INFECTION-ENHANCING ANTIBODY RESPONSES TO GENETIC VARIANTS OF DENGUE VIRUS SEROTYPE 2 IN HEALTHY ADULTS IN BANGKOK, THAILAND

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ABSTRACT

Dengue virus serotype 2 (DENV2) has been strongly associated with severe dengue manifestations, and its genotypic diversity may influence infection outcomes through antibody-dependent enhancement (ADE). This study aimed to preliminary detect the infection-enhancing activity of antibodies against three genotypically distinct DENV2 variants Asian I, Sylvatic, and Cosmopolitan among healthy individuals residing in Bangkok, Thailand. A total of 30 serum samples were examined of the level of infection-enhancing antibodies using a luciferase-based ADE assay against DENV2 reporter representing three genetic variants in the same conditions. The Sylvatic genotype revealed the highest ADE activity, followed by Asian I genotype, while the Cosmopolitan genotype exhibited minimal enhancement. Significant differences in ADE responses were observed across genotypes ($p < 0.05$). Peak enhancement was observed in age groups 30-49 years. This study highlights the role of DENV2 genotypic diversity in modulating ADE activity of population residing in dengue endemic area. The elevated enhancement response to the Sylvatic and Asian I genotype suggests increased risk for severe outcomes upon secondary infections in endemic populations. Genotype-specific functional assays can be valuable for guiding vaccine development and risk assessment strategies.

Keywords: Dengue, Antibody-dependent Enhancement, DENV Genotype, Seroprevalence

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INTRODUCTION

Dengue continues to pose a significant public health challenge worldwide. It is caused by the DENV, which is classified into four distinct serotypes: DENV1, DENV2, DENV3, and DENV4. Together, these serotypes are responsible for an estimated 390 million infections each year, leading to over 100 million symptomatic cases (Bhatt et al., 2013). Of these, around 5% develop into severe conditions such as dengue hemorrhagic fever (DHF) and dengue shock syndrome (DSS), both of which substantially increase dengue-related deaths (Bhatt et al., 2013; WHO, 2018).

Asia contributes to more than 70% of dengue cases worldwide, putting billions of people at risk as the disease continues to spread endemically across the region (Bhatt et al., 2013; Gwee, Chua, & Pang, 2021). Thailand is recognized as a dengue hyperendemic country, where all four DENV serotypes circulate simultaneously every year. Between 2003 and 2024 incidence rates often exceeded 100 cases per 100,000 population, underscoring the persistent burden of the disease (Division of Vector Born Disease, 2020, 2025; Nonyong et al., 2021). DENV2 has been most frequently associated with fatal cases during recent outbreaks (Poltep et al., 2021).

DENV exhibit genetic variability over time and across geographic regions (Holmes, 1998). The genetic diversity among DENV strains alters the antigenic characteristics of viral proteins, impacting how the host's immune system responds and how effective the produced antibodies are during infection. Both serotype-specific and cross-reactive antibody responses have been identified, playing roles in either neutralizing the virus or enhancing subsequent infections (Cardosa, Porterfield, & Gordon, 1983; Guy & Jackson, 2016; Morrone & Lok, 2019; Shukla, Ramasamy, Shanmugam, Ahuja, & Khanna, 2020). Antibody-dependent enhancement (ADE) is a phenomenon where cross-reactive, sub-neutralizing antibodies promote viral entry into host cells during a secondary infection with a different serotype or strain. This mechanism has been suggested as a major factor contributing to the heightened severity of dengue during secondary infections (Bell, Katzelnick, & Bedford, 2019; de Alwis et al., 2014; Guzmán et al., 2000).

In regions where dengue is endemic, most people encounter multiple DENV strains, frequently through asymptomatic infections. These repeated exposures lead to the development of cross-reactive antibodies that exist at sub-neutralizing levels, increasing the likelihood of ADE and severe disease when reinfection occurs (Katzelnick et al., 2017; Vandepitte et al., 2019). Among the four DENV serotypes, DENV2 is most often linked to severe clinical manifestations. This serotype is divided into six distinct genotypes: Asian I, Asian II, Asian/American, American, Cosmopolitan, and Sylvatic. Genetic variations in the prM and E proteins across these genotypes are estimated at around 9.1% and 8.5%, respectively (Martinez et al., 2020; Shrivastava et al., 2018). Changes in circulating genotypes have been linked to shifts in disease severity, as evidenced by the replacement of the DENV2 American genotype with the Asian I genotype in the Americas, which correlated with increased severity (Guzmán et al., 2000; Messina et al., 2014).

Recent evidence suggests that the DENV2 Cosmopolitan genotype is becoming increasingly prevalent in Thailand (Poltep et al., 2021; Uttayamakul et al., 2020). In Bangkok, the predominant circulating DENV2 genotypes from 2015 to 2020 were Cosmopolitan and Asian I, with sequence similarities observed in strains from neighboring countries including Laos, Cambodia, Taiwan, Indonesia, Singapore, and China (Poltep et al., 2021). Bangkok's ecological and urban conditions support intense DENV transmission and the co-circulation of multiple genotypes. However, limited data exist on the prevalence of infection-enhancing antibodies, which are critical for assessing the risk of severe secondary infections. This limitation is partially due to technological challenges in detecting such antibodies.

To address this gap, this study employed a single-round infectious particle (SRIP) reporter virus system incorporating prM and E proteins from three DENV2 strains representing distinct

genotypes: DENV2/Thailand/1964-16681 (Asian I), DENV2/Malaysia/2008-DKD-811 (Sylvatic), and DENV2/India/1974-Poona-742295 (Cosmopolitan) (Junjhon et al., unpublished results). A luciferase-based ADE assay was used to measure infection-enhancing antibody activity in healthy individuals residing in Bangkok (Junjhon et al., 2021). Findings are expected to contribute to a deeper understanding of how genotypic diversity of DENV2 influences the risk of severe disease, thereby informing public health strategies in dengue-endemic regions.

RESEARCH METHODOLOGY

Study design

This was a cross-sectional study to preliminary evaluate the infection-enhancing antibody responses against the three DENV2 genotypic variants. Serum samples were collected from 30 healthy adult individuals residing in Bangkok who underwent routine health examinations in 2022. Informed consent was obtained for the use of leftover serum samples, with collaboration from the Center for Public Health and Academic Services, Faculty of Public Health, Mahidol University. Ethical approval for this study was obtained from the Human Research Ethics Committee, Faculty of Public Health, Mahidol University, through an exemption review (approval number 75/2565).

Serum samples

Serum samples containing neutralizing antibodies (NAbs) against all four DENV serotypes, as previously identified using the focus reduction neutralization test (FRNT) (Junjhon et al., unpublished data), were heat-inactivated at 56°C for 30 minutes before use. These samples were subsequently examined for the levels of infection-enhancing antibodies against three antigenically diverse DENV2 reporter strains (Junjhon et al., 2021), using a luciferase (Luc)-based enhancement assay (Junjhon et al., 2021).

Cells and viruses

The Human erythroleukemic cell lines, K562 was used in Luc-based enhancement assay for infection-enhancing antibodies detection. This cell line was seeded in RPMI 1640 containing 10% (v/v) FBS, 2 mM L-glutamine (Gibco BRL), 100 units/ml penicillin and 100 µg/ml streptomycin (Gibco BRL) in the presence of 5% CO2 at 37°C.

DENV2 reporters expressing premembrane (prM) and envelope (E) proteins of three genotypic variants were generated previously (Junjhon et al., 2021). Briefly, a plasmid containing full-length cDNA of DENV2 strain 16681 reporter was further modified by replacing the prME fragment with each of DENV2 strains; DENV2/Malaysia/2008-DKD-811 and DENV2/India/1974-Poona-742295 (Table 1.) (Junjhon et al., 2021). These DENV2 reporters replicate for only one cycle of infection because of lacking its capsid (C) gene in the DENV2 reporter genome. However, Lucia luciferase released from infected cells can be used as an infective parameter for DENV replication, facilitating robust detection of antibody-dependent enhancement against DENV2 genetic variants by Luc-based ADE assay.

Table 1 Virus reporters displaying genetic variants of DENV2 used in Luc-based ADE assay.

Type	Location	Year	GenBank identity number	Genotype Holmes and Twiddy nomenclature
DENV-2	Thailand	1984	U87411	Asian I
DENV-2	Malaysia	2008	FJ467493	Sylvatic
DENV-2	India	1974	FJ538920	Cosmopolitan

Detection of luciferase activity

Lucia luciferase, generated through the Luc-based ADE assay, was measured using the QUANTI-Luc detection kit (Invivogen, San Diego, USA). A 50 µl volume of substrate, diluted 1:4, was mixed with an equal volume of the infected culture supernatant in a 96-well microplate.

The luciferase signal was then immediately detected using a VICTOR™ X series multi-label plate reader. The luciferase activity was expressed as relative light units (RLU).

Luc-based ADE assay

The Luc-based ADE assay was employed to assess the infection-enhancing antibodies, measured by the peak increase in secreted luciferase activity compared to a no-serum control at specific serum dilutions. The assay was conducted using K562 cells. Serum samples were first heat-inactivated at 56 °C for 30 minutes to inactivate complement proteins. These sera were then serially diluted (4-fold) in RPMI 1640 medium supplemented with 2% FBS and dispensed into U-bottom 96-well plates (30 µl per well). Equal volumes of DENV2 reporter were mixed with the diluted sera and incubated at 37 °C for 1 hour. Afterward, 2×10^5 K562 cells were added to each well and incubated at 37 °C for 2 hours to allow infection. Post-incubation, the cells were washed twice with 150 µl of 2% FBS-RPMI by centrifugation at 900 × g for 7 minutes to eliminate unbound virus-antibody complexes. Fresh medium (70 µl of 2% FBS-RPMI) was then added, and the cells were incubated at 37 °C with 5% CO_2 for 48 hours. Culture supernatants were finally collected, and luciferase activity was measured as an indicator of infection.

Statistical analysis

A total of 30 serum samples were analyzed to evaluate infection-enhancing antibody responses against three genetically distinct DENV2 variants. All statistical analyses were conducted using IBM SPSS Statistics software, version 17 (IBM Corp., USA). Friedman's test was applied to compare relative fold enhancement titers among the three variants. The Mann-Whitney U test was used to assess sex-based differences, while the Kruskal-Wallis H test evaluated variations across age groups for each DENV2 strain. Statistical significance was defined as a p-value less than 0.05.

RESEARCH RESULTS

Thirty sera of healthy adults residing in Bangkok were examined for the presence of infection-enhancement antibody responses. The study population consisted of 15 males and 15 females, aged between 23 and 64 years, with a mean age of 44 years. The highest infection-enhancement activity was observed with the Sylvatic genotype, with 29 out of 30 serum samples (96.66%) demonstrating enhancement. The fold enhancement values observed ranged from 0.92 to 50.22, with a median of 4.14 (Table 2). The second highest ADE activity was detected against the DENV2/Thailand/1964-16681 strain (Asian-I genotype), where 25 out of 30 samples (83.33%) exhibited enhanced infection, with fold enhancement values ranging from 0.43 to 4.20 and a median of 2.12 (Table 2). Notably, only one serum sample (3.33%) showed ADE activity against the DENV2/India/1974-Poona-742295 strain (Cosmopolitan genotype), with a fold enhancement value of 1.19 (Tables 2).

Table 2 Number of serum samples exhibiting infection-enhancing activity and the levels of enhancement against three DENV2 variants.

DENV2 reporters	Total serum tested	No. of ADE-positive serum	No. of ADE-negative serum	Fold enhancement		
				Range	Median	Std.
16681 (Asian-I)	30	25	5	0.43-4.20	2.120	1.02
Malaysia (Sylvatic)	30	29	1	0.92-50.22	4.14	16.18
India (Cosmopolitan)	30	1	29	0.12-1.19	0.242	0.24

When analyzing the differences in infection-enhancing levels among all serum samples collectively against the three DENV2 variants, a statistically significant lower fold-enhancement was observed for Cosmopolitan genotype compared to both Asian-I genotype

and Sylvatic genotype. However, no statistically significant difference in fold-enhancement levels was found between Sylvatic and Asian-I genotype (Figure1).

To test whether the magnitude of infection-enhancement was associated with sex group, fold enhancement values were compared within females and males. This study demonstrated significant differences in the levels of infection-enhancement activity against three DENV2 genotypes within male and female groups. In the male group, the highest median fold enhancement was observed for Asian-I genotype at 2.30, followed by Sylvatic genotype at 1.70, and the lowest was Cosmopolitan genotype at 0.39. In contrast, in the female group, the highest median fold enhancement was found for Sylvatic genotype at 8.21, followed by Asian-I genotype at 1.59, and the lowest was Cosmopolitan genotype at 0.41 (Figure 2).

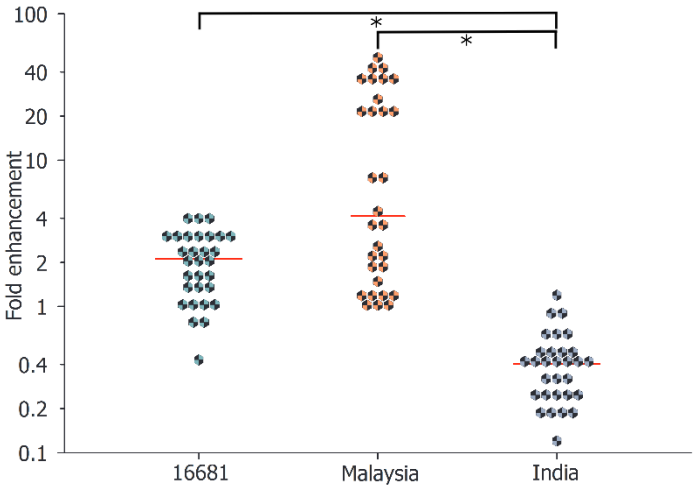

Figure 1 Comparison of fold enhancement levels against three genotypic variants of DENV2, DENV2/Thailand/1964-16681 (Asian-I genotype), DENV2/Malaysia/2008-DKD-811 (Sylvatic genotype), and DENV2/India/1974-Poona-742295 (Cosmopolitan genotype). Dots display the fold enhancement values from individual and the median from 30 samples. Statistical analysis was performed using the Friedman test. Statistical significance: *p < 0.05.

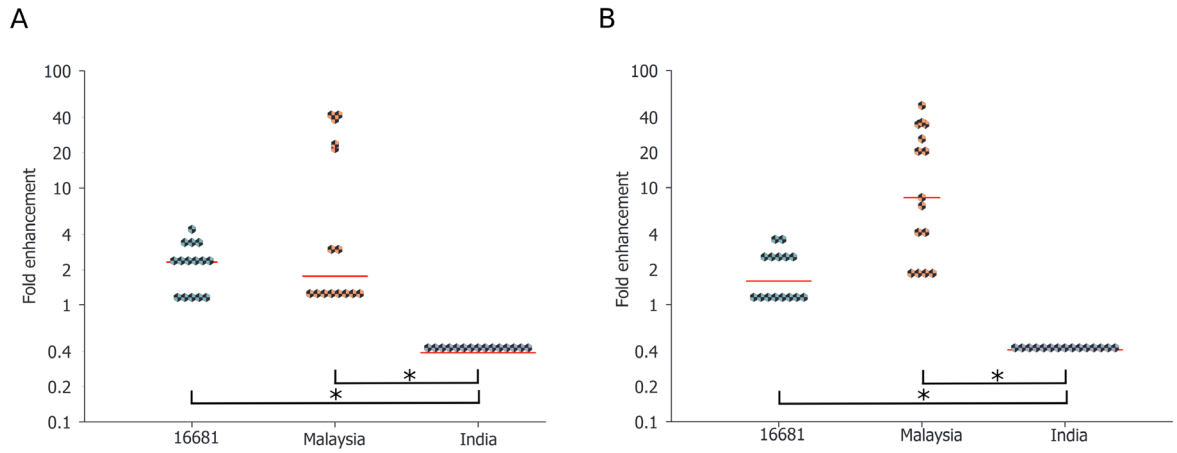

Figure 2 Comparison of fold enhancement levels against DENV2/Thailand/1964-16681 (Asian-I genotype), DENV2/Malaysia/2008-DKD-811 (Sylvatic genotype), and DENV2/India/1974-Poona-742295 (Cosmopolitan genotype) within sex groups: (A) Male group (n = 15) and (B) Female group (n = 15). Statistical analysis was performed using the Friedman test. Statistical significance: *p < 0.05.

Comparison between sex groups revealed females tended to exhibit significantly higher fold enhancement against the Sylvatic genotype compared to male sera (8.21 vs. 1.70). In contrast, for the Asian-I genotype, male sera showed a slightly higher median fold enhancement than female sera (2.30 vs. 1.59). When comparing the fold enhancement levels of each DENV2 variant between sex groups, both male and female groups exhibited significantly higher fold enhancement against Asian-I genotype, and Sylvatic genotype compared to Cosmopolitan genotype (p value < 0.05) (Figure 2). However, no statistically significant differences in fold enhancement levels were observed between males and females for each individual DENV2 variant (Figure 3).

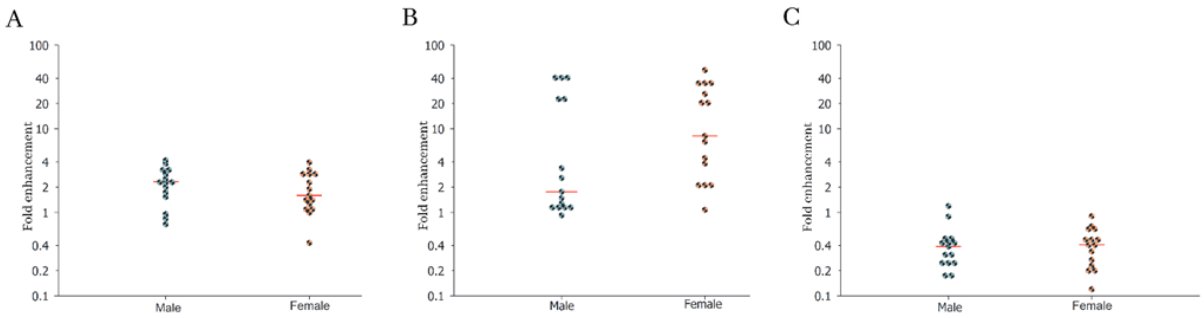

Figure 3 Comparison of fold enhancement levels of DENV2 strains between sex groups: (A) DENV2/Thailand/1964-16681 (Asian-I genotype), (B) DENV2/Malaysia/2008-DKD-811 (Sylvatic genotype), and (C) DENV2/India/1974-Poona-742295 (Cosmopolitan genotype). Statistical analysis was performed using the Mann-Whitney U test. Statistical significance: *p < 0.05.

To investigate the relationship between fold enhancement levels of three DENV2 variants across different age groups, serum samples were categorized into five age ranges: 20-29, 30-39, 40-49, 50-59, and over 60 years. The highest fold enhancement level against Sylvatic genotype was observed in the 40-49 years age group (median = 20.45), followed by the 20-29 years group (median = 18.32). In contrast, the 30-39, 50-59, and over 60 years age groups exhibited lower fold enhancement levels, with medians of 3.92, 4.78, and 2.80, respectively. However, similar fold enhancement levels against Asian-I genotype were observed across all age groups, with median fold enhancement values ranging from 1.31 to 2.84 (Figure 4). Comparison of fold enhancement levels among different age groups for each of the three DENV2 variants revealed that the fold enhancement level against Sylvatic genotype was significantly higher than that against Cosmopolitan genotype in all age groups except for those over 60 years old (Figure 4). Nevertheless, no significant differences in fold enhancement levels were found among the age groups when comparing each DENV2 variant individually (Figure 5).

DISCUSSION & CONCLUSION

This study provides a preliminary data on infection-enhancement activity in healthy adults in Bangkok, Thailand. The finding revealed that the Sylvatic genotype elicited the strongest enhancement activity, followed by the Asian-I genotype, whereas the Cosmopolitan genotype showed minimal enhancement across all age and sex groups. This predominance of infection-enhancement activity against the Sylvatic strain, particularly among younger and middle-aged adults (20-49 years), suggested the presence of sub-neutralizing, cross-reactive antibodies from prior exposure recognized this Sylvatic strain. These antibodies likely contributed to increased viral entry into Fc receptor-bearing K562 cells a hallmark of ADE.

Interestingly, although the Sylvatic genotype has rarely been reported in human outbreaks in Asia compared to the widely circulating Asian I and Cosmopolitan genotypes in Thailand (Cardosa et al., 2009; Phadungsombat et al., 2018; Poltep et al., 2021), it showed the highest ADE activity in this study. Genetic surveillance data from 2016-2020 confirm that Bangkok's population has been primarily exposed to Asian I and Cosmopolitan genotypes, with no documented local circulation of Sylvatic strains (Phadungsombat et al., 2018; Poltep et al., 2021). However, the considerable genetic divergence observed in the Sylvatic strain, particularly in the E protein with up to 13 amino acid differences compared to the Asian I Thailand 1974 reference may explain the observed cross-reactive, sub-neutralizing antibody responses (Martinez et al., 2020).

This cross-reactive antibody response, even without prior direct exposure to the Sylvatic strain, may drive ADE upon encountering Sylvatic virus in laboratory tests. These findings highlight that populations without prior Sylvatic exposure can still exhibit high ADE levels, underscoring the importance of studying the relationship between viral genetic diversity and cross-immunity. Such interactions may play a critical role in disease severity, especially during secondary infections or the emergence of new viral genotypes in partially immune populations (Bell et al., 2019; Guzmán et al., 2000; Katzelnick et al., 2017).

The limited enhancement observed against the Cosmopolitan genotype, despite its increasing circulation in Thailand (Poltep et al., 2021) may be due to the distance of antigenic similarity between the strain tested and the strain that has been circulating currently, leading to fewer cross-reactive antibodies capable of facilitating ADE. This observation agreed with the previous finding when observed low virus neutralization activity against the Cosmopolitan strain within the same population (Pingkul et al., unpublished results). Alternatively, the structural configuration of its envelope proteins may result in lower affinity interactions with pre-existing antibodies, thus diminishing enhancement potential (Martinez et al., 2020).

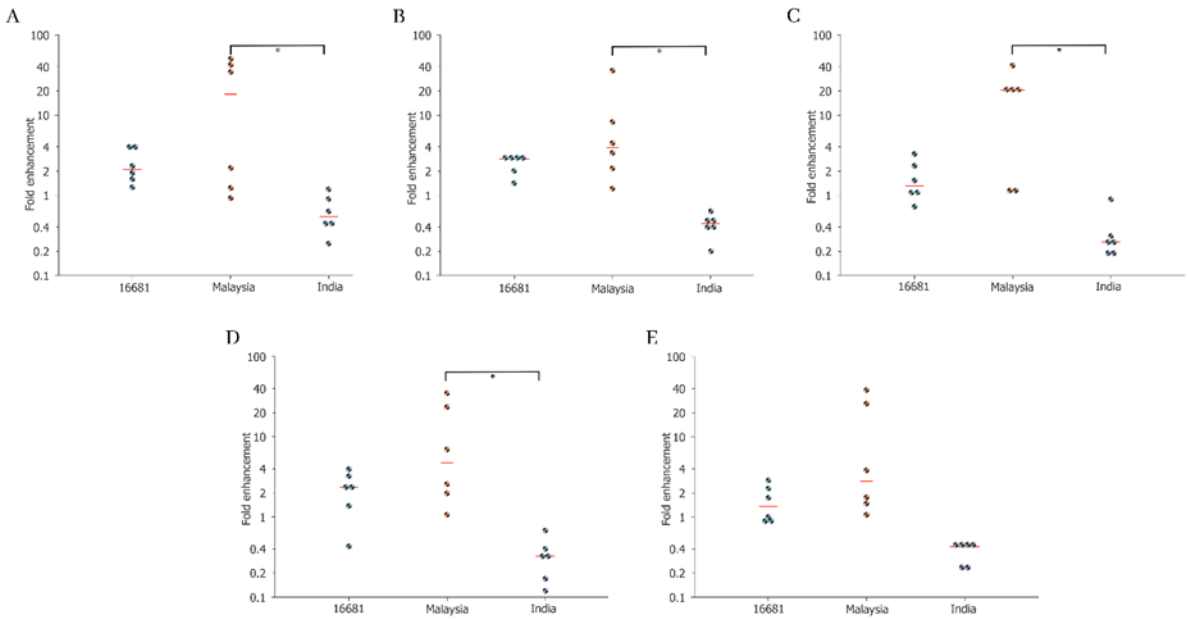

Figure 4 Comparison of fold enhancement levels within age groups. Median fold enhancement values were determined for each age category: A) 21-30 years, B) 31-40 years, C) 41-50 years, D) 51-60 years, and E) over 60 years. Differences among the three DENV2 variants, DENV2/Thailand/1964-16681 (Asian-I genotype), DENV2/Malaysia/2008-DKD-811 (Sylvatic genotype), and DENV2/India/1974-Poona-742295 (Cosmopolitan genotype) within each age group were tested using the Friedman test. Statistical significance: *$p < 0.05$.

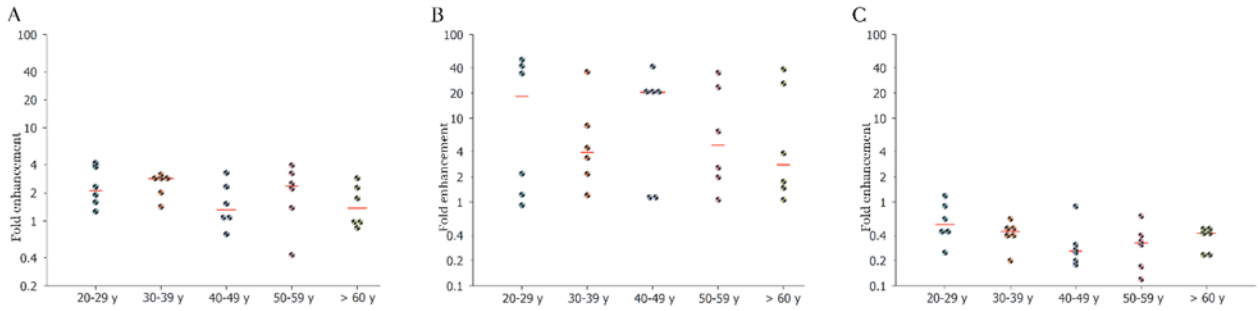

Figure 5 Comparison of fold enhancement levels across different age groups for each DENV2 variant: A) DENV2/Thailand/1964-16681 (Asian-I genotype), B) DENV2/Malaysia/2008-DKD-811 (Sylvatic genotype), and C) DENV2/India/1974-Poona-742295 (Cosmopolitan genotype). Differences in median fold enhancement levels among age groups were analyzed using the Kruskal-Wallis H test. Statistical significance: *$p < 0.05$.

Infection-enhancement activity often associated with heterologous serotype of secondary DENV infection. However, this study highlighted the ADE with homologous serotype of DENV secondary infection as shown by the infection-enhancement activity detected for the Sylvatic and Asian I genotype by the DENV2 infection background. This was consistently observed with the previous finding that suggested grouping DENV by serotype is too coarse to understand the antigenic property of the virus and the implement with disease outcome (Katzelnick et al., 2015). The antigenic distance of different strains of DENV is more relevant to emphasize the relationship between the antibody responses and virus strains, which could link to the disease outcome.

The current study also demonstrated a phenomenon commonly observed with ADE during heterotypic secondary DENV infections in dengue-endemic areas. Individuals with an antibody background from prior DENV1, DENV3, or DENV4 infections exhibited significant ADE at varying levels when exposed to DENV2 genotypes, particularly the Sylvatic and Asian I strain. These patterns highlight the complexity of dengue immunity in endemic populations, where repeated, heterotypic, and genotypically diverse exposures shape an antibody landscape that is both protective and enhancement prone.

Furthermore, when directly comparing individuals with a single DENV2 infection to those with multiple DENV exposures (including prior DENV2 infection), the study found that sera from the multiple exposure group exhibited markedly higher fold enhancement levels against DENV2 genotypes, especially Sylvatic and Asian I. In contrast, sera from the single DENV2 infection group, although showing enhancement, demonstrated relatively lower fold enhancement levels. These observations suggest that repeated DENV exposures, particularly heterotypic ones across serotypes or genotypes, may expand the pool of cross-reactive, sub-neutralizing antibodies that fuel more robust ADE responses. Importantly, even among individuals with homologous serotype immunity (such as a DENV2 background), the genotypic diversity within DENV2 can significantly influence the magnitude of enhancement, underscoring the intricate interplay between viral genetic variation and the host immune response.

In summary, this study highlighted the crucial influence of genotypic diversity and host immune background on shaping ADE responses. The notably greater enhancement associated with the Sylvatic and Asian-I genotypes underscores the importance of ongoing monitoring of circulating DENV genotypes and conducting thorough antigenic characterization. Further study with relevant population size should be included to uncover the strong integrity of the finding. Hence, these findings have major implications for vaccine design and evaluation, particularly in assessing the risk of vaccine-induced ADE. Genotype-specific functional assays,

such as the Luc-based ADE assay employed here, combined with detailed neutralizing antibody profiling, may serve as valuable tools in identifying high-risk population subsets and guiding public health strategies to mitigate severe dengue risks.

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Data Availability Statement: The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

Conflicts of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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