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Human heavy chain antibody genes elicited in Thai dengue patients during DENV2 secondary infection.

Nipa Thammasonthijarern¹, Wilarat Puangmanee¹, Pimolpachr Sriburin³, Subenya Injampa⁴, Supawat Chatchen³, Weerapong Phumirattanaprapin⁵, Chonlatip Pipattanaboon⁶, Pongrama Ramasoota¹², Pannamthip Pitaksajjakul¹²*

¹Department of Social and Environmental Medicine, Mahidol University, Thailand.
²Center of Excellence for Antibody Research, Faculty of Tropical Medicine, Mahidol University, Thailand, 10400.
³Department of Tropical Pediatrics, Faculty of Tropical Medicine, Mahidol University, Thailand, 10400.
⁴Faculty of Medicine, King Mongkut’s Institute of Technology Ladkrabang, Bangkok, Thailand, 10520.
⁵Department of Clinical Tropical Medicine, Faculty of Tropical Medicine, Mahidol University, Thailand, 10400.
⁶Department of Microbiology, Faculty of Medicine, Khon Kaen University, Thailand, 40002.

*Corresponding author: Pitaksajjakul Pannamthip

Faculty of Tropical Medicine, 420/6 Ratchawithi Road, Ratchathewi, Bangkok, Thailand, 10400
Tel. +662-354-9100, Fax. +662-354-9139
E-mail: pannamthip.pit@mahidol.ac.th
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Summary

Dengue is one of the most serious mosquito-borne viral diseases occurring in humans. From their complexity of 4 serotypes, the ideal vaccine for dengue should be able to stimulate cross-neutralizing antibodies. Recently, genetic-based immune response have been studied for guiding of vaccine design for several viral pathogens. Despite a recent approval of dengue vaccine, the information of genetics-based immune response against dengue virus (DENV) are still limited. Consequently, we aimed to determine the profiles of immunoglobulin heavy chain gene from DENV2 infected patients. The immunoglobulin heavy chain variable region genes (IGHV) were amplified from peripheral blood mononuclear cells of DENV2 secondary infected patients over the acute, convalescence and recovery phases. Antibody heavy chain genes were sequenced by next-generation sequencing (NGS), and analyzed to identify correlations with neutralizing and enhancing activities of the serum samples. IGHV1-69, 3-23, and 3-30 were frequently discovered in our Thai DENV2 infected patients. Our findings provided the new data on the human B cell response during secondary DENV2 infections in Thai dengue patients that provide a supportive information for dengue vaccine design and therapeutics development.

1. Introduction

Dengue is a mosquito-borne viral infection that caused by dengue virus (DENV) and become a major public health problem. Primary DENV infections in humans from any serotypes generate antibodies that produce lifelong protection against future homotypic infection, but only temporary immunity to the others. Consequently, most severe dengue cases occur in patients experiencing secondary heterotypic infections, and these are linked with non- or
sub-neutralizing antibodies that form a complex with the virus and infect phagocytes via Fc receptor, leading to higher viral loads and enhanced infection. This phenomenon is called antibody-dependent enhancement (ADE).

Despite the first vaccine against DENV is recently licensed (1), its lower efficacy, studied from the phase IIb and phase III clinical trials, was observed in dengue naïve comparing to sero-positive individuals. For this reason, the understanding of human immune response after natural infection, especially for secondary infection, that mimic the immune response inducing after vaccination of dengue pre-exposure, can help design for dengue vaccine and therapeutic development.

Recently, the preferential immunoglobulin heavy chain variable region genes (IGHV) genes responded to human diseases have been widely studied. The antibody germline characterization of several anti-viral monoclonal antibodies (MAbs) have been reported e.g. HIV-1 (2-5), Zika (6), hepatitis C (7, 8), influenza (2) and DENV (9-11). For DENV, genetic information on the human monoclonal antibodies (HuMAbs) isolated from secondary acute DENV2 infections in Thai patients have been identified (9). One study has reported the marked difference of CDRH3 from dengue patients during the three phases (10). Also, the comparison of germline genes usage of DENV-neutralizing antibodies between natural infection and vaccine immunization have been described (12). However, it is unclear how antibody repertoires of patients experiencing DENV infections have changed during different infection phases and in different individuals. Here, using NGS, we have identified the B cell antibody populations from three DENV2 infected patients to discover the antibody profile during three different stages of infection, comparing within and between different individual, aiming to provide a fundamental knowledge of the genetic antibody response to
DENV infection, by comparing with dengue unexposed donors. It is anticipated that the compositions and dynamics of rising heavy chain variable genes (IGHV) derived from natural DENV infections in Thai dengue patients together with their neutralizing activity can be further applied for therapeutic development and dengue vaccine design.

2. Materials and methods

2.1. Ethic

The research protocols for human samples were approved by the Ethics Committee of the Faculty of Tropical Medicine (FTM), Mahidol University (MU) (protocol number FTM ECF-019-05). Informed consent was obtained from all patients and donors before enrollment.

2.2. Blood sample collection

Blood samples of DENV2 infected patients during three infection phases from each individual were collected on days 1 to 6 (acute phase), days 7 to 14 (convalescent), and more than 4 weeks after fever onset (recovery) at the Hospital for Tropical Diseases, FTM, MU, Thailand. Four healthy donors lacking dengue infections for the previous 6 months were included as seronegative samples for comparison with the dengue patients. All collected healthy donor serum samples were confirmed for the absence of dengue antibody titer by ELISA.

2.3. PBMCs isolation and RNA extraction

PBMCs were isolated using Ficoll paque (GE Healthcare Bio-sciences AB, Uppsala, Sweden). The PBMCs at approximately $1 \times 10^6$ cells were lysed in 1 ml Trizol reagent (Invitrogen Corp., Carlsbad, California, USA) to isolate total RNA (13), according to the
manufacturer’s protocols. Complementary DNA (cDNA) was synthesized using oligo (dT) primer from the Superscript III First-Strand System (Invitrogen Corp., Carlsbad, California, USA), according to the manufacturer’s protocols.

2.4. Dengue serotyping

Viral RNA were isolated from plasma samples using QIAamp Viral RNA kit (Qiagen, Hilden, Germany) according to the manufacturer’s instructions. Viral RNA was converted to cDNA, using Superscript III First-Strand System (Invitrogen Corp., Carlsbad, California, USA) for dengue serotyping by nested multiplex PCR (14). The resulting PCR products with 482, 119, 290 and 392 bp were characterized as DENV serotypes 1, 2, 3, and 4, respectively (14, 15).

2.5. Cell lines and dengue viruses

Vero cells were cultured in minimal essential medium (MEM) (GE Healthcare UK Ltd., Buckinghamshire, UK) with 10% fetal bovine serum (FBS). The DENV strains used in this study were Mochizuki strain of DENV1, 16681 strain of DENV2, H87 strain of DENV3, and H241 strain of DENV4. All DENVs were propagated in C6/36 cells, and maintained in Leibovitz’s L-15 medium (Gibco, Grand Island, New York, USA), supplemented with 10% FBS and 0.3% BACTO Tryptose Phosphate Broth (Sigma-Aldrich, Missouri, USA).

2.6. Enzyme-linked immunosorbent assay (ELISA)

Dengue specific IgM and IgG capture ELISA was performed to determine immune status of DENV2 patients by using acute and convalescence sera (16). Samples were identified as primary, or secondary infection when the IgM/IgG ratio was higher or lower than 1.8, respectively. Also, this ELISA was used to check antibodies specific to dengue virus from
healthy serum samples. All healthy samples were confirmed as negative for dengue IgG comparable with negative control.

2.7 Reactivity of human serum with DENV by western blot

DENV1-4 infected C6/36 cell lysates were used as an antigen. The DENV lysates were heated at 95°C for 5 min in non-reducing loading buffer and separated the protein component by 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis with protein marker (Spectra™ Multicolor Broad Range Protein Ladder, ThermoFisher Scientific). Proteins were electro-transferred to PVDF membranes (Amersham™ Hybond™ P 0.45, GE Healthcare) and blocked with 5% skimmed milk for overnight before incubation with the serum samples (diluted 1:3,000) from dengue patients at the acute, convalescent and recovery stages and a monoclonal antibody specific for the envelope protein (anti-E; 23-1B3B9) (17), NS1 protein (anti-NS1; D25-4 D4C3) (17), and prM protein (anti-prM; 2H2) (diluted 1:3) for 2 h. After washing, the membranes were incubated with HRP-conjugated goat anti-human IgG (AP112P, Merck Millipore, California, USA) for 1 h. The interaction signal was developed with ECL Prime chemiluminescent substrate (Amersham™ ECL Prime Western Blotting Detection Reagent, GE Healthcare) and detected the signal by the ImageQuant™ LAS 4000 mini (GE Healthcare).

2.8 Neutralization assay using Focus Reduction Neutralization Test (FRNT)

FRNT was performed using Vero cell. The amount of virus was adjusted to obtain approximately 100-150 foci/well (9). Each serotype of DENVs was individually mixed with serially diluted serum (5-fold serial dilution) and incubated at 37 °C for 1 h. Each mixture was added to Vero cell monolayer in 96-well cell culture plate and incubated for 2 h. Overlay medium composing of 2% Carboxymethyl cellulose and 2% FBS in MEM medium was
added to the cells and incubated for 2 d for DENV4 and for 3 d for DENV1, 2, and 3. The cells were fixed with 3.7% formaldehyde/PBS, followed by 0.1% Triton X-100/PBS. Infected foci were stained with anti-DENV HuMAbs, followed by anti-human IgG Alexa Flour 488 (1:1,000). Foci were counted and compared with no antibody control to calculate NT$_{50}$.

**2.9 Antibody Dependent Enhancement assay**

ADE activity was assessed on K562 cells (18). Serum at serial four-fold dilutions and viruses (16681 strain of DENV2) were mixed in 10% FBS RPMI medium in 96-well poly-L-lysine-coated plates (Corning Inc., New York, USA) and incubated at 37 °C. After 2 h, 50 µl of $2 \times 10^6$ cells/ml K562 cells were added to virus-antibodies complexes and incubated at 37 °C under 5% CO$_2$ for 48 h. The cells were fixed and stained as previously described. Infected cell counts obtained from the test were compared with the mean infected cell counts obtained from the four negative controls set in the same experiment.

**2.10 Next generation sequencing of variable Ig HC**

The cDNA was generated from seropositive and seronegative PBMCs samples. The VH genes were PCR-amplified using primers specific to the published sequences for VH1-VH6 framework regions (19). The amplified VH fragment at approximated 450-500 bp were gel-purified and used for VH library construction. The purified DNA (100-1000 ng) samples were qualified using Qubit3.0 machine. Agilent 2100 was then used to determine the insert size and nucleic acid concentration of the resulting library samples. Sequencing was performed on Illumina MiSeq platform, in a 2x300 bp paired-end configuration, with 50,000 reads per sample (Vishuo, Thailand). Base calling was performed by Illumina bcl2fastq 2.17
software based on index information. The number of reads and quality score (Q30) were also counted.

2.11 Sequence processing and data analysis

High-throughput sequences data was analyzed with IMGT/HighV-QUEST v1.5.7 online bioinformatics software (http://www.imgt.org/HighV-QUEST/) and R package bcRep program (20). The percent IGHV usage of unexposed donor was the average among 4 samples. The different of IGHV usage among the sample of dengue patients and unexposed donor were statistically tested using online statistical software (MEDCALC; https://www.medcalc.org/calc/comparison_of_proportions.php). Additionally, the difference of ADE activity among three patients and sample collection phases were statistical assessed using “Mann-Whitney U test”.

3 Results

3.1 Dengue patient samples

Nine samples from 3 dengue patients that identified as DENV2 infection were selected in this study. All 3 patients were identified as secondary infections by ELISA. The detail of patients samples are summarized in Table 1.

3.2 Binding activity of human sera with dengue virus

All serum samples showed cross-reactivity to all 4 serotypes of DENV. Among 3 phases, antibodies to envelope protein were mainly detected in the acute phase of the three patients, with an approximated size at 50 kDa, whereas NS1-specific antibodies were mostly detected during the convalescence and recovery phases. prM-specific antibodies were detected during all three infection phases of three dengue patients (Fig. 1).

3.3 Immunoglobulin HC gene usage
After sequencing, two major VH gene family; VH1 and VH3 were predominantly found (Fig. 2). Among VH1 and VH3, IGHV1-69, IGHV3-23 followed by 3-30 were highly expressed. (Fig. 3). For P1, IGHV3 was presented in all three phases, with IGHV3-30 (13%), IGHV3-11 (13%) and IGHV3-23 (12%) is the highest heavy chain gene found in the acute, convalescence and recovery phases, respectively. Similarly, IGHV3-23 was also found as the highest frequently used heavy chain gene in all three phases of P4 (11%, 30% and 13% during acute, convalescence and recovery phase, respectively). In contrast, the IGHV usage for P3 was more diverse, with IGHV1-69 (32%), IGHV1-46 (12%) and IGHV3-23 (6%) being highly expressed during the acute, convalescence, and recovery phases, respectively (Fig. 3). We then compared the percentage of IGHV usage of dengue patients with dengue unexposed donors, which shown that 5% of IGHV1-69, and 10% of IGHV3-23 and 3-30 were found (Fig. 3). The rising of 3 IGHV (IGHV1-69, 3-23, and 3-30) were significantly raised in dengue patients with p-value < 0.05 (Fig. 3). Despite IGHV1-69 was significantly increased during the acute phase in P3 (approximately 6 times higher than that of the healthy donors), a similar trend was not observed for P1 and 4, in that IGHV1-69 did not exceed 10%.

3.4 FRNT assay

The total number of infected foci were counted and compared with the foci number obtained from the wells of no antibody. Polyclonal antibodies in all serum samples could neutralized all serotypes of DENV (1-4), not only for the infecting serotype (DENV2), even though lesser extent of neutralizing activity for serotypes 1, 3, and 4 were observed. The highest neutralizing titer were observed during convalescence phase for all patients (Fig. 4).

3.5 ADE assay
The balancing of neutralization and enhancement activity of all serum samples against DENV2 were studied in K562 cell carrying Fc gamma receptor, which was more represented the situation in vivo (21), than those in Vero cell. In accordance with the FRNT, we observed less ADE activity during convalescence phase for all dengue patient samples. Considering for the samples of the same infection phase of all patients, we observed higher enhancement from all 3 phases of P3 sample, which is significantly differented from P1 and P4 sample (\(p\)-value < 0.01) (Fig. 5).

**Discussions**

While antibodies producing during primary infection are mostly type-specific, the immune response during secondary infection are serotype-cross reactive (22). In this study, all serum samples that derived from secondary DENV2 infected patients showed cross-reactivity to all 4 serotypes of dengue virus with the highest neutralizing activity against DENV2. Strong reactivity with E protein were observed from all dengue patients at an acute phase, but in later time, higher amount of anti-prM and anti-NS1 was detected (Fig. 1).

The discovery of immunoglobulin genetic information, responded to dengue infection is a recent information to advance the development of vaccine and therapeutics (12, 23). In this study, at the acute phase, by comparing with the basic profile of seronegative donor (\(p\)-value < 0.01), we found high frequency of IGHV1-69 in P3, and IGHV3-23 and 3-30 in P1 and P4. The high frequency of IGHV1-69 in P3 (32%) during acute phase (\(p\)-value < 0.01), comparing with the frequency obtained from dengue unexposed donors, is in accordance with our previous report of HuMAbs (9) and also correlated well with antibody repertoire that found in naturally DENV infection with clinical manifestations (11), and
associated with IGHV1 higher expression level observed in plasmablasts from patients with acute dengue infections (24). However, this IGHV was also found in non-flavivirus like Hepatitis C (25, 26) and influenza virus (2). For IGHV3, two IGHVs (3-23 and 3-30) usage were significantly presented in all three phases of P1 and P4, ranged from 7-30% ($p$-value < 0.01) by comparing with dengue un-exposed donors (approximately 9% of IGHV3-23 and 8% of IGHV3-30) as well as baby cord blood samples (approximately 4.94% for IGHV3-23 and 3.10% for IGHV3-30) (27). Noticeably, the higher usage of IGHV3 at recovery phase were observed for all 3 patients, whereas they are different earlier (Fig. 3). It might be assumed that this IGHV3 were selected to stay longer, possibly due to their better avidity.

By determining the usage of IGHV with serum neutralizing activity, all samples showed cross-neutralizing activity against four serotypes of DENV, with the highest neutralizing titer observed during convalescence phase (Fig. 4). Link with IGHV usage during this infection phase, IGHV3-11, and 3-23 for P1, IGHV1-46, 3-21, and 3-30 for P3, and IGHV3-23, and 3-30 for P4 were highly expressed over other IGHVs (Fig. 3). This higher expression of VH3 family might be correlated, at least in part, with high neutralizing titer that determined from serum samples. This finding was also correlated well with our previous reports of HuMAbs, which IGHV3-23 was shared 26% from 19 HuMAbs (9).

However, from the study in K562 cell, it was found that, when comparing the samples derived from the same infection phase, P3 serum especially at acute phase showed statistically higher enhancing activity comparing with serum samples from P1 and P4, (Fig. 5). Considering this activity with type of germline used, we found less used of IGHV1-69 (2-9%), and higher used of IGHV3-23 (7-30%) in samples P1 and P4 that showed higher NT and lower ADE activity (Fig. 2, Fig. 5). It was also noticed that serum sample from acute
phase of P3, which showed higher NT titer in Vero cell (Fig. 4), showed higher enhancement activity in K562 cell (\textit{p-value} < 0.04). It was implied that the antibody population that existed in acute serum sample of P3 (majority in IGHV1-69), can bind to dengue virus and showed good neutralizing activity in Vero cell, but it may not be the population that showed protective activity \textit{in vivo}.

We supposed that IGHV1-69 possibly existed as a circulating HC, which is stored as a memory B cell that can rapidly responded to both DENV and other viral pathogens during the early phase of infection (2, 28-31). We suspected that the increasing of IGHV1-69 observed during acute phase of P3 sample probably generated against conserved envelop protein of other envelop viruses, since it was also found in antibodies specific to conserved epitopes of other viral pathogens, such as influenza (32, 33), and hepatitis C virus (8). Considering to the usage of IGHV germline linked with target protein and epitopes, we supposed that IGHV1-69 and 3-23 that majorly found in this study might targeted to the fusion loop epitopes of E protein, similar to our previous report of our isolated HuMAbs (9, 34), as this type of epitope are conserve and predominantly found in human immune response (35). In addition, these two IGHV was rarely found in envelop-dimer epitope (EDE) type HuMAbs (36). Nevertheless, since our study did not select antibody population against specific target protein, the antibodies specific to other proteins like NS1 or prM could be included in the repertoires, even though it considered as a minor population as shown by western blot analysis. For this reason, study of antibody repertoires after selection with specific target proteins should be considered for further study.
By comparing with other studies, however, there are some differentiations of IGHV usage existed, which might resulted from the different immune status of the patients, and different geographic regions.

Despite all of blood samples were obtained from the same dengue infected serotype (DENV2) and disease severity (DF) at similar time points of sample collections, some variations in the antibody profiles were found. This variation might resulted from their own baseline immune status, their unknown previous infection, and their genetic background. Thus, our findings provide insight into reshaping of antibody repertoires of different individuals over time, and also emphasize the need for through characterization of an individual’s immune status (37). Even though the sample size in this study was small, variation of antibody repertoires in different individual highlight the potential problems of using a universal dengue vaccine in people without previous knowledge of their immune status (37). So, there is a critical need to study the antibody repertoire in a larger amount of dengue patient’s samples to obtain an accurate picture of the immune profiles of Thai dengue patients. We considered that our findings could provide a new data on the human B cell response during secondary DENV2 infections in Thai dengue patients that probably useful for further study of dengue vaccine development and therapeutic interventions.

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Conflicts of interests:
The authors have declared that no competing interests existed.

Abbreviations
ADE: Antibody-dependent enhancement, CDRH3: Complementary determining region 3 of heavy chain gene, DENV: Dengue virus, FRNT: Focus reduction neutralization test, IGHV: Immunoglobulin heavy chain variable region genes, NGS: Next generation sequencing, NT: Neutralization test, PBMC: Peripheral blood mononuclear cell.

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Table 1. Serum panel characteristics. The table showed details of each dengue patients including age, infecting serotypes, severity, and time of blood sample collection. Target proteins of each serum sample were also described.

| Case number | Patient age | Infection | Serotype | Severity | Acute Day of collection | Convalescence Day of collection | Recovery Day of collection | WB                  |
|-------------|-------------|-----------|----------|----------|-------------------------|-------------------------------|-----------------------------|----------------------|
| P1          | 24          | Secondary | DENV-2   | DF       | Day 5                   | Day 14                        | Day 60                      | E, NS1, prM          |
| P3          | 18          | Secondary | DENV-2   | DF       | Day 5                   | Day 14                        | Day 66                      | E, NS1, prM          |
| P4          | 42          | Secondary | DENV-2   | DF       | Day 3                   | Day 14                        | Day 80                      | E, NS1, prM          |

WB, Western blot; E, envelope protein; NS1, Non-structural protein; prM, pre-membrane protein.
Fig. 1. Western blots of serum samples from DENV2 infected patients among three phases; acute, convalescence and recovery phases for three patient P1, 3 and 4 (A-C).
Fig. 2. Preferential IGHV usage based on the bcRep analysis. IGHV families were obtained from productive sequence of P1, 3 and 4 (A-C).
Figure legends

Fig. 1. Western blots of serum samples from DENV2 infected patients among three phases; acute, convalescence and recovery phases for three patient P1, 3 and 4 (A-C).

Fig. 2. Preferential IGHV usage based on the bcRep analysis. IGHV families were obtained from productive sequence of P1, 3 and 4 (A-C).

Fig. 3. Heatmap of estimated IGHV gene usage of healthy donors and DENV2 infected patients. The values represent the percent of IGHV usage displayed by the different colors. The percent usage of some IGHV have been indicated in the heatmap.

| Patients IGHV | Donors | Acute 1 | Conv 1 | Recov 1 | Acute 3 | Conv 3 | Recov 3 | Acute 4 | Conv 4 | Recov 4 |
|--------------|--------|---------|--------|---------|---------|--------|---------|---------|--------|---------|
| IGHV1-2      |        |         |        |         |         |        |         |         |        |         |
| IGHV1-8      |        |         |        |         |         |        |         |         |        |         |
| IGHV1-18     |        |         |        |         |         |        |         |         |        |         |
| IGHV1-46     |        |         |        |         |         |        |         |         |        |         |
| IGHV1-58     |        |         |        |         |         |        |         |         |        |         |
| IGHV1-69     | 5      | 6       | 2      | 5       | 9       | 3      | 2       |         |        |         |
| IGHV3-7      |        |         |        |         |         |        |         |         |        |         |
| IGHV3-11     |        |         |        |         |         |        |         |         |        |         |
| IGHV3-21     |        |         |        |         |         |        |         |         |        |         |
| IGHV3-23     | 9      | 7       | 10     | 12      | 2       | 4      | 6       | 11      | 30     | 13      |
| IGHV3-30     | 8      | 13      | 6      | 4       | 3       | 7      | 5       | 7       | 13     | 6       |
| IGHV3-30-3   |        |         |        |         |         |        |         |         |        |         |
| IGHV3-33     |        |         |        |         |         |        |         |         |        |         |
| IGHV3-43D    |        |         |        |         |         |        |         |         |        |         |
| IGHV3-48     |        |         |        |         |         |        |         |         |        |         |
| IGHV3-69-1   |        |         |        |         |         |        |         |         |        |         |
| IGHV3-73     |        |         |        |         |         |        |         |         |        |         |
| IGHV3-74     |        |         |        |         |         |        |         |         |        |         |
| IGHV4-38-2   |        |         |        |         |         |        |         |         |        |         |
| IGHV4-39     |        |         |        |         |         |        |         |         |        |         |
| IGHV4-55     |        |         |        |         |         |        |         |         |        |         |
| IGHV5-10-1   |        |         |        |         |         |        |         |         |        |         |
| IGHV5-51     |        |         |        |         |         |        |         |         |        |         |
| IGHV6-1      |        |         |        |         |         |        |         |         |        |         |

<5% 6-10% 11-15% 16-20% 21-25% 26-30% >30%

Fig. 4. Neutralizing activity (FRNT 50) for each serum samples from DENV2 infected patients among three different phases to dengue virus serotype 1-4 are shown for each individual.

Fig. 5. Antibody dependent enhancement (ADE) activity studied in K562 cell. Serum samples from all dengue patients of all infection phases were tested and compared between the same infection phases of different patient samples using the Mann–Whitney U test. Dotted lines indicate cut-off values for differentiating neutralizing and enhancing activity from the average plus three times the SD of the number of infected cells (mean ±3SD) derived from no antibody control.
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