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Clinical, Virological, and Cytokine Profiles of Children Infected with Dengue Virus during the Outbreak in Southern Vietnam in 2017

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Abstract. Dengue virus (DENV) infection is a major cause of morbidity and mortality in Vietnam, and the incidence is higher and more consistent in the southern part of the country. This study investigated the circulation of DENV serotypes, viremia levels, immunological status, and cytokine levels, with disease severities among children infected in 2017 in Ho Chi Minh City, Southern Vietnam. Acute and convalescent serum samples were collected from clinically diagnosed dengue children. They were confirmed to have DENV infection by NS1 antigen, IgM and IgG ELISAs, virus isolation, and conventional and real-time RT-PCR. Measurement of 10 cytokine levels was performed in the serum samples. All the children were dengue IgM positive; 28% and 72% of them had primary and secondary DENV infections, respectively, whereas 54% of those with secondary infection were children with dengue with warning signs and with severe dengue. Any or mixed infection of the four serotypes of DENV RNA was detected in 58 children. Twenty DENV strains (DENV-1 = 16 and DENV-4 = 4) were isolated. Levels of IFN-γ, TNF-α, MCP-1, IL-10, and IL-6 were significantly higher in severe dengue cases. We report the predominance of DENV-1 over other serotypes in the 2017 dengue outbreak in Southern Vietnam. Our data showed that cytokine expressions were correlated with dengue pathogenesis and may help in identifying an effective therapeutic strategy.

INTRODUCTION

The global incidence of dengue (DEN) has grown dramatically in recent decades.[1] The mosquito-borne dengue viruses (DENVs) of the genus *Flavivirus* are positive-sense single-stranded RNA viruses and can be transmitted to humans by the bite of infected *Aedes* mosquitoes.[2] Infection with any of the DENV serotypes can result in asymptomatic manifestation or a wide range of clinical manifestations, from mild-to-severe dengue infection.[3] The 2009 WHO criteria classify DEN according to levels of severity: DEN without warning signs (DwoWS), DEN with warning signs (DwWS), and severe DEN (SD).[4]

There are several mechanisms to explain the severe forms of DENV infection. They include antibody-dependent enhancement of infection, virulence of DENV strain, cell-mediated immune response, and quantity and type of cytokines during infection.[5] It has been observed in several studies that secondary DENV infection by a DENV serotype different from the first infecting serotype is more likely to produce severe disease.[6–11] Severe DEN is a leading cause of serious illness and death among children in some Asian and Latin American countries.[2] A number of studies have shown cytokine profiles related to DEN pathogenesis.[12–14] Studies to elucidate cytokine storm hypothesis by analyzing sera of severe DEN patients in Vietnam, India, and Cuba have shown elevated levels of IFN-γ, TNF-α, and IL-10 in patients with an increased severity of dengue infection.[15–17]

Dengue has caused a substantial health and economic burden in Vietnam, with the number of reported cases varying significantly year by year. Between 2007 and 2016, the average number of reported cases per year was 90,844.[18] Dengue outbreaks tend to be larger and more frequent in the southern provinces, with the incidence of infection typically peaking between June and October.[18] According to a 2017 dengue report from the National Institute of Hygiene and Epidemiology in Hanoi (Vietnam), the reported number of dengue cases in Southern Vietnam was 81,626 with 32 deaths; 33,729 of these cases with seven deaths were from Ho Chi Minh City. From 2007, DENV-1 was the dominant serotype in dengue outbreaks in Southern Vietnam. However, there has been limited information about cytokine profiles in Vietnamese children during acute and convalescent phases of DENV infection. In this study, we did a clinical, virological, and cytokine analyses of acute- and convalescent-phase serum samples of children with different types of disease severity and infection (primary or secondary) during the dengue outbreak in 2017 in Ho Chi Minh City, Southern Vietnam.

MATERIALS AND METHODS

Samples, patients, and ethical approval. During the 2017 DEN outbreak, paired acute and convalescent serum samples were collected from 76 clinically diagnosed dengue pediatric patients (aged 0–17 years), who were admitted to Children's Hospital No (1), Ho Chi Minh City, Southern Vietnam. Acute and convalescent serum samples were obtained from days 3 to 7 and days 8 to 19 after the onset of fever, respectively. Laboratory tests such as hematocrit test (Hct), tourniquet test, platelet (PLT) counts, and SD bioline NS1 antigen rapid test were carried out by using acute serum samples.[19] Based on the clinical findings supported by laboratory tests, patients were diagnosed and classified based on DEN severity according to the criteria of the 2009 WHO guideline (WHO,

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2009). Serum samples were also collected from a healthy control group composed of schoolchildren (aged < 16 years) who were negative to DEN diagnostic tests (DENV NS1 antigen and IgM) and found to have no other observable diseases. The study was approved by the Ethics Committee of National Institute of Hygiene and Epidemiology in Vietnam (08061924-7).

Serological confirmation. Serological confirmation of DENV infection was performed by the in-house DENV IgM capture ELISA,[20] detection of NS1 antigen, and in-house DENV IgG indirect and capture ELISA.[21,22] In the DENV IgM capture ELISA, optical density (OD) was read at 492 nm and a P/N (positive control or sample OD/negative control OD) ratio ≥ 2 was considered as positive. NS1 antigen from serum samples was detected by DEN NS1 antigen rapid test (SD bioline, Suwon, Korea). The DENV IgG indirect and capture ELISA, and DENV IgG ELISA (Vircell, Granada, Spain) were used to determine primary and secondary DENV infections.[21,23] A sample with a titer ≥ 1:29,000 was considered to be from a patient with a secondary DENV infection.[21] A sample with a titer < 1:29,000 was considered to be from a patient with a primary DENV infection.[21]

DENV isolation and serotyping. All acute serum samples were inoculated onto *Aedes albopictus* clone C6/36 mosquito cells contained in flat culture tubes. Infected cells were incubated at 28°C for 7 days in Eagle's minimum essential medium supplemented with 2% fetal calf serum and 0.2 mM of nonessential amino acids.[24] The infected culture fluid (ICF) from each tube was collected, aliquoted, and stored at −80°C until use.

RNA was extracted from the ICF by using the viral RNA Mini Kit (Quiagen, Hilden, Germany) according to the manufacturer's instruction. To screen the ICF which contained DENV, RT-PCR was carried out using one-step RT-PCR following the manufacturer's instruction (Takara, Shiga, Japan). Conventional RT-PCR was carried out by using primer sets (Supplemental Table S1) for the detection of DENV and another primer set for the determination of specific DENV serotypes (Supplemental Table S1).[25,26]

Quantification of DENV genome levels. Viral RNA was directly extracted from 140 μL of the patient's acute serum by using the same kit to extract RNA from the ICF. A volume of 5 μL of RNA was used for quantitative real-time RT-PCR (qRT-PCR). Amplification of the envelope gene was performed using a total of 20 μL of reaction mixture (5 μL of TaqMan master mix, 9 μL of nuclease water, 0.3 μL of 100 pmol of forward and reverse primers, and 0.4 μL of probe with DENV serotype–specific primers, Supplementary Table S2) using TaqMan Fast Virus 1-Step Master Mix (Life Technologies, Carlsbad, CA) and following the protocol from a previous report.[27,28] Ten-fold serial dilutions of standard cDNA (10^8–10^2 genome copies) were applied for quantification of viral genome levels.[28] The detection limit for viral genome was 100 copies. The viral genome levels were expressed as $\log_{10}$ genome copies/mL.

Quantification of DENV viremia levels. Focus formation assay was performed to determine DENV viremia levels in patient sera.[29] Serially diluted serum samples were inoculated onto Vero cells which were then incubated at 37°C for 2 hours followed by the overlaying of 1.25% methylcellulose 4,000 in 2% FCS MEM. After incubation at 37°C for 3 days, the cells were fixed with 4% paraformaldehyde phosphate buffer and permeabilized with 1% Nonidet P-40 in phosphate-buffered saline without magnesium and calcium (PBS-). This was followed by sequential adding at 1-hour interval of Blockace, pooled human sera containing high-titered anti-*flavivirus* IgG and HRP-conjugated goat antihuman IgG (American Qualex, San Clemante, CA). To visualize positive results, the substrate 3,3′-diaminobenzidine tetrahydrochloride (DAB; Wako, Tokyo, Japan) was added. The stained foci of cells were counted under the microscope to calculate focus-forming units per mL (FFU/mL) for the virus titer.

Identification and quantification of cytokines. IFN-γ, TNF-α, IL-1β, IL-12P40, IL-2, IL-6, IL-10, IL-4, MCP-1, and IL-8 levels from acute- and convalescent-phase serum samples of children were measured by using Milliplex Map kit (Human Cytokine/Chemokine Magnetic Bead Panel 60K, EMD Millipore, Billerica, MA) according to the manufacturer's instructions.[30] Analysis was carried out on the Bio-Plex 200 instrument, and data were analyzed by Bio-Plex Manager software (version 6).

Statistical analysis. Data were analyzed by using SPSS for Windows, version 22.0 (IBM Corp., Armonk, NY). Continuous variables were presented as median (IQR, interquartile range) and categorical variables as absolute number (*n*) and percentage (%). The comparison of continuous variables was performed by the Mann–Whitney *U* test between two groups and the Kruskal–Wallis test among three or more groups, whereas the chi-squared test (or Fisher's exact test, as appropriate) was used to compare the categorical variables. Spearman's correlation was used to examine the correlation between two continuous variables. An alpha level of 0.05 was used for all statistical tests. A two-tailed *P*-value less than 0.05 was considered statistically significant.

RESULTS

Characteristics of the study population. The characteristics of the study population are shown in Table 1. According to the 2009 WHO criteria, 19 (25%), 36 (47%), and 21 (28%) of the 76 pediatric patients were diagnosed to have DwoWS, DwWS, and SD, respectively. Forty-seven (62%) of them were males and 29 (38%) were females, with an average age of 8.5 years. Significant difference in age was found between DwoWS, DwWS, and SD groups (Table 1, Figure 1A). The levels of white blood cell (WBC), Hct, and PLT were measured by using acute-phase serum samples of children. No significant differences were found in the WBC count and Hct levels among the three clinical severity groups. However, a significantly higher WBC count was found in the SD group than in the DwoWS and DwWS groups (Figure 1B). Platelet count was significantly lower in the SD group than in the DwoWS and DwWS groups (Figure 1C). A significantly lower count of PLT was found in patients with secondary infection than in those with primary DENV infection (Figure 1D). Significant differences were found in clinical symptoms such as purpura, abdominal pain, hemorrhagic manifestation, respiratory difficulties, ascites, and pleural effusion among the three groups (Table 1).

Serological examination, infecting virus serotypes, and viremia levels. Forty-five of the 76 children were DENV NS1 positive. Sixteen, 21, and eight of them were from the DwoWS, DwDW, and SD groups, respectively (Table 2). Measurement of RNA in serum samples by qRT-PCR showed that 58 of the 76 children were infected with specific DENV serotypes as follows: 37 children with DENV-1, two children with DENV-2, one child with DENV-3, 15 children with DENV-4, one child with both DENV-1 and DENV-3, two children with both DENV-1 and DENV-4, and one child with both DENV-2 and

TABLE 1
Demographic, clinical, and laboratory information of the study population subgrouped according to different degrees of severity of dengue infection

Variables	Severity grades			*P*-value
	DwoWS	DwWS	SD	
Number of children	19	36	21	
Age (years), median (IQR)	9 (7–13.2)	10 (7–13)	8 (4.5–8.7)	**0.040**
Gender (Male:female)	14:05	22:14	11:10	
WBC ($\times 10^3$), median (IQR)	2.6 (2.2–3.4)	3.3 (2.6–4.3)	4.3 (3.3–4.6)	0.060
Hct (%), median (IQR)	38.9 (36.6–41.3)	40.4 (37.5–44)	36.75 (33.9–38.3)	0.461
PLT ($\times 10^3$), median (IQR)	110 (97.7–122)	98.0 (68.0–121)	18.5 (13.2–41.2)	**0.0001**
Torniquet test positive	2 (25.0)	6 (75.0)	0 (0.0)	**0.002**
Fever	19 (26.4)	34 (47.2)	19 (26.4)	0.470
Muscle pain	11 (35.5)	17 (54.8)	3 (9.7)	0.232
Headache	4 (19.0)	15 (71.5)	2 (9.5)	0.097
Rash	7 (19.4)	19 (52.8)	10 (27.8)	0.468
Purpura	14 (26.9)	30 (57.7)	8 (15.4)	**0.002**
Vomiting	7 (17.9)	20 (51.3)	12 (30.8)	0.340
Nausea	9 (19.1)	25 (53.2)	13 (27.7)	0.270
Joint pain	16 (32.0)	27 (54.0)	7 (14.0)	0.277
Abdominal pain	0 (0.0)	24 (64.9)	13 (35.1)	**0.0001**
Haemorrhagic manifestation*	0 (0.0)	8 (72.7)	3 (27.3)	0.083
Respiratory difficulties	1 (7.1)	4 (28.6)	9 (64.3)	**0.004**
Ascites	0 (0.0)	1 (12.5)	7 (87.5)	**0.001**
Pleural effusion	0 (0.0)	2 (18.2)	9 (81.8)	**0.0001**

WBC = white blood cell; Hct = hematocrit test; PLT = platelets; DwoWS = dengue without warning signs; DwWS = dengue with warning signs; SD = severe dengue. Numbers in the parentheses indicate percentage, unless stated otherwise. All comparisons for categorical variables between/among the groups are performed by using the chi-square test or Fisher's exact test as appropriate, whereas the comparison for continuous variables is performed by using the Kruskal–Wallis test (for more than two groups) and the Mann–Whitney *U* test (between two groups). $P < 0.05$ is considered significant.

DENV-4. All of the 76 (100%) children were DENV IgM positive, whereas 21 (28%) and 55 (72%) of them had primary and secondary infections, respectively. Among those with secondary infection, 41 children had DwWS and SD, whereas 12 children had DwoWS. No significant difference was found among the three groups in terms of the type of infection (primary and secondary DENV infections) and DENV RNA detection (Table 2).

Dengue virus strains were isolated from 20 children who, with the exception of one, were NS1 positive (Table 3). Of the 20 DENV isolates, 16 were DENV-1, six of which were from

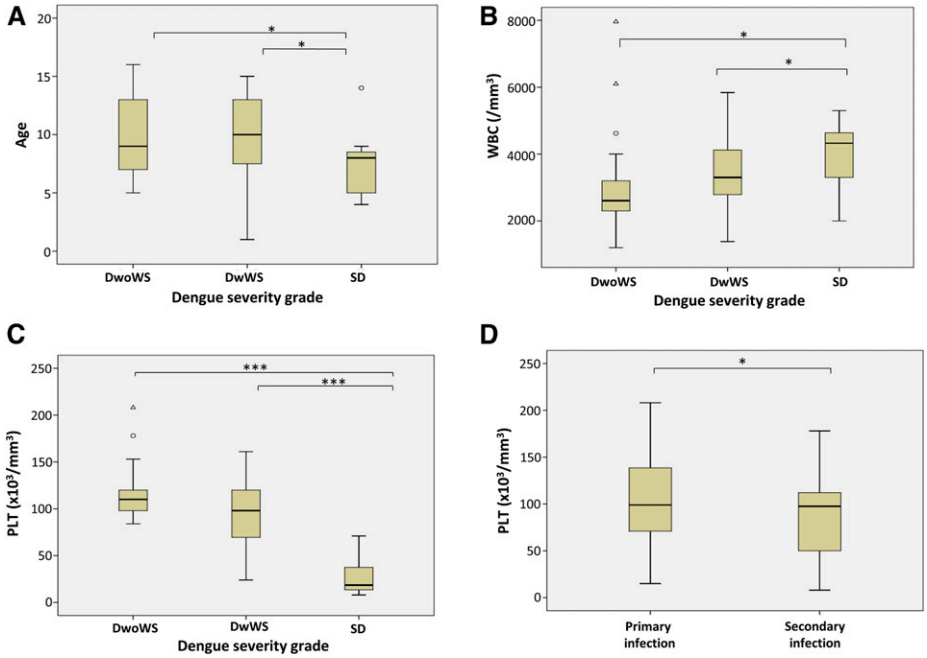

FIGURE 1. Distribution of age of patients and values of hematological parameters according to the different degrees of dengue severity or types of infection. All comparisons among the three groups of children are performed by using the Kruskal–Wallis test. *Comparison between two groups is performed by using the Mann–Whitney *U* test (**A**–**D**). *P*-value less than 0.05 is considered as statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Box plots show median values (horizontal line in the box), 25–75% interquartile range (lower-upper limits of the box), additional upper and lower whiskers (represent data outside the IQR including the maximum and minimum values), and outliers (circles or triangles for extreme). DwoWS = dengue without warning signs; DwWS = dengue with warning signs; SD = severe dengue; WBC = white blood cell; PLT = platelets. This figure appears in color at www.ajtmh.org.

TABLE 2
Diagnostic profiles of children with different dengue severity grades in Vietnam, 2017

Laboratory diagnosis assays	Total	Severity grades			*P*-value
		DwoWS	DwWS	SD	
DENV NS1 Ag positive	45	16 (35.6%)	21 (46.7%)	8 (17.8%)	**0.011**
Virus Isolation					
DENV-1	16	6 (37.5%)	10 (62.5%)	0	0.648
Primary	6	2	4	0	
Secondary	10	4	6	0	
DENV-4	4	2 (50.0%)	2 (50.0%)	0	–
Secondary	4	2	2	0	
DENV RNA detection*					
DENV-1	40	9	19	12	0.549
DENV-2	3	2	0	1	
DENV-3	1	0	1	0	
DENV-4	18	4	8	6	
DENV IgM positive	76	19	36	21	–
DENV IgG detection					
Primary	21	5 (23.8%)	12 (57.1%)	4 (19.0%)	0.540
Secondary	55	14 (25.5%)	24 (43.6%)	17 (30.9%)	–

* DENV serotypes are detected by real-time RT-PCR. All comparisons for categorical variables between/among the groups are performed by using the chi-square test or Fisher's exact test as appropriate, whereas the comparison for continuous variables is performed by using the Kruskal–Wallis test (for more than two groups) and the Mann–Whitney *U* test (between two groups). $P < 0.05$ is considered significant.

patients with DwoWS and 10 from DwWS. The six patients had primary infection, and 10 had secondary infections, respectively. Four DENV-4 isolates were from two patients with DwoWS, and two from patients with DwWS. These four patients had secondary infection. Two children (sample ID 14 and 20) with DENV-1 isolates were detected by real-time PCR to have RNA from both DENV-1 and DENV-4. One child (sample ID 1) with DENV-4 isolate had DENV RNA levels from both DENV-2 and DENV-4 (Table 3). We measured the DENV genome level (log_{10} copies/mL) and viremia levels (FFU/mL) using serum samples of children with DENV isolates. Because of volume limitation, virus titration was performed starting with a 100-time dilution to each of the 20 serum samples with DENV isolates. Of the 20 DENV isolates, viremia levels were detected only in 13 children, and seven samples were found to have an undetectable DENV level (< 100) (Table 3).

Age distribution. The distribution of children from different age-groups as to the types of infection showed that seven and five children in the 1- to 4-year-old age-group, five and 20 children in the 5- to 8-year-old age-group, five and 13 children in the 9- to 12-year-old age-group, and three and 15 children in the 13- to 16-year-old age-group had primary and secondary infections, respectively, in each age-group. The most number of affected children was in the 5- to 8-year-old age-group with secondary infection (Figure 2). Although the association between age-group and immune response (primary/secondary infection) is not statistically significant ($P = 0.057$), there is a clear trend particularly of increasing secondary infection

TABLE 3
Characteristics of DENV strains isolated from children with different clinical severities and types of DENV infection in Vietnam in 2017

ID	Clinical severity	Type of infection	NS1	Virus isolation serotype	Real-time PCR (log_{10} genome copies/mL)			Virus titer (log_{10}FFU/mL)
					DENV-1	DENV-2	DENV-4	
1	DWoWS	Sec	+	DENV-4	–	4.1*	4.3*	2.9
2	DwWS	Sec	+	DENV-1	5.9	–	–	3.8
3	DwWS	Pri	+	DENV-1	5.8	–	–	3.8
4	DWoWS	Sec	+	DENV-4	–	–	5.1	–
5	DwWS	Sec	+	DENV-1	4.0	–	–	–
6	DwWS	Sec	+	DENV-1	–	–	–	–
7	DWoWS	Sec	+	DENV-1	6.1	–	–	4.7
8	DwWS	Sec	+	DENV-1	5.9	–	–	4.5
9	DwWS	Sec	+	DENV-4	–	–	5.8	–
10	DwWS	Sec	–	DENV-1	5.5	–	–	–
11	DwWS	Pri	+	DENV-1	5.1	–	–	2.9
12	DwWS	Sec	+	DENV-1	6.3	–	–	4.4
13	DWoWS	Sec	+	DENV-1	6.0	–	–	2.7
14	DWoWS	Pri	+	DENV-1	8.0*	–	3.9*	–
15	DwWS	Sec	+	DENV-4	–	–	7.3	4.8
16	DwWS	Pri	+	DENV-1	7.8	–	–	7.0
17	DWoWS	Pri	+	DENV-1	5.7	–	–	3.1
18	DwWS	Pri	+	DENV-1	7.7	–	–	5.9
19	DWoWS	Sec	+	DENV-1	–	–	4.7	–
20	DWoWS	Sec	+	DENV-1	6.1*	-	4.3*	3.1

DENV = dengue virus; FFU = focus-forming unit; Pri = primary infection; Sec = secondary infection.
* Mixed infection of DENV.

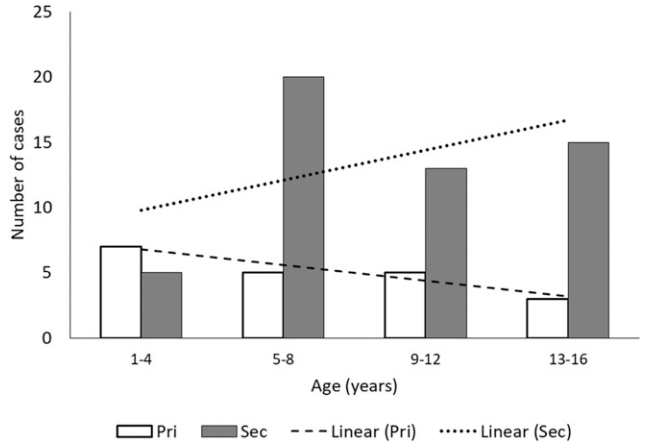

FIGURE 2. Age distribution of children with primary or secondary infection.

with the increasing age and decreasing primary infection with the increasing age (Figure 2).

Pro-inflammatory cytokine profile and clinical parameters. Serum samples from only 58 of the 76 dengue-positive children and from a group of nine healthy children (control) were subjected to cytokine analysis (Figures 3 and 4). Because of inadequate volume, serum samples from 18 of 76 dengue-positive children were not included. Of the 58 children 17, 22, and 19 belonged to the DwoWS, DwWS, and SD groups, respectively. Ten different cytokines were measured: pro-inflammatory (IFN-γ, TNF-α, IL-1β, IL-12P40, and IL-2), anti-inflammatory (IL-10), pro- and anti-inflammatory cytokines (IL-6 and IL-4), and two chemokines (MCP-1 and IL-8). The IFN-γ levels were significantly higher in both acute- and convalescent-phase serum samples of patients belonging to the three clinical severity groups than in the control group (Figure 3A). The IFN-γ levels in the convalescent-phase serum samples of the DwWS and SD groups were significantly higher than those of the DwoWS group. The IFN-γ levels in the acute-phase serum samples of the DwoWS and DwWS groups were significantly higher than those in the convalescent phase. The TNF-α levels were significantly higher in the acute-phase serum samples of the DwWS group and in both acute- and convalescent-phase serum samples of the SD group than those of the control group (Figure 3B). During the convalescent phase, TNF-α levels were significantly increased in the SD group as compared with the DwoWS and DwWS groups. The levels of IL-1β were significantly increased in the convalescent-phase serum samples of the DwWS group compared with those of the control group (Figure 3C). IL-12P40 levels were significantly higher in both phases of serum samples of patients in the three clinical severity groups than those of the control group (Figure 3D). There were no detectable IL-2 levels in both the three dengue severity groups and the control group.

Anti-inflammatory and pro- and anti-inflammatory cytokines, chemokine profiles, and clinical parameters. The levels of pro- and anti-inflammatory cytokine IL-6 were significantly higher in the acute-phase serum samples of the DwWS group and in both phases of serum samples of the SD group than the control group (Figure 4A). IL-6 was significantly increased in both phases of serum samples of the SD group compared with the DwoWS and DwWS groups. The levels of

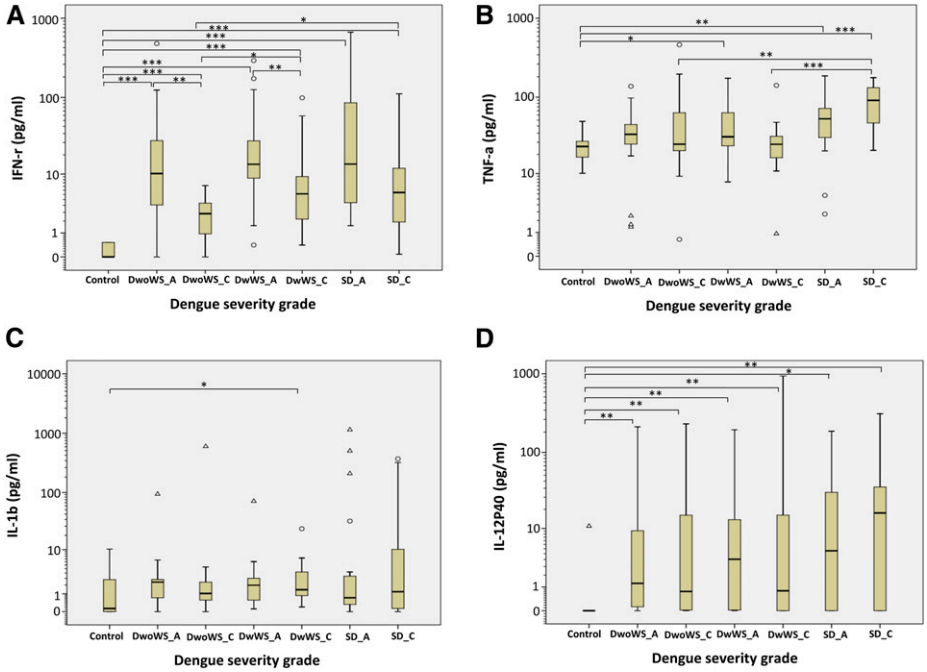

FIGURE 3. Serum levels of pro-inflammatory cytokines in children with different degrees of dengue severity compared with healthy controls. All comparisons among three or more groups are performed by using the Kruskal–Wallis test. *Comparison between two groups is performed by using the Mann–Whitney *U* test. *P*-value less than 0.05 is considered as statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Box plots show median values (horizontal line in the box), 25–75% interquartile range (lower-upper limits of the box), additional upper and lower whiskers (represent data outside the IQR including the maximum and minimum values), and outliers (circles or triangles for extreme). DwoWS-A,C = acute and convalescent phase of dengue without warning signs ($n = 17$); DwWS-A,C = acute and convalescent phase of dengue with warning signs ($n = 22$); SD-A,C = acute and convalescent phase of severe dengue ($n = 19$). This figure appears in color at www.ajtmh.org.

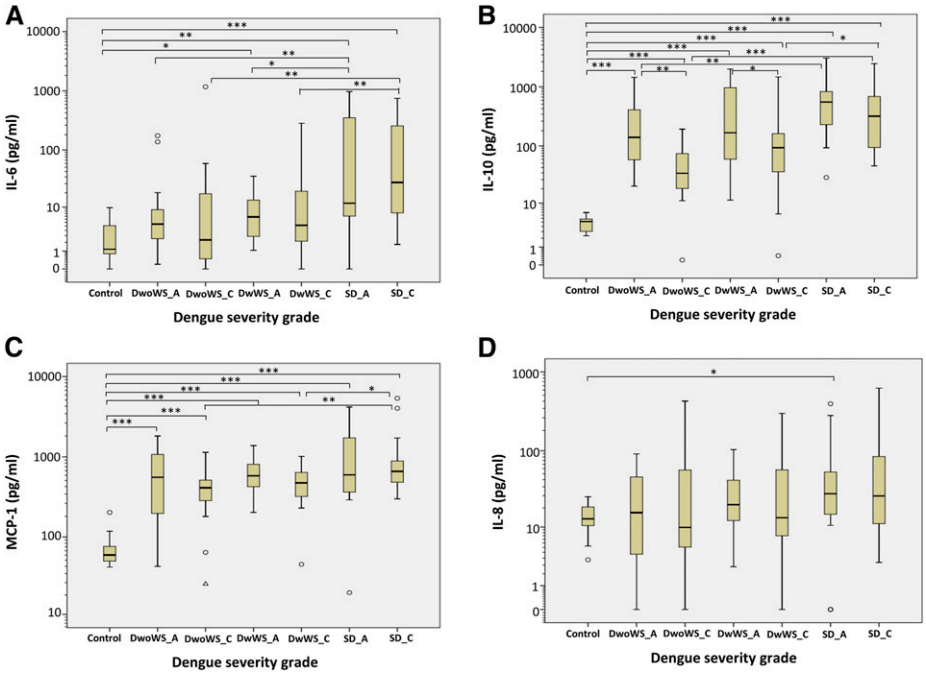

FIGURE 4. Serum levels of anti-inflammatory cytokines in children with different degrees of dengue severity compared with healthy controls. All comparisons among three or more groups are performed by using the Kruskal–Wallis test. *Comparison between two groups is performed by using the Mann–Whitney *U* test. *P*-value less than 0.05 is considered as statistically significant (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$). Box plots show median values (horizontal line in the box), 25–75% interquartile range (lower-upper limits of the box), additional upper and lower whiskers (represent data outside the IQR including the maximum and minimum values), and outliers (circles or triangles for extreme). DwoWS-A,C = acute and convalescent phase of dengue without warning signs ($n = 17$); DwWS-A,C = acute and convalescent phase of dengue with warning signs ($n = 22$); SD-A,C = acute and convalescent phase of severe dengue ($n = 19$).This figure appears in color at www.ajtmh.org.

anti-inflammatory cytokine IL-10 were significantly higher in both phases of serum samples of the three clinical severity groups than those of the control group (Figure 4B). Particularly, the IL-10 levels were significantly higher in the SD group than in the DwoWS group during the acute phase of infection. Moreover, IL-10 level in convalescent-phase serum samples of the SD group was significantly higher than that of the DwoWS and DwWS groups. In the DwoWs and DwWS groups, IL-10 levels were significantly higher in the acute-phase than in the convalescent-phase serum samples. The levels of chemokine MCP-1 were significantly higher in both phases of the serum samples of the DwoWS, DwWS, and SD groups than those of the control group (Figure 4C). During the convalescent phase, MCP-1 levels in the SD group were significantly higher than those in the DwoWS and DwWS groups. The levels of chemokine IL-8 were significantly higher in acute-phase serum samples of the SD group than those in the control group (Figure 4D). The level of pro- and anti-inflammatory cytokine IL-4 productions in the dengue groups of children and the control group was mostly undetectable. The *P*-values on levels of pro- and anti-inflammatory cytokines in children having DwoWS, DwWS, SD, and healthy controls are described in Supplemental Table S3.

Association of pro-inflammatory, anti-inflammatory, pro- and anti-inflammatory cytokines and chemokines with primary and secondary DENV infections. We compared the cytokines and chemokine levels among patients with primary DENV infection, secondary DENV infection, and healthy controls (Table 4). During the acute phase, the levels of IFN-γ, IL-6, IL-10, and MCP-1 were significantly higher in patients with primary and secondary infections than those in healthy controls. Similarly, the levels of IFN-γ, IL-6, IL-10, MCP-1, and IL-12P40 were significantly higher in patients with primary and secondary infections than those in healthy controls during the convalescent phase. Notably, levels of IFN-γ and IL-6 in convalescent sera were significantly higher in patients with secondary infection than those in patients with primary infection.

DISCUSSION

Our study described the serological, molecular, and immunological status of children infected with DENV during the dengue outbreak in 2017 in Ho Chi Minh City, Southern Vietnam. All patients in this study were DENV IgM positive; 27.6% and 72.3% of them had primary and secondary DENV infections, respectively. Similar to the previous studies involving Vietnamese children and adults, the number of patients with secondary infection in this study was higher than that of those with primary infection.[31–33] In the present study, there were more patients with secondary infection in DwWS and SD groups than those with primary infection. Other studies have also shown that severe DENV infection is chiefly seen in secondary infection.[7,34] In our study, the number of NS1-positive patients was significantly higher in the DwoWS and DwWS groups than that of those in the SD group. A total of 20 DENV were isolated from DwoWS and DwWS patients, 19 of whom were NS1 positive. The less number of NS1 (8 of 21) positive and no DENV isolation among the SD group might be because of collection of blood at a later time after the onset of illness because patients sought primary health care first before going to a referral hospital.

TABLE 4

Comparison of cytokines and chemokine levels in acute and convalescent sera of children with primary and secondary infections, Vietnam, 2017

Cytokine or chemokine	Control (*N* = 9)	Primary infection (*N* = 15)	Secondary infection (*N* = 43)	*P*-value
Acute sera				
IFN-γ	0.0 (0.0–0.5)	8.7 (3.5-16-8)	14.3 (5.6–79.8)	**0.0001**
IL-10	4.3 (2.5–5.2)	155.2 (36.3–409.2)	332–7 (102.5–928.6)	**0.001**
IL-12P40	0.0 (0.0–0.0)	6.8 (0.1–20.1)	1.9 (0.0–18.9)	0.08
IL-1b	0.1 (0.0–2.4)	1.2 (0.7–2.3)	1.5 (0.4–3.8)	0.18
IL-6	1.1 (0.7–6.3)	5.3 (2.5–17.2)	7.3 (2.8–22.1)	**0.024**
IL-8	12.9 (7.9–19.6)	18.2 (12.0–49.2)	22.1 (7.4–46.2)	0.31
MCP-1	59.3 (45.8–97.2)	687.2 (203.0–1,179.9)	558.0 (381.7–992.4)	**0.0001**
TNF-α	23.2 (16.4–27.3)	34.1 (22.2–86.7)	34.8 (25.3–63.3)	0.07
Convalescent sera				
IFN-γ	0.0 (0.0–0.5)	5.9 (3.5–12.9)	3.2 (1.1–6.8)	**0.0001***
IL-10	4.3 (2.5–5.2)	94.4 (74.3–144.2)	75.3 (29.0–332.1)	**0.0001**
IL-12P40	0.0 (0.0–0.0)	4.2 (0.0–42.1)	1.0 (0.0–24.0)	**0.011**
IL-1b	0.1 (0.0–2.4)	1.3 (0.5–4.6)	1.1 (0.4–2.9)	0.15
IL-6	1.1 (0.7–6.3)	30.5 (3.7–59.4)	5.6 (1.1–27.6)	**0.013***
IL-8	12.9 (7.9–19.6)	37.5 (8.7–86.1)	12.9 (6.4–56.9)	0.37
MCP-1	59.3 (45.8–97.2)	471.1 (412.5–890.5)	482.8 (321.0–660.6)	**0.0001**
TNF-α	23.2 (16.4–27.3)	43.5 (25.7–132.2)	30.1 (20.4–78.1)	0.08

Cytokine concentrations are expressed in pg/mL. Cytokines and chemokines levels are expressed as median (IQR) for each groups (control, primary, and secondary infection). All comparisons within groups are performed by using the Kruskal–Wallis test, and comparison between two groups is performed by the Mann–Whitney test. Range of numbers in parentheses beside the median refers to minimum–maximum values.

* Between patients with primary and secondary infections, level of IFN-r ($P = 0.02$) and IL-10 ($P = 0.04$) are significantly different.

As DENV qRT-PCR results showed, the most dominant serotype was DENV-1 (52.6%), followed by DENV-4 (23.6%), DENV-2 (4.8%), and DENV-3 (1.3%). Based on virus isolation results, DENV-1 (21%) was dominant, followed by DENV-4 (5.2%). Interestingly, DENV-1 was the dominant serotype in the 2017 dengue outbreak and had persistently dominated for about one decade in Southern Vietnam. For further studies, a molecular analysis and phenotypic and genotypic characterization will be carried out for DENV-1 and DENV-4 isolates in this study.

IFN-γ is produced by T cells and NK cells and activated monocytes and macrophages.[35] In our study, increased production of IFN-γ levels was observed during the acute phase of infection of the SD group, compared with the DwoWS and DwWS groups. High levels of IFN-γ were observed in dengue patients from Asia and Latin America and were associated with severity.[36] Similar levels of IFN-γ were found in the DwoWS and the DwWS groups in the present study, and these results were similar with those of another study in Thai children.[37] TNF-α is secreted by monocytes, and it enhances vascular permeability and coagulation-activating effects.[12] TNF-α has been reported to be associated with severity and has higher concentration values for patients with dengue haemorrhagic fever than those with dengue fever in Brazil and Thailand.[12,38–40] Our finding showed that TNF-α was significantly increased in the DwsWS and SD groups compared with the controls. IL-1β is released by mononuclear monocytes activation with IFN-γ.[32] Studies have shown that significant increase or highest level of IL-1β occurs in SD patients compared with those with mild dengue in Brazil and Vietnam.[32,41] However, our result showed that the IL-1β level was not significantly different among the DwoWS, DwWS, SD, and control groups.

IL-12 induces the production of IFN-γ by T or NK cells.[42] In the present study, the role of IL-12 in DENV infection was assessed by measuring the circulating levels of the p40-subunit of IL-12 (IL-12 p40). A significant increased level of IL-12p40 was observed in the clinical severity groups compared with controls. However, in another study, no significant IL-12-p40 response was observed in dengue patients, compared with controls.[43] The concentration levels of some cytokines such as IL-1β, IL-8, and IL-12P40 were not significantly different among DwoWS, DwWS, and SD groups in our study. IL-6, pro- and anti-inflammatory cytokine, is secreted by T cells, macrophages, and NK cells and influences antigen-specific immune responses and inflammatory reactions.[12] Some studies have described increased levels of IL-6 in SD versus DwoWS and control groups, whereas others failed to show the same results.[12,31,41,44] In our study, IL-6 levels were significantly higher in SD versus the DwoWS and DwWS groups during the acute phase of infection.

With IL-10, other studies have shown increased level in the DwWS and SD groups compared with the DwoWS group.[17,45] IL-10 has been shown to be associated with other virus infections such as influenza and to be related to neurological complications.[46] Our findings confirmed the higher IL-10 level in the SD group versus the other two clinical severity groups. In the case of MCP-1, a significant difference was noted in the three severity groups compared with the controls, and the highest MCP-1 level was found in the SD group. This MCP-1chemokine has been associated with permeability changes in endothelial cells.[47] Chemokine IL-8, which is produced by monocytes, endothelial cells, and hepatocytes, enhances vascular permeability and activates the innate immune system. Elevated levels of IL-8 have been observed in DSS patients compared with not-severe dengue patients and healthy controls.[48,49] Our results also showed similar results, as IL-8 levels being significantly higher in the SD group than in the control group.

It has been shown that the DENV nonstructural protein NS1 elicits inflammatory cytokine production and that the endothelial cell monolayer leaks via Toll-like receptor four activation of mouse macrophages and human peripheral blood mononuclear cells.[50–52] Dengue NS1 has been noted to be directly toxic, damaging endothelial cells and producing lethal vascular permeability in mouse models.[52,53] Furthermore, anti-dengue NS1 compounds and antibodies prevent this toxic damage.[53] Because of the limitation of serum volume in our

study, we were not able to measure NS1 level and analyze its correlation with the inflammatory cytokines and disease severity. Further studies are needed to clarify cytokine hypothesis to severe dengue pathogenesis.

In conclusion, we report the predominance of DENV-1 over other serotypes in the 2017 dengue outbreak in Southern Vietnam. Our study showed that the levels of IFN-γ, TNF-α, MCP-1, IL-10, and IL-6 were significantly higher in children with severe dengue. The findings support monitoring of changes in the characteristics of the circulating DENV to generate more robust epidemiological data. The cytokine profiles from this study could provide future discovery and validation of biomarkers for the prediction of severe dengue infection.

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