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Research article

Full-genome dengue virus sequencing in mosquito saliva shows lack of convergent positive selection during transmission by *Aedes aegypti*

Sebastian Lequime,[1,2,3,*,†,‡] Vaea Richard,[4,†,§] Van-Mai Cao-Lormeau,[4,††] and Louis Lambrechts[1,2,**]

[1]Insect-Virus Interactions Group, Department of Genomes and Genetics, Institut Pasteur, 28 rue du Docteur Roux, 75015 Paris, France, [2]Centre National de la Recherche Scientifique, Unité de Recherche Associée 3012, 25-28 rue du Docteur Roux, 75015 Paris, France, [3]Université Pierre et Marie Curie, Cellule Pasteur UPMC, 4 place Jussieu, 75005 Paris, France and [4]Unit of Emerging Infectious Diseases, Institut Louis Malardé, BP 30, 98713 Papeete, Tahiti, French Polynesia

[*]Corresponding author: E-mail: sebastian.lequime@kuleuven.be

[†]Contributed equally to this work.

[‡]http://orcid.org/0000-0002-3140-0651

[§]http://orcid.org/0000-0001-8132-0740

[††]http://orcid.org/0000-0001-5606-7480

[**]http://orcid.org/0000-0001-5958-2138

Abstract

Like other pathogens with high mutation and replication rates, within-host dengue virus (DENV) populations evolve during infection of their main mosquito vector, *Aedes aegypti*. Within-host DENV evolution during transmission provides opportunities for adaptation and emergence of novel virus variants. Recent studies of DENV genetic diversity failed to detect convergent evolution of adaptive mutations in mosquito tissues such as midgut and salivary glands, suggesting that convergent positive selection is not a major driver of within-host DENV evolution in the vector. However, it is unknown whether this conclusion extends to the transmitted viral subpopulation because it is technically difficult to sequence DENV genomes in mosquito saliva. Here, we achieved DENV full-genome sequencing by pooling saliva samples collected non-sacrificially from 49 to 163 individual *Ae. aegypti* mosquitoes previously infected with one of two DENV-1 genotypes. We compared the transmitted viral subpopulations found in the pooled saliva samples collected in time series with the input viral population present in the infectious blood meal. In all pooled saliva samples examined, the full-genome consensus sequence of the input viral population was unchanged. Although the pooling strategy prevents analysis of individual saliva samples, our results demonstrate the lack of strong convergent positive selection during a single round of DENV transmission by *Ae. aegypti*. This finding reinforces the idea that genetic drift and purifying selection are the dominant evolutionary forces shaping within-host DENV genetic diversity during transmission by mosquitoes.

Key words: *Aedes aegypti*; dengue virus; mosquito saliva; virus evolution.

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1. Introduction

With an estimated 390 million infections each year (Bhatt et al. 2013), dengue viruses (DENVs) are the most important arthropod-borne viruses (arboviruses) affecting humans. DENVs are mosquito-borne RNA viruses (*Flavivirus*, Flaviviridae) that exist as four related serotypes (DENV-1 to DENV-4), which are phylogenetically discrete and for the most part antigenically distinct (Katzelnick et al. 2015). Each serotype can be further divided into different genotypes based on phylogenetic clustering. For instance, five (Weaver and Vasilakis 2009) to six (Pyke et al. 2016) DENV-1 genotypes have been described to date. DENVs are mainly transmitted among humans by the yellow fever mosquito, *Aedes aegypti* (Lambrechts et al. 2010). Mosquitoes become infected after taking an infectious blood meal from a viremic person. The virus first infects the midgut and then spreads systemically to the rest of the insect‘s body. DENV transmission to another human host occurs during a subsequent blood meal when viral particles are released into saliva several days after the initial infectious blood meal.

Owing to their poor replication fidelity, high replication rate, and large population sizes, DENVs, like other RNA viruses, evolve as a diverse population of variants within their host. This 'swarm' of variants is considered critical for pathogenesis, viral fitness (both replicative fitness and transmission fitness), and adaptive potential of RNA viruses (Domingo et al. 2012). Understanding the evolutionary forces shaping the within-host genetic diversity of arboviruses in their vector is important because adaptation of novel virus variants during transmission may result in emergence events (Brault et al. 2004; Moudy et al. 2007; Tsetsarkin et al. 2007). Upon infection of the mosquito vector, within-host arbovirus populations may undergo not only natural selection but also genetic drift due to population bottlenecks that occur during traversal of anatomical barriers (Forrester et al. 2014; Franz et al. 2015; Lambrechts and Lequime 2016). Recent studies established that within-host DENV evolution in the vector is primarily driven by genetic drift and purifying selection (Sessions et al. 2015; Sim et al. 2015; Lequime et al. 2016). Importantly, there was no evidence for convergent adaptive mutations (i.e. that would be consistently positively selected) during mosquito infection because consensus changes (frequency >50%) in the DENV genome were either undetectable (Sim et al. 2015) or random (Lequime et al. 2016).

A significant limitation of these earlier studies is the lack of information on the DENV subpopulation that is ultimately transmitted to the next host. Some of these studies examined the viral genetic diversity in mosquito salivary glands as a whole, but the DENV subpopulation present in salivary secretions was not investigated. DENV genomes detected in salivary glands may not be representative of the viral particles released in saliva because active viral replication in salivary glands may increase the proportion of defective genomes (Raquin and Lambrechts 2017) and/or because only a variable fraction of the viral population present in the salivary glands may be released in saliva (Grubaugh et al. 2016, 2017). The importance of studying the transmitted viral subpopulation is exemplified by adaptive mutations associated with the emergence of chikungunya virus variants with epidemic potential that were significantly enriched in saliva but not in the corresponding mosquito bodies and salivary glands (Stapleford et al. 2014).

Arguably, the main reason underlying this knowledge gap is the technical difficulty to sequence DENV genomes directly from *Ae.* mosquito saliva samples. Mosquito saliva is typically collected by an *in vitro* forced salivation method (Aitken 1977), but this technique only recovers minute amounts of virus, varying from a few ten to a few hundred infectious viral particles (Poole-Smith et al. 2015). Virus detection in mosquito saliva generally relies on tissue-culture assays (Bian et al. 2010; Vazeille et al. 2010; Lambrechts et al. 2012) or prior amplification by intrathoracic mosquito inoculations followed by molecular detection (Nguyen et al. 2013; Whitehorn et al. 2015). Such amplification methods may lead to changes in the genetic composition of the arboviral population (Sessions et al. 2015; Stapleford et al. 2016), and even result in spurious signals of adaptive evolution (McWhite et al. 2016). This technical limitation has been a major hurdle to extend conclusions obtained from analysis of within-host DENV genetic diversity in mosquito midguts and salivary glands.

Here, we report the first full-genome sequencing of DENV-1 in mosquito saliva samples without prior biological amplification of the viral population. We used a non-sacrificial method to collect mosquito saliva in time series (van den Hurk et al. 2007) followed by high-throughput sequencing of pooled saliva samples without any manipulation other than sequencing library preparation. We successfully obtained a full-length consensus genome sequence for several pooled saliva samples, and for one of them sequencing depth was sufficient to analyze the pooled viral population genetic diversity. Our technical achievement allowed a relevant assessment of convergent positive selection during a single round of DENV transmission by *Ae. aegypti* mosquitoes.

2. Materials and methods

2.1 Ethics statement

Virus isolates used in this study were previously obtained from the serum of anonymized patients in 2008 and 2013. As these viruses were isolated in cell culture for diagnostic purposes (unrelated to this study), informed consent of the patients was not necessary because the isolates were no longer considered human samples.

2.2 Virus and mosquitoes

Two DENV-1 isolates from French Polynesia, PF08/080108-88 (Genotype IV) and PF13/190813-45 (Genotype I), were obtained from the serum of infected patients in 2008 and 2013, respectively. Each isolate was amplified in *Aedes albopictus* C6/36 cells (Igarashi 1978) (ATCC CRL-1660, USA) by three (PF13/190813-45) or five (PF08/080108-88) successive passages. The final cell culture supernatant was concentrated using Centricon Plus-70 centrifugal filter devices (Millipore, Germany) as described previously (Richard et al. 2015). The supernatant was mixed 5:1 with heat-inactivated foetal bovine serum (FBS; Life Technologies, USA) and stored at -80 °C until use. To estimate viral titer, serial ten-fold dilutions of virus stock were inoculated onto C6/36 cells in a 96-well plate. After 7 days of incubation, virus was detected by indirect immunofluorescence (Richard et al., 2015) and the 50 per cent tissue-culture infectious dose ($TCID_{50}$/mL) was calculated using the method of Reed and Muench (1938).

A laboratory colony of *Ae. aegypti* initially collected from Toahotu, Tahiti, French Polynesia was used between the 9th and the 12th generations (Experiment A) and between the 11th and the 14th generations (Experiment B). Eggs were hatched under low pressure in tap water. Larvae, pupae, and adult mosquitoes were reared as described previously (Richard et al. 2016)

in a climatic chamber (Sanyo MLR-351 H, Japan) set at 27 °C, 80 per cent relative humidity and under a 12:12 h light-dark cycle.

2.3 Experimental infections

The entire experimental approach is summarized in Fig. 1. Mosquitoes were fed on a blood meal containing either a DENV-1 genotype I (Gen. I) isolate or a DENV-1 genotype IV (Gen. IV) isolate. The experiment was repeated twice (referred to as Experiments A and B). An artificial infectious blood meal was offered to 4- to 5-day-old (Experiment A) or 5- to 6-day-old (Experiment B) mosquitoes previously starved and water-deprived for 24 hours. The infectious blood meal consisted of a viral suspension mixed with washed bovine cells and adenosine triphosphate (A6419, Sigma-Aldrich, USA) at a final concentration of 5 mM. The volume of viral suspension was adjusted to reach a final titer of 10^8 $TCID_{50}$/mL in the blood meal. The infectious blood meal was provided to mosquitoes through a Parafilm-M membrane stretched over artificial feeders maintained at 37 °C by a water-circulating system (Fig. 1A). After 30 minutes, fully engorged females were sorted and placed into individual plastic containers to avoid horizontal DENV transmission during sugar feeding (Doggett et al. 2001; van den Hurk et al. 2007). Mosquitoes were maintained in the climatic chamber for 14 days under the conditions described above. In order to collect mosquito saliva non-sacrificially, a strip of filter paper (coffee filter paper no 4; Carrefour, France) was placed on the nylon mesh covering the incubation containers and soaked at its extremity in a 10 per cent (m/v) sugar solution (Fig. 1B).

At 7, 10, 13, and 14 days after oral exposure to DENV-1, surviving mosquitoes were counted and the strips of filter paper were collected and replaced with new ones. At each time point, all strips corresponding to the same virus isolate were pooled together in NucliSENS lysis buffer (bioMérieux, France) (Fig. 1C). Samples were vigorously vortexed during 20 seconds and incubated at room temperature (20°–25 °C) for 10 minutes to release nucleic acids from the filter paper. Nucleic acids were extracted with the NucliSENS miniMAG system (bioMérieux) according to the manufacturer‘s instructions. Viral RNA was detected by real-time reverse transcription polymerase chain reaction (RT-PCR) on a CFX96 Touch Real-Time PCR Detection System instrument using iScript One-Step RT-PCR Kit for Probes (Bio-Rad Laboratories, France) with primers and probe as described previously (Johnson et al. 2005; Aubry et al. 2012).

After 14 days of incubation, 18 (Experiment A) or 20 (Experiment B) surviving mosquitoes were collected in individual microtubes and stored at -80 °C until processing. Each mosquito was homogenized with metal beads for 4 minutes at 20 Hz (Mixer Mill Retsch MM301, Germany) in cell-culture medium supplemented with 20 per cent FBS. Homogenates were clarified by 5-minute centrifugation at 20,000 *g*. Nucleic acids were extracted from supernatants and real-time RT-PCR was performed as described above to determine the proportion of infected mosquitoes.

2.4 Virus sequencing

Total RNA was DNAse treated (Turbo DNAse; Life Technologies, USA), purified with magnetic beads (Agencourt RNAClean; Beckman Coulter, USA) and reverse transcribed using Transcriptor High Fidelity cDNA Synthesis Kit and random hexamers (Roche Applied Science, Germany). Second strand was synthetized in a single reaction with *Escherichia coli* DNA ligase (New England Biolabs, USA), *E. coli* DNA polymerase I (New England Biolabs), *E. coli* RNAse H (New England Biolabs) in second-strand synthesis buffer (New England Biolabs). Resulting dsDNA was purified with magnetic beads (Agencourt AMPure XP, Beckman Coulter) and its concentration was measured by fluorometric quantification (Quant-iT PicoGreen dsDNA, Invitrogen, USA).

Sequencing libraries were prepared using Nextera XT DNA Library Preparation Kit (Illumina, USA), multiplexed and sequenced in single end on an Illumina NextSeq 500 platform using a mid-output 150-cycle v2 kit (Illumina). Sequencing reads were demultiplexed using bcl2fastq v2.15.0 (Illumina). Raw sequences were deposited in the NCBI Sequence Read Archive (Bioproject accession number PRJNA382618). After demultiplexing, reads were trimmed to remove Illumina adaptor sequences

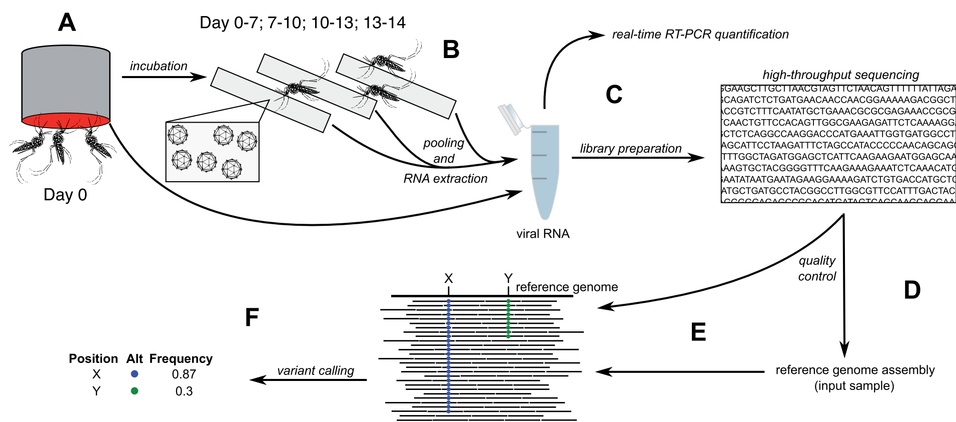

Figure 1. Schematic representation of the experimental approach. (A) Mosquitoes were exposed to an infectious blood meal containing 10^8 $TCID_{50}$/mL of either one of two DENV-1 isolates (Gen. IV and Gen. I). (B) Fully blood fed mosquitoes were sorted and incubated individually with permanent access to strips of filter paper soaked in 10 per cent sucrose solution to collect saliva samples non-sacrificially. Strips of filter paper were collected and replaced at 7, 10, 13, and 14 days after exposure. (C) Strips of filter paper were pooled by virus isolate and time point. Total RNA was extracted and purified from the pooled saliva samples and from the blood-meal samples, and the amount of viral RNA was estimated by real-time RT-PCR. The remainder of RNA was subjected to library preparation and high-throughput sequencing. (D) Sequencing reads from the blood-meal samples were trimmed according to their quality and used to assemble full-length viral genomes *de novo*. (E) After quality control, sequencing reads from the pooled saliva samples were aligned to the previously assembled reference genome. (F) For each nucleotide position of the reference viral genome, variants were called based on sequencing quality and depth. For each sample, variants with a frequency >50 per cent would become the consensus sequence.

and remaining reads shorter than 32 nucleotides were discarded using Trimmomatic v0.33 (Bolger et al., 2014).

Full-length DENV genomes were reconstructed using a two-step procedure (Fig. 1D). First, reads from the input (blood meal) samples were assembled *de novo* using Ray v2.0.0 (Boisvert et al. 2012). The largest contig with DENV homology was extended in 3' and 5' using the full DENV-1 genome of the closest BLAST hit (accession numbers DQ672561 and FJ432719 for PF08/080108-88 and for PF13/190813-45, respectively). Second, this chimeric sequence was used as the reference to map the reads from input samples using Bowtie v2.1.0 (Langmead and Salzberg 2012). The alignment file was converted, sorted, and indexed using Samtools v0.1.19 (Li et al. 2009). Coverage and sequencing depth were assessed for each input sample using bedtools v2.17.0 (Quinlan and Hall 2010). Only nucleotides with $>10\times$ of sequencing depth were retained to generate the consensus sequence and correct the chimeric sequence. Final full-length genome sequences for both DENV-1 isolates were deposited to GenBank (accession numbers KY926848 and KY926849 for isolates PF13/190813-45 and PF08/080108-88, respectively).

After quality control, reads from saliva samples were mapped to the newly assembled DENV genome sequences using Bowtie v2.1.0 (Langmead and Salzberg 2012) (Fig. 1E). The alignment file was converted, sorted, and indexed using Samtools v0.1.19 (Li et al. 2009), and the coverage and sequencing depth were assessed for each sample using bedtools v2.17.0 (Quinlan and Hall 2010). Single nucleotide variants (SNVs) and their frequency were called using LoFreq* v2.1.1 (Wilm et al. 2012) (Fig. 1F).

2.5 Statistical analyses

All statistical analyses were performed in the statistical environment R v3.3.2 (R Core Team 2008) using the packages car (Fox and Weisberg 2011), MASS (Venables and Ripley 2002), pscl (Jackman 2015), plyr (Wickham 2011), and stringr (Wickham 2017). Figures were prepared using the package ggplot2 (Wickham 2009).

Infection prevalence was analyzed as a binary response variable by logistic regression and analysis of deviance as a function of experiment (1 degree of freedom), virus genotype (1 degree of freedom), and their interaction. Real-time RT-PCR cycle threshold (Ct) values were analyzed as continuous response variables by analysis of variance (ANOVA), as a function of experiment, virus genotype, day of sampling (1 degree of freedom), and their interactions up to the second order. Mean depth of coverage was analyzed as continuous response variable by ANOVA, as a function of pool size (number of strips).

Models including interactions were analyzed with type-III analysis of deviance (prevalence) or ANOVA (Ct values), whereas models without interactions were analyzed with type-II analysis of deviance (prevalence) or ANOVA (Ct values). Non-significant terms ($P > 0.05$) were removed in a stepwise fashion to obtain the minimum adequate model. McFadden's pseudo R^2 was calculated as a goodness-of-fit metric for logistic regression. Validity of ANOVA was assessed at each step by examination of quantile–quantile (Q–Q) plots of residuals and formal assessment of their normal distribution by a Shapiro–Wilk test.

The impact of mosquito transmission on pooled viral genetic diversity was assessed by comparing SNV frequencies between input (blood meal) and output (saliva) DENV populations for one pooled saliva sample with a sequencing depth of about $1{,}000\times$. For each SNV, the absolute value of the change in frequency during mosquito transmission was computed. The statistical significance of the change in SNV frequency was tested by using two technical replicates (blood meal of Experiments A and B for isolate PF08/080108-88) to estimate measurement error. The mean change in SNV frequency during mosquito transmission was compared with the mean measurement error in SNV frequency using ANOVA. To satisfy model assumptions, absolute values of changes in SNP frequencies were normalized by a Box-Cox power transformation, of which parameter λ was estimated by computing and selecting the highest log-likelihood ($\lambda = 0.163$). Validity of the model assumptions was assessed by the examination of Q–Q plots of residuals and formal assessment of their normal distribution by a Shapiro–Wilk test.

3. Results

3.1 Experimental mosquito infection

In two separate experiments (denoted A and B), *Ae. aegypti* mosquitoes were exposed to an infectious blood meal containing an expected titer of 10^8 $TCID_{50}$/mL of a DENV-1 isolate belonging to either genotype IV (Gen. IV) or genotype I (Gen. I). In Experiment A, 51 (Gen. I) and 52 (Gen. IV), and in Experiment B, 163 (Gen. I) and 110 (Gen. IV) female mosquitoes were fully blood fed. After blood feeding, mosquitoes were individually incubated for 14 days and provided with strips of filter paper soaked in sucrose solution. At 7, 10, 13, and 14 days after exposure, the strips were replaced and live mosquitoes were counted to assess survival. Strips of filter paper were pooled by experiment and virus isolate to represent the entire cohort of mosquitoes at each time point. The survival rate was >95 per cent at 14 days after virus exposure with the exception of mosquitoes exposed to the Gen. I isolate in Experiment A, for which only 53 per cent survived until Day 14 post exposure. No obvious explanation was identified for this higher mortality rate. Mosquitoes exposed to both isolates in Experiment A were derived from the same generation of the same population and were maintained under identical conditions before and after the infectious blood meal. Overall, the pools of saliva samples represented groups of 49–163 individual mosquitoes at each time point.

The proportion of infected mosquitoes was determined by real-time RT-PCR 14 days after virus exposure. Both the virus isolate ($P < 0.0001$) and the experiment ($P = 0.0001$) significantly influenced the infection prevalence. The proportion of infected mosquitoes 14 days after exposure was significantly higher ($P = 0.0009$) in Experiment B than in Experiment A and significantly higher ($P < 0.0001$) for the Gen. IV isolate than for the Gen. I isolate (Fig. 2).

The amount of viral RNA was estimated in the input (blood meal) and output (pools of saliva collected on strips of filter paper) samples by real-time RT-PCR. Ct values ranged from 8.9 to 15.7 for input samples. In Experiment A, Gen. I output samples resulted in Ct values ranging from 29.7 to 33.7, whereas the Gen. IV output samples resulted in Ct values ranging from 19.5 to 24.0 (Table 1). In Experiment B, Gen I. output samples resulted in Ct values ranging from 19.9 to 21.7, whereas the Gen. IV output samples resulted in Ct values ranging from 20.1 to 22.7 (Table 1). Virus isolate ($P < 0.0001$), experiment ($P < 0.0001$), and their interaction ($P < 0.0001$) significantly influenced Ct values of saliva samples. The significant interaction was primarily driven by the higher Ct values of the Gen. I isolate in Experiment A. As mentioned above, mosquito mortality was unexpectedly high in this group (47% on Day 14) and the proportion of infected mosquitoes (16.7%) was too low for a meaningful analysis; therefore, the Gen. I isolate in Experiment A was excluded from further study.

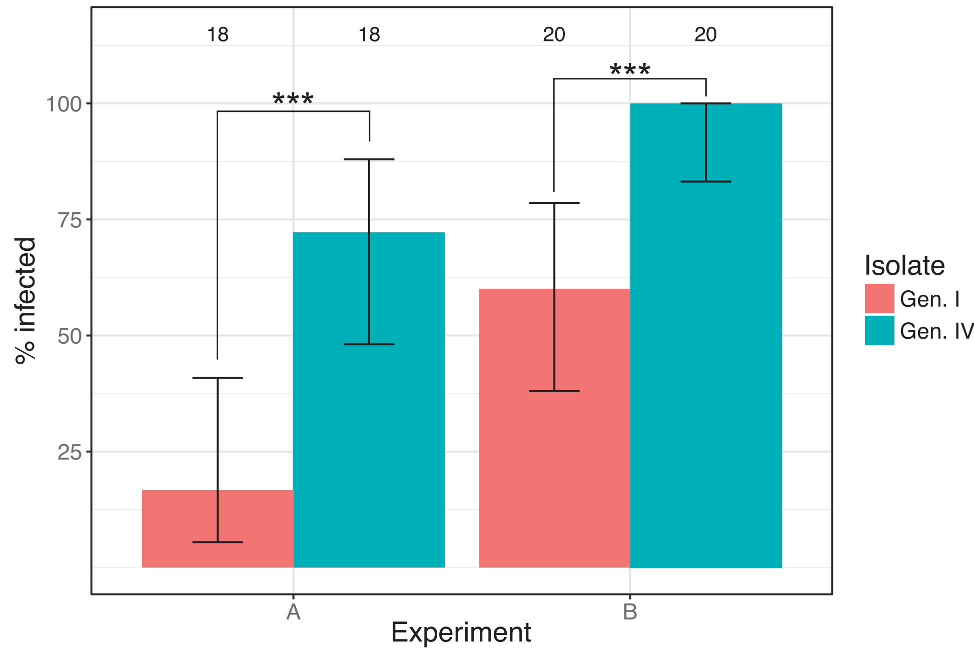

Figure 2. Percentage of DENV-infected individuals by experiment and virus isolate. Bars show the percentage of DENV-positive mosquitoes and their 95 per cent confidence intervals 14 days after virus exposure. Numbers above the bars represent the sample size. ***$P < 0.001$.

Table 1. Virus sequencing summary.

Experiment	Virus isolate	Time point (days)	Ct value	Total reads	Uniquely aligned	Mean depth (in X)	% positions >10×	% positions >100×
A	Gen. IV	0	15.7	2,528,392	2,092 ,887	20959.18	100.00	100.00
		7	24.04	1,770 ,271	11,818	84.32	99.82	23.54
		10	**19.47**	**1,691 ,572**	**117,364**	**949.44**	**100.00**	**99.91**
		13	22.11	2,148 ,477	13,970	97.04	99.91	44.79
		14	22.02	477,134	12,998	107.89	99.81	56.38
A	Gen. I	0	8.9					
		7	29.69					
		10	30.32			Not sequenced		
		13	31.53					
		14	33.65					
B	Gen. IV	0	9.6	2,149 ,085	1,849 ,117	20085.23	100.00	100.00
		7	22.7	1,599 ,062	14,670	98.34	99.83	44.73
		10	22.1	577,148	7,916	65.88	99.65	2.99
		13	22.6	1,238 ,164	4,531	39.15	99.56	0
		14	20.1	2,472 ,130	31,546	242.04	100.00	98.96
B	Gen. I	0	11.4	3,683 ,138	2,252 ,061	19226.51	100.00	100.00
		7	21.7	2,769 ,240	151	1.37	0	0
		10	20.9	1,615 ,345	4,162	29.39	98.81	0
		13	20.7	1,061 ,725	2,332	18.42	92.71	0
		14	19.9	356,125	9,736	78.99	99.82	16.89

Time points are in days post virus exposure. Day 0 represents input (blood-meal) samples, whereas other time points represent output (pooled saliva) samples. The pooled saliva sample with sufficient sequencing depth to analyze viral population genetic diversity is indicated in bold font.

Among the remaining infected samples (i.e. infected mosquitoes of the Gen. I isolate in Experiment A and all infected mosquitoes in Experiment B), Ct values did not significantly differ between experiments or virus isolates.

3.2 Viral genetic diversity

The three blood-meal samples analyzed yielded an average of 2,064,688 uniquely aligned DENV-1 reads resulting in a mean sequencing depth of 20,090×. Eleven of the twelve saliva samples sequenced yielded between 2,332 and 117,364 reads (median: 11,818) that uniquely aligned to the reference DENV-1 genomes (Table 1). Mean sequencing depth ranged from 18.42 to 949.44× (median: 84.32×) for these eleven samples and was not influenced by the number of strips in the pool. One sample only had 151 uniquely aligned reads to the reference DENV-1 genome, resulting in a mean sequencing depth of about 1× (Table 1). Reconstruction of the full-genome consensus

sequence was possible for the eleven well-covered samples, which had >10× of sequencing depth on 92.71 per cent to 100 per cent (median: 99.82%) of the viral genome.

Deep coverage of input (blood-meal) samples allowed detection of low-frequency SNVs down to 0.5 per cent (Supplementary Table S1). Among the eleven pooled saliva samples that were successfully sequenced, the consensus sequences for all samples were exactly identical to that of the corresponding input virus.

One output sample (Experiment A, Gen. IV, 10 days post exposure) had sufficient sequencing depth (about 1,000×) to allow analysis of minority variants present in the pooled viral population (Table 1). All SNVs detected in this saliva sample were also found in the corresponding blood-meal sample. Using two technical replicates of the blood-meal samples to estimate measurement error, there was a statistically significant increase ($P < 0.0001$) in the absolute change of SNV frequency between input and output viral populations for this sample. However, the absolute change in frequency was always <4 per cent (Fig. 3).

4. Discussion

In this study, we combined high-throughput sequencing and a non-sacrificial mosquito salivation technique (van den Hurk et al. 2007) to successfully sequence, for the first time, full-length DENV genomes from pooled saliva samples without prior biological amplification in cell culture or *in vivo*. Sequencing depth was sufficient to reconstruct full-length DENV genomes for the vast majority (eleven out of twelve) of the pooled saliva samples tested. The viral genome sequences that we reconstructed from the saliva samples represent the most frequent nucleotide at each position in the transmitted DENV subpopulation from the entire cohort of mosquitoes over time. The pooling strategy allowed evaluation of the effect of mosquito transmission on the collective viral population, however it did not allow the analysis of viral populations on the individual mosquito level. In all pooled saliva samples and at all time points examined, the consensus genome sequence of the transmitted viral subpopulation did not differ from the consensus sequence of the original viral population present in the infectious blood meal.

Because the pooled saliva samples likely contain the saliva from a large number of mosquitoes, the lack of detectable changes in the consensus viral sequence does not preclude transmission of different virus variants, possibly reaching consensus level, by each individual mosquito. A new variant would only have been detected if its frequency exceeded 50 per cent in the collective viral population. That could have occurred if a large proportion of mosquitoes transmitting this variant at intermediate or high frequency had contributed to the pooled saliva sample at a given time point. Alternatively, it could have occurred if a small number of mosquitoes transmitting this variant at high frequency would have disproportionately contributed to the pooled saliva sample at a given time point. In both cases, such a variant reaching consensus level in the collectively transmitted DENV subpopulation would likely reflect adaptive evolution driven by convergent positive selection. Therefore, the overall lack of detectable consensus changes supports the hypothesis that convergent positive selection was not a major driver of within-host evolution in our experimental model of DENV transmission by *Ae. aegypti*. This observation is consistent with our previous study of the within-host evolution of DENV-1 populations in *Ae. aegypti*. Although different virus variants reached consensus level (frequency >50%) in the salivary glands of individual mosquitoes, they were not shared among different mosquitoes (Lequime et al. 2016). Because of the random nature of variants reaching consensus level at the mosquito individual level, pooling individual samples, like in this study, is expected to recapitulate the consensus sequence at the mosquito population level.

Likewise, random changes in the viral consensus sequence were observed in salivary glands and saliva samples from individual *Culex tarsalis* and *Culex quinquefasciatus* mosquitoes infected by West Nile virus (Grubaugh et al. 2016). In the same system, however, a recent study found convergent changes in the viral consensus sequence from two or more individual mosquitoes (Grubaugh et al. 2017). Interestingly, similar convergent mutations in the viral consensus sequence were detected when pooling saliva samples from about 20 individual *Ae. aegypti* or *Aedes albopictus* mosquitoes infected with chikungunya virus (Vega-Rúa et al. 2015). In another study, evolutionary convergence of chikungunya virus mutations found in the saliva of individual *Ae. aegypti* was observed for some of the viral strains tested but not all (Stapleford et al. 2014). Together, these studies indicate that the strength of convergent positive selection

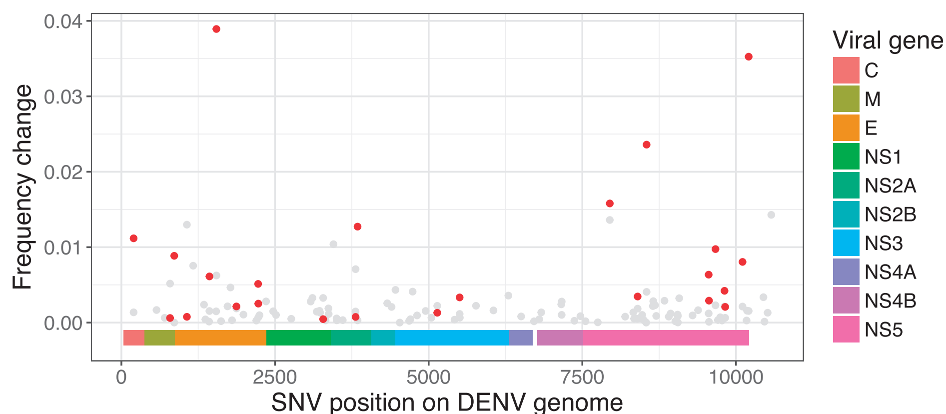

Figure 3. Change in minority variant frequency during transmission by mosquitoes. The absolute change in frequency between input (blood-meal and output (pooled saliva) samples is shown for all SNVs detected along the DENV genome. Red circles represent shared SNVs between input and saliva samples for the Gen. IV virus isolate on day 10 in Experiment A. Gray circles represent shared SNVs between two technical replicates of the input sample, as an estimate of measurement error. C, capsid protein; M, membrane glycoprotein; E, envelope glycoprotein; NS1, non-structural glycoprotein 1; NS2A, non-structural protein 2 A; NS2B, non-structural protein 2B; NS3, non-structural protein 3 (protease/helicase); NS4A, non-structural protein 4A; NS4B, non-structural protein 4B; NS5, non-structural protein 5 (RNA-dependent RNA polymerase).

during transmission may vary among mosquito-virus systems. Such variation was recently illustrated in a study of *Ae. albopictus* infected with chikungunya virus, in which genetically distinct mosquito populations exhibited differences in the selection patterns of an advantageous viral mutation (Vazeille et al. 2016).

Our study examined two different DENV-1 genotypes, both of which exhibited a lack of convergent positive selection in the same mosquito population, despite significant phenotypic variation in their infectivity to mosquitoes. Such differences in infectivity among DENV strains have been documented many times (Armstrong and Rico-Hesse 2003) and are often conditional on the mosquito genetic background in the form of genotype-by-genotype (G $\times$ G) interactions (Lambrechts et al. 2009). It is possible that different patterns of viral genetic diversity would be observed in different mosquito-virus pairings. In our previous study, we did not observe differences in the level of genetic drift or natural selection between three mosquito genotypes; however, the amount of viral genetic diversity was higher in one of the mosquito genotypes (Lequime et al. 2016). Further studies will be necessary to assess the significance of G $\times$ G interactions for within-host virus evolution.

In addition, the evolutionary forces acting on within-host DENV populations may depend on experimental conditions such as the infectious blood-meal titer. A lower blood-meal titer resulted in a stronger population bottleneck for Venezuelan equine encephalitis virus in *Culex* mosquitoes (Forrester et al. 2012). A tight population bottleneck is expected to promote genetic drift and consequently reduce the relative influence of natural selection (Moya et al. 2000) although exceptions have been observed (Miyashita and Kishino 2010). Conversely, the high blood-meal titer that we used in the present study, similar to other studies (Grubaugh et al. 2015, 2016), is expected to minimize the effect of genetic drift and maximize the efficiency of natural selection. Thus, the lack of convergent positive selection that we observed is unlikely to result from a lack of sensitivity.

Sequencing depth was too shallow to explore minority variants present in the transmitted viral subpopulation for all but one pooled saliva sample. In the single pooled saliva sample with sufficient sequencing depth to analyze minority variants, all variants present in the infectious blood meal were found in the transmitted viral subpopulation. Although our analysis detected that their frequency had shifted, the absolute difference in variant frequency did not exceed 4 per cent between the input and output viral population. Therefore, although they were detectable changes in variant frequency, their magnitude was very modest. This result suggests that although the within-host repertoire of virus variants is significantly reshuffled at the individual mosquito level (Sim et al. 2015; Lequime et al. 2016), the average genetic composition of the transmitted viral subpopulation appears to be relatively stable.

In conclusion, this study showed that the consensus genome sequence of the DENV subpopulation collectively transmitted by a group of mosquitoes previously fed on the same infectious blood meal was genetically identical to that of the input viral population. This finding suggests a lack of strong convergent positive selection during a single round of DENV transmission by *Ae. aegypti*. It extends to the transmitted DENV subpopulation our previous conclusion that genetic drift and purifying selection are likely the dominant evolutionary forces shaping within-host DENV genetic diversity in *Ae. aegypti* (Lequime et al. 2016). Deciphering the evolutionary forces that modulate the frequency of within-host DENV variants during transmission contributes to advance our understanding of DENV adaptation and evolution.

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Supplementary data

Supplementary data are available at *Virus Evolution* online.

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