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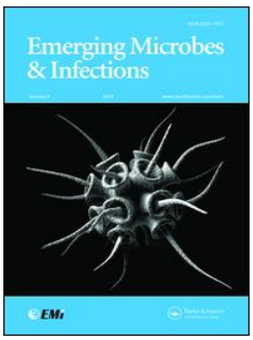

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Potential role of vector-mediated natural selection in dengue virus genotype/lineage replacements in two epidemiologically contrasted settings

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Dengue virus (DENV) evolutionary dynamics are characterized by frequent DENV genotype/lineage replacements, potentially associated with changes in disease severity and human immunity. New Caledonia (NC) and Cambodia, two contrasted epidemiological settings, respectively experienced a DENV-1 genotype IV to I replacement in 2012 and a DENV-1 genotype I lineage 3 to 4 replacement in 2005-2007, both followed by a massive dengue outbreak. However, their underlying evolutionary drivers have not been elucidated. Here, we tested the hypothesis that these genotype/lineage switches reflected a higher transmission fitness of the replacing DENV genotype/lineage in the mosquito vector using *in vivo* competition experiments. For this purpose, field-derived *Aedes aegypti* from NC and Cambodia were orally challenged with epidemiologically relevant pairs of four DENV-1 genotype I and IV strains from NC or four DENV-1 genotype I lineage 3 and 4 strains from Cambodia, respectively. The relative transmission fitness of each DENV-1 genotype/lineage was measured by quantitative RT-PCR for infection, dissemination, and transmission rates. Results showed a clear transmission fitness advantage of the replacing DENV-1 genotype I from NC within the vector. A similar but more subtle pattern was observed for the DENV-1 lineage 4 replacement in Cambodia. Our results support the hypothesis that vector-driven selection contributed to the DENV-1 genotype/lineage replacements in these two contrasted epidemiological settings, and reinforce the idea that natural selection taking place within the mosquito vector plays an important role in DENV short-term evolutionary dynamics.

Keywords: Dengue virus, genotype/lineage replacement, *Aedes aegypti*, transmission fitness, competition assay

Introduction

Dengue virus (DENV) is the most important arthropod-borne viral pathogen affecting humans worldwide [1]. Forms of dengue fever are broad, ranging from asymptomatic to life-threatening, sometimes resulting in hemorrhagic manifestations (even fatal) [2]. DENV is transmitted to humans through infected *Aedes* mosquitoes, primarily *Aedes aegypti*.

DENV is a positive-sense, single-stranded RNA virus belonging to the *Flaviviridae* family (*Flavivirus* genus). Four genetically distinct serotypes (DENV-1 to -4), sharing approximately 65% amino acid identity, can be distinguished [3]. Each serotype can be subdivided in several genotypes, displaying less than 6% nucleotide sequence divergence [4,5]. Phylogenetic analyses based on the DENV envelope gene sequence identified 5 genotypes for DENV-1, namely genotype I to V, representing of different geographic origins [4]. Moreover, each genotype can be subdivided into multiple lineages mainly based on the analysis of the whole genome sequence.

Phylogenetic analyses of DENV genetic diversity have revealed that DENV short-term evolutionary dynamics are characterized by rapid turnover of DENV genotypes and lineages [6-8]. These replacement events can lead to the extinction of the circulating DENV genotype/lineage, or its replacement by a new genotype or lineage [6,7,9-16]. Such mechanism has been documented for all DENV serotypes. Indeed, in the 1980s and 1990s, DENV-3 lineage replacements were observed in Sri Lanka and Thailand [6,14,15], whereas DENV-4 lineage replacements were identified in Puerto Rico [7]. Further, DENV-1 lineage or genotype replacements were observed in South-East Asia and in South/Latin America [6,9-11,17,18]. DENV-2 lineage or genotype

replacements occurred in the South Pacific region in the 1970s, and in America and Asia during the last decades [12,13,16,19,20].

These genotype/lineage switches have important epidemiological implications. Indeed, both epidemiological observations and *in vitro* studies suggest that distinct genotypes have a different potential to cause severe dengue epidemics [21]. For example, the appearance of hemorrhagic forms in the Americas in 1981 was associated with the introduction of a DENV-2 Southeast Asian genotype that supplanted the native American genotype [12,22]. Furthermore, recent study indicated that protection from homologous DENV re-infection may sometimes be incomplete, although virological complementary studies are needed to be performed in order to confirm this observation [23]. In the same manner, another study showed that the immune response may be variable within the same serotype [24]. The mechanisms underlying the DENV genotype/lineage turnover, however, remain poorly understood. Some studies have suggested that these dynamics result from stochastic events [9,15]. Conversely, other studies proposed that this turnover arises from differences in viral fitness, such as higher viremia in humans [13] or higher transmissibility in mosquitoes [10,25-27]. Indeed, a significantly higher replicative index of the replacing DENV strain was observed in some DENV lineage replacements as early as initial infection establishment in *Ae. aegypti*. The increase of viral titer in the midgut was associated with a greater dissemination efficiency, suggesting a higher viral transmission rate and/or a shorter extrinsic incubation period [25,26]. Despite the complex epidemiological pattern of dengue, the turnover of DENV genotypes and lineages is observed in all affected regions irrespective of their epidemic, endemic or even hyper-endemic status. Here, we investigated these replacements in two epidemiologically contrasted countries: New Caledonia (NC) (epidemic) and Cambodia (hyper-endemic).

In NC, DENV circulation has been characterized by an epidemic transmission of a single dominant DENV serotype/genotype for three to five years, with subsequent replacement by another serotype/genotype. This epidemiological profile has evolved over the last fifteen years, with more frequent epidemics, co-circulation of several arboviruses and an unusual persistence of DENV-1 [28] (**Supplementary Figure 1)**. Phylogenetic analyses using DENV-1 strains isolated between 2001 and 2017 showed that NC experienced a replacement of the genotype IV with the genotype I in 2012 [28,29]; this introduction being associated with a major outbreak in 2013 (more than 10,000 cases diagnosed), with uncommon complications. Since 2018, DENV-1 genotype I has been replaced by DENV-2 in NC [30].

In contrast, Cambodia is a dengue endemic country (10,000 to 60,000 annual cases). All four DENV serotypes co-circulate each year although the predominant serotype has alternated mainly between DENV-1, DENV-2, and DENV-3 over the last decades [31] (**Supplementary Figure 2)**. DENV-1 co-circulated with other serotypes as a minor serotype between 2000 and 2009 [11,32], and strains isolated over this period belong to genotype I grouped into four lineages (L1 to L4) with specific dynamics [11]. Indeed, three lineage replacements were observed: in 2000-2003 L2 displaced L1; in 2002-2003 L3 displaced L2; in 2005-2007, L4 replaced L3 [11]. In 2011, DENV-1 was detected throughout the country and resulted in a high magnitude outbreak in 2012 [33]. Unlike DENV-3, which was sporadically detected after having caused a major outbreak in 2007, DENV-1 continued to circulate as a dominant serotype until 2015.

Interestingly, these genotype/lineage replacements were associated with periods of low DENV-1 circulation in both Cambodia and NC [11,29,33]. Periods of low virus transmission could favor the emergence of previously rare variants randomly replacing dominant ones, resulting in a ladder-like tree topology [7,34-36]. The genotype/lineage

replacements observed in both countries are inconsistent with this scenario because a newly introduced genotype/lineage replaced the resident one after a period of co-circulation [11,29]. They are more consistent with an adaptive replacement reflecting the higher fitness of the invasive genotype/lineage relative to the resident one. However, the selective forces involved have not been identified yet. Here, we investigated whether the DENV genotype/lineage replacement events observed in these two epidemiologically contrasted settings could reflect viral fitness differences in the mosquito vector.

Materials and Methods

Viruses

Thirty-three epidemic DENV-1 strains were selected in NC from 2009 to 2017 and 21 DENV-1 strains were selected in Cambodia from 2005 to 2016. Viruses were isolated from human serum by three successive passages in *Aedes albopictus* (C6/36) cells, maintained in Leibovitz's L-15 medium (Sigma-Aldrich, Saint Louis, MO, USA) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Gibco, ThermoFisher Scientific, Paisley, UK) and 10% tryptose phosphate broth (Gibco, ThermoFisher Scientific, Paisley, UK). After five days of incubation at 28°C, supernatants were collected. For each DENV-1 strain selected for competition assays, viral titer was determined by immuno-fluorescent focus assay [37] using the anti-DENV complex antibody, clone D3-2H2-9-21 (Millipore, Temecula, CA, USA), and was expressed as focus-forming unit per milliliter (FFU/mL). If necessary, two concentration methods were employed to reach the appropriate viral titer. Viruses were either centrifuged using Vivaspin 6 centrifugal concentrator (Sartorius, Stonehouse, UK) or added with

Polyethylene Glycol (PEG, Sigma-Aldrich, Germany) and centrifuged at 1800 rpm for 1 hour.

Whole-genome sequencing and phylogenetic analysis

Whole-genomes of the 54 DENV-1 strains selected in this study were obtained by high-throughput sequencing of cell-culture supernatants [38]. The resulting whole-genomes were deposited in GenBank (**Supplementary Table 1**). Additionally, five whole-genome sequences from Cambodia already available in GenBank (FJ639684, FJ639687, HM181944, FJ639685, and GU131893) were included in the analysis. Phylogenetic analysis of DENV-1 was conducted on whole-genome sequences from NC and Cambodia with reference sequences from GenBank representing the five genotypes of DENV-1. Alignment was done using the MAFFT software [39]. The best substitution model for the alignment sequence was determined in W-IQ-Tree tool using the Model selection option, resulting in the GTR + G4 + I as the best model. The whole-genome phylogenetic tree was constructed by the Maximum Likelihood method based on the best substitution model with 1.000 bootstrap replicates in W-IQ-Tree tool [40]. DENV-3 (AY858048) was used as the out-group.

Quantitative RT-PCR

Viral RNA from all samples (body, head/legs/wings, and saliva) was extracted with the QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany). The detection and quantification of DENV-1 genotypes I and IV (DENV-1 GI and GIV) and DENV-1, genotype I, lineages 3 and 4 (DENV-1 L3 and L4) was performed by specific TaqMan quantitative RT-PCR assays with the Superscript III Platinum One-Step RT-qPCR kit (ThermoFisher Scientific, Carlsbad, CA, USA). Genotype or lineage-specific primers and probes, respectively targeting the NS2B or E gene for New Caledonian or

Cambodian strains, were used (**Supplementary Table 2**). Amplifications were performed on either a LightCycler 480 instrument II (Roche, Basel, Switzerland) or a Bio-Rad CFX96 Real-Time Detection System (Bio-Rad, Hercules, CA, USA) using the following program: one cycle (50°C, 15 min; 95°C, 2 min) followed by 45 cycles (95°C, 15 s; 57°C, 1 min).

Mosquitoes

Ae. aegypti larvae and pupae were collected at the end of the hot season (May 2018 in NC and April 2019 in Cambodia) and reared to the adult stage in laboratory. Adults were then maintained with access to 10% sucrose solution *ad libitum* at 28°C ± 1°C, 80% ± 10% relative humidity and 12:12h light:dark cycle. F1 and F2 generations were successively produced by sib-mating and collective oviposition following blood feeding.

In vivo competition assay

Two DENV-1 strains representatives of each genotype (GI and GIV) or lineage (L3 and L4) were selected from the phylogenetic tree generated (**Figure 1**) to measure their relative fitness by competition assays in mosquitoes. Five- to seven-days-old nulliparous *Ae. aegypti* F2 females were challenged with infectious blood meals at a final concentration of 5.10^6 FFU/mL with two initial FFU ratios (DENV-1 GI/GIV relative percentage: 50/50 and 10/90, or DENV-1 L3/L4: 50/50) (**Supplementary Table 3 and Supplementary Figure 3**). For each competition *Ae. aegypti* from NC and Cambodia were orally challenged with the selected DENV-1 strains from NC and Cambodia respectively as previously described [37]. At 7 and 14 days post challenge (pc), 30 *Ae. aegypti* mosquitoes randomly collected were subjected to a forced salivation. Briefly, after removing wings and legs, their proboscis was inserted into a

20-µL filter tip filled with 5 µL FBS for 30 minutes. The mixture was subsequently supplemented with 45 µL of Leibovitz's L-15 medium and stored at -80°C. After decapitation, the body and head of each mosquito were mechanically homogenized for 2 minutes at 2000 rpm using a mini-BeadBeater 96 (BioSpecProducts, Bartlesville, OK, USA) with metal beads in 200 µL of Leibovitz medium supplemented with 2% FBS, 10% tryptose phosphate broth and antibiotics/antifungals (100 units/mL of penicillin, 0.1 mg/mL of streptomycin and 0.25 µg/mL amphotericin B, Gibco, ThermoFisher Scientific, Carlsbad, CA, USA) in NC. In Cambodia, the body and head/legs/wings were stored in 400 µL phosphate buffered saline (PBS) supplemented with 10 % FBS, 1% Penicillin/Streptomycin, 1% Amphotericin B (GIBCO, Waltham MA, USA) until homogenization. The homogenization was performed using a MagNA Lyser (Roche, Basel, Switzerland) with ceramic beads at 6,500 rpm for 50 sec. The lysates were then centrifuged at 10,000 g for 5 minutes and stored at -80°C. Detection of viral RNA from body, head and saliva for each competition assay was performed by two specific quantitative RT-PCR experiments as described above. The viral infection profile for the three vector competence indices (infection, dissemination and transmission rates) was determined as follows: i) the infection rate is the number of mosquitoes displaying a DENV positive body among all tested mosquitoes, ii) the dissemination rate is the number of mosquitoes with an infected head divided by the number of mosquitoes having a DENV positive body, and iii) the transmission rate is the number of mosquitoes with a DENV positive saliva divided by the number of mosquitoes with an infected head (**Supplementary Figure 3**).

Statistical analysis

Competition assays were analyzed statistically by detecting each genotype/lineage in individual mosquitoes and comparing their relative frequency for infection,

dissemination, and transmission indices. The 2 x 2 contingency tables of presence/absence for both competing viruses were compared by McNemar's chi-squared test for paired nominal data. All statistical analyses and data plotting of competition assays were performed with R v3.6.1 software [41], considering *p*-values < 0.05 as statistically significant.

Ethics statement

In NC, the use of DENV strains isolated from the serum of anonymized patients was granted by the Consultative Ethics Committee of New Caledonia 26.11.2019 and by the Comité de Protection des Personnes Sud-Est II (N° Eudract 2019-A03114-53). In Cambodia, the use of DENV strains obtained from patients enrolled in the National Program for Dengue Surveillance by Ministry of Health of Cambodia was approved by National Ethics Committee for Health Research (N^{o} 264NECHR).

Results

Phylogenetic analysis

Fifty-four whole genomes of DENV-1 strains from NC and Cambodia clustered within two of the five DENV-1 genotypes with 85% (45/54) within genotype I (**Figure 1**). Five lineages were observed inside the genotype I. The 21 Cambodian strains from this study belonged to genotype I. They fell into three (L3, L4 and L5) of the five distinct lineages among other Cambodian strains reported previously. DENV-1 strains in lineage L3 circulated between 2002 and 2007, L4 between 2003 and 2016 and L5 between 2007 and 2016. Twenty-five New Caledonian strains collected from 2012 to 2017 clustered in lineage L5 along with five Cambodian strains collected in 2014, 2015 and 2016 (**Figure**

1). The last 8 strains from NC collected between 2009 and 2012, fell into genotype IV, and are closely related to a virus sampled in French Polynesia in 2001.

Phylogenetic analyses indicated that the last lineage replacement occurred in Cambodia between 2005 and 2007 with an extinction of DENV-1 lineage L3 and an expansion of L4. Lineage L4 then circulated until 2016 and co-circulated with lineage L5 since 2015. However, no extinction of lineage L4 was observed during the period (**Figure 1**). Among the 21 Cambodian strains, 5 belong to this co-circulation period with 3 assigned to lineage L3 and 2 to lineage L4. Genotype replacement also occurred in NC in 2012 with a co-circulation of both genotypes during this period, as observed previously [29]. Among the 33 NC DENV strains collected for this study, 9 belong to this co-circulation period with 3 strains falling into genotype IV and 6 into genotype I. For *in vivo* competition assays, 2 strains of each genotype from NC (GI and GIV) or lineage from Cambodia (L3 and L4) isolated during these periods of co-circulation (2012 for NC and 2005-2007 for Cambodia) that favored the expansion of the newly introduced genotype (GI for NC) or lineage (L4 for Cambodia) were selected (**Figure 1**).

DENV-1 genotypes (GI and GIV) competition assays

Co-infection of *Ae. aegypti* mosquitoes with DENV-1 GI and GIV from NC were performed using two strains of each DENV-1 genotype (GI.a, GI.b, GIV.c, GIV.d). DENV-1 was detected in mosquitoes either as mono-infection of GI or GIV or as co-infection with GI and GIV from 7 days pc, regardless of the initial ratio or viral combination (**Figures 2 and 3; Supplementary Figure 3**).

With the 50:50 infectious ratio, infection rates ranged from 43.3% to 100% and from 76.7% to 100% at 7 and 14 days pc, respectively. A significant predominance of DENV-1 GI relative to DENV-1 GIV was observed at 7 days pc for the two viral

combinations with the GI.b isolate (*p*-values = 0.001 and < 0.001, for GI.b & GIV.c and GI.b & GIV.d combinations, respectively). At 14 days pc, a third viral combination also showed a significantly higher infection with DENV-1 genotype I (*p*-values = 0.004, = 0.002 and = 0.002, for GI.a & GIV.d, GI.b & GIV.c and GI.b & GIV.d, respectively) (**Figure 2A**). The same profile was observed for dissemination rates, with a significantly higher dissemination of DENV-1 GI for two and three out of the four viral combinations at 7 and 14 days pc, respectively (*p*-values < 0.001 at 7 days pc for GI.b & GIV.c and GI.b & GIV.d, and = 0.01, <0.001, < 0.001 at 14 days pc for GI.a & GIV.d, GI.b & GIV.c and GI.b & GIV.d respectively) (**Figure 2B**). Viral transmission was detected at 7 and 14 days pc, ranging from 9% to 21.4% and 41.2% to 79.3%, respectively (**Figure 2C**). At 7 days pc, only the viral combination GI.b & GIV.d showed a transmission advantage for DENV-1 GI compared to DENV-1 GIV (*p*-values = 0.04). The DENV-1 GI was significantly better transmitted compared to DENV-1 GIV at 14 days pc for the four combinations (*p*-values = 0.01, = 0.04, = 0.003 and = 0.003 for GI.a & GIV.c, GI.a & GIV.d, GI.b & GIV.c and GI.b & GIV.d, respectively).

With the 10:90 infectious ratio in favor of DENV-1 GIV, results were more heterogeneous. Infection rates ranged from 50% to 90% irrespective of the day pc. A significant predominance of DENV-1 GIV infection compared to DENV-1 GI was observed for the two viral combinations containing the GIV.c isolate regardless of the day pc (*p*-values = 0.004, < 0.001 and = 0.001, = 0.001 for GI.a & GIV.c and GI.b & GIV.c at 7 and 14 days pc, respectively). Furthermore, no DENV-1 GI only infection was detected in these two viral combinations at 7 and 14 days pc. Conversely, the two viral combinations containing the GIV.d isolate showed a significant higher infection with DENV-1 GI irrespective of the GI isolate and the day post challenge (*p*-values = <0.001, = 0.02 and < 0.001, =0.001 for GI.a & GIV.d and GI.b & GIV.d at 7 and 14

days post challenge respectively) (**Figure 3A**). Although DENV-1 GIV was in excess in these two viral combinations, no DENV-1 GIV only infected mosquitoes were detected, except at 14 days pc for GI.b & GIV.d. Dissemination rates ranged from 93.3% to 100% and 80% to 100% at 7 and 14 days pc, respectively. The same profile as for the infection rates was observed for all viral combinations. Heads from the combination GI.b & GIV.c at 7 days pc were not analyzed, since only DENV-1 GIV was detected in infected mosquitoes (bodies). Interestingly, for the GI.a & GIV.c viral combination, some infected mosquito heads were only positive for DENV-1 GI despite the significant predominance of DENV-1 GIV (**Figure 3B**). Viral transmission was assessed at 14 days pc for the four viral combinations. Transmission rates were close to 50%, except for GI.b & GIV.c, which was 33%. DENV-1 GI was significantly better transmitted compared to DENV-1 GIV for only one out the four viral combinations (*p*-value = 0.01 for GI.a & GIV.d). Transmission of DENV-1 GI, however, was also detected in two other viral combinations although no significant difference was detected. No DENV-1 GI transmission was observed for GI.b & GIV.c (**Figure 3C**).

DENV-1 genotype I lineages (L3 and L4) competition assays

Cambodian *Ae. aegypti* mosquitoes were infected with a mixture of DENV-1 L3 (L3.a, L3.b) and L4 (L4.c, L4.d) at 50:50 ratio. All the four viral combinations showed an infection in mosquitoes from 7 days pc whatever the initial ratio or viral combinations (**Figures 4**). DENV-1 was detected in mosquitoes either as mono-infection of L3 or L4 or as co-infection with L3 and L4.

The infection rates were high (ranging from 86.7% to 100%) irrespective of the pc. Although DENV-1 L4 strains seemed to be mostly detected at higher percentage compared to L3 in mosquitoes at 7 days pc and at 14 days pc, the difference was significant only for combination L3.b & L4.c at 14 days pc (*p*-value = 0.023) (**Figure**

4A).The dissemination profile was similar to that of infection rate with a tendency to a greater dissemination of DENV-1 L4 in three out of the four viral combinations at 7 and 14 days pc. However, the percentage of mosquitoes infected with L4 was significantly higher than that of L3 only for the combination L3.b and L4.c (*p*-values = 0.004 and = 0.026 at 7 and 14 days pc, respectively) (**Figure 4B**). Likewise, the transmission rate of DENV-1 L4 was significantly higher than that of L3 for the combination L3.b and L4.c at 7 and 14 days pc (*p*-values = 0.045 and = 0.023, respectively) (**Figure 4C**).

Discussion

A typical feature of DENV epidemiology is the fluctuation in dengue incidence and serotype prevalence. In addition, rapid turnover of DENV genotypes and lineages is frequently observed in DENV evolutionary dynamics. NC and Cambodia experienced such replacements [11,29]. Detailed phylogenetic analyses performed on DENV-1 strains isolated in both countries between 2005 and 2017 confirmed that NC experienced a genotype replacement in 2012: DENV-1 GI rapidly displaced DENV-1 GIV [29]. All DENV-1 GI from NC belong to lineage 5 along with Cambodian strains isolated between 2014-2016. Our results also confirmed that Cambodia experienced lineages replacements in 2002-2003 (L2 displaced L1) and 2005-2007 (L4 displaced L3) [11]. Whereas Cambodia has experienced frequent DENV lineage or genotype replacements [11], the DENV-1 genotype replacement observed in 2012 is the first described in NC [28].

To date, few studies have examined the evolutionary mechanisms driving DENV genotype/lineage replacement events, and specifically focusing on the potential role of natural selection. A previous study did not detect a significant difference in infection, dissemination and transmission rates between DENV-1 GI or DENV-1 GIV

in *Ae. aegypti* from NC orally challenged by the two genotypes separately [37]. In competition assays, however, a fitness advantage of DENV-4 on DENV-1 was shown in *Ae. aegypti* while no difference was observed between the corresponding mono-infections, highlighting the value of assessing viral fitness in mixed infection experiments [42]. More generally, competition assays are the gold standard in viral fitness studies because they eliminate host-to-host variation that can reduce the power of experiments with individual viral strain infections, and because the viral ratio can be assayed with more accuracy than individual virus titers [43-45]. In this study, we experimentally tested the hypothesis that genotype/lineage replacement events observed in NC and Cambodia were associated with enhanced transmissibility of the replacing genotype/lineage relative to the resident ones. Our assessment relied on competition experiments in mosquitoes *in vivo*, in which we monitored the relative proportions of the competing DENV genotype/lineage to determine their relative transmission fitness.

The results obtained in this study support the hypothesis that DENV-1 genotype or lineage replacement observed in NC and Cambodia have been driven, at least in part, by viral fitness differences in the vector. Indeed, a markedly higher transmission fitness was observed for DENV-1 GI relative to GIV in NC. Provided at equal titer, three out of the four viral combinations showed a competitive advantage for DENV-1 GI for the infection and dissemination rates at 14 days pc. Moreover, all four viral combinations showed a higher fitness of DENV-1 GI at 14 days pc for the ultimate stage of vector competence: the transmission rate. Interestingly, in mixed infection with 10-fold less amount of DENV-1 GI, a higher transmission fitness of DENV-1 GI relative to GIV was observed. This higher transmission fitness could result from a better replication fitness from the invasive DENV genotype at the early stage of mosquito infection. Although the difference is more subtle between DENV-1 lineage L3 and L4, a slightly

higher transmission fitness for DENV-1 L4 over L3 was observed in the same manner in Cambodia. Our results are in accordance with a previous study showing that a DENV-1 clade replacement in Thailand was associated with enhanced mosquito transmission [10]. Further, a previous study showed that the lineage replacement of the DENV-2 Asian-American genotype in Nicaragua was associated with a higher replicative index of the replacing NI-2B lineage over NI-1 lineage in *Ae. aegypti* mosquitoes orally infected with both lineages as soon as 3 days post-infectious blood-meal [25]. A difference in the production of subgenomic flaviviral RNA fragments (sfRNA), that inhibit the interferon expression or disrupt the mosquito immunity, may explain the higher transmission fitness. Indeed, DENV lineage replacements in Puerto-Rico and Nicaragua were recently associated with increased production of sfRNAs by the invasive lineage in the human host [46]. Likewise, another study focusing on DENV lineage replacement in Puerto-Rico showed a link between higher epidemiological fitness and increased production of sfRNAs in the vector [47]. Thus, this viral factor seems to play an important role in the epidemiological fitness of DENV [48,49].

Further studies are needed to complete our results and identify the mechanisms underlying the observed fitness differences. Indeed, we demonstrated a better transmission fitness of the invasive DENV-1 genotype I in the natural course of infection in the vector. All the results, however, were expressed as RNA copies per mosquito, which do not directly reflect the concentration of infectious viruses. DENV are known to produce a large amount of viral defective genomes that could confound the results if their relative amount differed between DENV genotypes/lineages. DENV epidemiologic fitness, defined as the capacity of a DENV strain to become dominant in the field, relative to other DENV strains, depends on the combination of replicative fitness and transmission fitness [50]. Our experimental design does not allow us to

investigate the replicative fitness strictly speaking as we did not measure the capacity of the DENV used in this study to produce infectious progeny in the vector. Moreover, in Cambodia, we cannot exclude the non-mutually exclusive alternative hypothesis that the lineage replacement could also result from a stochastic event due to genetic drift [9,51]. In a same way, a purifying selection or vector genetic factors may also have contributed to genotype/lineage replacement in both countries [35,51,52]. Furthermore, impact of population herd-immunity were not assessed in this study although it may have partly conditioned this genotype replacement observed in NC. Indeed recent analyses suggest that antigenic heterogeneity may exist within each DENV serotype [24]. Another study demonstrated an association between higher lineage replicative fitness and reduced antigenicity, weak B and T cell stimulation and weak host immune system interactions [53]. Finally, selective forces occurring in the human host may also be important drivers shaping the DENV epidemiologic fitness. Indeed, higher transmission fitness of the invasive genotype observed within the vector may not be preserved in the human host [54]. In NC, we can suppose, however, that the selection of DENV-1 GI in the mosquito, in relation with the enhanced mosquito transmission potential, had an impact on transmission with a higher probability of human-to-mosquito transmission as described previously [13]. Indeed, a major outbreak occurred in 2013 following this genotype replacement, and the circulation of DENV-1 GI was maintained until 2017 [28].

Although other selective and stochastic forces may also drive DENV evolution, our study provides evidence that vector-driven selection may have contributed to the DENV-1 genotype/lineage replacement that occurred in NC and to a smaller extent, in Cambodia. To our knowledge, few studies have assessed the transmission fitness of epidemiologically relevant DENV strains by their native vector. We demonstrated the

higher transmissibility of the invading DENV strain using competition experiments that included analyzes of mosquito saliva, representing the ultimate step of DENV transmission from the mosquito to the human host. At the beginning of the vaccine era, our work highlights the needs for a better understanding of the evolutionary mechanisms driving DENV genotype/lineage replacements. These data are crucial as they may impact vaccine strategies by the complexity of their antigenic properties.

Author contributions: Conceptualization: OOC, VD, LL, MDR; Formal analysis: OOC, TOP; Funding Acquisition MDR; Investigation OOC, TPO, FA, SD, SR, DG, SI, MM; Resources SB, PD, NP, VBS; Writing – Original Draft Preparation: OOC; Writing – Review & Editing: OOC, TPO, VD, LL, MDR. All authors carefully revised the manuscript.

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Declaration of interest statement: The authors declare no competing interests.

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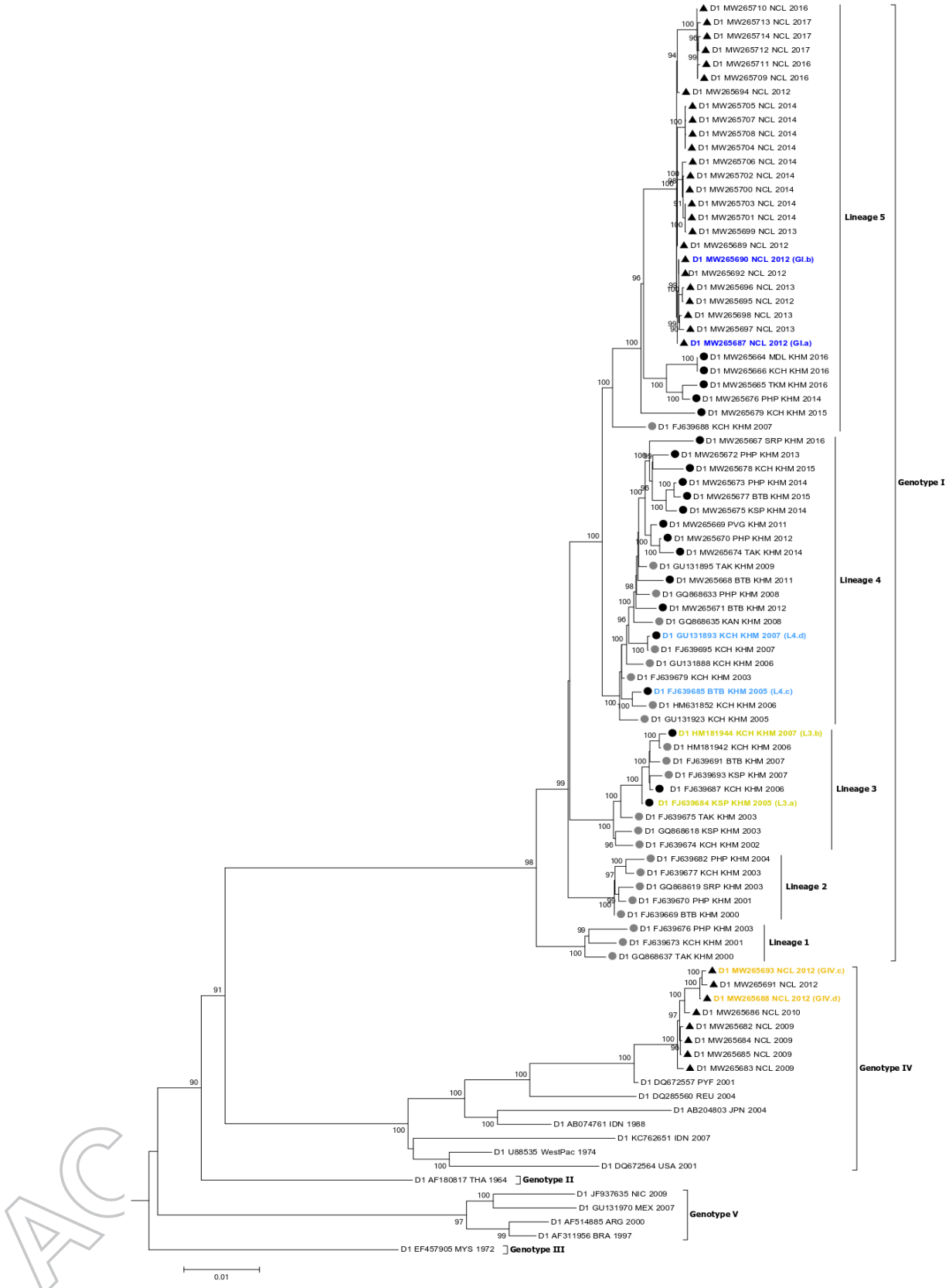


Figure 1: Phylogenetic tree of whole genomes of DENV-1 strains. Cambodia: strains included in our study (black circle); in previous studies (grey circle). Strains from NC: black triangle. Two DENV-1 strains representatives of each genotype (GIV in orange;

GI in blue) from NC or lineage (L3 in yellow; L4 in sky-blue belonging to genotype I) from Cambodia were selected for *in vivo* competition assays.

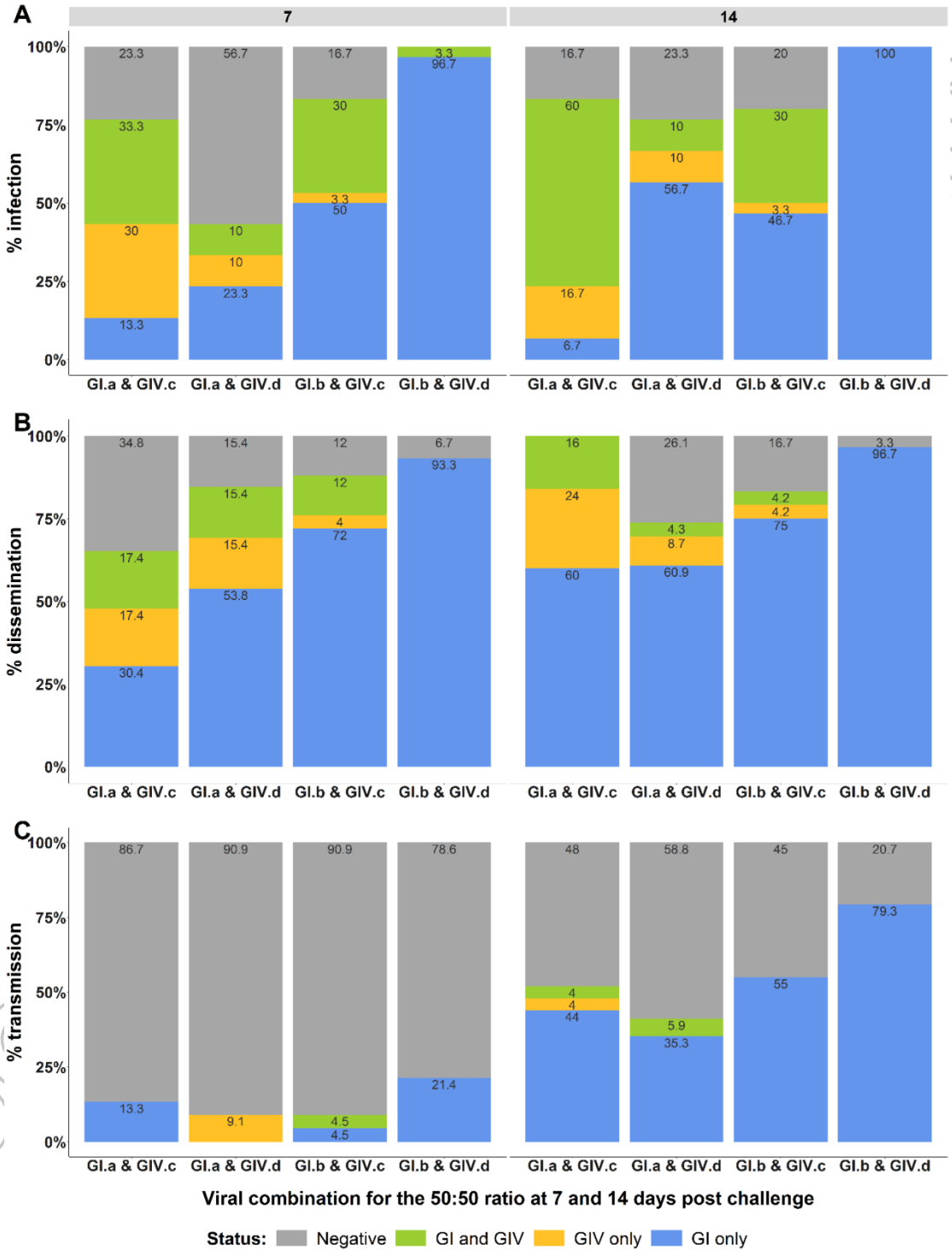


Figure 2: Infection, dissemination and transmission rates of New Caledonian *Ae. aegypti* mosquitoes observed with the 50:50 infectious ratio. Infection rates (A),

dissemination rates (B) and transmission rates (C) are represented. Status GI/GIV, GI or GIV corresponds to the detection by RT-qPCR of both targets, only GI or only GIV respectively in each mosquito compartment (body, head and saliva). No amplification detected for negative status.

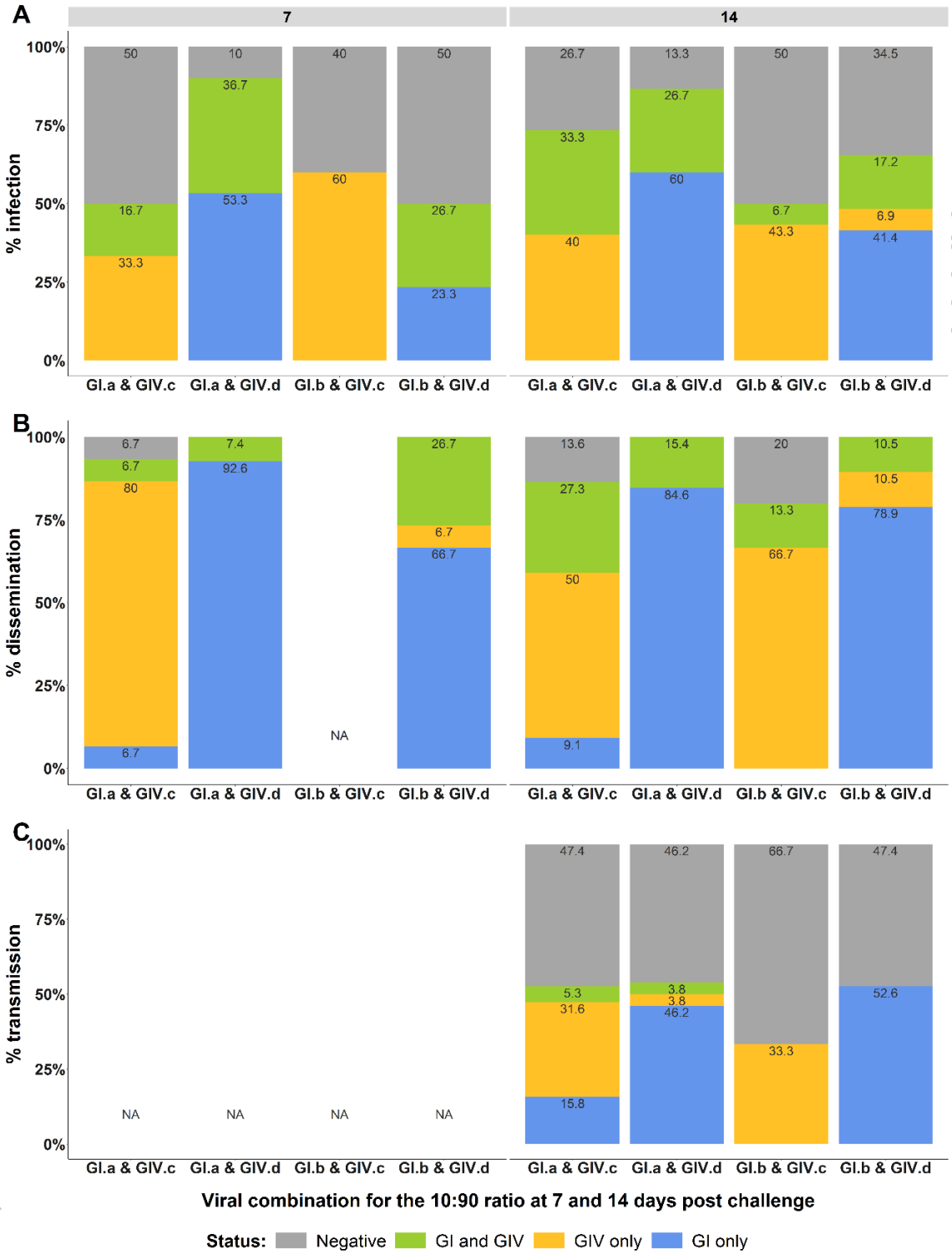


Figure 3: Infection, dissemination and transmission rates of New Caledonian *Ae. aegypti* mosquitoes measured with the 10:90 infectious ratio. Infection rates (A) dissemination rates (B) and transmission rates (C) are represented. Status GI and GIV, GI or GIV corresponds to the detection by RT-qPCR of both targets, only GI or only

GIV respectively in each mosquito compartment (body, head and saliva). No amplification detected for negative status. NA: not analyzed.

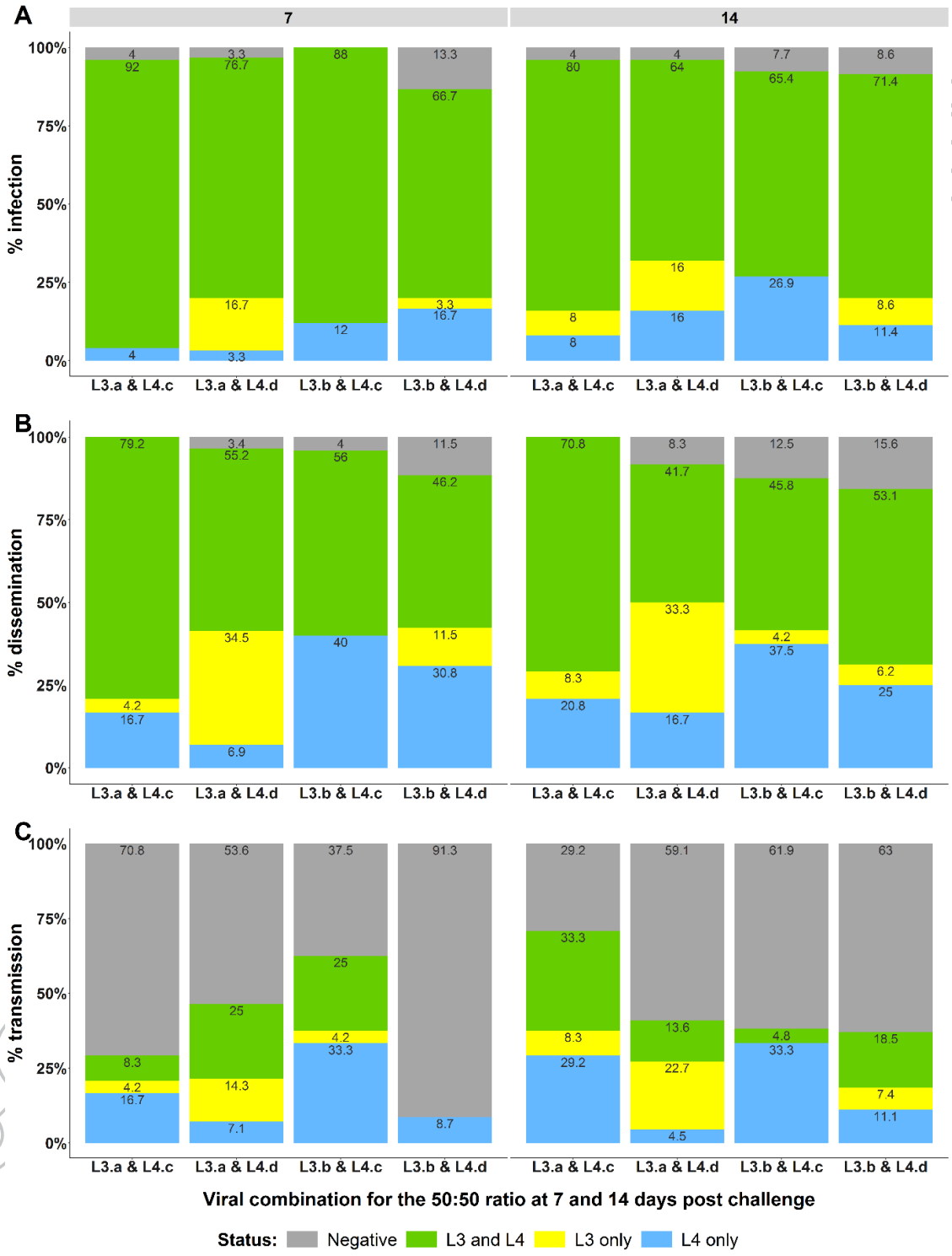
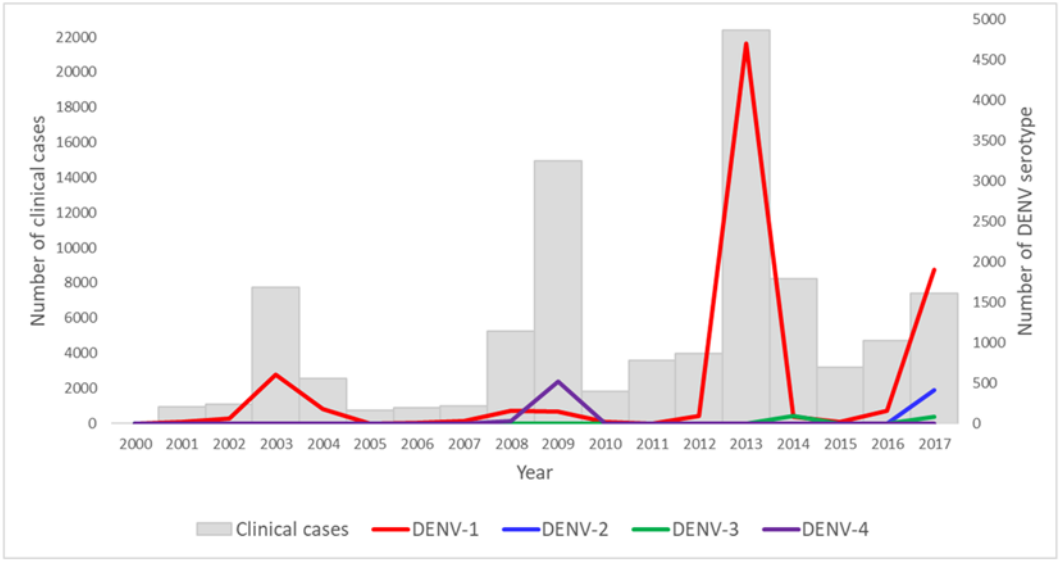


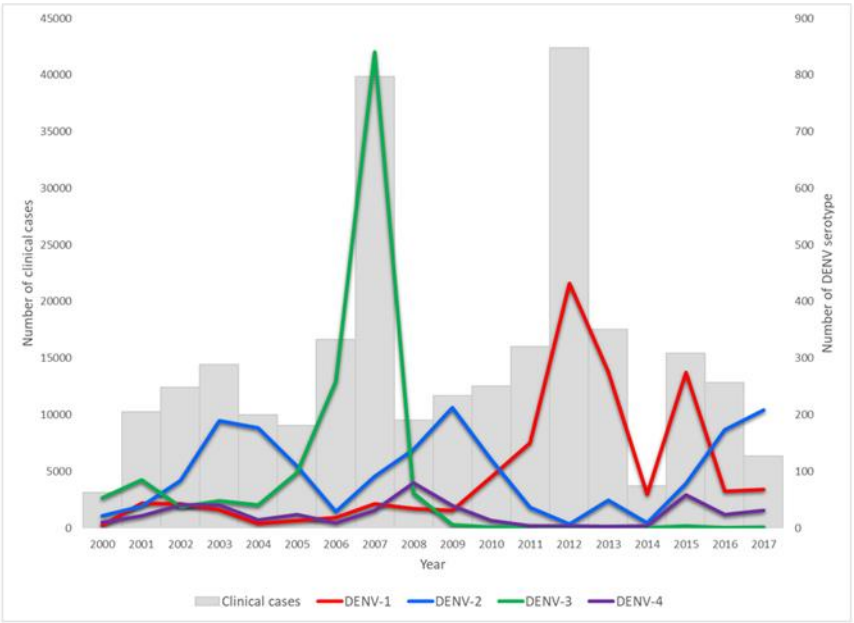
Figure 4: Infection, dissemination and transmission rates of Cambodian *Ae. aegypti* mosquitoes detected with the 50:50 infectious ratio. Infection rates (A), dissemination rates (B) and transmission rates (C) are represented. Status L3 and L4, L3

or L4 corresponds to the detection by RT-qPCR of both targets, only L3 or only L4 respectively in each mosquito compartment (body, head and saliva). No amplification detected for negative status.

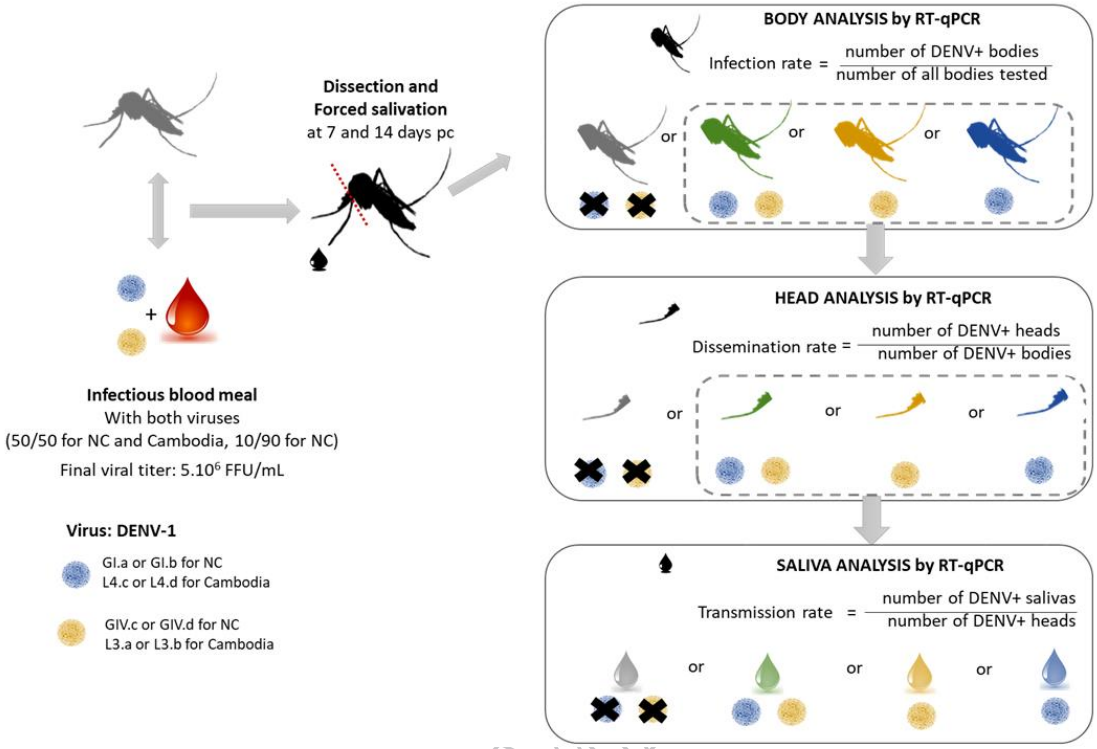
Supplementary Figure 1: Dengue epidemic profile in New Caledonia, 2000-2017



Supplementary Figure 2: Dengue epidemic profile in Cambodia, 2000-2017



Supplementary Figure 3: Experimental design of the in vivo competitive assay and determination of viral infection profiles (infection, dissemination and transmission rates)



Supplementary Table 1: GenBank accession number of all Dengue virus 1 used in the alignment.

Number	Name of sequence	ID Genbank	Province	Country	Year
1	D1_AF514885_ARG_2000	AF514885		Argentina	2000
2	D1_AF311956_BRA_1997	AF311956		Brazil	1997
3	D1_MW265664_MDL_KHM_2016	MW265664	Mondulkiri	Cambodia	2016
4	D1_MW265665_TKM_KHM_2016	MW265665	Tbaung Khmom	Cambodia	2016
5	D1_MW265666_KCH_KHM_2016	MW265666	Kampong Cham	Cambodia	2016
6	D1_MW265667_SRP_KHM_2016	MW265667	Seam Reap	Cambodia	2016
7	D1_FJ639669_BTB_KHM_2000	FJ639669	Battambong	Cambodia	2000
8	D1_FJ639670_PHP_KHM_2001	FJ639670	Phnom Penh	Cambodia	2001
9	D1_FJ639673_KCH_KHM_2001	FJ639673	Kampong Cham	Cambodia	2001
10	D1_FJ639674_KCH_KHM_2002	FJ639674	Kampong Cham	Cambodia	2002

11	D1_FJ639675_TAK_KHM_2003	FJ639675	Takeo	Cambodia	2003
12	D1_FJ639676_PHP_KHM_2003	FJ639676	Phnom Penh	Cambodia	2003
13	D1_FJ639677_KCH_KHM_2003	FJ639677	Kampong Cham	Cambodia	2003
14	D1_FJ639679_KCH_KHM_2003	FJ639679	Kampong Cham	Cambodia	2003
15	D1_FJ639682_PHP_KHM_2004	FJ639682	Phnom Penh	Cambodia	2004
16	D1_FJ639684_KSP_KHM_2005	FJ639684	Kampong Speu	Cambodia	2005
17	D1_FJ639685_BTB_KHM_2005	FJ639685	Battambong	Cambodia	2005
18	D1_FJ639687_KCH_KHM_2006	FJ639687	Kampong Cham	Cambodia	2006
19	D1_FJ639688_KCH_KHM_2007	FJ639688	Kampong Cham	Cambodia	2007
20	D1_FJ639691_BTB_KHM_2007	FJ639691	Battambong	Cambodia	2007
21	D1_FJ639693_KSP_KHM_2007	FJ639693	Kampong Speu	Cambodia	2007
22	D1_FJ639695_KCH_KHM_2007	FJ639695	Kampong Cham	Cambodia	2007
23	D1_GQ868618_KSP_KHM_2003	GQ868618	Kampong Speu	Cambodia	2003
24	D1_GQ868619_SRP_KHM_2003	GQ868619	Seam Reap	Cambodia	2003
25	D1_GQ868633_PHP_KHM_2008	GQ868633	Phnom Penh	Cambodia	2008
26	D1_GQ868635_KAN_KHM_2008	GQ868635	Kandal	Cambodia	2008
27	D1_GQ868637_TAK_KHM_2000	GQ868637	Takeo	Cambodia	2000
28	D1_GU131888_KCH_KHM_2006	GU131888	Kampong Cham	Cambodia	2006
29	D1_GU131893_KCH_KHM_2007	GU131893	Kampong Cham	Cambodia	2007
30	D1_GU131895_TAK_KHM_2009	GU131895	Takeo	Cambodia	2009
31	D1_GU131923_KCH_KHM_2005	GU131923	Kampong Cham	Cambodia	2005
32	D1_HM181942_KCH_KHM_2006	HM181942	Kampong Cham	Cambodia	2006
33	D1_HM181944_KCH_KHM_2007	HM181944	Kampong Cham	Cambodia	2007
34	D1_HM631852_KCH_KHM_2006	HM631852	Kampong Cham	Cambodia	2006
35	D1_MW265668_BTB_KHM_2011	MW265668	Battambong	Cambodia	2011
36	D1_MW265669_PVG_KHM_2011	MW265669	Prey Veng	Cambodia	2011
37	D1_MW265670_PHP_KHM_2012	MW265670	Phnom Penh	Cambodia	2012
38	D1_MW265671_BTB_KHM_2012	MW265671	Battambong	Cambodia	2012
39	D1_MW265672_PHP_KHM_2013	MW265672	Phnom Penh	Cambodia	2013

40	D1_MW265673_PHP_KHM_2014	MW265673	Phnom Penh	Cambodia	2014
41	D1_MW265674_TAK_KHM_2014	MW265674	Takeo	Cambodia	2014
42	D1_MW265675_KSP_KHM_2014	MW265675	Kampong Speu	Cambodia	2014
43	D1_MW265676_PHP_KHM_2014	MW265676	Phnom Penh	Cambodia	2014
44	D1_MW265677_BTB_KHM_2015	MW265677	Battambong	Cambodia	2015
45	D1_MW265678_KCH_KHM_2015	MW265678	Kampong Cham	Cambodia	2015
46	D1_MW265679_KCH_KHM_2015	MW265679	Kampong Cham	Cambodia	2015
47	D1_DQ672557_PYF_2001	DQ672557		French Polynesia	2001
48	D1_AB074761_IDN_1988	AB074761		Indonesia	1988
49	D1_KC762651_IDN_2007	KC762651		Indonesia	2007
50	D1_AB204803_JPN_2004	AB204803		Japan	2004
51	D1_EF457905_MYS_1972	EF457905		Malaysia	1972
52	D1_GU131970_MEX_2007	GU131970		Mexico	2007
53	D1_MW265682_NCL_2009	MW265682	Nouméa	New Caledonia	2009
54	D1_MW265683_NCL_2009	MW265683	Nouméa	New Caledonia	2009
55	D1_MW265684_NCL_2009	MW265684	Nouméa	New Caledonia	2009
56	D1_MW265685_NCL_2009	MW265685	Mont_Dore	New Caledonia	2009
57	D1_MW265686_NCL_2010	MW265686	Houailou	New Caledonia	2010
58	D1_MW265687_NCL_2012	MW265687	Nouméa	New Caledonia	2012
59	D1_MW265688_NCL_2012	MW265688	Ponérihouen	New Caledonia	2012
60	D1_MW265689_NCL_2012	MW265689	Ouegoa	New Caledonia	2012
61	D1_MW265690_NCL_2012	MW265690	Mont_Dore	New Caledonia	2012
62	D1_MW265691_NCL_2012	MW265691	Nouméa	New Caledonia	2012
63	D1_MW265692_NCL_2012	MW265692	Nouméa	New Caledonia	2012
64	D1_MW265693_NCL_2012	MW265693	Maré	New Caledonia	2012
65	D1_MW265694_NCL_2012	MW265694	Dumbéa	New Caledonia	2012
66	D1_MW265695_NCL_2012	MW265695	Nouméa	New Caledonia	2012

67	D1_MW265696_NCL_2013	MW265696	Nouméa	New Caledonia	2013
68	D1_MW265697_NCL_2013	MW265697	Nouméa	New Caledonia	2013
69	D1_MW265698_NCL_2013	MW265698	Ile des Pins	New Caledonia	2013
70	D1_MW265699_NCL_2013	MW265699	Dumbéa	New Caledonia	2013
71	D1_MW265700_NCL_2014	MW265700	Mont_Dore	New Caledonia	2014
72	D1_MW265701_NCL_2014	MW265701	Nouméa	New Caledonia	2014
73	D1_MW265702_NCL_2014	MW265702	Nouméa	New Caledonia	2014
74	D1_MW265703_NCL_2014	MW265703	Nouméa	New Caledonia	2014
75	D1_MW265704_NCL_2014	MW265704	Nouméa	New Caledonia	2014
76	D1_MW265705_NCL_2014	MW265705	Koné	New Caledonia	2014
77	D1_MW265706_NCL_2014	MW265706	Hienghène	New Caledonia	2014
78	D1_MW265707_NCL_2014	MW265707	Nouméa	New Caledonia	2014
79	D1_MW265708_NCL_2014	MW265708	Nouméa	New Caledonia	2014
80	D1_MW265709_NCL_2016	MW265709	Nouméa	New Caledonia	2016
81	D1_MW265710_NCL_2016	MW265710	Mont_Dore	New Caledonia	2016
82	D1_MW265711_NCL_2016	MW265711	Koné	New Caledonia	2016
83	D1_MW265712_NCL_2017	MW265712	Nouméa	New Caledonia	2017
84	D1_MW265713_NCL_2017	MW265713	Nouméa	New Caledonia	2017
85	D1_MW265714_NCL_2017	MW265714	Nouméa	New Caledonia	2017
86	D1_JF937635_NIC_2009	JF937635		Nicaragua	2009
87	D1_DQ285560_REU_2004	DQ285560		Reunion	2004
88	D1_AF180817_THA_1964	AF180817		Thailand	1964
89	D1_DQ672564_USA_2001	DQ672564		United States	2001
90	D1_U88535_WestPac_1974	U88535		Western Pacific	1974

Supplementary Table 2: Primers and probes used for real-time quantitative RT-PCR assays for the detection of DENV-1 genotypes I and IV and DENV-1, genotype I, lineages 3 and 4. Primers were shared between systems and only the probes differed. Efficacy of the RT-qPCR and specificity of the probes were tested against the different lineages and genotypes used in this study.

Oligonucleotide	Sequence (5'→3')	ORF position (KDH0030A)
DENV_NC_F	GGCATGCTAATAGCATGTTATG	4142-4162
DENV_NC_R	CTTCATGGTTCCATCATCTTG	4270-4290
DENV_NC_GI	FAM-CAGCCGACCTATCATTA-BHQ1	4178-4194
DENV_NC_GIV	FAM-CGGCCGATTTATCACTG-BHQ1	4178-4194
DENV_CAM_F	TGGAACCGTTCTAGTGC	1791-1807
DENV_CAM_R	GTTCTGCCTCAATGTTGAC	1933-1951
DENV_CAM_L3	FAM-ACTCAGAATGGAAGACTGATAACAGC-BHQ1	1876-1901
DENV_CAM_L4	HEX-ACCCAGAATGGGAGATTGATAACAGC-BHQ2	1876-1901

Supplementary Table 3: List of DENV-1 strains used for in vivo competition assays. Mix of viruses were as follows, for NC: GI.a/GIV.c, GI.a/GIV.d, GI.b/GIV.c, GI.b/GIV.d and for Cambodia: L3.a/L4.c, L3.a/L4.d, L3.b/L4.c, L3.b/L4.d.

DENV-1 strain	Genotype/ Lineage	Country	Titer used in 50/50 ratio	Titer used in 10/90 ratio
DENV-1 GI.a	Genotype I	NC	$2.5.10^6$ FFU/mL	$0.5.10^6$ FFU/mL
DENV-1 GI.b	Genotype I	NC	$2.5.10^6$ FFU/mL	$0.5.10^6$ FFU/mL
DENV-1 GIV.c	Genotype IV	NC	$2.5.10^6$ FFU/mL	$4.5.10^6$ FFU/mL
DENV-1 GIV.d	Genotype IV	NC	$2.5.10^6$ FFU/mL	$4.5.10^6$ FFU/mL
DENV-1 L3.a	Lineage 3	Cambodia	$2.5.10^6$ FFU/mL	NT
DENV-1 L3.b	Lineage 3	Cambodia	$2.5.10^6$ FFU/mL	NT
DENV-1 L4.c	Lineage 4	Cambodia	$2.5.10^6$ FFU/mL	NT
DENV-1 L4.d	Lineage 4	Cambodia	$2.5.10^6$ FFU/mL	NT

NT: not tested