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Title

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Author(s)

VASQUEZ VELASQUEZ, CLARA ALEJANDRA

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Alpha tryptase allele of Tryptase 1 (TPSAB1) gene associated with Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) in Vietnam and Philippines

Clara Vasquez Velasquez a, Arthur Dessi Roman a,c, Nguyen Thi Phuong Lan b, Nguyen Tien Huy c, Edelwisa Segubre Mercado d, Fe Esperanza Espino e, Ma Lucila M. Perez f, Vu Thi Que Huong g, Tran Thi Thuy h, Vo Dinh Tham i, Cao Thi Phi Nga j, Tran Thi Ngoc Ha k, Josie M. Bilar f, Jemimah Dawn P. Bajar o, Benilda Q. Baello f, Mihoko Kikuchi k, Michio Yasunami a, Kouichi Morita l, Naohiro Watanabe m, Juntra Karbwang n, Kenji Hirayama a,c,*

a Department of Immunogenetics, Institute of Tropical Medicine (NEKKEN), and Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
b Immunology and Molecular biology, Pasteur Institute, Ho Chi Minh City, Vietnam
c Department of Clinical Product Development, Institute of Tropical Medicine (NEKKEN), and Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
d Molecular Biology Laboratory, Research Institute for Tropical Medicine, Marinduque City, Philippines
e Medical Department, Research Institute for Tropical Medicine, Manila City, Philippines
f Office of Research Development, Philippine Children’s Medical Center, Quezon City, Philippines
g Arbovirus Laboratory, Pasteur Institute, Ho Chi Minh City, Vietnam
h Hospital No. 2, Ho Chi Minh City, Vietnam
i Center for Preventive Medicine, Vinh Long Province, Vietnam
j Department of Virology, Institute of Tropical Medicine (NEKKEN), and Graduate School of Biomedical Sciences, Nagasaki University, Nagasaki, Japan
k Department of Tropical Medicine, Jikei University School of Medicine, Tokyo, Japan

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Dengue Shock Syndrome (DSS)

A B S T R A C T

We previously reported, significantly higher levels of Chymase and Tryptase in early stage plasma of DSS patients prior to the occurrence of shock suggesting a possible role of mast cells in dengue pathogenesis. To further investigate, we analyzed CMA1 promoter SNP (rs1800875) and TPSAB1 gene alleles, which encode the Human Chymase and α- and β- tryptase 1 enzymes respectively, for susceptibility to Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS) in patients from hospitals in Vietnam (Ho Chi Minh City and Vinh Long) and the Philippines. While the CMA1 promoter SNP (rs1800875) was not associated with DHF/DSS, the homozygous form of α-tryptase allele was associated with DSS patients in Vinh Long and the Philippines (OR = 3.52, p < 0.0001; OR = 3.37, p < 0.0001, respectively) and with DHF in Ho Chi Minh City (OR = 2.54, p = 0.0084). Also, a statistically significant association was observed when DHF and DSS were combined in Vinh Long (OR = 1.5, p = 0.034) and the Philippines (OR = 2.36, p = 0.0004); in Ho Chi Minh City when DHF and DSS were combine an association was observed, but it was not statistically significant (OR = 1.5, p = 0.0505). Therefore, the α-tryptase might have a possible effect on the susceptibility to severe form of Dengue infection.

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1. Introduction

Dengue infection causes a wide spectrum of clinical presentation, from asymptomatic and mild Dengue fever (DF), to the most serious forms: Dengue Hemorrhagic Fever (DHF) and Dengue Shock Syndrome (DSS). It is a global emerging arboviral illness with the number of severe cases rapidly increasing about fivefold within the past 20 years. In Southeast Asian and Latin American countries, majority of the severe cases occur among the pediatric population [1].

DHF is characterized by plasma leakage, thrombocytopenia, coagulation abnormalities, and/or hemorrhage while DDS is accompanied by hypovolemic shock. The pathogenesis of DDS/DHF is not clear. Increased vascular permeability is generally recognized as the hallmark of these severe dengue forms. Currently, no targeted treatments exist to counterbalance the vascular leakage that occur during these severe complicated episodes, partly due to the lack of understanding of the mechanisms of Dengue virus (DV)-induced vascular leakage [2,3].

It has been suggested that increased vascular permeability, which has been implicated in the pathogenesis of asthma and other allergic diseases, is also responsible for the complications in severe Dengue infections. Mast cell activation is closely linked with local or systemic increases in vascular permeability in allergic diseases. Interestingly, recent studies in mouse models suggest that degranulation of mast cells are also involved in Dengue virus infection making them possible targets for novel therapeutic and preventive strategies [4–6]. Chymase and Tryptase are the major proteins specifically stored inside mast cells. They are secreted in the process of degranulation, which is a reflection of the magnitude of mast cell activation. These enzymes have indeed been reported to participate in the allergic process due to their vasoactive, proinflammatory and chemotactic actions [7–9]. We previously reported that on admission, plasma levels of Tryptase and Chymase are significantly elevated among DHF and/or DSS patients compared with that on admission, plasma levels of Tryptase and Chymase are significantly elevated among DHF and/or DSS patients compared with healthy control children was performed at the Children's Hospital No. 2 in Ho Chi Minh City (HCMC) (n = 448) and the Center for Preventive Medicine in the Vinh Long Province (VL) (n = 513) during 2002 to 2005 in Vietnam. The inclusion criteria at the entry point in the hospital were age 6 months to 15 years old, Kinth ethnicity, and must not be related with each other. The patients were diagnosed with dengue using standard procedures for titration of anti-DV IgM and IgG antibodies, virus isolation, and RT-PCR for determination of viral. DV infection was defined by previously established serologic criteria for IgM/IgG Elisa's to DV (DEN 1–4) and Japanese encephalitis virus (kit from Pasteur Institute HCMC) in paired sera, collected in at least three days interval [27]. IgM/ IgG ELISAs were considered positive if the ratio of optical density (OD) of test sera to OD of negative control sera was ≥2.3 [28]. The cases were diagnosed as secondary infection when DV IgM-to-IgG ratio was <1.8 [27]. Buffy coat samples were used to extract genomic DNA by using the QIAamp DNA blood kit (Qiagen, Germany). The 1998 WHO classification criteria [28] were applied to classify patients into DF, DHF and DDS. Our classification met the requirement of the simplified classification system of Integrated Management of Childhood Illness (IMCI), which is based on plasma leakage as a hallmark of severe dengue (DHF/DDS) [29].

146 patients from Ho Chi Minh City and 218 from Vinh Long Province were considered DDS patients, 107 patients and 97 were DHF patients, respectively. Vinh Long Province have a higher percentage of secondary infection (65%) of 315 patients cases confirmed compared to 47% (120 patients) in 253 confirmed cases both DHF and DDS from Ho Chi Minh City.

Healthy unrelated school children living in HCMC (n = 195) and VL (n = 198), who had no symptoms of Dengue virus infection were recruited as a background population control group for the genetic study and titration of IgM were done according to the standard protocol for MAC-ELISA. In these control groups, 2 cases (1.02%) in HCMC and 13 cases (6.6%) in VL were seropositive by MAC-ELISA.

Philippine subjects: This study included children aged 5–15 years old of Filipino Malay ethnicity (n = 155) from two hospitals in Metro Manila, the Philippines—the Research Institute for Tropical Medicine (RITM), located in Alabang, Muntinlupa City, and the Philippine Children's Medical Center (PCMC) in Quezon City, from June 2008 to December 2009. Diagnostic tests used to determine dengue infection included detection of viral RNA by RT-PCR and titration of IgM and IgG antibodies by ELISA (PanBio Dengue IgM and IgG Capture Antibody test, Inverness Medical Innovations, Queensland, Australia). The definition of secondary infection was the same used in the Vietnam population. The 1998 WHO classification criteria [28] was likewise used to classify the Dengue severity. Patients who went into shock (DSS cases) accounted for majority of the patients (121% or 77.6%), while DHF cases were 34 patients. Most of the confirmed cases both DHF and DDS had secondary infection (123% or 80%).

Unrelated children from a community in Quezon City and a meningococcemia vaccine efficacy study in Muntinlupa City formed the background population group (n = 268), which sera were also tested for Dengue IgG antibodies using the PanBio Dengue IgG Capture Antibody test by ELISA (Inverness Medical Innovations, Queensland, Australia) and 10 cases (4%) were seropositive for IgG antibodies.

2. Subjects and methods

2.1. Study subjects

Vietnamese subjects: The Patients were enrolled in the study previously reported [4]. Briefly, the case control study including...
of all participants and assent from participants more than 9 years old were obtained upon enrollment to the study.

2.3. CMA1: the promoter region of CMA1 at position –1903G/A substitution

Genomic DNA was extracted from EDTA-Blood and stored at −20 °C as described elsewhere [30]. The promoter region of CMA1 at position −1903 G/A substitution (rs1800875) was investigated using primer pairs: CMA1 Forward Primer: 5'-GGA-AATGTTAGGACATACCCGATC-3' and CMA1 Reverse Primer: 5'-AATCCGGAGCGTGAACCTCTTGTC-3'. Polymerase chain reaction (PCR) amplifications were performed in a final volume of 30 μl using 10 pmol/μl of forward and reverse primer (CMA1F, CMA1R). 10X buffer with 25 mM containing MgCl₂, 2.5 mM of dNTPs, 10 ng DNA template and 2 unit of Takara Ex Taq enzyme (Takara Bio, Inc., Shiga, Japan). PCR amplification was then performed for these specimens using Thermal cycler (Biometra, Fr) programmed for 94 °C for 5 min as initial denaturation, 34 cycles of 15 s at 94 °C for denaturation, 60 °C annealing for 15 s, 15 s extension at 72 °C, and a final extension for 10 min at 72 °C. The product from PCR was confirmed by 2% agarose gel electrophoresis.

The restriction cutting of CMA1 gene promoter region at position −1903 for alternative allele G/A substitution was used for restriction fragments length polymorphisms (RFLP) method. The Amplified DNA fragments were genotyped by restriction digestion with enzyme BstXI (New England Biolabs, MA, USA). Digested fragments were determined by electrophoresis using 2% agarose gels, stained with ethidium bromide, and visualizing with UV trans-illuminator. RFLP genotype of individuals were typed in the GG genotype (Takara Bio. Inc., Shiga, Japan). PCR amplification was then performed for these specimens using Thermal cycler (Biometra, Fr) programmed for 94 °C for 5 min as initial denaturation, 34 cycles of 15 s at 94 °C for denaturation, 60 °C annealing for 15 s, 15 s extension at 72 °C, and a final extension for 10 min at 72 °C. The product from PCR was confirmed by 2% agarose gel electrophoresis.

2.7. Statistical analysis

Hardy-Weinberg equilibrium, linkage disequilibrium (LD), and haplotype analysis were calculated using PyPopWin320.7.0 software (University of Berkely, Berkely, CA) [32]. The strength of association of the given genetic factors with disease severity was assessed using odds ratio (OR) and the significance of association was evaluated by two-sided Fisher’s exact test using StatsDirect statistical software (ver.2.8.0, Stats direct Ltd. UK).

3. Results

3.1. Gene frequency of the SNP at position −1903 G/A of the promoter region of Chymase gene (CMA1)

For CMA1 gene −1903 G/A SNP (rs1800875), the distribution of A and G alleles was not found to be significantly different between cases and controls in both populations from Vietnamese and the Philippines (Table 1). The genotype frequencies fit to the Hardy-Weinberg equilibrium in the total subjects. Direct sequencing of the PCR products from 180 control samples from Vietnam confirmed the amplification of the target promoter region containing the expected BstXI restriction site at position −1903. α- and β-tryptase allele frequency by the detection of 10 bp deletion in intron 4 at position 1239–1249 bp of Tryptase gene (TPSAB1).

The gene frequency of the α-tryptase in Vietnam and the Philippines, was fitted to the Hardy–Weinberg equilibrium (Table 2).

As shown in Table 3, the α allele in homozygous form was significantly associated with the development of DHF compared with healthy controls (OR = 2.54, p = 0.0084) at HCMC. Development of DSS was significantly associated with the homozygous α-allele compared with healthy controls in patients from VL and the Philippines (OR = 3.52, p < 0.0001 and OR = 3.37, p < 0.0001, respectively), furthermore when immune response was considered, those with secondary infection and confirmed DSS have a significant increase of susceptibility compared with healthy controls (VL; OR = 4.44, p < 0.0001 and the Philippines; OR: 3.77, p < 0.0001). When the group of DSS and DHF patients were combined and compared with healthy controls, association of these severe forms with the α allele was noted to be significant in patients from VL and the Philippines (OR = 2.63, p = 0.0001 and OR = 3.06, p < 0.0001 respectively); however, this association did not reach statistical significance among HCMC subjects (OR = 1.64; p = 0.11). A similar
tendency for association was observed when the dominant α allele contribution model was used to compare those with severe forms of Dengue with healthy controls in HCMC, VL and the Philippines (Table 3).

4. Discussion

Our study showed no significant association between CMA1 –1903G allele and Dengue severity. Several studies have looked into the association of this allele with allergic diseases. A positive association between CMA1 –1903G allele and Japanese patients with atopic eczema, but not in those with asthma, atopic dermatitis or rhinitis, has been reported [21,22]. The same allele was also associated with total serum levels of IgE (<500 IU/mL) among atopic or rhinitis, has been reported [21,22]. The same allele was also associated with total serum levels of IgE (<500 IU/mL) among atopic eczema patients [23]. In an Egyptian study, this allele was associated with asthmaic children [14]. On the other hand, no significant association of this allele was observed in several studies associated among Caucasian patients with asthma and atopic dermatitis [11–13]. Such inconsistent results suggest that CMA1 –1903 G/A SNP (rs1800875) may not be a strong marker for susceptibility to allergic diseases. Similarly, the results of our study seem to show that this allele is less likely to play a major role in the progression to and pathogenesis of severe dengue infections. Although chymase levels have been found to be elevated in the serum of severe dengue patients [10], this increase may not be attributable to the genotypes of the SNP studied or other mechanisms in transcription, translation and gene expression regulation might be responsible.

Because we found a significant association between severe forms of Dengue fever and the homozygous α-tryptase allele of the TPSAB1 gene as shown in Table 3, the expression of α-tryptase in mast cells or in basophils [24] is suspected to affect susceptibility to DHF and DSS. α- and β- tryptase are soluble proteases, and proteolytic activation leads them to assemble into a mature tetrameric form and storage in mast cell granules, however it has been considered this process occurs with β-tryptase and confine to α-tryptase, which is continuously secreted, as a result of a deletion in the TPSAB1 gene [9,19]. In our previous study, using the same plasma samples from Vietnamese patients, plasma levels of tryptase increased significantly in DHF and DSS thus suggesting that degranulation of mast cells is a key process in the pathogenesis of severe Dengue [10]. Note, however, that the tryptase in serum, whose levels had been found to be elevated in that study, included both α-tryptase and β-tryptase. Our present observation of increased susceptibility to severe dengue in those with the homozygous form of α-tryptase allele might suggest that expression of this allele might not be constitutive as previously believed. The elevation in total serum plasma, believed to be largely β-tryptase mediated, may in fact be attributable to the effects of α-tryptase. In a study involving Caucasian families with at least two asthmatic siblings (per family) reported a significant association of α-tryptase allele and the disease severity in asthma [25]. This study also reported that more than one copy of α alleles may be present in one gene and that the presence of more copies are associated with increased atopy severity. Similarly, a study involving healthy subjects reported significantly higher levels of plasma tryptase in those with genotypes having at least one α-tryptase allele than in those without [26]. Taken together, α-tryptase allele may contribute to the degree of vascular leakage, and consequently to the severity of a disease via mechanisms that are still not well elucidated. This may possibly be related to the plasma levels of α-tryptase or that α-tryptase might be a competitive inhibitor to β-tryptase tetramerization inside the granules in the setting of severe dengue.

At this point, we are not able to demonstrate the plasma levels of α-tryptase. However, the finding of the strong effect of homozygous α-tryptase on the pathogenesis of DHF and DSS encourages further investigation of the mast cell activation mechanism during the acute phase of dengue infection. With previous studies reporting a significant association of the α-allele with atopy, it would be
Table 3
Association of TPSAB1 alleles α and β with the severe forms of Dengue fever (DHF, DSS) patients and healthy controls in Vietnam and the Philippines.

<table>
<thead>
<tr>
<th>TPSAB1</th>
<th>αα vs ββ</th>
<th>HCMC</th>
<th>αα αβ vs ββ</th>
<th>Metro Manila</th>
<th>αα vs ββ</th>
<th>HCMC</th>
</tr>
</thead>
<tbody>
<tr>
<td>DSS vs controls</td>
<td>15/146 vs 19/195</td>
<td>1/06</td>
<td>69/218 vs 23/198</td>
<td>52/121 vs 49/268</td>
<td>91/146 vs 110/195</td>
<td>165/218 vs 122/198</td>
</tr>
<tr>
<td>OR</td>
<td>3.52</td>
<td>&gt;0.9999</td>
<td>3.37</td>
<td>1.3</td>
<td>1.94</td>
<td>1.23</td>
</tr>
<tr>
<td>95%CI</td>
<td>0.48–2.3</td>
<td>&lt;0.0001</td>
<td>2.03–5.56</td>
<td>0.81–2.03</td>
<td>1.24–1.03</td>
<td>1.23–1.74</td>
</tr>
<tr>
<td>p value</td>
<td>&gt;0.9999</td>
<td>&gt;0.9999</td>
<td>0.95–9.6</td>
<td>0.42–1.39</td>
<td>0.9–1.81</td>
<td>0.03–1.2</td>
</tr>
<tr>
<td>DSS with primary infection vs controls</td>
<td>7/70 vs 19/195</td>
<td>1/03</td>
<td>17/77 vs 23/198</td>
<td>52/141 vs 23/198</td>
<td>23/141 vs 76/198</td>
<td>18/94 vs 89/268</td>
</tr>
<tr>
<td>OR</td>
<td>2.15</td>
<td>&gt;0.9999</td>
<td>3.13</td>
<td>1.94</td>
<td>2.12</td>
<td>0.0052</td>
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<tr>
<td>95%CI</td>
<td>0.35–2.71</td>
<td>&gt;0.9999</td>
<td>0.95–9.6</td>
<td>0.42–1.39</td>
<td>0.9–1.81</td>
<td>0.03–1.2</td>
</tr>
<tr>
<td>p value</td>
<td>&gt;0.9999</td>
<td>&gt;0.9999</td>
<td>0.95–9.6</td>
<td>0.42–1.39</td>
<td>0.9–1.81</td>
<td>0.03–1.2</td>
</tr>
<tr>
<td>DSS with secondary infection vs controls</td>
<td>8/76 vs 19/195</td>
<td>0.82</td>
<td>52/141 vs 23/198</td>
<td>43/94 vs 49/268</td>
<td>29/76 vs 85/195</td>
<td>18/94 vs 89/268</td>
</tr>
<tr>
<td>OR</td>
<td>4.44</td>
<td>&gt;0.9999</td>
<td>2.18–6.5</td>
<td>0.44–1.42</td>
<td>0.07–0.54</td>
<td>0.01</td>
</tr>
<tr>
<td>95%CI</td>
<td>0.4–2.76</td>
<td>&gt;0.9999</td>
<td>0.95–9.6</td>
<td>0.42–1.39</td>
<td>0.9–1.81</td>
<td>0.03–1.2</td>
</tr>
<tr>
<td>p value</td>
<td>&gt;0.9999</td>
<td>&gt;0.9999</td>
<td>0.95–9.6</td>
<td>0.42–1.39</td>
<td>0.9–1.81</td>
<td>0.03–1.2</td>
</tr>
<tr>
<td>DHF vs controls</td>
<td>23/107 vs 19/195</td>
<td>1.089</td>
<td>12/97 vs 23/198</td>
<td>11/34 vs 49/268</td>
<td>75/107 vs 110/195</td>
<td>58/97 vs 122/198</td>
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<tr>
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<td>2.54</td>
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<td>1.81</td>
<td>0.31</td>
<td>0.48</td>
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<td>0.4–2.76</td>
<td>&gt;0.9999</td>
<td>0.95–9.6</td>
<td>0.42–1.39</td>
<td>0.9–1.81</td>
<td>0.03–1.2</td>
</tr>
<tr>
<td>p value</td>
<td>&gt;0.9999</td>
<td>&gt;0.9999</td>
<td>0.95–9.6</td>
<td>0.42–1.39</td>
<td>0.9–1.81</td>
<td>0.03–1.2</td>
</tr>
<tr>
<td>DHF with primary infection vs controls</td>
<td>18/45 vs 19/195</td>
<td>0.0084</td>
<td>3/33 vs 23/198</td>
<td>3/5 vs 49/268</td>
<td>17/63 vs 85/195</td>
<td>0 vs 89/268</td>
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<tr>
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<td>&gt;0.9999</td>
<td>0.05</td>
<td>0.24–0.92</td>
<td>0.51–2.65</td>
<td>0.0099</td>
</tr>
<tr>
<td>95%CI</td>
<td>0.4–2.76</td>
<td>&gt;0.9999</td>
<td>0.05</td>
<td>0.24–0.92</td>
<td>0.51–2.65</td>
<td>0.0099</td>
</tr>
<tr>
<td>p value</td>
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<td>&gt;0.9999</td>
<td>0.05</td>
<td>0.24–0.92</td>
<td>0.51–2.65</td>
<td>0.0099</td>
</tr>
<tr>
<td>DHF with secondary infection vs controls</td>
<td>5/44 vs 19/195</td>
<td>1.2</td>
<td>8/20 vs 49/268</td>
<td>15/44 vs 85/195</td>
<td>25/63 vs 76/198</td>
<td>4/29 vs 89/268</td>
</tr>
<tr>
<td>OR</td>
<td>2.64</td>
<td>&gt;0.9999</td>
<td>3.06</td>
<td>1.7</td>
<td>1.06</td>
<td>0.32</td>
</tr>
<tr>
<td>95%CI</td>
<td>0.4–2.76</td>
<td>&gt;0.9999</td>
<td>0.61–4.3</td>
<td>0.31–1.39</td>
<td>0.56–1.96</td>
<td>0.08–0.97</td>
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<tr>
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<td>&gt;0.9999</td>
<td>0.61–4.3</td>
<td>0.31–1.39</td>
<td>0.56–1.96</td>
<td>0.08–0.97</td>
</tr>
<tr>
<td>DSS vs DHF</td>
<td>15/146 vs 23/107</td>
<td>0.42</td>
<td>52/121 vs 11/34</td>
<td>52/121 vs 11/34</td>
<td>91/146 vs 75/107</td>
<td>165/218 vs 58/97</td>
</tr>
<tr>
<td>OR</td>
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<td>&gt;0.9999</td>
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<td>0.7</td>
<td>2.09</td>
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<td>0.66–3.91</td>
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<td>1.21–3.6</td>
<td>0.13–1.86</td>
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<td>&gt;0.9999</td>
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<td>1.21–3.6</td>
<td>0.13–1.86</td>
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<tr>
<td>DSS + DHF vs controls</td>
<td>38/253 vs 19/195</td>
<td>1.64</td>
<td>63/155 vs 49/268</td>
<td>166/253 vs 110/195</td>
<td>223/315 vs 122/198</td>
<td>128/155 vs 179/268</td>
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<td>1.02–2.23</td>
<td>1.42–3.99</td>
</tr>
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<td>p value</td>
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<td>0.0001</td>
<td>0.0505</td>
<td>0.034</td>
<td>0.0004</td>
</tr>
</tbody>
</table>

Bold letter shows the significant number.

TPSAB1, tryptase 1 gene; DHF, Dengue Hemorrhagic Fever; DSS, Dengue Shock Syndrome; VL, Vinh Long Providence; HCMC, Ho Chi Minh City; OR, odds ratio; p value, Exact Fisher’s two sided; 95%CI, Fisher exact 95% confidence interval, using StatsDirect statistical software (ver2.8.0, Stats Direct Ltd. UK).
important to elicit the history of atopy or any allergic conditions and rule them out as a potential, confounding factor.

Author Contributions
Study was conceived and designed by CV, MK and KH. The experiments were performed by CV, MK, ADR, CV, MK, and KH analyzed the data. CV, ADR and KH wrote the manuscript. All authors contributed the data collection and approved the final version for publication.

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