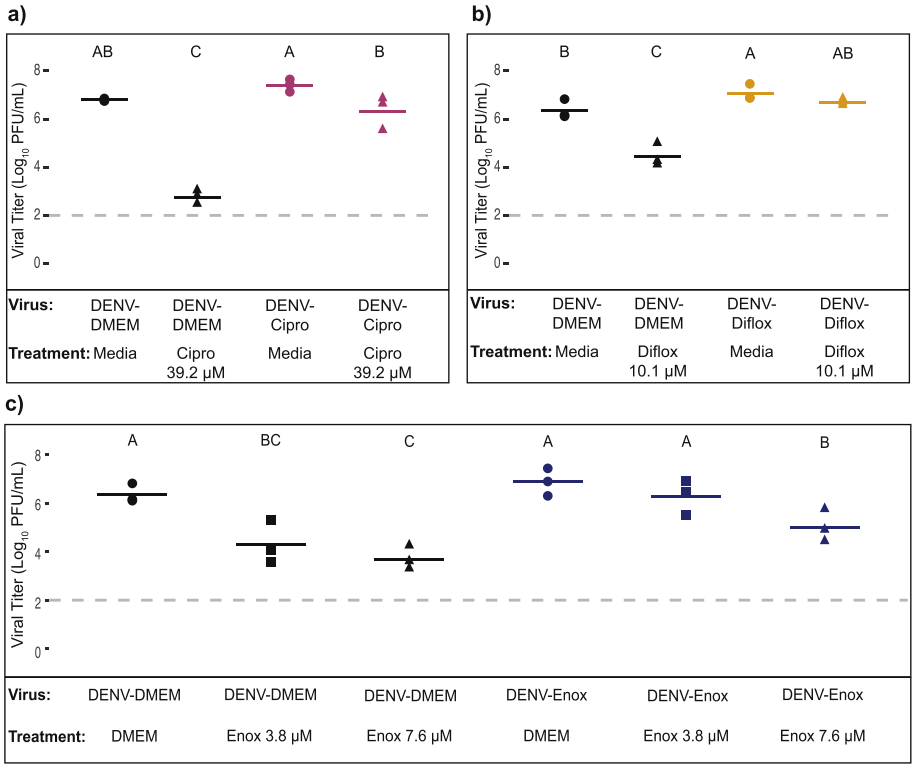

Fig. 2. DENV-4 resistance to ciprofloxacin was detected after 7 passages and difloxacin after 10 passages, but not to enoxacin. **a)** Viral titers for passage 7 DENV-Cipro (pink) and DENV-DMEM (black) after treatment with media (circles) or 39.2 μM (2x EC_{50}) ciprofloxacin (triangles). Two-way ANOVA $F(1,8) = 40.7$, $P = 0.0002$. **b)** Viral titers for passage 10 DENV-Diflox (yellow) and DENV-DMEM (black) after treatment with media (circles) or 10.1 μM (EC_{50}) difloxacin (triangles). Two-way ANOVA $F(1,8) = 13.7$, $P = 0.006$. **c)** Viral titers for passage 10 DENV-Enox (blue) and DENV-DMEM (black) after treatment with media (circles), 3.8 μM (0.5x EC_{50}, squares) or 7.6 μM (EC_{50}, triangles) enoxacin. Two-way ANOVA $F(2,12) = 1.9$, $P = 0.19$. Groups that do not share a letter are significantly different by Tukey pairwise comparisons ($P < 0.05$). Solid lines indicate mean and dashed line indicates the limit of detection. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

Table 1
Nucleotide and amino acid changes for coding mutations identified through deep sequencing in the DENV-Cipro, DENV-DMEM viruses from passage 7 as well as DENV-P.

Gene	Nucleotide change	Amino acid change	Present in DENV-Cipro (P7)			Present in DENV-DMEM (P7)			DENV-P	Possible function
			A	B	C	A	B	C		
5′ UTR	A26G	NA	–	–	–	X	–	–	–	HEK293 adaptation
	A31G	NA	X	–	–	–	–	–	–	HEK293 adaptation
	U40C	NA	–	–	X	–	–	X		HEK293 adaptation
E	G981C	V15L	–	X	X	–	–	–	–	Ciprofloxacin resistance
	A1918G	E327G	X	X	X	X	X	X	–	HEK293 adaptation (Lin et al., 2014; Añez et al., 2009)
	A2188C	E417A	X	–	–	–	–	–	–	Ciprofloxacin resistance
NS2B	C4387U	S85F	X	X	–	–	X	–	–	HEK293 adaptation
NS4B	U6864C	F13L	X	X	–	–	X	–	–	HEK293 adaptation
	C7129U	P101L	–	–	X	X	–	X	–	HEK293 adaptation (Hanley et al., 2003)
	C7141U	T105I	X	X	–	–	X	–	–	HEK293 adaptation (Pletnev et al., 2002)
	G7182C	G119R	–	–	–	–	X	–	–	HEK293 adaptation
	C7546U	A240V	–	X	X	–	–	X	–	HEK293 adaptation (Blaney et al., 2001)
3′ UTR	U10290C	NA	X	X	–	–	X	–	–	HEK293 adaptation
	U10352C	NA	X	X	–	–	X	–	–	HEK293 adaptation

X: Mutation present at at least 0.5 frequency.
-: Mutation not present at at least 0.5 frequency.

Table 2
Nucleotide and amino acid changes for envelope gene identified with Sanger sequencing in the DENV-Diflox, DENV-Enox, DENV-DMEM viruses from passage 10 as well as DENV-P.

Gene	Nucleotide change	Amino acid change	Present in DENV-Diflox (P10)			Present in DENV-Enox (P10)			Present in DENV-DMEM (P10)			DENV-P	Possible function
			A	B	C	A	B	C	A	B	C		
E	A1918G	E327G	X	X	X	X	–	X	X	X	X	–	HEK293 adaptation (Lin et al., 2014; Añez et al., 2009)
	A2188C	E417A	–	X	–	–	–	–	–	–	–	–	Difloxacin resistance

X: Mutation detected.
-: Mutation not detected.

control viruses (Table 1). No consensus mutations were identified in DENV-P compared to the reference sequence (AY648301). Silent mutations within the open reading frame are listed in Table S1.

To assess amino acid conservation across the flaviviruses at positions 15 and 417 in E, we generated a multiple sequence alignment of mosquito-borne, tick-borne, no known vector, and insect-only flaviviruses (Fig. S3). The valine as position 15 is conserved across most mosquito-transmitted flaviviruses, but a leucine occurs in this position in DENV-1 and DENV-3. Position 417 is a glutamic acid in DENV-4 and the tick-borne flaviviruses, an aspartic acid in DENV-1, DENV-2, DENV-3 and the other mosquito-transmitted viruses analyzed, a serine in the two tick-borne flaviviruses analyzed and a threonine in the single insect-only flavivirus analyzed. Notably, an alanine was not detected in this position in any of the viruses analyzed.

3.3. Ciprofloxacin-resistance does not increase interpopulation viral diversity

RNA viruses are capable of rapid intrapopulation diversification due to their high mutation rates (Eigen, 1993; Lauring and Andino, 2010). Populations with variants that cover more sequence space, i.e. more unique variants, are more likely to see minor variants become major variants if those variants increase viral fitness. Variant frequencies of the DENV-Cipro, DENV-DMEM, and DENV-P viral populations were used to evaluate the impacts of ciprofloxacin resistance on inter-population diversity. Additionally, variant frequency and sequencing coverage were used to calculate Shannnon entropy to evaluate complexity of the viral populations.

Overall there was no consistent difference between DENV-Cipro, DENV-DMEM, or DENV-P lineages in diversity. Mean frequency of variants was significantly different across the seven virus populations (Fig. 3a); however pairwise comparisons revealed that the mean variant frequency of only one DENV-Cipro replicate (CB) was significantly higher than the DENV-P population (6.0-fold increase) and one DENV-DMEM replicate population (MC) (3.3-fold increase) (Fig. 3a). Genetic complexity, evaluated using Shannon entropy, was also different for the 7 populations (Fig. 3b), but pairwise comparisons indicate that the only significant difference was between two DENV-Cipro replicates.

Variation in variant frequency among lineages for individual genes was also assessed. The variants for all three viral populations were distributed across the genome (Fig. 4). There was no significant interaction between viral population (DENV-Cipro, DENV-DMEM, DENV-P) and gene in level of Shannon entropy (Two-way ANOVA: $F(18, 331) = 1.02$, $P = 0.44$), indicating that difference in diversity among genes were similar across viral populations. Pairwise analyses for individual genes or UTRs showed that the mean variant frequency of DENV-Cipro populations was higher than that of DENV-P in the 5' UTR, envelope, and NS4B (Fig. 5, Table S2). The mean variant frequency of DENV-Cipro was higher than DENV-DMEM in NS2B and NS4B (Fig. 5, Table S2).

3.4. Cross-resistance between ciprofloxacin and difloxacin does not extend to enoxacin

We evaluated whether DENV resistance to one fluoroquinolone can confer resistance to another. As shown in Fig. 6, the efficacy of difloxacin and ciprofloxacin to suppress DENV-Cipro and DENV-Diflox, respectively, was significantly diminished. Remarkably, enoxacin retained its ability to suppress viral replication of both DENV-Cipro and DENV-Diflox. However, there was some evidence of nascent cross-resistance to enoxacin. The diminution of titer for DENV-DMEM treated with enoxacin (3.7 log decrease) was substantially greater than that for DENV-Cipro (2.1 log decrease). Similarly, diminution of titer for DENV-DMEM treated with enoxacin (3.1 log decrease) was greater than that of DENV-Diflox (2.0 log decrease).

3.5. In the absence of ciprofloxacin, DENV-Cipro viruses are more fit than DENV-DMEM viruses in two lines of human cells but not Vero or mosquito cells

Next we tested whether the acquisition of drug resistance impacted viral fitness, which often decreases with evolution of resistance (Domingo et al., 2019). Multicycle replication curves (Fig. 7) revealed that, in the absence of ciprofloxacin, both DENV-DMEM and DENV-Cipro viruses gained fitness in the HEK-293 cells in which they were passaged relative to DENV-P, and that DENV-Cipro lineages reach equivalent peak titer to DENV-DMEM lineages but declined in titer more slowly (Fig. 7). Viral titers of DENV-Cipro and DENV-DMEM were an average of 2.7 log and 2.5 log higher, respectively, than DENV-P from days 1– 3 p.i. Viral titers of DENV-Cipro were greater than DENV-DMEM on days 3, 4, and 6 p.i.

In HuH-7 cells, replication of DENV-Cipro reached a higher peak titer by day 2 post-infection than DENV-DMEM and sustained that titer for the 12 days over which sampling was conducted (Fig. 7). Viral titers of DENV-Cipro were greater than DENV-DMEM on days 2–12 p.i. In African green monkey kidney cells (Vero), replication of DENV-P, DENV-Cipro, and DENV-DMEM lineages were all remarkably similar and no differences in viral titer were detected (Fig. 7). In contrast to mammalian cells, DENV-P replicated to higher levels in mosquito C6/36 cells compared to DENV-Cipro and DENV-DMEM (Fig. 7).

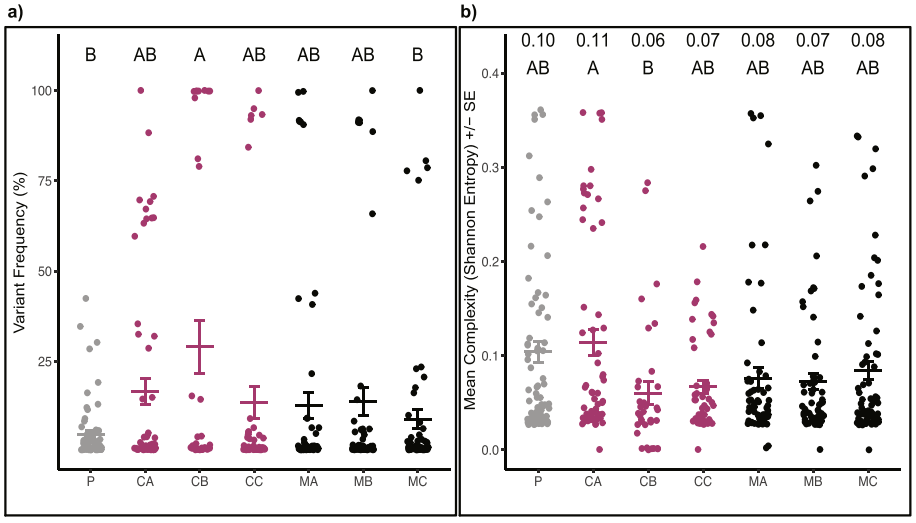

Fig. 3. Ciprofloxacin-resistance does not alter frequency of DENV-4 variants or genetic complexity. **a)** Frequency of occurrence for DENV-4 variants for DENV-P (P; grey), DENV-Cipro (CA, CB, CC; pink), and DENV-DMEM (MA, MB, MC; black) populations (ANOVA $F(6, 394) = 3.51$, $P = 0.002$). **b)** Overall genetic complexity of DENV-Cipro (CA, CB, CC; pink), DENV-DMEM (MA, MB, MC; black) and DENV-P (P; grey) populations ($F(6, 394) = 3.03$, $P = 0.007$). Mean Shannon Entropy values are indicated for each replicate. Groups that do not share a letter are significantly different by Tukey pairwise comparisons ($P < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

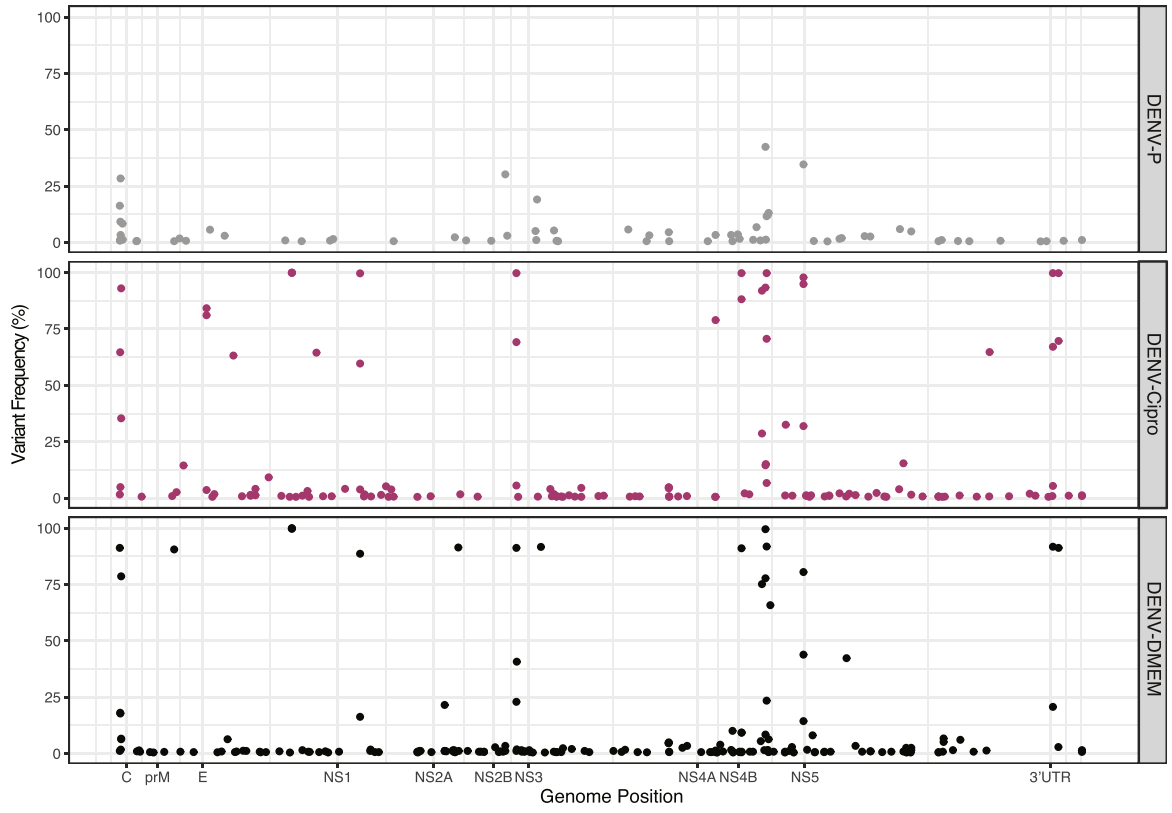

Fig. 4. Variants in the DENV-Cipro and DENV-DMEM populations, pooled by passage treatment, are distributed across the DENV-4 genome. The frequency of occurrence for DENV-4 variants by genome position for DENV-P (grey), DENV-Cipro (pink), and DENV-DMEM (black). The start of each gene and untranslated region are indicated. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

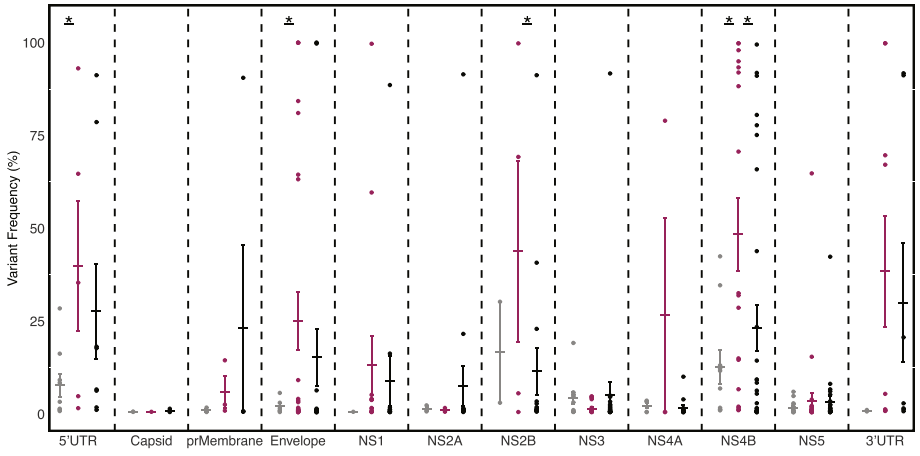

Fig. 5. DENV-Cipro populations experienced higher frequency of variants in the 5′ UTR, envelope, and NS4B. The frequency of occurrence for DENV-4 variants from DENV-P (grey), DENV-Cipro (pink), and DENV-DMEM (black) within each gene and UTR. Differences were detected with a two-way ANOVA ($F_{(22, 365)} = 1.12$, $P = 0.32$) and Tukey pairwise comparisons. Full pairwise statistics for differences within and between genes by treatment are in Table S2. *$P < 0.05$. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.6. DENV-cipro virions are more stable than DENV-DMEM virions

DENV-Cipro and DENV-DMEM achieved similar high titers in HEK-293 cells, but DENV-Cipro titer decline more slowly, suggesting that this virus might have enhanced stability. Thus stability of each lineage was quantified over the course of 30 h, during which both DENV-Cipro and DENV-DMEM showed remarkably greater stability than DENV-P. DENV-Cipro virions were also significantly more stable than DENV-DMEM virions, although this difference only manifested at the last sampling point of the 30-h test (Fig. 8). At 30 h, mean viral titers for DENV-Cipro were 15% less than their initial values at time 0, whereas DENV-DMEM viral titers were reduced by 52% (Fig. 8).

3.7. Neither DENV-Cipro nor DENV-DMEM infected live mosquitoes

To evaluate the impacts of ciprofloxacin-resistance on viral infection and dissemination, *Ae. aegypti* were fed on bloodmeals containing one of each of the three replicates of DENV-Cipro or DENV-DMEM or the single replicate of DENV-P and infection was assessed 10 days post-feeding. Mortality ranged from 5.3% to 63.2% and was not different among viruses (ANOVA $F_{(2,6)} = 1.3$, $P = 0.3$); most mosquitoes that died simply failed to revive after being chilled for sorting. Of the survivors that fed on DENV-P, 24.3% became infected with an average titer of 2.25 $\log_{10}$PFU/body, however, none of mosquitoes that fed on bloodmeals containing DENV-Cipro or DENV-DMEM became infected (Table 3; $\chi^2 =$

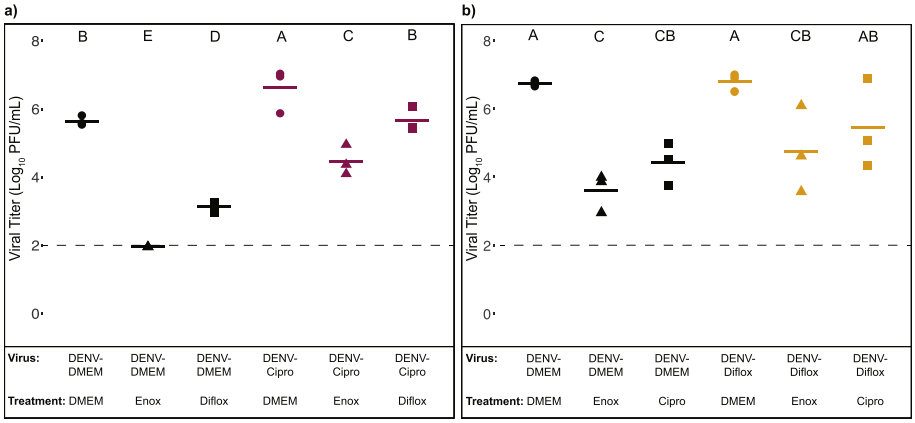

Fig. 6. DENV-Cipro is cross-resistant to difloxacin, but not enoxacin and DENV-Diflox is cross-resistant to ciprofloxacin, but not enoxacin. **a)** DENV-Cipro (pink) and DENV-DMEM (black) titers after treatment with media (circles, 7.6 μM enoxacin (triangles), or 10.1 μM difloxacin (squares). Two-way ANOVA F (2,12) = 8.7, P = 0.005. **b)** DENV-Diflox (yellow) and DENV-DMEM (black) titers after treatment with media (circles), 7.6 μM enoxacin (triangles), or 19.6 μM ciprofloxacin (squares). Two-way ANOVA F (2,12) = 0.8, P = 0.48. Groups that do not share a letter are significantly different by Tukey pairwise comparisons (P < 0.05). Dashed line indicates the limit of detection. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

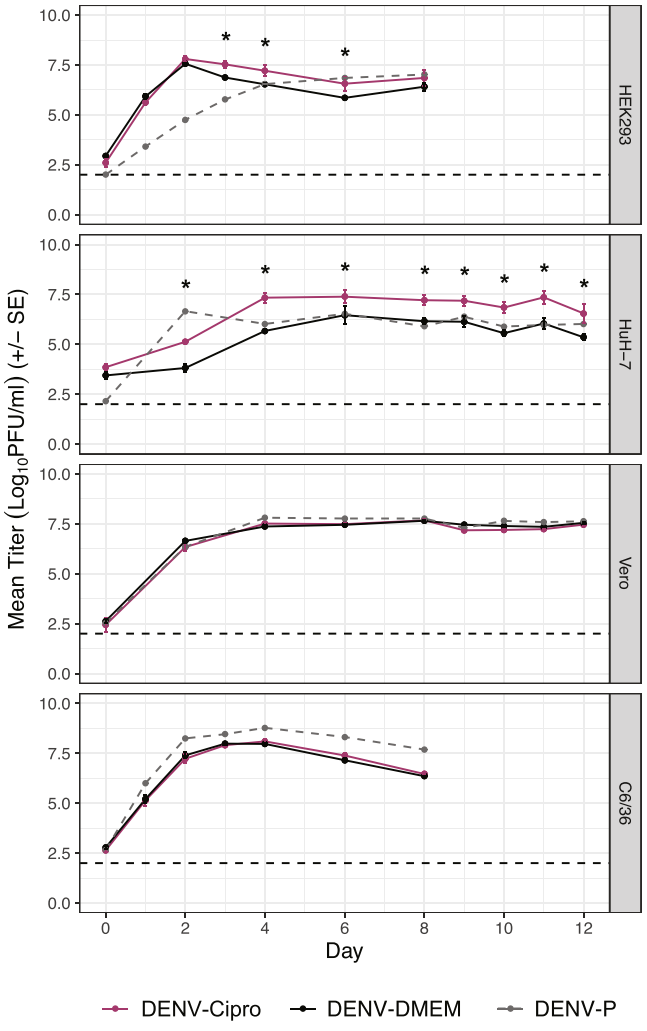

Fig. 7. Replication kinetics of DENV-Cipro (pink solid line), DENV-DMEM (black solid line), and DENV-P (grey dashed line) in HEK-293 (repeated measures ANOVA, F (6,24) = 6.1, P < 0.0006), HuH-7 (repeated measures ANOVA, F (8,32)(= 1.3, P = 0.26), Vero (repeated measures ANOVA, F (8,32) = 1.0, P = 0.43), or C6/36 (repeated measures ANOVA. F (6,24) = 0.6, P = 0.71) cells. Dotted line indicates the limit of detection. *P < 0.05 from Tukey pairwise comparisons. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

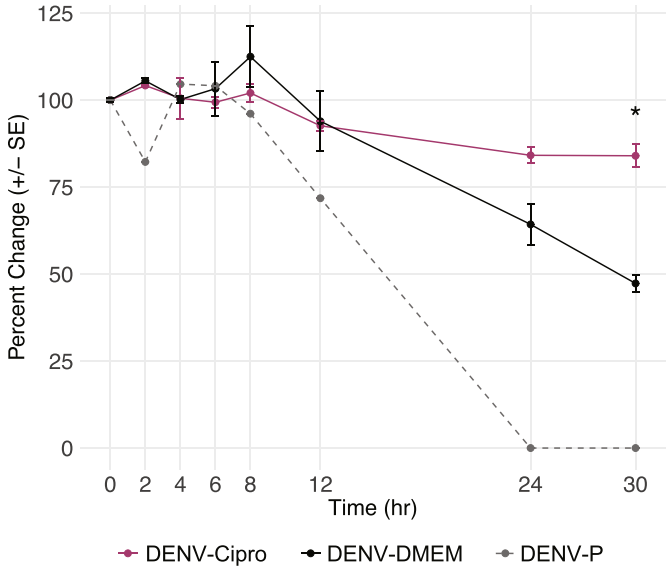

Fig. 8. Virion decay as measured by the percent change from the initial viral titer of DENV-Cipro (pink solid line), DENV-DMEM (black solid line) and DENV-P (grey dashed line). Repeated measures ANOVA, F (7,28) = 1.8, P = 0.13 with Tukey pairwise comparisons. *P < 0.05. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

22.0, df = 2, P < 0.0001). Of the infected mosquitoes that fed on bloodmeals spiked with DENV-P, 45% of the heads were also infected with an average titer of 2.69 $\log_{10}$PFU/head (Table 3), while, as expected based on bodies, the heads of all the mosquitoes who fed on DENV-Cipro or DENV-DMEM were negative for infection.

4. Discussion

We recently demonstrated the efficacy of three fluoroquinolones, ciprofloxacin, difloxacin and enoxacin, against a panel of flaviviruses in cultured cells and A129 mice (Scroggs et al., 2020). In the current study, we investigated the barrier to evolution of resistance and cross-resistance to these drugs, their mechanisms-of-action, and the fitness consequences of resistance. DENV-4 was passaged in triplicate in the presence or absence of each drug until resistance was detected or to a predetermined endpoint of 10 passages.

We found that DENV-4 evolved resistance to ciprofloxacin within seven passages and difloxacin within ten passages but the virus failed to evolve resistance to enoxacin within ten passages, suggesting a higher barrier to resistance against enoxacin than the other two drugs. The

Table 3
Infection of DENV-Cipro, DENV-DMEM, and DENV-P in *Aedes aegypti* mosquitoes 10 days after feeding with an infectious artificial bloodmeal

Virus	Replicate	Number Fed	Bloodmeal titer pre-feeding[a]	Bloodmeal titer post-feeding[a]	% Mortality (n)	% Body Infected (n)	Mean titer (±SE) in infected bodies [b]	% Head Infected (n)	Mean titer (±SE) in infected heads [b]
DENV-Cipro	A	34	5.96	5.61	50.0 (17)	0 (0)	–	–	–
	B	29	6.66	5.52	37.9 (11)	0 (0)	–	–	–
	C	30	5.62	4.34	33.3 (10)	0 (0)	–	–	–
DENV-DMEM	A	28	5.96	3.48	28.6 (8)	0 (0)	–	–	–
	B	30	6.05	4.11	26.7 (8)	0 (0)	–	–	–
	C	19	6.01	3.90	5.3 (1)	0 (0)	–	–	–
DENV-P	A	19	6.58	4.45	63.2 (12)	28.6 (2)	1.88 (0.9)	50.0 (1)	2.19
	B	31	6.43	4.58	16.1 (5)	19.2 (5)	2.69 (0.7)	60.0 (3)	2.41 (0.7)
	C	24	6.45	4.00	33.3 (8)	25.0 (4)	2.19 (0.4)	25.0 (1)	3.48

[a] $\log_{10}$ PFU/mL.
[b] $\log_{10}$ PFU/designated mosquito tissue.

variation in the rate of evolution of resistance could be due to stochasticity in evolution, particularly as our definition of resistance required that all three replicates within a treatment evolve resistance, but variation in resistance evolution could also reflect differences among the drugs in mechanism-of-action. In our previous study of these drugs, time-of-addition assays revealed that enoxacin suppresses intermediate life cycle stages of ZIKV, such as translation of the polyprotein and replication of the genome while ciprofloxacin and difloxacin suppress ZIKV during both early and intermediate life cycles stages, suggesting that ciprofloxacin and difloxacin may share a mechanism-of-action that is different than that of enoxacin (Scroggs et al., 2020). Supporting this contention, ciprofloxacin resistance conferred resistance to difloxacin and vice versa, but neither resistance to ciprofloxacin nor difloxacin conferred resistance to enoxacin according to our *a priori* definition of resistance. However, there were more subtle difference in the responses of the virus lineages to enoxacin that did suggest that resistance to ciprofloxacin or difloxacin did engender some degree of cross-resistance to enoxacin. Differences in biochemical activities among the three drugs are documented (Tang et al., 2002); in particular, while enoxacin strongly enhances RNAi, ciprofloxacin and difloxacin have a moderate and marginal effect on RNAi, respectively (Shan et al., 2008). Cross-resistance does raise concerns about the long-term sustainability of ciprofloxacin and difloxacin as flaviviral inhibitors, but combination therapies with other antiviral compounds that are not similar in structure and do not share a mechanism-of-action may mitigate these concerns.

To gain further insight into the antiviral mechanism-of-action, we sequenced the whole genome of each of the three DENV-Cipro viruses, three DENV-DMEM viruses, and DENV-P. We discovered two coding mutations, both in the envelope glycoprotein, that were unique to the DENV-Cipro viruses. These mutations produced a V15L substitution in two replicates and an E417A substitution in one replicate. E417A was also detected in one replicate of the DENV-Diflox viruses, but neither substitution was detected in the DENV-Enox viruses. DENV-Cipro viruses were sequenced by Illumina while DENV-Diflox and DENV-Enox viruses were analyzed via Sanger sequencing, thus it is possible that the mutations detected in the DENV-Cipro lineages occurred in these two groups at frequencies below the level of detection. Also, resistance to difloxacin was not as complete as resistance to ciprofloxacin, as resistance was confirmed at the EC_{50} (10.1 μM), but not at 2x EC_{50} (20.2 μM). We note that although these findings are suggestive, reverse genetics will be needed to formally confirm the role of these mutations in resistance.

Envelope dimers form the herringbone protein coat of the virion (Chambers et al., 1990). Upon acidification of the endosome, genome release is initiated when domain II folds out at the hinge point located between domain I and II, forming contact points with the cellular vesicle (Modis et al., 2003). V15L occurred in the envelope protein in domain I, a β-barrel that organizes two envelope proteins into a dimer (Modis et al., 2003), while E417A occurred in domain III. The conservative change of valine to leucine at amino acid position 15 is unlikely to cause a major change in protein structure. We have previously shown that ciprofloxacin, difloxacin, and enoxacin suppress DENV-1 (Scroggs et al., 2020), despite the fact that all five DENV-1 genotypes possess a leucine at position 15 (GenBank: MN945984, AF180818, DQ285561, AF425627, ARX70560), suggesting that epistatic interactions between the amino acid at position 15 and other positions within the DENV-4 envelope protein likely shapes the resistance phenotype. In contrast, replacement of a negatively charged glutamic acid at amino acid 417 with a hydrophobic alanine could potentially shift protein structure and function; all vector-borne flaviviruses analyzed possessed a negatively charged amino acid at this position and none of the 13 flaviviruses analyzed possessed an alanine. Additionally, a panel of 9 mutations were shared by resistant and control viruses, many of which have been previously shown to enhance replication in mammalian cells (Blaney et al., 2001; Lin et al., 2014; Hanley et al., 2003; Pletnev et al., 2002; Añez et al., 2009).

Fluoroquinolones have been suggested to inhibit HCV by interfering with the viral helicase (Khan et al., 2012). We did not detect any consensus mutations in NS3, the gene that encodes the DENV-4 helicase, suggesting that ciprofloxacin does not interact with this protein. Fluoroquinolones are known to enhance RNAi (Brackney et al., 2009, 2015). We did not find an increase in the frequency of variants or the genetic diversity of the DENV-Cipro populations. Brackney et al. (2009) have shown that activation of RNAi led to enhanced diversity of West Nile virus. Thus, the lack of association between ciprofloxacin resistance and viral population diversity suggests that ciprofloxacin does not suppress viral replication by enhancing RNAi.

Our findings do suggest that ciprofloxacin and difloxacin may antagonize virus binding or entry. Fluoroquinolones are produced as a byproduct during chloroquine synthesis and share similarities in structure (Fig. 1) (Emmerson and Jones, 2003), and the action of chloroquine may offer a clue about how ciprofloxacin and difloxacin could affect DENV entry. Chloroquine has been shown to suppress multiple viruses including DENV, ZIKV, chikungunya virus, and poliovirus (Shiryaev et al., 2017; Li et al., 2017; Delvecchio et al., 2016; Farias et al., 2013, 2014; Khan et al., 2010; Zeichardt et al., 1985; Kronenberger et al., 1991) likely by blocking endosome acidification and thereby inhibiting virus entry (Delvecchio et al., 2016; Khan et al., 2010; Savarino et al., 2003; Browning, 2014). Interest in chloroquine as an antiviral recently spiked due to its potential utility against the novel coronavirus, SARS-CoV-2, that has caused a massive and ongoing pandemic. Unpublished data suggests that like chloroquine, ciprofloxacin neutralizes the pH level within the lumen of the *trans*-Golgi network of bronchial epithelial cells (Poschet et al., 2020). As antiviral assays are conducted to determine the efficacy of chloroquine to suppress coronaviruses, it would be prudent to evaluate the ability of fluoroquinolones to suppress coronaviruses as well.

Alternatively, mutations in envelope could increase the stability of the protein and thereby slow virion degradation. The envelope protein

of ZIKV and the ZIKV virion as a whole are more stable and infectious at high temperatures than DENV (Kostyuchenko et al., 2016); this thermostability of ZIKV depends on hydrophobic-hydrophilic interactions in the αβ helix of domain II of E (Xie et al., 2018). In this study, both ciprofloxacin-resistant and media control virions were dramatically more stable than the parent virion, and ciprofloxacin-resistant virions were slightly more stable than the media controls. Moreover, both DENV-Cipro and DENV-DMEM had higher stability than that reported for wild type ZIKV (Gallichotte et al., 2017). Both DENV-Cipro and DENV-DMEM share a E327G mutation in domain III of E, which is the most likely cause of their enhanced stability. Añez et al. found that passage of DENV-4 in fetal rhesus lung (FRhL) cells resulted in the same E327G mutation, and that DENV-4 carrying this mutation exhibited an increase in replication and infectivity in FRhL cells compared to its parent (Añez et al., 2009). To bind to a target cell the DENV E protein interacts with heparan sulfate (Chen et al., 1997) and the E327G mutation increases DENV-4 binding affinity for heparan sulfate (Añez et al., 2009). Molecular modeling further predicted that the E327G mutation increased the net positive charge on the surface of the virion and suggested that the mutation created a new heparan sulfate binding site (Añez et al., 2009). The increase in virion stability of DENV-Cipro and DENV-DMEM is likely due to the increased positive charge on the surface of the virion. Importantly, when two rhesus macaques were infected with the DENV-4 passaged in FRhL cells, neither monkey had detectable levels of viremia or antibodies, indicating that a glutamic acid at position 327 is favorable for monkey infection even if a glycine does increase infection, replication, and heparan sulfate binding in cell culture (Añez et al., 2009).

A third possibility is that V15L and/or E417A could increase overall fitness to overcome suppressive effects of ciprofloxacin. The fitness of DENV-Cipro, DENV-DMEM and DENV-P viruses in the absence of the drug was assessed in four different cell lines. Under these conditions, viral titers of DENV-Cipro viruses were greater than those of the DENV-DMEM viruses in HEK-293 and HuH-7 cells. This gain in fitness in the absence of ciprofloxacin contradicts our hypothesis that resistance evolved due to a specific drug evasion. Intriguingly, replication of the three virus lineages were indistinguishable in Vero cells, a cell line that lacks an interferon response (Desmyter et al., 1968; Emeny and Morgan, 1979). It is not clear from our data whether the gain in fitness seen in HEK-293 and HuH-7 cells, but not Vero cells is dependent on the lack of interferon and/or is an artifact of adaptation to human cells. Importantly, we also do not know how this gain in fitness will impact infection *in vivo*.

Further, fitness of the DENV-Cipro and DENV-DMEM viruses were equivalent, but lower than that of DENV-P, in C6/36 cells, a mosquito cell line that lacks a functional RNAi response (Brackney et al., 2010) and both lineages failed to infect live *Ae. aegypti* mosquitoes fed on artificial bloodmeals spiked with these viruses. The failure of the passaged viruses to infect mosquitoes is likely due to the suite of mutations in NS4B that they accrued; some of these mutations have previously been associated with mammalian adaptation and loss of mosquito infectivity (Blaney et al., 2001; Lin et al., 2014; Hanley et al., 2003; Pletnev et al., 2002; Añez et al., 2009). This finding is consistent with the trade-off hypothesis, which states that a gain in fitness in the mammalian host will negatively impact fitness in the mosquito vector (Deardorff et al., 2011; Vasilakis et al., 2009; Ciota et al., 2013). Our data underscore the complexity of viral fitness and how the patterns are likely influenced by a combination of drug resistance-associated and cell culture adaptation mutations.

Fluoroquinolones show some promise for repurposing as antiflavivirals, but this study also suggests several lines of inquiry that should be pursued to further evaluate the consequences of evolution of fluoroquinolone resistance. While the barrier to resistance to enoxacin was relatively high, DENV-4 evolved resistance to ciprofloxacin and difloxacin within ten passages at or near the EC_{50} of each drug. Furthermore, resistance to either ciprofloxacin or difloxacin confers cross-resistance to the other drug. Unexpectedly, mutations associated with resistance occurred in the envelope gene of the virus; the phenotypic impact of these E mutations should be formally assessed using reverse genetics in a future study. These mutations were associated with substantial gains in virion stability and viral fitness, but the degree to which the increase in fitness is attributable to the envelope mutations associated with resistance and how the increase in fitness in human cells will play out *in vivo* remains unknown. Future experiments to interrogate evolution of resistance to these fluoroquinolones *in vivo* are needed to better assess their potential and perils as antiflaviviral therapies.

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CRediT authorship contribution statement

Stacey L.P. Scroggs: Conceptualization, Methodology, Formal analysis, Investigation, Project administration, Validation, Data curation, Visualization, Writing - original draft, Writing - review & editing. **Jordan T. Gass:** Investigation, Writing - original draft, Writing - review & editing. **Ramesh Chinnasamy:** Resources, Writing - review & editing. **Steven G. Widen:** Investigation, Resources, Writing - review & editing. **Sasha R. Azar:** Investigation, Writing - review & editing. **Shannan L. Rossi:** Writing - review & editing. **Jeffrey B. Arterburn:** Funding acquisition, Resources, Supervision, Writing - review & editing. **Nikos Vasilakis:** Funding acquisition, Resources, Supervision, Writing - review & editing. **Kathryn A. Hanley:** Conceptualization, Methodology, Funding acquisition, Formal analysis, Validation, Supervision, Writing - original draft, Writing - review & editing.

Declaration of competing interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2020.09.004.

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