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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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ABSTRACT
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 3–4 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.

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 titre 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 (10,000–60,000 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 favour 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 titre 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 titre. 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 programme: 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 12:12 h 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, Thermo-Fisher 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 s. 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
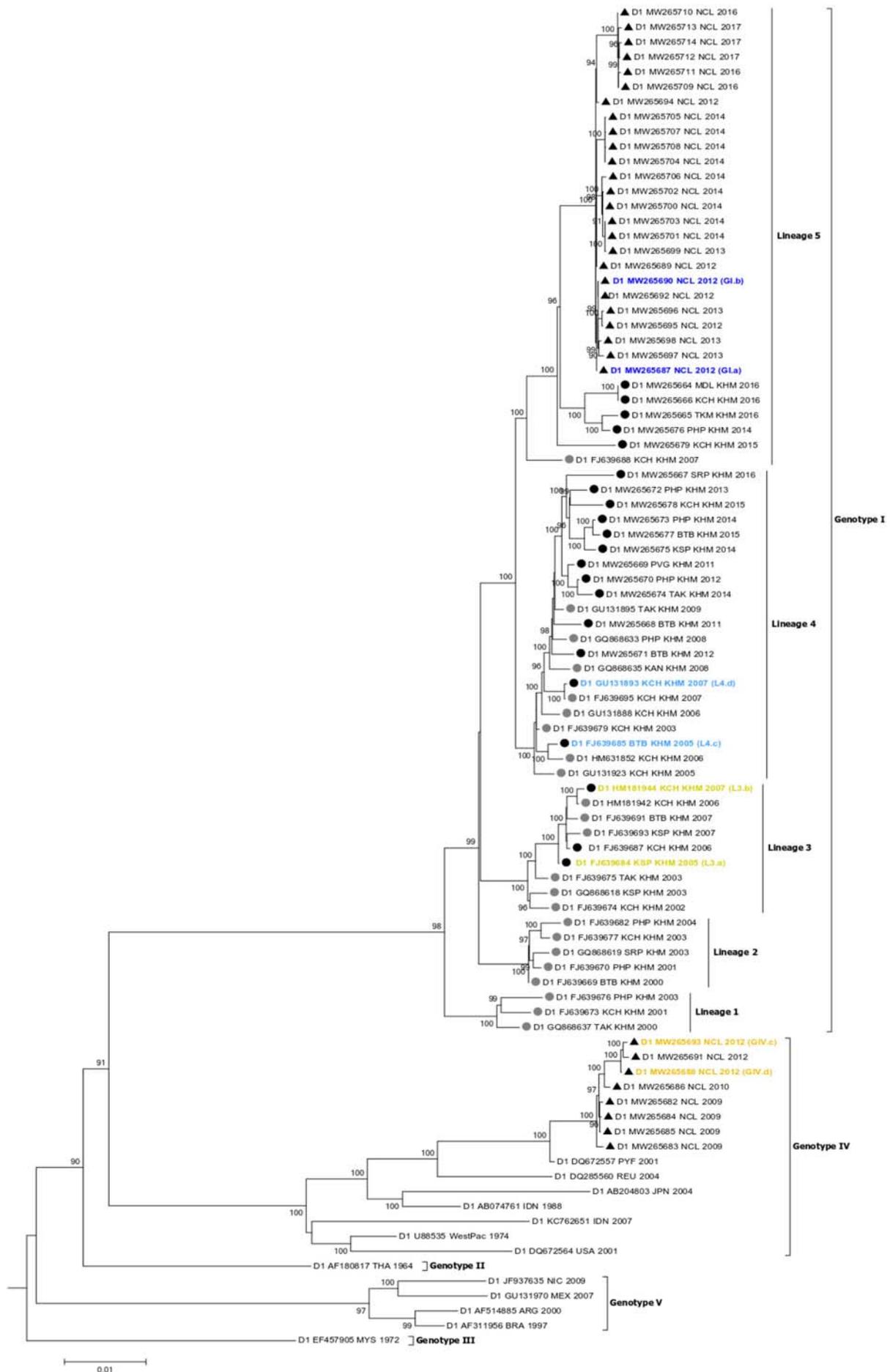
Figure 1. Phylogenetic tree of whole genomes of DENV-1 strains. Cambodia: strains included in our study (black circle); in previous studies (grey circle). Strains from NC: black triangle. Two DENV-1 strains representatives of each genotype (GIV in orange; GI in blue) from NC or lineage (L3 in yellow; L4 in sky-blue belonging to genotype I) from Cambodia were selected for *in vivo* competition assays.

(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 × 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° 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 eight 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, five 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, nine belong to this co-circulation period with three strains falling into genotype IV and six into genotype I. For *in vivo* competition assays, two 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 favoured 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
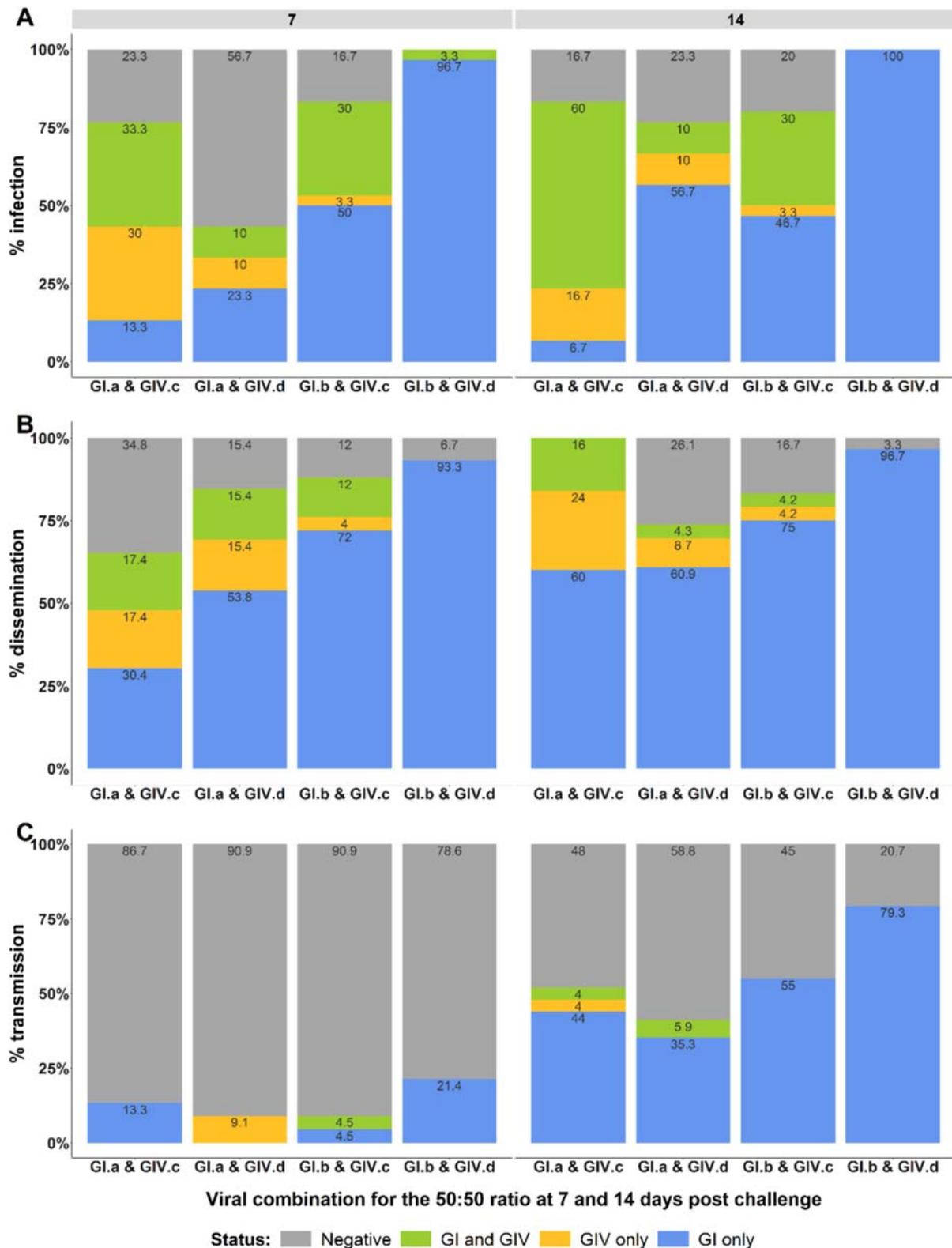
Figure 2. Infection, dissemination and transmission rates of New Caledonian *Ae. aegypti* mosquitoes observed with the 50:50 infectious ratio. Infection rates (A), dissemination rates (B) and transmission rates (C) are represented. Status GI/GIV, GI or GIV corresponds to the detection by RT-qPCR of both targets, only GI or only GIV respectively in each mosquito compartment (body, head and saliva). No amplification detected for negative status.

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 favour of DENV-1 GIV, results were more heterogeneous. Infection
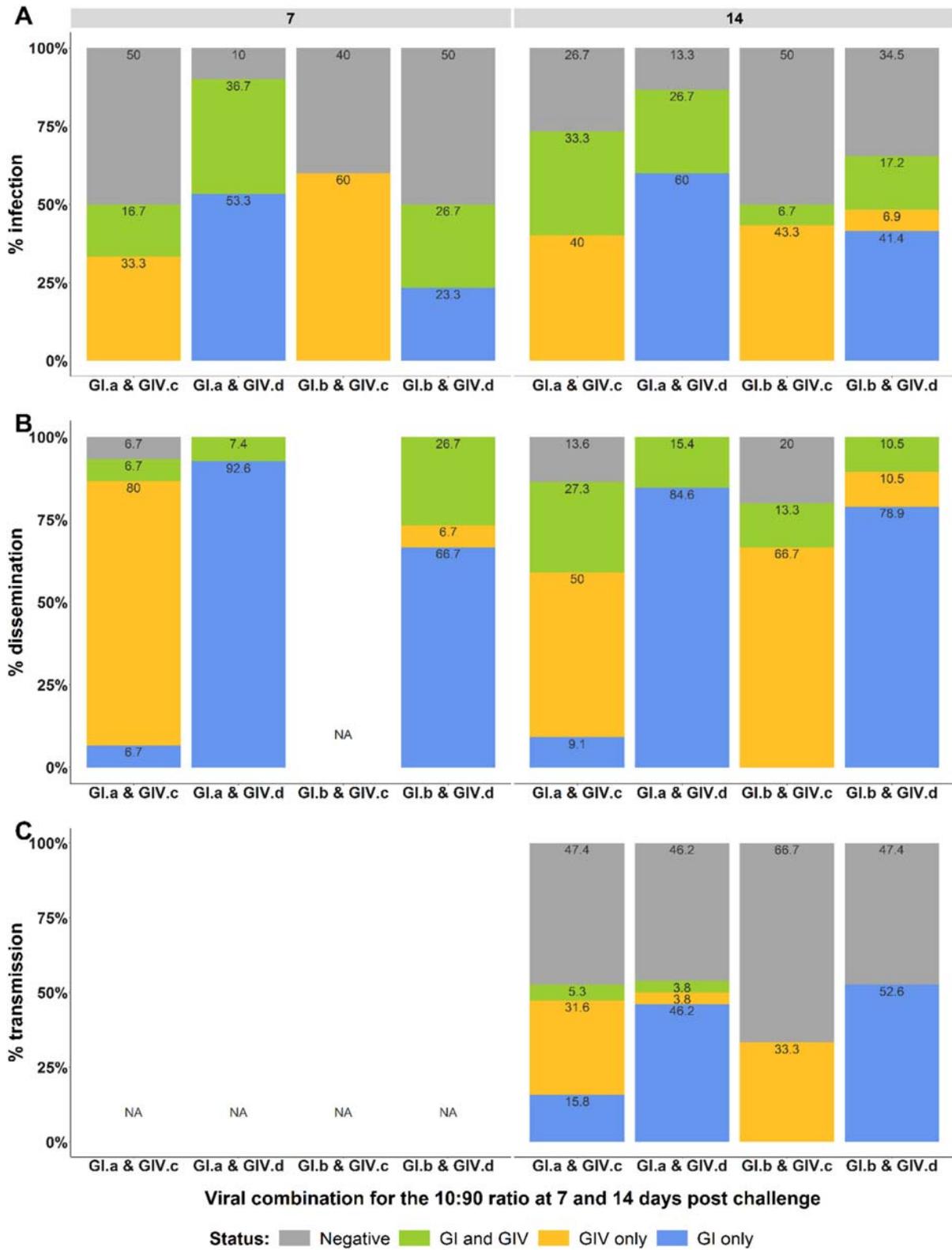
Figure 3. Infection, dissemination and transmission rates of New Caledonian *Ae. aegypti* mosquitoes measured with the 10:90 infectious ratio. Infection rates (A) dissemination rates (B) and transmission rates (C) are represented. Status GI and GIV, GI or GIV corresponds to the detection by RT-qPCR of 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.

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 (Figure 4; Supplementary Figure 3). 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 and 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 titres [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
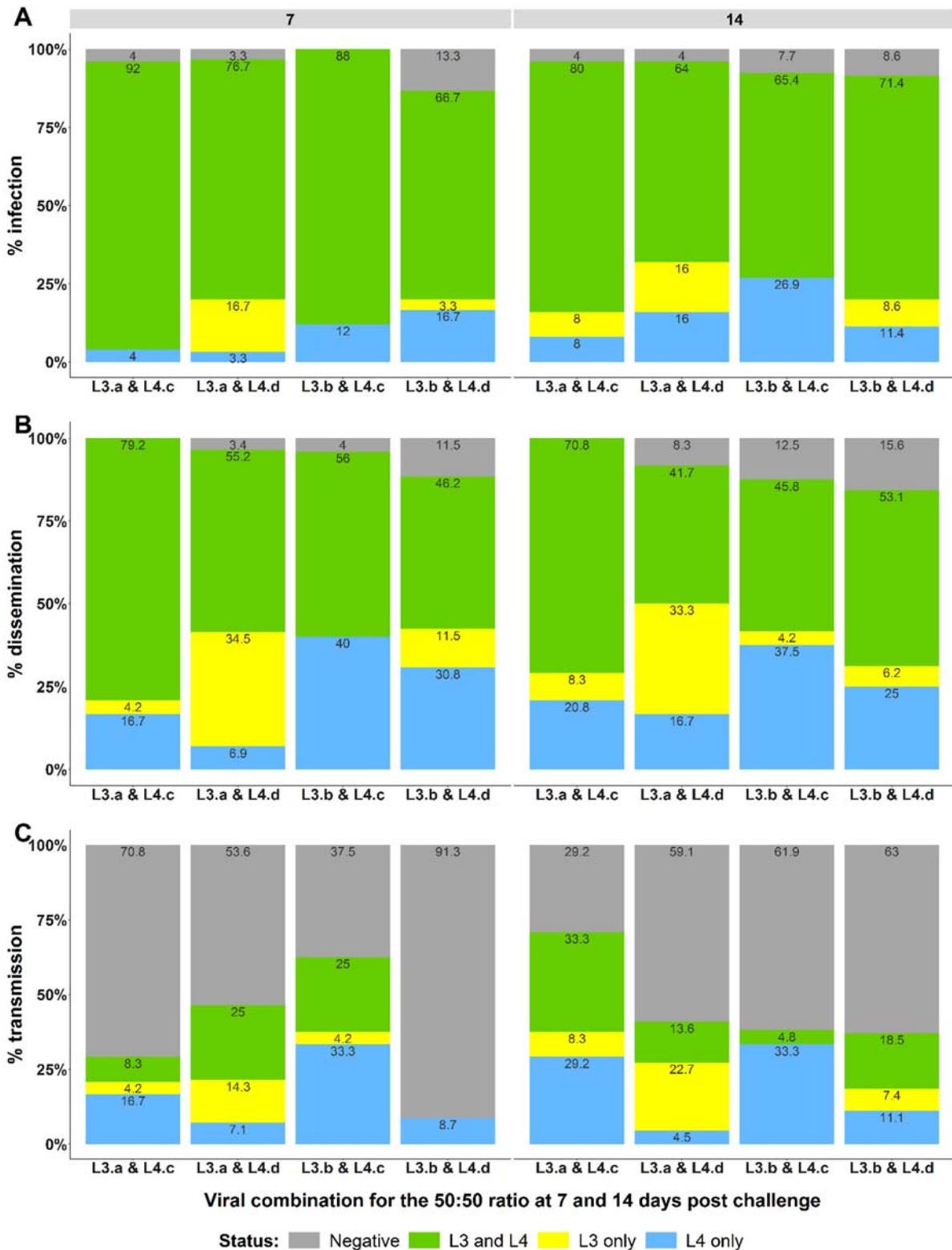
Figure 4. Infection, dissemination and transmission rates of Cambodian *Ae. aegypti* mosquitoes detected with the 50:50 infectious ratio. Infection rates (A), dissemination rates (B) and transmission rates (C) are represented. Status L3 and L4, L3 or L4 corresponds to the detection by RT-qPCR of both targets, only L3 or only L4 respectively in each mosquito compartment (body, head and saliva). No amplification detected for negative status.

fitness was observed for DENV-1 GI relative to GIV in NC. Provided at equal titre, 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

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Disclosure statement

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