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Supplementary Information for

Dengue type 1 viruses circulating in humans are highly infectious and poorly neutralized by human antibodies

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This PDF file includes:

SI Methods
Figs. S1 to S8
Tables S1 to S3

SI Methods

Patient Blood plasma

Blood was collected from patients in BD Vacutainer® plastic EDTA tube and centrifuged to get plasma as supernatant. Plasma was stored in multiple aliquots at -80˚C till further use.

Cells Lines and Viruses

C6/36 cells were grown in Gibco minimal essential medium (MEM) at 32°C. Vero 81 cells and furin over-expressing Vero cells were grown in Gibco Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM-F-12) supplemented with sodium bicarbonate (Gibco) at 37°C. K562 cells were grown in Roswell Park Memorial Institute (RPMI) 1640 medium at 37°C. All media were additionally supplemented with 5% fetal bovine serum, 1X Gibco non-essential amino acids, 2mM L-glutamine, 100 U/ml penicillin, and 100 µg/ml streptomycin. Furin overexpressing Vero cells were maintained with 7% fetal bovine serum and 5µg/ml blasticidin. All cells were incubated in 5% CO_2. DENV1 viruses from patient plasma were isolated as explained in the next section. Multiple aliquots of P1 isolates were stored at -80˚C till further use.

Isolation of virus from clinical samples

We inoculated 15µl of plasma with 185 µl of media containing 2% heat inactivated fetal bovine serum (FBS) in 6-well plates and incubated at 37˚C for Vero and furin over-expressing Vero cells and 32˚C for C6/36 cells in 5% CO_2 incubator for 90 minutes. We added above infection media up to 2ml, collected supernatant after cytopathic effect was observed on day 6-8 post-infection, and called this Passage 0 (P0). We again inoculated respective confluent cells in 75cc flasks with 500ul of P0 stocks to make P1 working stocks.

Digital drop PCR

We isolated RNA from 140µl of sample using QIAamp Viral RNA Mini Kit from Qiagen using manufacturer's protocol. Cell culture DENV1 samples were spiked with 1:1 ratio of normal human plasma. cDNA was made using isolated RNA as template and SuperScript® III Reverse Transcriptase from Invitrogen. Bio-Rad Digital drop PCR was performed with various dilutions of cDNA using forward primer 5'-ATCTGTTCAGCCGTTCCAG-3', reverse primer 5'- TGACAGCATGTCTTCTGTTGTC-3', and probe FAM-5'-TGGGTTCCAACCAGCCGCACCACTT-3'- Iowa Black quencher for NS5 region of DENV1 according to manufacturer's protocol. Briefly, PCR reaction ingredients were mixed with oil droplets in Bio-Rad's automated droplet generator instrument AutoDG™. PCR reaction was performed using thermal cycler and fluorescence in each droplet was read on Bio-Rad's QX200™ Droplet reader machine in FAM settings. Reactions were run in technical duplicates and back calculated to get exact copies of DENV1 RNA in samples.

Vero infectious titre assay

Vero cells ($3x10^4$) were grown in a 24-well plate for 48 hours and used at about 100% confluency. 100µl of ten-fold serially diluted DENV samples were infected onto the Vero monolayer for 90 minutes at 37˚C in 5% CO_2. Cell culture DENV samples were spiked with equal amount of normal human plasma in the first ten-fold dilution. Infected cells were overlayed with methyl cellulose and kept at 37˚C in 5% CO_2 until foci formation. The monolayer was stained on day 5 post infection using a combination of mouse 2H2 and 4G2 mAbs and HRP labeled anti-mouse secondary antibody followed by addition of KPL TrueBlue™ Peroxidase Substrate from Sera Care. Foci were counted manually.

Antibody Dependent Enhancement Assay

We incubated 167ng/ml concentration of 2G3 (anti-prM) monoclonal antibody with DENV1 from Vero cells, furin over-expressing Vero cells and plasma for 1 hour at 37℃. Cell culture viruses were incubated along with an equal amount of normal human plasma. the immune complex was infected at an M.O.I (multiplicity of infection) of 1 on K562 cells for 90 minutes at 37℃ and 5% CO_2. After 24 hours, infected cells were washed with PBS, fixed with 4% formaldehyde, permeabilized and stained with 2H2 mAb labelled with Alexa 488. Infected cell population was checked and analyzed using a flow cytometer machine.

Neutralization Assay

Vero cell monolayer was grown for 48 hours until about 100 % confluency. Various dilutions of mAbs or sera were made and treated with about 50 focus forming units of different viruses in duplicates for 1hour at 37˚C. The mAb-virus complex was inoculated on the Vero cell monolayer and kept for 90 minutes at 37˚C and 5% CO_2. Infection media was removed and methyl cellulose overlay medium was added. Cells were incubated for 5 days at 37˚C and 5% CO_2. Monolayer was stained with a combination of 2H2 and 4G2 monoclonal antibodies as primary antibody and peroxidase conjugation was used in secondary antibody followed by color development using KPL TrueBlue™ Peroxidase Substrate (Sera Care). Sigmoidal curves were generated using Prism v4.0 (GraphPad Software). The 50% neutralization titer ($Neut_{50}$) was calculated as the dilution that neutralizes 50% of the DENV. An equal amount of normal human plasma was spiked in cell culture virus while performing the assay.

Library Preparation and Sequencing plasma and C6/36 passage 1 DENV1

RNA was isolated from 140µl of human plasma and their cell culture isolates sample using QIAamp Viral RNA Mini Kit from Qiagen using manufacturer's protocol. Library preparation was done using NuGen Trio RNAseq technology and sequencing was performed on Illumina HiSeq system.

Data analysis for plasma and C6/36 passage 1 DENV1

Sequencing data were analyzed using CLC Genomic Workbench version 11 (Qiagen). Sequencing reads were aligned to KM204119 reference dengue genome. We used Low Frequency Variant Detection (Minimum frequency = 2%, Minimum central base quality = 20) from CLC package for each analyzed strain. Then, we filtered unique non-present in plasma C6/36 variants and selected all non-reference alleles which had frequency of variants higher than 5% and coverage higher than 500x.

Library Preparation and Sequencing of plasma and Vero passage 1 DENV1

RNA was extracted from 150 µL plasma or Vero passaged virus using the Direct-zol™ RNA Miniprep kit (Zymo Research) according to manufacturer's instructions. Sequencing libraries were prepared in triplicate for each plasma and Vero passage using Nextera XT (Illumina) and sequenced on an Illumina NextSeq500 system.

Data analysis for plasma and Vero passage 1 DENV1

Sequencing data was analyzed using an in-house pipeline. Reads were quality trimmed using Trimmomatic [1]. Plasma virus reads were aligned to a Sri Lankan DENV1 reference sequence (HQ891314.1) in order to generate the consensus sequence of the plasma samples. We then aligned the reads from all samples to the plasma virus consensus sequence and called variants using LoFreq [2]. Variants present in all three technical replicates of Vero passaged virus and not present in plasma samples are shown in Table 2.

1. Bolger AM, Lohse M, Usadel B. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics. 2014;30(15):2114-20.
2. Wilm A, Aw PP, Bertrand D, Yeo GH, Ong SH, Wong CH, et al. LoFreq: a sequence-quality aware, ultra-sensitive variant caller for uncovering cell-population heterogeneity from high-throughput sequencing datasets. Nucleic acids research. 2012;40(22):11189-201.

Statistical Analyses

To compare specific infectivity differences and monoclonal antibody neutralization among plasma DENV1 samples and their cell culture isolates, we used two way ANOVA with Tukey's multiple comparisons test. Two way ANOVA was performed to compare maturation state and antibody neutralization differences between plasma DENV1 samples and their clinical isolates. Error bars in bar diagrams and neutralization curves indicate range of three and two technical replicates, respectively. Each data point in neutralization curve and bar diagram is the mean two and three technical replicates, respectively, in a single experiment. Generation of graphs and statistical analyses was done using GraphPad Prism 7.

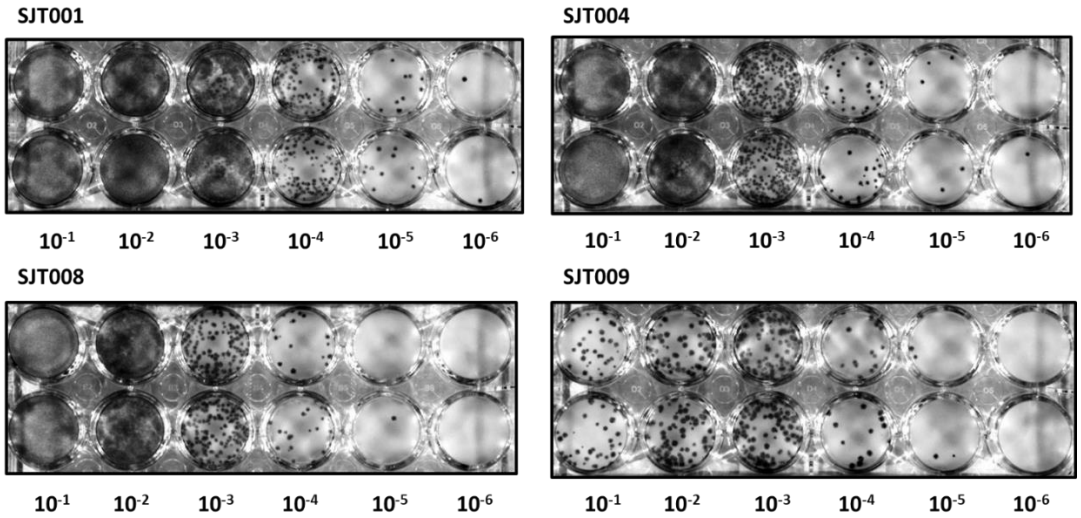

Figure S1: Patient plasma DENV1 foci on Vero Cells. *Tenfold dilutions of human patient blood plasma were infected on 48 hour grown confluent Vero cell monolayer. Foci were visualized by using a combination of 2H2 and 4G2 monoclonal antibodies as primary antibody and peroxidase conjugated secondary antibody followed by color development with TrueBlue™ peroxidase substrate. Images shown (SJT001, SJT004, SJT008 and SJT009) are few selected samples showing foci representative of DENV1 in human blood plasma. Numbers below each image represents the dilution of plasma sample.*

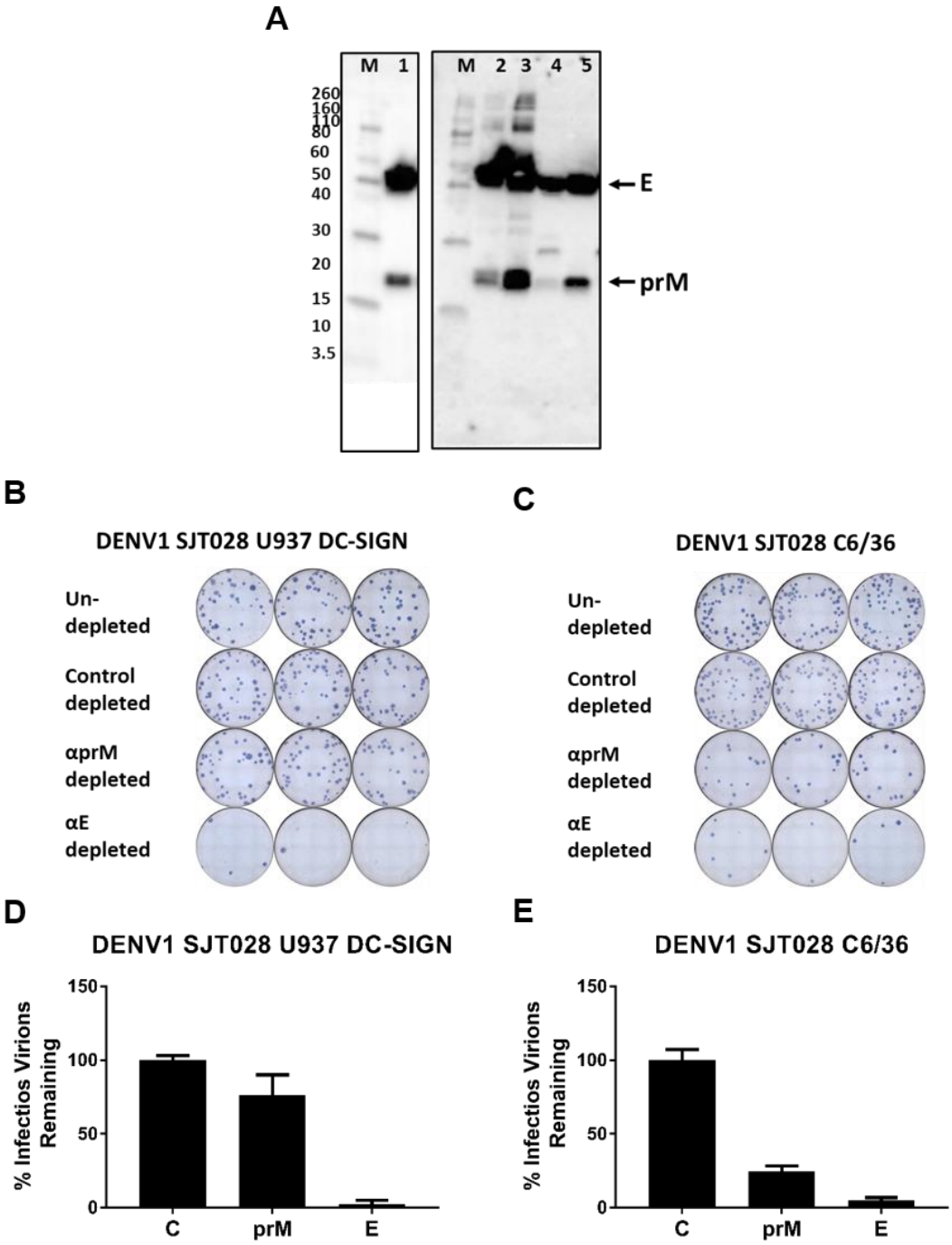

***Figure S2: Validation of Depletion Assay**. **A)** Western blot was done with DENV E and prM specific mAbs. DENV1 SJT028 clinical isolate was grown in C6/36 cells (Lane 1), Vero cells (Lane 2 & 3), U937 DC-SIGN cells (Lane 4) and ammonium chloride added U937 DC-SIGN cells (Lane 5). U937 DC-SIGN grown virus produced more mature (less-prM) virus but when grown in the presence of ammonium chloride produces more immature virus similar to virus produced by C6/36 and Vero cells. Lanes M show markers with molecular weight shown on left. **B)-E):** DENV1 SJT028 virions from U937 DC-SIGN (B & D) and C6/36 cells (C & E) were depleted using magnetic beads coated with an E*

specific mAb 1M7, prM specific mAb 2H2 and DENV3 type specific mAb 8A1 (Control depletion; C). After removal of specific virus populations using mAb coated magnetic beads, the remaining infectious virions were detected using a Vero cell based infectious focus assay. Depletion data corroborates the Western blot data. PrM mAb depleted about 25% of infective virus from U937 derived DENV1 and more than 75% from C6/36 derived DENV1. Error bar indicates range of three technical replicates.

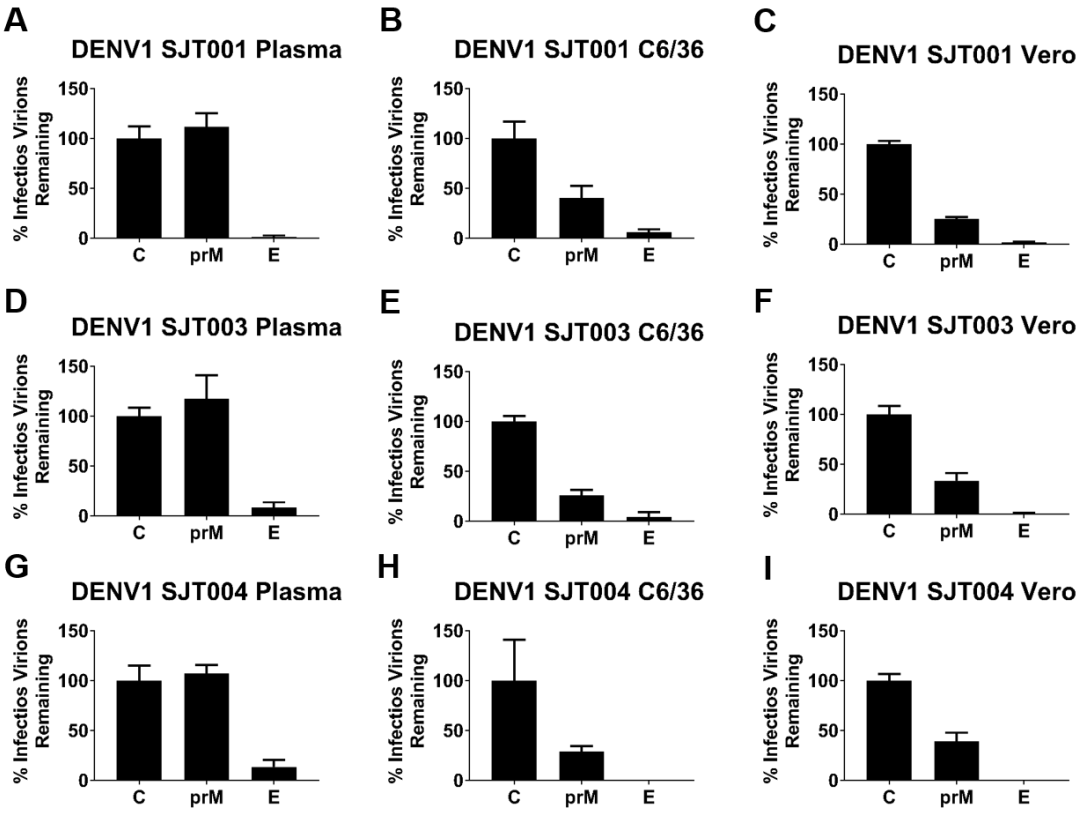

Figure S3: Depletion of remaining blood plasma DENV with maturation sensitive and maturation insensitive monoclonal antibodies. *Blood plasma DENV1 (A, D and G) and their clinical isolate on C6/36 (B, E and H) and Vero cells (C, F and L) were depleted with a DENV3 Envelope (E) specific monoclonal antibody (mAb), 8A1 (Negative Control), anti-prM mAb (2H2) and anti-E mAb (1M7) respectively. The depleted samples were infected on Vero cell monolayer in a 24 well plate format. Error bar indicates range of three technical replicates.*

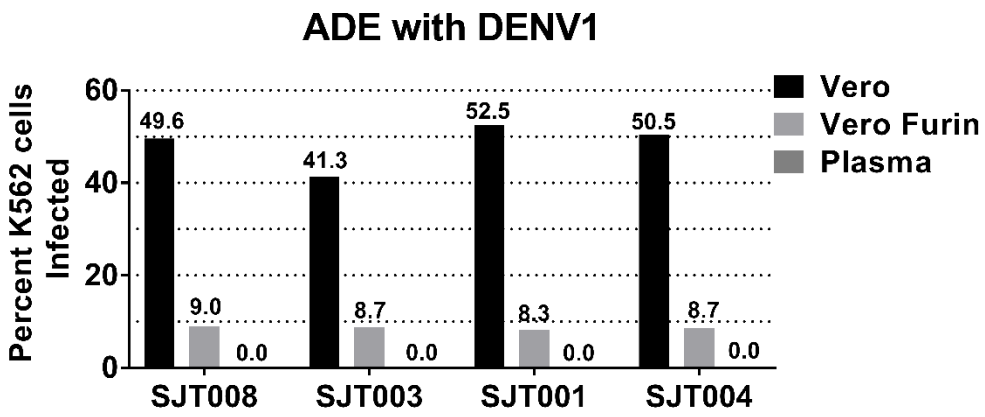

***Figure S4**: **Antibody dependent enhancement of DENV1.** SJT008, SJT003, SJT001 and SJT004 DENV1 derived from Vero cells, furin over-expressing Vero cells and plasma were allowed to react with 167ng/ml of 2G3 (human anti-prM) monoclonal antibody for 1hour at 37℃ and infected on to K562 cells at an m.o.i. of 1. Infected cells were stained after 24 hours and checked for infectivity on flow cytometer. The results are from a single technical point in a single experiment.*

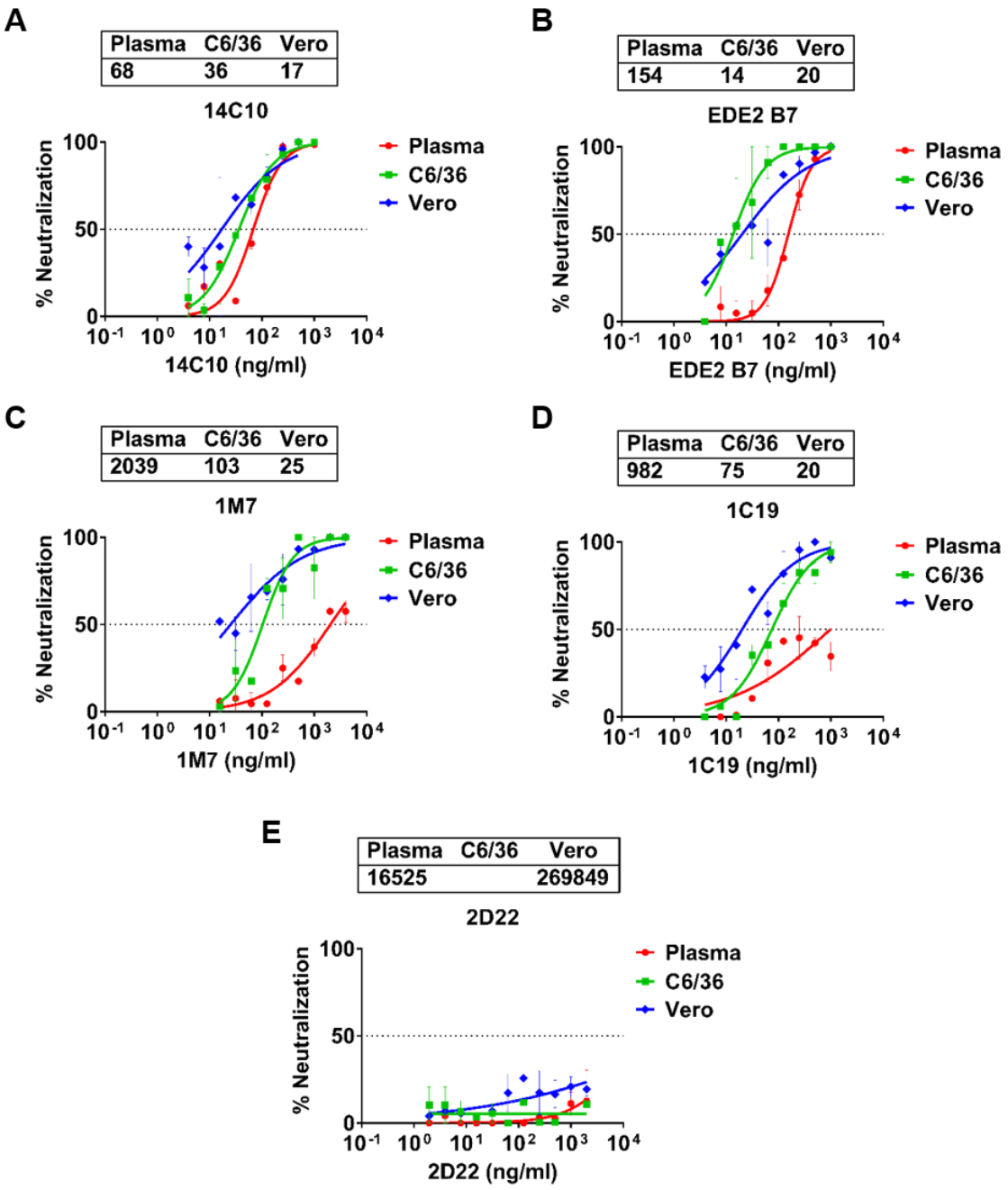

***Figure S5**: **Neutralization of blood plasma SJT004 DENV1, its C6/36 and Vero clinical isolate.** Neutralization curves for human mAbs were plotted by using GraphPad Prism. Table shows neutralization 50 values of viruses in ng/ml concentration. Each data point is the mean of percent neutralization for two technical replicates in a single experiment. Error bar indicates range of two technical replicates.*

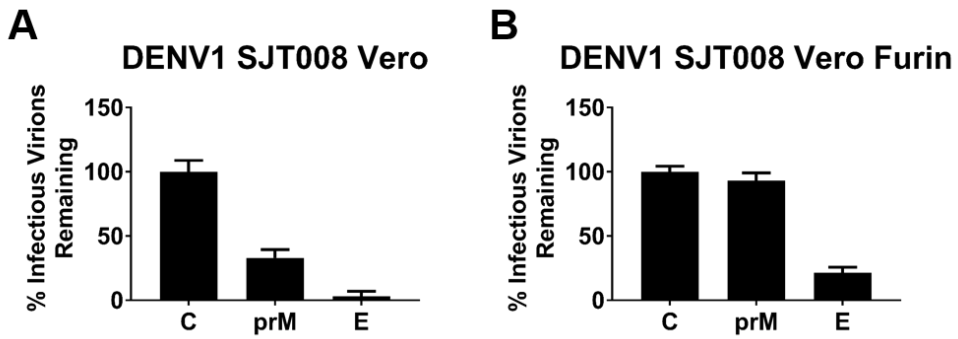

***Figure S6**: **DENV virions produced in furin over-expressing Vero cells are mature.** Maturation state of DENV1 isolate from patient SJT008 grown in regular **(A)** and furin overexpressing **(B)** Vero cells. The virus preparations were incubated with magnetic beads coated with a negative control mAb (C), Envelope (E) specific 1M7 mAb and preMembrane (prM) specific 2H2 mAb. After removal of specific virus populations using mAb coated magnetic beads, the remaining infectious virions were detected using a Vero cell based infectious focus assay. The virions harvested from cells over-expressing furin were not depleted by the prM mAb coated beads indicating that the particles were mature compared to virions harvested from regular Vero cells. Error bar indicates range of three technical replicates.*

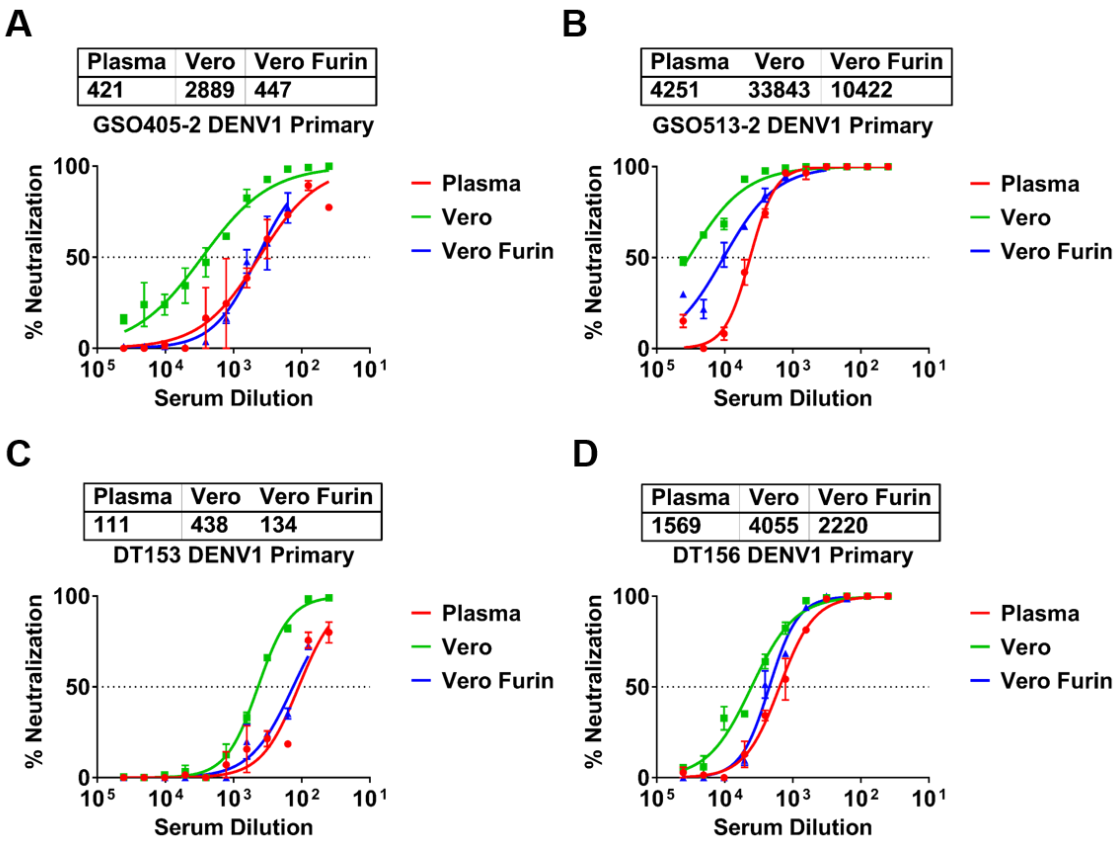

***Figure S7**: **Neutralization by type specific DENV1 primary sera of blood plasma SJT008 DENV1, its Vero and Furin-overexpressing Vero clinical isolate**. Neutralization curves for primary DENV1 infected sera were plotted by using GraphPad Prism. Tables on top of each graph shows neutralization 50 values of viruses. Each data point is the mean of percent neutralization for two technical replicates in a single experiment. Error bar indicates range of two technical replicates.*

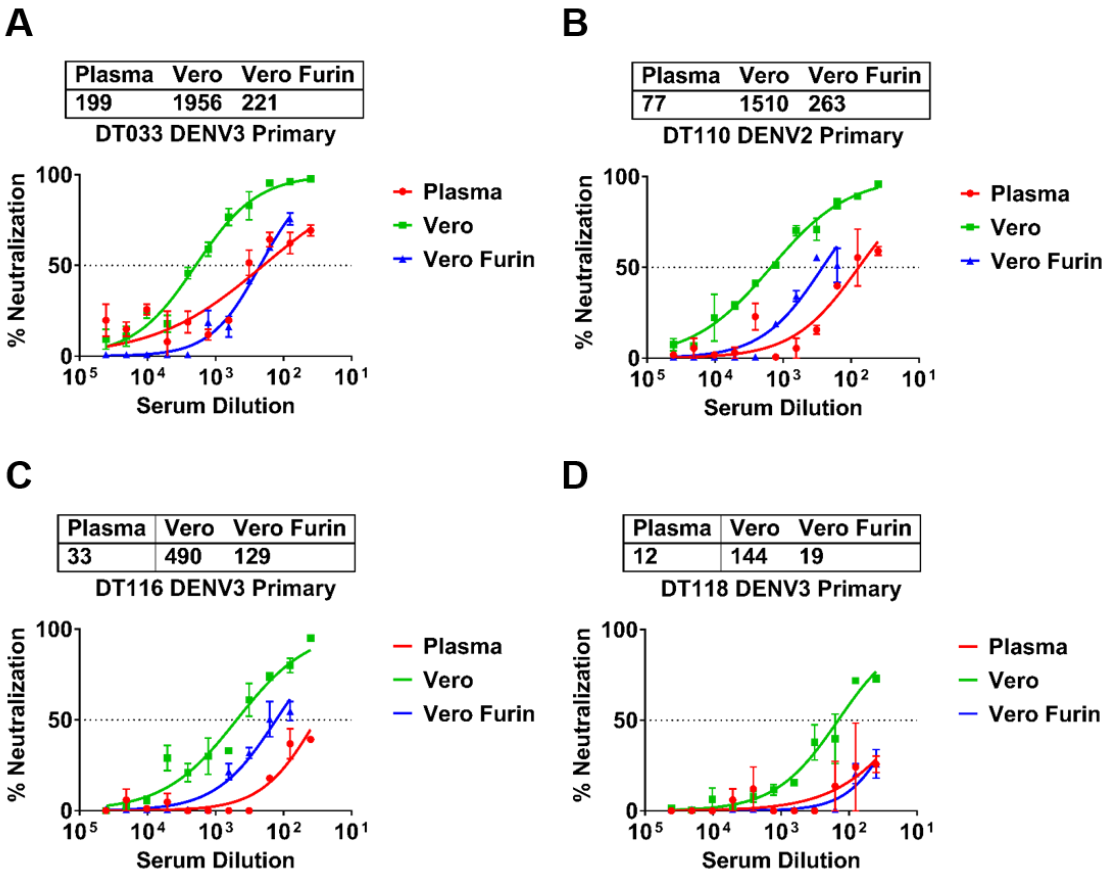

***Figure S8**: **Neutralization by cross reactive heterotypic sera of blood plasma SJT008 DENV1, its Vero and Furin-overexpressing Vero clinical isolates.** Neutralization curves for primary DENV2 and DENV3 infected sera were plotted by using GraphPad Prism. Tables on top of each graph shows neutralization 50 values of viruses. Each data point is the mean of percent neutralization for two technical replicates in a single experiment. Error bar indicates range of two technical replicates.*

Table S1: DENV infectious particle to RNA ratio of plasma viruses and their mosquito and mammalian cell derived clinical isolates. Infectious titre and RNA copies in plasma samples and their C6/36 and Vero cell derived cell culture isolates were performed by Vero infection assay (Figure S1) and digital drop PCR respectively. FFU=focus forming units; GC=genome copies; SI=specific infectivity.

Sample ID	Characteristics	Plasma	C6/36	Vero
SJT001	FFU/ml	2.E+07	9.E+04	3.E+07
	GC/ml	9.E+08	1.E+10	5.E+11
	SI	**2.E-02**	**8.E-06**	**5.E-05**
SJT003	FFU/ml	9.E+06	1.E+05	7.E+06
	GC/ml	5.E+08	1.E+10	2.E+11
	SI	**2.E-02**	**8.E-06**	**3.E-05**
SJT004	FFU/ml	5.E+06	3.E+05	9.E+06
	GC/ml	9.E+08	2.E+10	6.E+10
	SI	**5.E-03**	**2.E-05**	**1.E-04**
SJT005	FFU/ml	4.E+06	3.E+05	9.E+06
	GC/ml	2.E+09	2.E+10	1.E+11
	SI	**2.E-03**	**1.E-05**	**6.E-05**
SJT008	FFU/ml	2.E+06	5.E+04	1.E+07
	GC/ml	6.E+08	4.E+10	1.E+11
	SI	**3.E-03**	**1.E-06**	**9.E-05**
SJT009	FFU/ml	2.E+06	1.E+05	1.E+07
	GC/ml	8.E+08	9.E+09	1.E+11
	SI	**2.E-03**	**1.E-05**	**1.E-04**

Table S2: List of DENV human mAbs used in neutralization assay against patient plasma DENV1 with their epitope specificities and characteristics.

mAb	Specificity	Comments
14C10	DENV1 EDI, EDI & II Hinge and DIII of a neighboring E protein	Quaternary; Proposed therapeutic candidate
EDE2 B7	Intra-dimer epitope	Quaternary
1M7	EDII fusion loop region	Conserved
1C19	EDII BC-loop and fusion loop	Conserved
2D22 (Negative Control)	DENV2 EDIII and EDII on two different monomers within a single dimer	Quaternary

Table S3: List of type specific (TS) and cross reactive (CR) primary convalescent sera used in neutralization assay against patient plasma DENV1 with their collection time post infection and infecting serotypes.

Serum ID	Collected Post Infection	Primary Infecting Serotype
405-2	60 days	DENV1
513-2	120 days	DENV1
DT153	4.5 Years	DENV1
DT156	15 Years	DENV1
DT110	10 Years	DENV2
DT033	4 months	DENV3
DT116	>3 Years	DENV3
DT118	1 Year	DENV3

Table S4: Sequencing results of plasma DENVs and their cell culture isolates: *Variations in DENV1 from human plasma samples after passaging SJT003 and SJT008 in C6/36 cells (top table), and SJT001 and SJT004 in Vero cells (bottom table). There were no variants found in case of SJT001.*

C6/36 Vs. Plasma DENV1

Sample ID	Region	Position	Type	Reference	Allele	Coverage	Frequency (%)	Mutation
SJT003	3'-UTR[1]	10277^ 10278	IN[2]	-	A[3]	187904	8.25	N.A.[4]
SJT003	3'-UTR	10618	SNV[5]	G[6]	T[7]	1378	12.99	N.A.
SJT003	3'-UTR	10621	SNV	C[8]	T	1143	83.46	N.A.
SJT003	3'-UTR	10624	SNV	C	T	1092	84.43	N.A.
SJT003	3'-UTR	10691	SNV	A	T	540	6.30	N.A.
SJT008	5'-UTR	69..70	MNV[9]	GT	AC	221130	18.51	N.A.
SJT008	3'-UTR	10277^ 10278	IN	-	A	221920	5.72	N.A.
SJT008	3'-UTR	10621	SNV	C	T	1227	80.03	N.A.

Vero P1 vs. Plasma DENV1

Sample ID	Region	Position	Type	Reference	Allele	Coverage	Frequency (%)	Mutation
SJT004	NS3	5705	IN DEL[10]	C	CA	7955	5.00	N.A.

UTR: untranslated region.
[2]IN: Insertion.
[3]A: adenine.
[4]N.A.: not applicable.
[5]SNV: single-nucleotide variation.
[6]G: guanine.
[7]T: thymine.
[8]C: cytosine.
[9]MNV: multi-nucleotide variation.
[10]IN-DEL: Insertion-Deletion.