International Journal of Infectious Diseases 139 (2024) 59–68

Contents lists available at ScienceDirect

International Journal of Infectious Diseases

journal homepage: www.elsevier.com/locate/ijid

Role of pre-existing immunity in driving the dengue virus serotype 2 genotype shift in the Philippines: A retrospective analysis of serological data

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ARTICLE INFO

Article history:
Received 7 September 2023
Revised 17 November 2023
Accepted 19 November 2023

Keywords:
Dengue
Antibody-dependent enhancement
Genotype shift
Neutralizing antibodies
Pre-existing immunity

ABSTRACT

Objective: The invasion of dengue virus (DENV)-2 Cosmopolitan genotype into the Philippines, where the Asian II genotype previously circulated challenges the principle of dengue serotype-specific immunity. Assessment of antibodies in this population may provide a mechanistic basis for how new genotypes emerge in dengue-endemic areas.

Methods: We evaluated the neutralizing antibody (nAb) and antibody-dependent enhancement (ADE) responses against the two genotypes using archived serum samples collected from 333 patients with confirmed dengue in Metro Manila, Philippines, before, during, and after the introduction of the Cosmopolitan genotype. We quantified nAb titers in baby hamster kidney (BHK-21) cells with or without the Fcγ receptor IIA (FcγRIIA) to detect the capacity of virus-antibody complexes to neutralize or enhance DENV.

Results: The nAb potency of the archived serum samples against the two genotypes was greatly affected by the presence of FcγRIIA. We found significant differences in nAb titers between the two genotypes in BHK-21 cells with FcγRIIA (P <0.0001). The archived serum samples were incapable of fully neutralizing the Cosmopolitan genotype, but instead strongly promoted its ADE compared to the Asian II genotype (P <0.0001).

Conclusion: These results reinforce the role of pre-existing immunity in driving genotype shifts. Our finding that specific genotypes exhibit differing susceptibilities to ADE by cross-reactive antibodies may have implications for dengue vaccine development.

Introduction

The dengue virus (DENV) consists of four distinct serotypes (DENV1-4) [1], with each serotype further classified into genetically distinct genotypes. Following primary infection with DENV, serotype-specific and cross-serotype antibodies provide protection

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https://doi.org/10.1016/j.ijid.2023.11.025

from re-infection. However, only serotype-specific neutralizing antibodies (nAbs) are thought to provide complete protection against re-infection with the same serotype. Cross-serotype antibodies with neutralizing activity against the remaining serotypes wane over time [2,3], and may eventually enhance secondary infection with a different serotype [4]. This phenomenon, termed antibody-dependent enhancement (ADE), may explain the increased risk of severe disease in secondary DENV infection [5]. Antibody-dependent enhancement of DENV infection has been reported *in vitro* [6,7], *in vivo* [8,9], and in natural human infection [10]. The ADE activity of antibodies is commonly evaluated by using cells expressing Fcγ receptors (FcγRs), which facilitate ADE by allowing entry and internalization of virus-immune complexes, leading to increased viremia in the target immune cells of DENV.

The current dogma that infection by one serotype provides complete and lifelong protection against re-infection with the same serotype has been challenged by recent *in vitro* studies, which demonstrate that genetically variable genotypes can have distinct influences on virus neutralization [11–13]. This phenomenon is supported by an epidemiological report of a large DENV-2 outbreak in Peru, which demonstrated that protection from DENV-2 re-infection may be incomplete [14] and also by an analysis of homotypic re-infections (infection with the same serotype) in a pediatric cohort study in Nicaragua [15]. The occurrence of homotypic re-infections may have profound epidemiological consequences, particularly during genotype shifts, in which endemic genotypes are completely replaced by new genotypes imported from another geographical region. DENV genotype shifts often result in large epidemics in dengue-endemic areas [16,17]. For example, in the Philippines, the 1998 DENV-2 epidemic recorded the highest dengue case fatality rate (2.6%) in Philippine history [18,19], which coincided with a genotype shift from the Asian II genotype to the Cosmopolitan genotype [20]. To date, there is no definitive mechanism known for how the Cosmopolitan genotype displaced the Asian II genotype and how this genotype has continued to circulate. We speculate that the emergence and rapid spread of the Cosmopolitan genotype in the Philippines may be attributed to a lack of robust immunity in the local population due to the presence of cross-serotype-enhancing antibodies derived from pre-existing exposure to dengue.

To test this hypothesis, we first performed an evolutionary analysis of DENV-2 in the Philippines to infer the genetic diversity and evolution of DENV-2, and to track the emergence of the Cosmopolitan genotype. This provided a comprehensive molecular epidemiological overview of the DENV-2 genotype shift in the Philippines. Additionally, we performed protein structure prediction to better understand the structural differences between these two genotypes and how these differences may affect their interaction with antibodies. We retrospectively evaluated the nAbs and ADE responses of archived serum samples, collected from 1995 to 2009, against the two genotypes using baby hamster kidney (BHK-21) cells with or without FcγRIIA to measure the capacity of serum samples to neutralize or enhance DENV infection. We utilized a replicon-based plasmid technology to generate DENV-2 single-round infectious particles (SRIPs) that differ only by the genotype sequence of the precursor membrane (prM) and envelope (E) proteins. SRIPs are pseudo-infectious virus particles capable of a single round of replication and closely mimic live virus replication without producing infectious progeny virus particles [21–23]. We demonstrated that the local population possessed robust immunity against the prior circulating Asian II genotype throughout the collection period, but lacked robust immunity against the invading Cosmopolitan genotype. These findings support our hypothesis that the Cosmopolitan genotype was able to efficiently expand due to the presence of cross-serotype ADE antibodies derived from endemic circulation of DENV in the Philippines.

Methods

Study population

We used 333 archived serum samples collected from 333 patients with confirmed DENV infection spanning from January 1995 to December 2009 in Metro Manila, Philippines (Supplementary Table 1; Supplementary Figure 1). These samples were obtained from the sample collection of the Research and Biotechnology Group, St. Luke's Medical Center, Quezon City, Philippines. Specifically, cases were initially selected according to the 1997 World Health Organization dengue guidelines. Samples collected during the febrile phase of DENV infection were subjected to standard reverse transcriptase-polymerase chain reaction (RT-PCR) as described previously [20].

Molecular clock analyses

We used the Bayesian analysis method to determine the evolutionary characteristics of DENV-2 isolates in the Philippines. We performed an alignment of the full-length DENV-2 E gene sequences using ClustalW 2.1 and manually edited the aligned sequences in Mesquite 3.3. We assessed the rate of nucleotide substitution and time to the most recent common ancestor (TMRCA) of the Cosmopolitan DENV-2 isolates using Bayesian inferences implemented in the Bayesian evolutionary analysis by sampling trees 2 software. We time-tagged each sequence based on year of isolation and determined the best-fit nucleotide substitution model by Bayesian information criterion in ModelTest-NG [24]. For our analysis, we employed the relaxed lognormal molecular clock and the Bayesian skyline coalescent tree prior. To ensure stationarity and convergence, we set the chain length of the Markov chain Monte Carlo to 100,000,000 with sampling frequency of 10000. We analyzed the resulting log files in Tracer 1.7.2 to ascertain the convergence of the chain. To present the evolutionary relationships, we generated the maximum clade credibility (MCC) tree using Tree Annotator and visualized the resulting MCC tree in FigTree 1.4.4. Support for each node on the tree was ascertained by Bayesian posterior probability values.

Protein structure prediction

We submitted the prM and E sequences of the representative Philippine Asian II and Cosmopolitan isolates to the Robetta protein structure prediction service (https://robetta.bakerlab.org) for single chain modeling using the RoseTTAFold [25] deep learning-based method, which utilizes multiple sequence alignments and templates of similar protein structures to achieve the best optimal structural prediction performance. We chose the best structure models based on the angstrom error estimate per residue parameter. We visualized the protein structures using ChimeraX [26,27] and superimposed the structures using the Needleman-Wunsch algorithm with the blocks substitution matrix scoring to determine the quality of the structural alignment between the predicted protein structures.

Cell lines

We maintained BHK-21 in Eagle's minimum essential medium (EMEM) (Wako) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (BioWest) and 100 U/mL penicillin-streptomycin solution (Sigma-Aldrich). We maintained BHK-21 cells that express the human FcγRIIA in EMEM supplemented with heat-inactivated 10% FBS and 0.5 mg/ml neomycin (G418, PAA Laboratories). We maintained human embryonic kidney 293T (HEK293T) cells in high glucose Dulbecco's modified Eagle's

medium with L-glutamine (DMEM, Wako) supplemented with heat-inactivated 10% FBS, 1X MEM non-essential amino acids (Gibco) and 100 U/mL penicillin-streptomycin solution. Cells were cultured at 37°C in a 5% CO_2 incubator.

Plasmid construction and production of SRIPs

We constructed the sub-genomic replicon plasmid from the DENV-1 D1/Hu/Saitama/NIID100/2014 strain genotype I (LC002828) containing the nano-luciferase gene (pCMV-D1-nluc-rep) and the expression plasmid for DENV-1 D1/Hu/Saitama/NIID100/2014 strain mature C consisting of 105 amino acids (pCAG-D1C) as described previously [23]. We constructed the pCAGGS-based expression plasmids encoding prM and E of DENV-2 Philippine Asian II (AY786372) and Cosmopolitan (AY786395) isolates from synthetic DNA (GENEWIZ). We sequenced the plasmids before use in transfection experiments. We transfected HEK293T cells grown in a 10-cm dish with three plasmids: 2.5 µg of replicon plasmid, 1.25 µg of capsid-expression plasmid, and 1.25 µg of prME-expression plasmid, using polyethylenimine Max (Cosmo-Bio) in Opti-MEM (Gibco) as described previously [23]. After 5-6 hours, we replaced the culture medium with fresh medium. At 2 days post-transfection, we replaced the medium with complete medium supplemented with 10 mM 4-(2-hydroxyethyl)-1-piperazineethane sulfonic acid buffer (Gibco). We harvested the standard SRIP preparations 3 days post-transfection, clarified the solutions through a 0.45-µM filter, and froze at -80°C until further use.

Neutralization and ADE assays using SRIPs

We performed the neutralization experiments using both FcγRIIA-negative and FcγRIIA-positive BHK-21 cells, whereas we performed ADE experiments using FcγRIIA-positive BHK-21 cells. First, we prepared the cells in 96-well plates at a density of 5.0 – 5.5 × 10^4 cells per well overnight. On the following day, we heat-treated the serum samples at 56°C for 30 min. Subsequently, we serially diluted the samples fourfold with EMEM supplemented with 2% FBS, mixed them with SRIPs at a 1:1 ratio, then incubated them at 37°C for one hour. We inoculated the SRIP-serum mixture onto plates containing cell monolayers and incubated them at 37°C in 5% CO_2 for 5-6 hours. Following this, we added fresh medium and further incubated them at 37°C in 5% CO_2 for 3 days. We included an SRIP control (no serum) and cell control (no SRIP, no serum) for each plate, testing each serum sample in duplicate technical replicates. After 3 days, we detected luciferase activity in SRIP-infected cells using the Nano-Glo® Luciferase Assay System (Promega). We expressed the neutralization titer as the serum dilution that reduced the luciferase signal by ≥50% (NT_{50}). We calculated the reduction of luciferase signal using the following formula:

$$\%\ \text{luciferase reduction} = 1 - \frac{RLU_t - RLU_c}{RLU_s - RLU_c} \times 100\%$$

We calculated fold enhancement by the following formula:

$$\text{fold enhancement} = \frac{RLU_t - RLU_c}{RLU_s - RLU_c}$$

RLU is the relative luminescence unit measured corresponding to the luciferase activity in SRIP-infected cells. RLU_t is the RLU of test serum samples, RLU_s is the RLU of SRIP controls, while RLU_c is the RLU of cell controls. We determined the peak enhancement titer (PET) by plotting the fold enhancement value on the y-axis and log-reciprocal serum dilution on the x-axis. We used a Gaussian distribution curve to fit the ADE titration curve. The data point corresponding to the amplitude was used to derive the log-reciprocal serum dilution and was reported as PET.

Antigenic cartography

We used the neutralization titers as the foundation for antigenic cartography. Neutralization titers below the detection limit were set to <10. Table distance D_{ij}, which was the distance between SRIP *i* and serum *j* derived from the corresponding neutralization titer, was calculated and projected onto a 2D map via ACMACS [28] (https://acmacs-web.antigenic-cartography.org).

Statistical analysis and graphical representations

We performed statistical analyses using GraphPad Prism 9 (GraphPad Software), with a 5% level of significance and two-tailed *P*-values. We prepared all figures in BioRender (http://biorender.com) for publication.

Results

Evolutionary analysis of the DENV-2 genotype shift in the Philippines

To date, a comprehensive molecular epidemiological picture of the DENV-2 genotype shift in the Philippines (Figure 1a) has not been conducted. Hence, we determined the evolutionary history, fluctuations in genetic diversity, and origin of DENV-2 Philippine isolates by conducting a molecular clock analysis using Bayesian methods. Based on the Bayesian skyline plot, which shows changes in genetic diversity of DENV-2 over time, we found that DENV-2 genetic diversity in the Philippines remained almost constant from 1983 to 1995. A slight increase in genetic diversity was observed between 1995 and 1996 followed by a decline in early 2000 coinciding with the emergence of Cosmopolitan genotype and subsequent displacement of Asian II genotype (Figure 1b and 1c). We also determined the estimated time of introduction and possible source of the Cosmopolitan genotype using E gene sequences isolated in the Philippines and its neighboring countries in Asia-Pacific. We found that the TMRCA of the dataset was in 1996, and Philippine isolates were closely related to older isolates from Australia, Singapore, and Thailand (Figure 1d).

Genetic and structural variations between the prM and E proteins of the Philippine Asian II and Cosmopolitan isolates used for SRIP construction

We generated SRIPs of two representative Philippine DENV-2 isolates, Asian II (AY786372) and Cosmopolitan (AY786395) (Figure 1b). These isolates were selected as they represent the conserved prM and E regions among the Philippine isolates for each genotype. The two representative Philippine isolates exhibited amino acid substitutions in their prM and E proteins. Specifically, they differed in nine amino acid residues within the prM, while they differed in six amino acid residues within E domain I (EDI) and EDII and six distinct amino acid residues within EDIII (Figure 2b). Amino acid changes in the E domains (E71, E129, E149, E164, E390) occurring on the tree branch leading to the Cosmopolitan genotype are present in all the Philippine DENV-2 Cosmopolitan isolates, as previously reported [29]. We performed structural alignment of the predicted prM and E protein structures between the two isolates and calculated their Root Mean Square Deviation (RMSD) values to determine the quality of the structural alignments; wherein RMSD is 0 Å for identical structures, and the value increases as the two structures become more different. The structural alignments between the prM of the two isolates were substantially different (RMSD = 4.800 Å) (Figure 2c), particularly in their pr polypeptides. In contrast, the predicted E proteins of the two isolates were not structurally

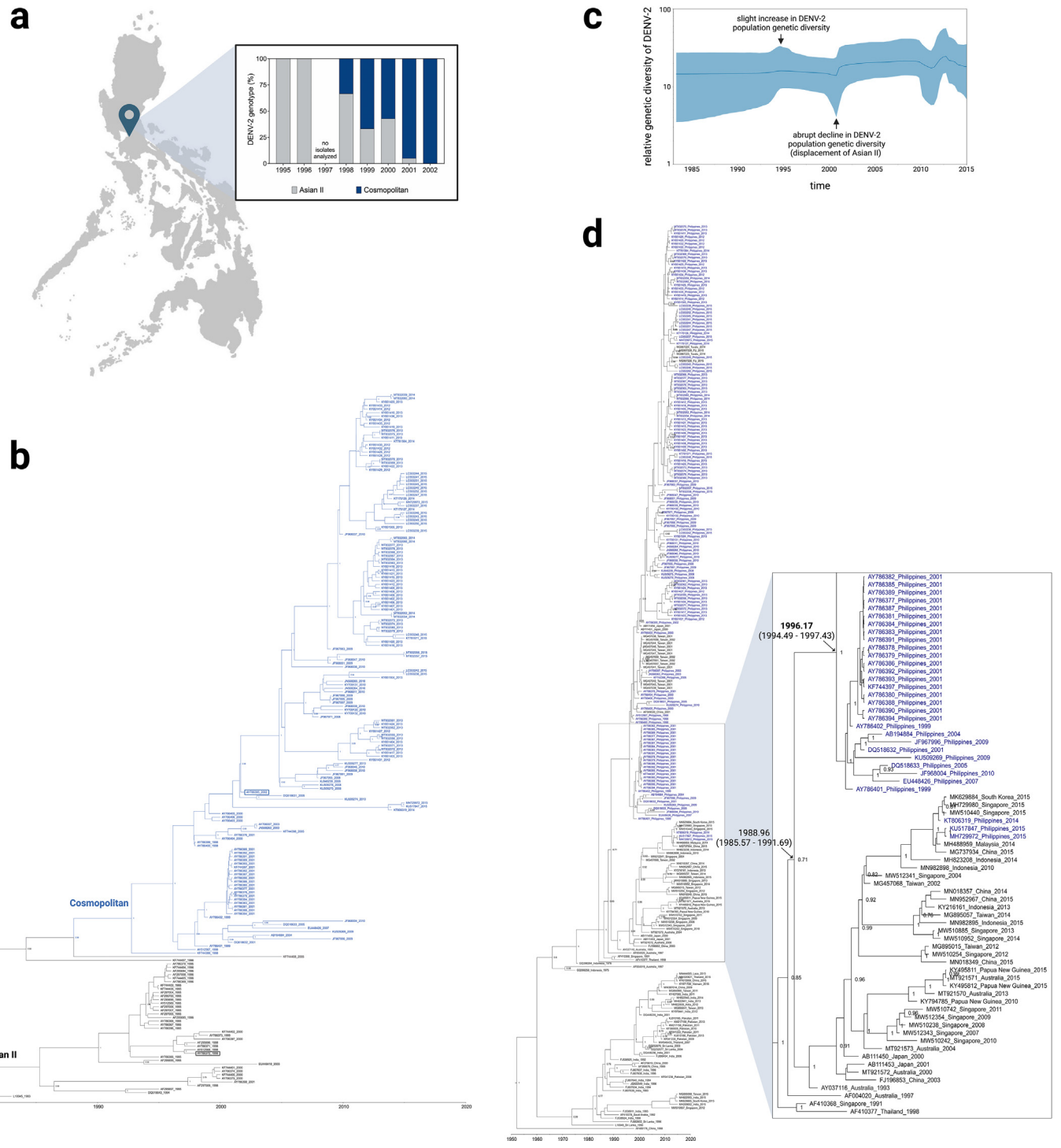
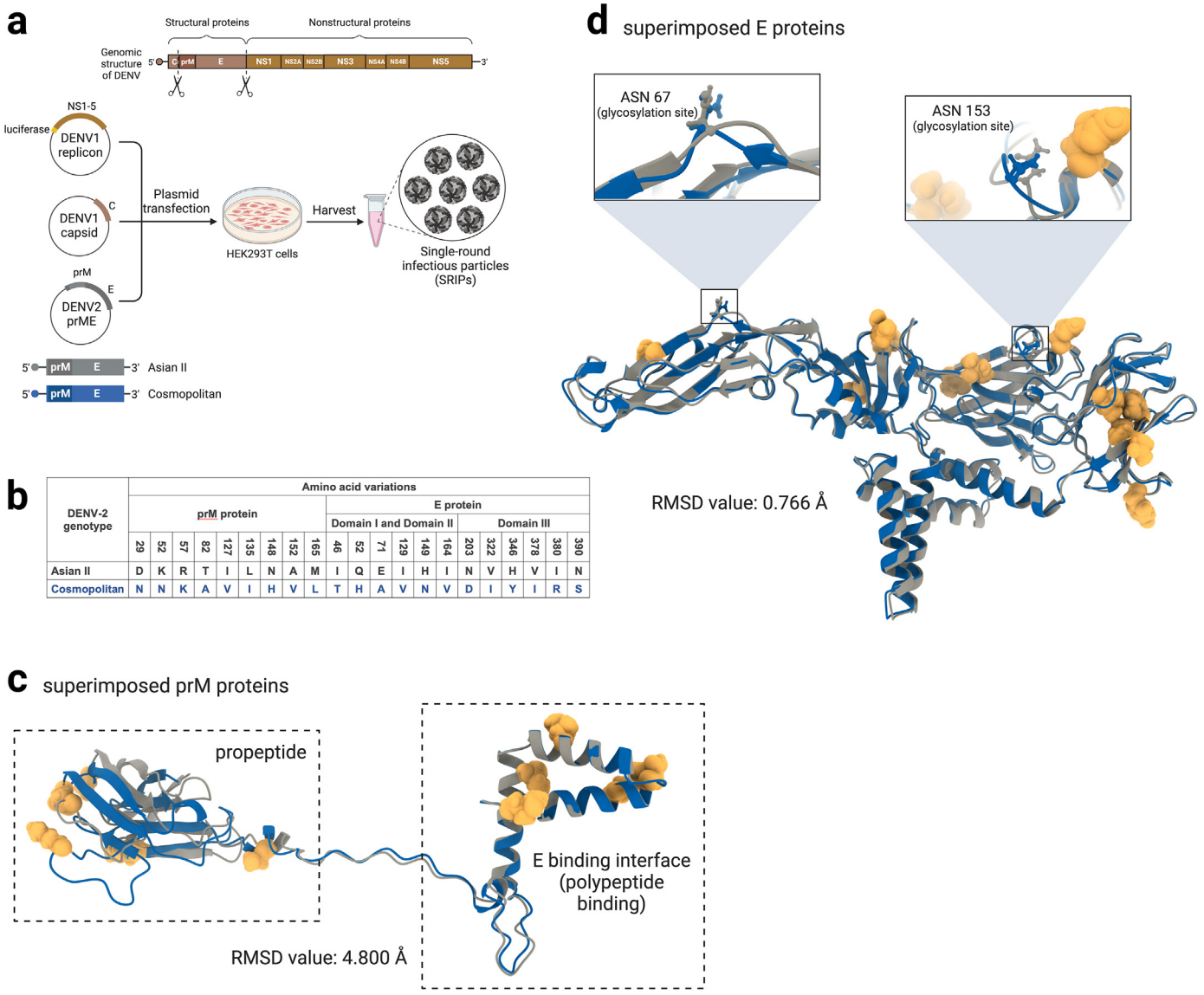


Figure 1. Molecular clock analysis of the DENV-2 genotype shift in the Philippines. **(a)** Prevalence of the DENV-2 Asian II and Cosmopolitan genotypes in Metro Manila, Philippines from 1995 to 2002 (Salda et al. [20]: cited with permission); Metro Manila represented by a map location pointer; data for creating the map were acquired from the Philippine geographic information system data website (www.philgis.org); created map in QGIS 3.6 and edited in BioRender. **(b)** MCC tree of 197 Philippine DENV-2 Asian II and Cosmopolitan isolates collected from 1995 to 2015; performed molecular clock analysis using the full-length E nucleotide sequences; Asian II and Cosmopolitan isolates used for preparing the DENV-2 SRIPs shown in bold letters; posterior values ≥ 0.7 shown on the nodes. **(c)** Bayesian skyline plot of 197 Philippine DENV-2 Asian II and Cosmopolitan isolates collected from 1983 to 2015; y-axis measures relative genetic diversity in log values, x-axis represents time in years, solid line represents the median estimate, blue shaded area represents the credibility interval based on 95% HPD interval. **(d)** Estimation of the time of introduction of the DENV-2 Cosmopolitan genotype in the Philippines using 263 Cosmopolitan isolates in the Philippines (datasets in blue) and the Asia-Pacific region; mean TMRCA of the Philippine DENV-2 Cosmopolitan isolates shown in principal nodes (mean and 95% HPD) as indicated by arrows, most recent common ancestor shown in bold brown letters; used BEAST2 to estimate divergence using the relaxed lognormal coalescent Bayesian skyline model; posterior values ≥ 0.7 shown on the nodes.
BEAST2, Bayesian evolutionary analysis by sampling trees 2; BHK-21, baby hamster kidney cells; DENV, dengue virus; HPD, highest probability density; SD, standard deviation; SRIP, single-round infectious particle; TMRCA, time to the most recent common ancestor.



b

DENV-2 genotype	Amino acid variations																				
	prM protein									E protein											
	Domain I and Domain II														Domain III						
	29	52	57	82	127	135	148	152	165	46	52	71	129	149	164	203	322	346	378	380	390
Asian II	D	K	R	T	I	L	N	A	M	I	Q	E	I	H	I	N	V	H	V	I	N
Cosmopolitan	N	N	K	A	V	I	H	V	L	T	H	A	V	N	V	D	I	Y	I	R	S

Figure 2. Genetic and structural variations between the DENV-2 Philippine Asian II and Cosmopolitan SRIPs. **(a)** Luciferase-expressing DENV-2 SRIPs produced via transfection of HEK293T cells with a mixture of three plasmids encoding the DENV-1 sub-genomic replicon containing the luciferase gene, the DENV-1 capsid, and the DENV-2 prME. **(b)** E protein amino acid positions that differ between the Asian II (grey) and Cosmopolitan (blue) isolates. **(c)** Superimposed predicted ribbon structures of the prM protein monomers of the DENV-2 Asian II (grey) and Cosmopolitan (blue) isolates. Amino acids that differ between the two isolates were mapped on the predicted prM monomers shown as grey spheres. **(d)** Superimposed predicted ribbon structures of the E protein monomers of the DENV-2 Asian II (grey) and Cosmopolitan (blue) isolates. Amino acids that differ between the two isolates were mapped on the predicted E monomers shown as grey spheres. The glycosylation sites of the E monomer, Asparagine (ASN) 67 and 153 were shown, and a slight difference on the glycosylation site conformation could be observed at ASN 153. The RoseTTAFold deep-learning method was used to predict the prM and E protein monomers and the monomers were superimposed in ChimeraX.
ADE, antibody-dependent enhancement; ASN, Asparagine; BHK-21, baby hamster kidney cells; DENV, dengue virus; prME, xxx; prM, xxx; SD, standard deviation; SRIP, single-round infectious particle.

different (RMSD = 0.766 Å) (Figure 2d). We also checked the predicted structures of other Asian II and Cosmopolitan isolates that harbor different mutations on their prM and E regions and found that they do not exhibit significant differences in their structures in comparison to the corresponding genotype isolate used in this study (Supplementary Figure 2). Given that prM protein is reported to be the major target of cross-reactive antibodies that have limited capacity to neutralize but have high capacity to enhance DENV infection, we speculate that structural differences in the prM of the two viruses may have consequences in their ADE by cross-reactive antibodies. Using the two isolates, we generated DENV-2 SRIPs to characterize serum samples collected from DENV-infected patients (Figure 2a; Supplementary Figure 3).

Antibody profiles of the archived serum samples against Asian II and Cosmopolitan from 1995 to 2009

To check whether Asian II and Cosmopolitan SRIPs exhibit differential neutralization throughout the collection period (1995–2009), we tested the nAb response of the archived serum samples (N = 333) using BHK-21 cells with or without the human FcγRIIA (Figure 3a). Yearly comparisons of the neutralization titers revealed that the archived serum samples exhibited strong nAb response to the Asian II SRIP, regardless of the presence of the FcγRIIA. In contrast, the archived serum samples showed a much lower nAb response to the Cosmopolitan SRIP in the presence of the FcγRIIA (Figure 3b). To determine the capacity of the archived serum samples (N = 333) to enhance DENV-2 infection *in vitro*,

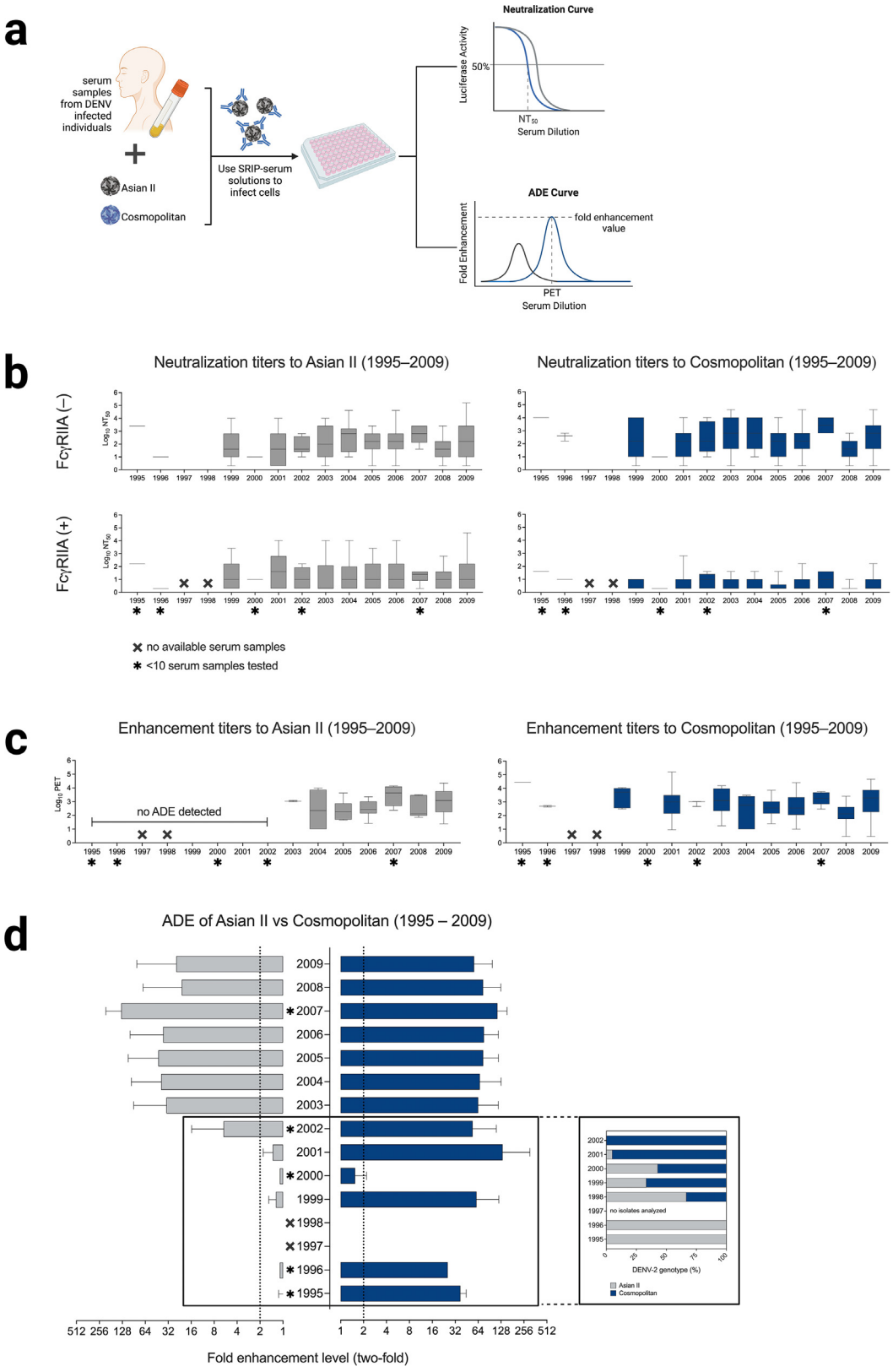


Figure 3. Neutralizing and enhancing antibody profiles of the archived serum samples to the Asian II and Cosmopolitan genotypes based on the year of infection. **(a)** Schematic diagram of the detection of neutralization and enhancement (ADE) titers by using luciferase-expressing DENV-2 SRIPs. **(b)** Comparison of the neutralization of Asian II and Cosmopolitan SRIPs by the archived serum samples ($N = 333$) collected from 1995 to 2009 (box and whiskers plotted as mean with SD) in the presence or absence of the human FcγRIIA. **(c)** Comparison of the enhancement (ADE) titers of the archived serum samples ($N = 333$) to Asian II and Cosmopolitan SRIPs from 1995 to 2009 (box and whiskers plotted as mean with SD). **(d)** Comparison of fold enhancement levels between Asian II and Cosmopolitan SRIPs from 1995 to 2009 (column bar graphs plotted as mean with SD); fold enhancement cut-off values set to 2.0 (shown as dotted lines); prevalence of DENV-2 Asian II and Cosmopolitan genotypes in Metro Manila, Philippines shown for years 1995 to 2002 (Salda et al. [20][32]: cited with permission). Statistical significance: $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.

ADE, antibody-dependent enhancement; BHK-21, baby hamster kidney cells; DENV, dengue virus; SD, standard deviation; SRIP, single-round infectious particle.

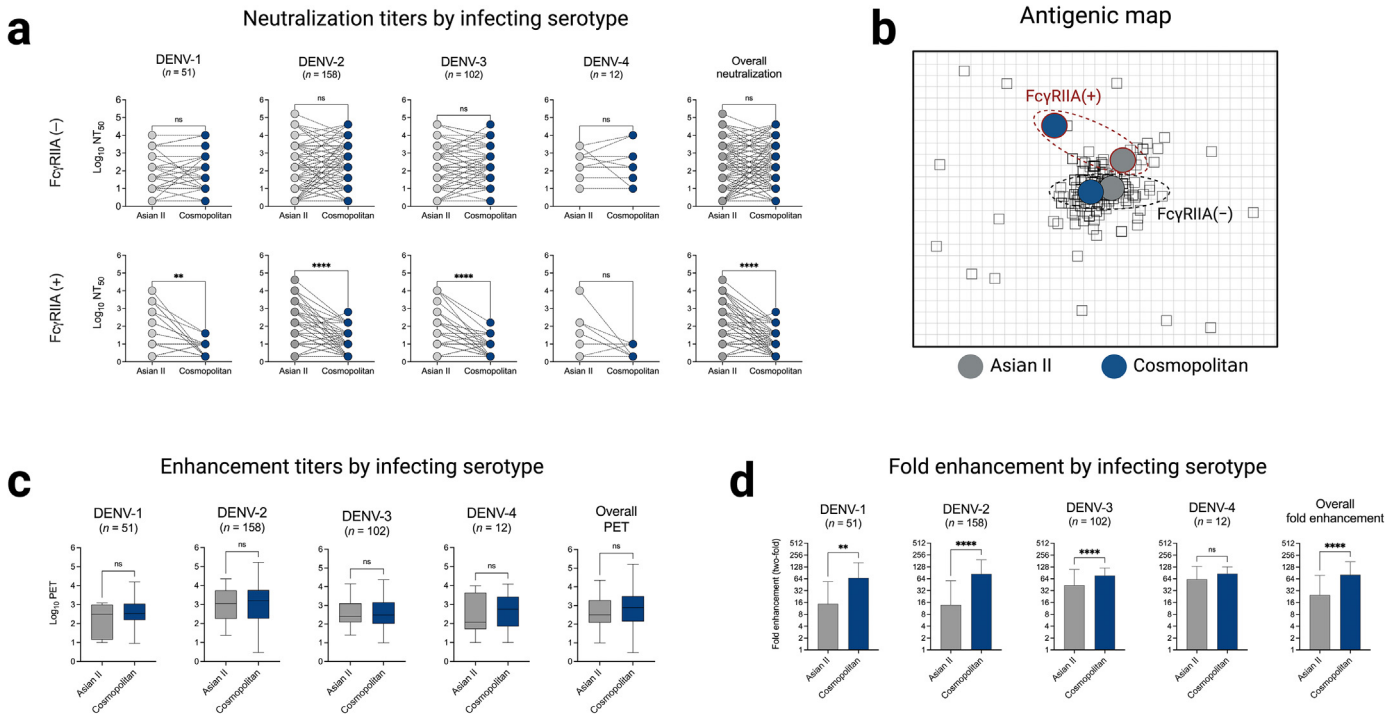


Figure 4. Neutralizing and enhancing antibody profiles of the archived serum samples to the Asian II and Cosmopolitan genotypes based on the infecting DENV serotypes. **(a)** Neutralization titers of the archived serum samples ($N = 333$) to Asian II and Cosmopolitan SRIPs categorized according to the infecting serotype; statistical significance determined by the nonparametric Mann-Whitney test; for <10 neutralization titers: a titer of 2 (about fourfold lower than the assay's limit of detection, 10) was entered for analysis. **(b)** Antigen map of DENV-2 Asian II and Cosmopolitan SRIPs titrated against antisera from infected patients in the Philippines; neutralization titers determined by using FcγRIIA-negative and FcγRIIA-positive BHK-21 cells; x and y axes represent antigenic distance, and each grid square corresponds to twofold dilution in the neutralization titer (antisera: squares; SRIPs: circles). **(c)** Enhancement (ADE) titers of the archived serum samples ($N = 333$) to Asian II and Cosmopolitan SRIPs categorized according to infecting serotype; statistical significance determined by the nonparametric Mann-Whitney test (box and whiskers plotted as mean with SD). **(d)** Fold enhancement levels of Asian II and Cosmopolitan SRIPs by the archived serum samples ($N = 333$) categorized according to infecting serotype; statistical significance determined by the nonparametric Mann-Whitney test (column bar graphs plotted as mean with SD). Statistical significance: $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$.
ADE, antibody-dependent enhancement; BHK-21, baby hamster kidney cells; DENV, dengue virus; SD, standard deviation; SRIP, single-round infectious particle.

we inoculated FcγRIIA-positive BHK-21 cells with the SRIP-serum mixture, following which, we performed an ADE assay to calculate the fold enhancement and PET (Figure 3a). The PET represents the specific serum dilution that most efficiently enhances DENV infection [10]. We started with a 1:10 dilution of serum sample to mimic serum antibody concentrations close to biological conditions. Yearly comparisons of the PET (Figure 3c) and the fold enhancement (Figure 3d) between the two SRIPs revealed that the Cosmopolitan SRIP was consistently enhanced by the archived serum samples throughout the collection period. In contrast, enhancement of the Asian II SRIP was mainly detected from 2002 to 2009. Out of the 333 archived serum samples, 79.0% exhibited ADE activity to the Cosmopolitan SRIP, while only 20.4% of the archived serum samples exhibited ADE activity to the Asian II SRIP at a fold enhancement cut-off of 2 (Figure 4b). Altogether, these findings suggest that the archived serum samples exhibited stronger protection to the Asian II genotype compared to the Cosmopolitan genotype throughout the collection period.

Neutralizing and infection-enhancing activities of pre-existing cross-serotype antibodies in archived serum samples

We next evaluated the impact of the DENV infecting serotype on the neutralization and enhancement of the two genotype viruses by stratifying the archived serum samples by the infecting serotype. Overall, the archived serum samples strongly neutralized Asian II SRIP, both with (mean $NT_{50} = 631.0$) or without (mean $NT_{50} = 2441.0$) FcγRIIA. In contrast, we found lower neutralization of the Cosmopolitan SRIP in FcγRIIA-positive BHK-21 cells (mean $NT_{50} = 13.5$), but not in FcγRIIA-negative BHK-21 cells (mean $NT_{50} = 2225.0$) (Supplementary Figure 4; Figure 4a). Based on the infecting serotype, the archived serum samples neutralized the Asian II SRIP more strongly than the Cosmopolitan SRIP in FcγRIIA-positive BHK-21 cells, but no difference was observed in FcγRIIA-negative BHK-21 cells (Figure 4a). However, for DENV-4 infected serum samples, no significant differences were observed in FcγRIIA-negative and FcγRIIA-positive BHK-21 cells, which may be attributed to the lesser number of serum samples (n = 12) tested compared to the other serotypes. Using the NT_{50} data, we constructed an antigenic map to further visualize the relationships between the SRIPs relative to the serum samples and in the presence or absence of FcγRIIA. We treated the NT_{50} of each serum sample as a measure of distance between the serum sample and SRIP, such that higher NT_{50} represented shorter distance between the serum sample and SRIP. The two SRIPs showed high antigenic similarity when assayed in FcγRIIA-negative BHK-21 cells, and the majority of the archived serum samples mapped closely to both SRIPs. However, when assayed in FcγRIIA-positive BHK-21 cells, the Cosmopolitan SRIP formed an antigenic outlier in the map, about 13-fold dilutions away from the next nearest SRIP (Figure 4b). We also checked the fold enhancement and ADE titers of the patient serum samples based on the infecting serotype and found that the mean PETs of the archived serum samples to the two SRIPs were not significantly different (Figure 4c) suggesting that serum dilutions (PET) at which ADE occurs is consistent between the two SRIPs but vary only in the magnitude of enhancement (Figure 4d). Overall, these findings indicate that the cross-reactive antibodies with ADE capacity present in the archived serum samples may have lowered the overall neutralization against the Cosmopolitan genotype and that these cross-

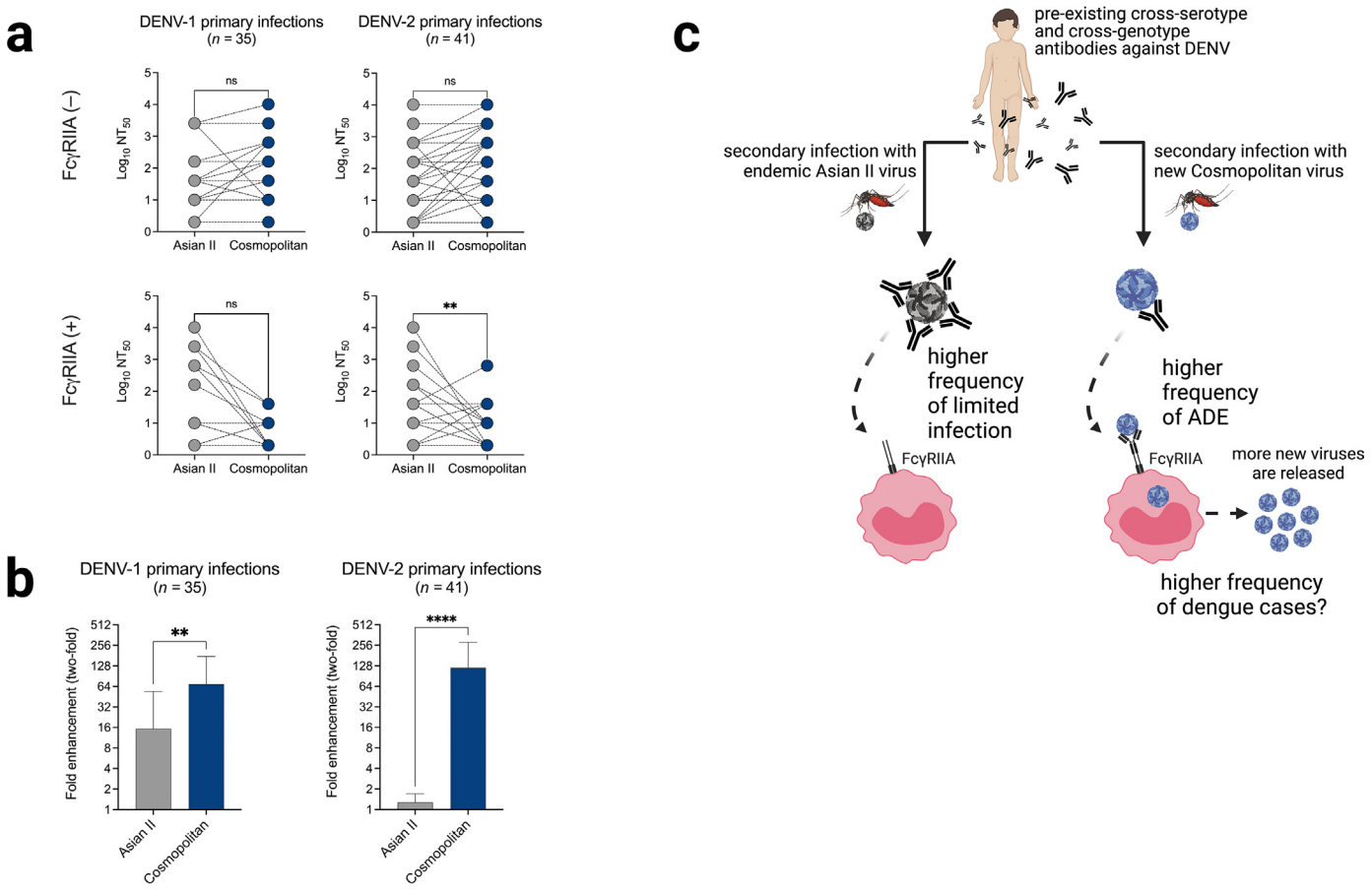


Figure 5. Neutralizing and infection-enhancing activities of cross-reactive antibodies in archived serum samples derived from DENV-1 and DENV-2 primary infections. **(a)** Neutralization titers of the archived primary DENV infections ($n = 76$) to the Asian II and Cosmopolitan SRIPs categorized based on the primary infecting serotype. Statistical significance determined by the nonparametric Mann-Whitney test; for <10 neutralization titers: a titer of 2 (about fourfold lower than the assay's limit of detection, 10) was entered for analysis. **(b)** Fold enhancement levels of the Asian II and Cosmopolitan SRIPs by the archived serum samples derived from primary DENV infections ($n = 76$) categorized according to the infecting serotype; statistical significance determined by the nonparametric Mann-Whitney test (column bar graphs plotted as mean with SD). Statistical significance: $^{ns}P > 0.05$, $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. **(e)** Hypothetical model explaining the documented DENV-2 genotype in the Philippines, which resulted in the massive DENV-2 epidemic in 1998.
DENV, dengue virus; SD, standard deviation; SRIP, single-round infectious particle.

reactive antibodies preferentially enhanced the Cosmopolitan virus *in vitro*.

Neutralizing and infection-enhancing activities of cross-reactive antibodies in archived serum samples derived from DENV-1 and DENV-2 primary infections

To demonstrate that the ADE of the Cosmopolitan genotype is mainly driven by antibodies generated from DENV-2 infection, we compared the neutralization and ADE of the Asian II and Cosmopolitan SRIPs using serum samples from patients who had primary DENV-1 (n = 35) and DENV-2 infections (n = 41) collected in the years 1995 to 2004. We found that the archived serum samples from either primary DENV-1 or DENV-2 infections, both neutralized the Asian II and Cosmopolitan SRIPs in BHK-21 cells without the FcγRIIA. However, in the presence of the FcγRIIA, serum samples derived from primary DENV-2 infections strongly neutralized the Asian II SRIP compared to the Cosmopolitan SRIP. In contrast, no difference was observed in serum samples derived from primary DENV-1 infections (Figure 5a). As expected, serum samples from primary DENV-1 infections enhanced both the Asian II and Cosmopolitan SRIPs. On the other hand, the serum samples from primary DENV-2 infections did not enhance the Asian II SRIP *in vitro* but only enhanced the Cosmopolitan SRIP (Figure 5b). These findings confirm that antibodies derived from DENV-2 infection preferentially enhanced the Cosmopolitan genotype compared to the Asian II genotype.

Discussion

We report in this study that the presence of cross-reactive antibodies with ADE potential derived from prior dengue immunity is a critical factor in the emergence of the Cosmopolitan genotype resulting in the 1998 DENV-2 epidemic in the Philippines. By performing molecular evolutionary analysis, we found that the displacement of the Asian II genotype was likely due to the emergence of the Cosmopolitan genotype. We determined that the virus likely emerged in 1996, 2 years before the first reported isolation of the Cosmopolitan virus. A similar Cosmopolitan virus caused a massive DENV-2 epidemic in Australia in 1993 [30] confirming the role of the emergence of the Cosmopolitan genotype in the DENV-2 epidemic situation in 1998.

Since the population in the Philippines at the time had no previous exposure to the Cosmopolitan genotype, the local population might have been highly susceptible to the new genotype resulting in its rapid invasion in the country. This is likely due to the preferential boost of cross-reactive antibodies with potent ADE potential induced by prior dengue exposure. These pre-existing cross-reactive antibodies may recognize and bind Cosmopolitan virus but are unable to neutralize it. As a consequence, ADE occurs resulting

in a higher frequency of dengue cases (Figure 5c). Our suggested mechanism is corroborated by significant differences in the structures of the predicted prM proteins of the two representative isolates, particularly in their pr polypeptides (Figure 2c), which are typically cleaved by furin protease during virus maturation. Cross-reactive antibodies that mainly target the prM protein do not neutralize DENV infection but potently promote ADE. The structural differences we observed in the prM of the two isolates may have played a role in the potent ADE of the Cosmopolitan virus. A noteworthy finding of our study is that the local population exhibited low nAb responses against the Cosmopolitan genotype, even after its continued persistence in the country. This could be explained through the "original antigenic sin" phenomenon [31], whereby individuals primed with a different serotype led to a lower nAb response toward the Cosmopolitan genotype. Secondary infections with the Cosmopolitan genotype may have preferentially boosted cross-reactive antibodies with non-neutralizing activity, which promotes original antigenic sin. Our results that patient serum samples derived from primary DENV-2 infections showed ADE activity to the Cosmopolitan genotype, but not to the Asian II genotype indicates that antibodies produced after DENV-2 infection showed stronger nAb response to the Asian II genotype than to the Cosmopolitan genotype after secondary infection by the Cosmopolitan genotype. This observation might have accounted for the rapid spread and prevalence of the Cosmopolitan genotype in the Philippines resulting in the displacement of the Asian II genotype. The findings we presented in this study may be the first indication that the occurrence of genotype shifts in dengue-endemic regions can be explained by reduced neutralizing activity coupled with enhanced susceptibility to ADE.

Although ADE has been proposed as a mechanism to explain severe dengue outcomes in secondary DENV infection, little work has been done to examine the implications of ADE in dengue vaccine development. That Sanofi Pasteur's *Dengvaxia* mimicked a primary DENV infection reinforced the importance of ADE evaluation in dengue vaccine development. Despite this, quantifying ADE is still not considered necessary in testing the efficacy of candidate dengue vaccines. In this study, we demonstrate that ADE can occur in secondary infections with the same serotype. As tetravalent dengue vaccine candidates are currently being developed using representative strains of each serotype, a comprehensive assessment of genotype-wide ADE should be performed to develop safer and more efficacious dengue vaccines. We argue that knowledge of both the neutralizing and ADE potential of serum antibodies is imperative in designing dengue vaccines, as this is especially important in devising strategies to protect vulnerable populations in the face of invading genotypes that may have higher susceptibility to ADE.

One caveat of our study is that the exact proportion of cross-reactive and serotype-specific antibodies and their contributions to neutralization and ADE after primary or secondary DENV infections remain unknown. This is primarily due to the lack of information on the infection status of the majority of the samples and limited sample volume to conduct subsequent antibody depletion experiments. Additionally, we do not have the genotyping data for all the DENV-2 infections. Future studies of well-documented cases with known primary and secondary infection serotypes from prospective cohort studies are needed to further understand the role of pre-existing DENV immunity in the occurrence of genotype shifts. Lastly, we were unable to fully characterize the viral kinetics and virulence of the DENV-2 Asian II and Cosmopolitan SRIPs used in this study, as only the prM and E regions are reflected in the SRIP system. Nonetheless, since we used an identical replicon backbone for the two genotypes, we were able to evaluate immunogenicity to prM and E without the effect of other steps, such as genome replication and release.

In conclusion, our findings support the role of pre-existing dengue immunity in driving the rapid and widespread introduction of new invading genotypes in dengue-endemic regions. Continuous monitoring of circulating genotypes and serotypes will be key in understanding the landscape and evolution of DENV populations and in further elucidating the roles of virological and serological factors in the occurrence of dengue outbreaks and epidemics.

Declaration of competing interests

The authors have no competing interests to declare.

Funding

This work was supported by the Japan Agency for Medical Research and Development (AMED: JP223Ja627004, JP23fk0108683, JP23fk0108656, 22wm0125006h003, 23wm0125006h004, 22fm0208101j0006, 23fk0108656h1201, 22ab0123456h001); e-Asia Joint Research Program under AMED (22jm0210079h0003 and JP23jm0210079h004); Strategic Center of Biomedical Advanced Vaccine Research and Development for Preparedness and Response: SCARDA under AMED (JP223fa627004h001 and JP23fa627004h002); Japan Society for the Promotion of Science (JSPS: 21K07059, 22kk0115); and the Naito Memorial Foundation. The funders of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the manuscript.

Ethics approval statement

Ethical approval for this study was obtained from the Institutional Review Boards of Nagasaki University (150917143 and 220915280) and St. Luke's Medical Center (SL-22139). Archived serum samples from the sample collection of the Research and Biotechnology Group of St. Luke's Medical Center were collected from subjects who provided written informed consent. Serum samples from children whose parents or legal guardians provided written informed consent were also collected.

Acknowledgments

We wish to thank the patients who provided serum samples for this study. We are also grateful to the research personnel in the Research and Biotechnology Group of St. Luke's Medical Center, especially to Lady Anne Suarez-Pangilinan, for the assistance received during the retrieval of the archived serum samples from the Dengue Serum Bank. We would also like to thank the members of the Department of Virology and the Department of Tropical Viral Vaccine Development, Institute of Tropical Medicine, Nagasaki University for providing technical advice and support.

Author contribution statement

KM conceived and supervised this study; JCB, MPSD, CCB, and KM designed this study; JCB, RS, and MM designed and produced the DENV SRIPs used for the antibody assays; JCB performed the neutralization and ADE experiments with support from DX. JCB analyzed and interpreted the data with support from KM. MPSD, RRM, FFN, and CCB provided the archived clinical samples with identified DENV serotypes. RS, MMNT, YT, and MLM provided resources for this study. KM, RS, and YT acquired funding for this study. JCB wrote the first draft of the manuscript with support from RC, CCB, and KM. All authors read and approved the final manuscript.

Data availability statement

All main data generated or analyzed during this study supporting the findings are available in the article file and supplementary information files. Other data that support the findings of this study are available from the corresponding author upon reasonable request. All authors had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.ijid.2023.11.025.

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