CLINICAL DIFFERENCES AMONG PCR-PROVEN DENGUE SEROTYPE INFECTIONS

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Abstract. The objective of this study was to compare the clinical spectra of the dengue serotypes proven by the PCR technique. This retrospective study reviewed the clinical information of dengue-infected patients who were admitted to northeastern provincial hospitals in Thailand from June to September 2002. Dengue infection and viral serotypes were confirmed by polymerase chain reaction (PCR). Paired anti-dengue immunoglobulin G (IgG) and IgM from paired sera were analyzed by enzyme-linked immunosorbent assay (ELISA). Ninety-nine PCR-proven dengue-infected Thai patients were studied. Their ages ranged from 3-30 years. They were infected with DEN1, DEN2, DEN3 and DEN4 in 21, 55, 12, and 12%, respectively. Twenty-two percent had primary and 78% had secondary infections. Dengue fever was the most common presentation for both primary (77.2%) and secondary infections (46.7%). The ratios of dengue fever:dengue hemorrhagic fever (DF:DHF) and non-dengue shock syndrome:dengue shock syndrome (non-DSS:DSS) for DEN2 was the lowest of the dengue serotypes. There was no difference in the duration of fever, percentage of hepatomegaly and bleeding among the serotypes in both DF and DHF. The trends in the white blood cells, lymphocyte and atypical lymphocyte counts in DEN3 were the highest, while those of DEN1 were the lowest of the dengue serotypes.

INTRODUCTION

Dengue fever (DF) and dengue hemorrhagic fever/dengue shock syndrome (DHF/DSS) are still the most important mosquito-borne viral diseases affecting humans worldwide, and constitute a major public health problem in tropical and subtropical regions (Gubler, 1998; Nimmanitaya, 2002). The four serotypes of dengue virus (DEN), a member of the Family Flaviviridae of positive-sense single-stranded RNA viruses, cause a broad spectrum of clinical manifestations in humans, ranging from acute febrile illness DF to the life-threatening DHF/DSS. Dengue fever is a self-limited, though debilitating, disease characterized by headache, retro-orbital pain, myalgia, arthralgia, rash, and in some cases, hemorrhagic manifestations. DHF is defined by hemorrhagic signs, thrombocytopenia and hemoconcentration, or other evidence of vascular leakage, which may progress to shock (DSS) and death (Nimmanitaya, 1987). Serological diagnosis of the dengue viruses is complicated by the existence of cross-reactive antigenic determinants shared by all four dengue virus serotypes and members of the flavivirus family. Even after a single exposure to a related flavivirus, convalescent patient sera usually contain detectable cross-reactive antibodies (Henchal and Putnak, 1990). Many reports in the literature have shown differences in clinical manifestations among the different dengue serotypes (Kalayanarooj *et al*, 2000; Vaughn *et al*, 2000), however, the serotypes proven by antibody detection may have a discrepancy with the real pathogen. The objective of this study was to compare the clinical spectra of the dengue serotypes proven by the PCR technique.

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MATERIALS AND METHODS

This study was a retrospective examination of dengue-infected patients aged less than 30 years who had sera available and were admitted to the Nongkhai, Nakhon Phanom and Mukdahan provincial hospitals, Thailand from June to September 2002. The diagnosis of dengue infection and infecting serotype were confirmed by polymerase chain reaction (PCR). Anti-dengue immunoglobulin G (IgG) and IgM from paired sera on admission and discharge were also analyzed by enzyme-linked immunosorbent assay (ELISA) to differentiate primary from secondary infections. The medical records were reviewed. Information on the duration of fever, bleeding presentation, hepatomegaly and tourniquet test were extracted. Complete blood counts, including the highest hematocrit, lowest white blood cell count, differential count, and lowest platelet count, were also reviewed. This information was used in the diagnosis of dengue fever and dengue hemorrhagic fever according to World Health Organization (WHO) criteria (WHO, 1997).

Statistical analysis was performed with the SPSS program. Fisher's exact test and the chi-square test were used to compare discrete variables, and the Student's *t*-test and ANOVA test were used for continuous variables with a significance level of 0.05 (two-tailed).

Clinical definitions

WHO diagnostic criteria were used to classify dengue-infected patients. Dengue fever is divided into classic dengue fever (DF) and dengue fever with unusual hemorrhagic manifestations. Classical dengue fever presents with thrombocytopenia and occasionally mild hemorrhage. Dengue hemorrhagic fever (DHF) is diagnosed when the patient has fever with thrombocytopenia and any sign of plasma leakage, such as hemoconcentration and hemorrhagic manifestation. Dengue shock syndrome (DSS) is diagnosed when the patient meets the DHF criteria plus either hypotension for age or narrow pulse pressure in the presence of clinical signs of shock. Thrombocytopenia is defined as a platelet count of less than 100,000/mm^3. Hemoconcentration is defined when the hematocrit rises 20% or more from the baseline value (WHO, 1997).

Enzyme-linked immunosorbent assay (ELISA)

The serum samples from each patient were tested for anti-dengue virus IgM and IgG antibodies by IgM and IgG capture ELISA, as described by Innis *et al* (1989).

Interpretation of results.

A ratio of anti-dengue IgM to IgG (if IgM >40 units) ≥1.8 was the criterion for primary dengue infection. A ratio of IgM to IgG <1.8 was the criterion for secondary infection.

Polymerase chain reaction (PCR)

Dengue viral RNA on patient sera was extracted using the Boom extraction method (Boom *et al*, 1990), then they were subjected to reverse transcriptase polymerase chain reaction (RT-PCR) using dengue-specific anti-sense D2 primer (Table 1) and their products were used as the template for the nested-PCR reaction using D1, TS1, TS2, TS3 and TS4 primers (Table 1), as described by Lanciotti *et al* (1992).

RESULTS

The medical records of 99 dengue-infected in-patients, confirmed by PCR and paired anti-dengue IgG and IgM ELISA tests, were recruited for this study. The mean (SD) age of the patients was 10.2 (3.9) years and the male:female ratio was 1.1:1.0.

The means (SD) for the first and second blood drawing days were of days 4.2 (1.5) and 7.8 (1.7), respectively. Dengue serotype 2 (DEN2) was the most frequent serotype found (54.5%) followed by DEN1 (21.2%), DEN3 (12.1%) and DEN4 (12.1%). The age, gender, and blood-draw days were not significantly different among the serotypes, as shown in Table 2. There was no fatality or unusual manifestations of dengue disease, such as liver failure or encephalopathy, in any of the cases reviewed.

Sequential infections were determined by ELISA, and 22 (22.2%) patients had primary, and 77 (77.8%) had secondary infections.

For primary infection, the most frequent causative serotype was DEN2 (45.5%), followed by DEN1 (40.9%) and DEN3 (13.6%), while there

Table 1
Primer for RT-PCR and nested PCR.

Primer	Sequence	Location	Target size (bp)
D1	5′-TCA ATA TGC TGA AAC GCG CGA GAA ACC G-3′	134-161	
D2	5′-TTG CAC CAA CAG TCA ATG TCT TCA GGT TC-3′	616-644	
TS1	5′-CGT CTC AGT GAT CCG GGG G-3′	568-586	482
TS2	5′-CGC CAC AAG GGC CAT GAA CAG-3′	232-252	119
TS3	5′-TAA CAT CAT CAT GAG ACA GAG C-3′	400-421	290
TS4	5′-CTC TGT TGT CTT AAA CAA GAG A-3′	506-527	392

During the RT-PCR step, D2 primer was used to amplify the dengue RNA, D1 *vs* TS1 (for dengue serotype 1), TS2 (serotype 2), TS3 (serotype 3) and TS4 (serotype 4).

Table 2
Epidemiological data of the study.

	Dengue serotype				
	DEN1	DEN2	DEN3	DEN4	Total
Number	21	54	12	12	99
Mean age (yr; SD)	9.9 (3.3)	10.4 (4.3)	10.3 (3.8)	9.6 (2.6)	10.2 (3.9)
Gender (M:F)	1.6:1.0	0.9:1.0	1.4:1.0	1.0:1.0	1.1:1.0
Means for 1st blood-draw specimen	4.2	4.2	3.6	4.8	4.2
(fever day; SD)	(1.1)	(1.4)	(1.4)	(1.0)	(1.5)
Means for 2nd blood-draw specimen	7.2	7.9	7.7	8.7	7.9
(fever day; SD)	(1.5)	(1.5)	(1.6)	(1.6)	(1.7)

Table 3
Spectrum of dengue infection by sequential infection and serotype.

	Dengue serotype				
	DEN1	DEN2	DEN3	DEN4	Total
Primary infection	9 (40.9%)	10 (45.5%)	3 (13.6%)	0 (0%)	22
DF	7/9 (77.8%)	7/10 (70%)	3/3 (100%)	0	17
DHF grade 1	1/9 (11.1%)	3/10 (30%)	0	0	4
DHF grade 2	1/9 (11.1%)	0	0	0	1
DHF grade 3	0	0	0	0	0
DHF grade 4	0	0	0	0	0
Secondary infection	12 (15.6%)	44 (57.1%)	9 (11.7%)	12 (15.6%)	77
DF	6/12 (50%)	18/44 (40.9%)	5/9 (55.6%)	7/12 (58.3%)	36
DHF grade 1	2/12 (16.7%)	6/44 (13.6%)	1/9 (11.1%)	1/12 (8.3%)	10
DHF grade 2	0	5/44 (11.4%)	3/9 (33.3%)	2/12 (16.7%)	10
DHF grade 3	3/12 (25.0%)	13/44 (29.5%)	0	2/12 (16.7%)	16
DHF grade 4	1/12 (8.3%)	2/44 (4.5%)	0	0	3
Total	21 (21.2%)	54 (54.6%)	12 (12.1%)	12 (12.1%)	99

Table 4
Clinical and laboratory findings for dengue fever by dengue serotype.

	DEN1 (n=13)	DEN2 (n=25)	DEN3 (n=8)	DEN4 (n=7)	p-value
Duration of fever (days)	6.6 (6-8)	6 (5-7)	6 (5-7)	6.9 (5-9)	0.493
Bleeding manifestations (%)	7.6	25	37.5	42.8	0.269
Positive tourniquet test (%)	42.8	76.2	100	100	0.088
Hepatomegaly (%)	NA	33.3	75	60	0.333
Highest hematocrit (%)	40.1 (38-43)	40.8 (40-42)	38.6 (36-42)	40.6 (38-43)	0.404
Lowest white blood cell count ($X10^9$/l)	2.6 (2-3)	3.6 (3-4)	5 (2-8)	2.9 (2-4)	0.038[a]
Lowest platelet count ($X10^9$/l)	129.1 (88-170)	103.4 (78-130)	109.1 (52-166)	84.9 (62-108)	0.442
Neutrophils ($X10^7$/l)	132.9 (94-172)	169 (121-218)	284 (5-563)	140 (90-190)	0.142
Lymphocytes ($X10^7$/l)	107.1 (75-139)	149.1 (114-183)	169.1 (84-254)	129.4 (75-184)	0.268
Atypical lymphocytes ($X10^7$/l)	3 (0-7)	15.6 (4-27)	30.5 (1-60)	13.8 (0-38)	0.127
Monocytes ($X10^7$/l)	13.6 (9-18)	18.9 (11-27)	35.2 (2-69)	10.4 (0-24)	0.106
Eosinophils ($X10^7$/l)	3.3 (1-6)	3.9 (1-7)	1.8 (0-4)	1.7 (0-4)	0.707
Basophils ($X10^7$/l)	3 (2-4)	2.2 (1-4)	8.8 (0-29)	6 (0-18)	0.343
Neutrophils (%)	49.4 (40-59)	45.6 (38-53)	46.8 (29-65)	47 (31-63)	0.943
Lymphocytes (%)	41.4 (33-49)	42.7 (36-49)	38.7 (22-55)	41.9 (32-52)	0.945
Atypical lymphocytes (%)	1.7 (0-4)	4 (2-6)	6.6 (0-14)	3.8 (0-11)	0.331
Monocytes (%)	5.1 (4-7)	6 (4-8)	6.3 (2-11)	3.9 (0-9)	0.764
Eosinophils (%)	1.2 (0-2)	1 (0-2)	0.5 (0-1)	0.6 (0-1)	0.588
Basophils (%)	1.2 (1-2)	0.8 (0-1)	1.1 (0-3)	2.8 (0-9)	0.291

( ) = 95% confidence interval
[a]p-value<0.05

were no primary DEN4 infections. Seventeen primary cases (77.3%) presented with dengue fever and only 5 (22.7%) had dengue hemorrhagic fever. All dengue hemorrhagic fever cases were in grades 1 and 2.

For secondary infection, DEN2 (57.1%) was also the most common causative serotype, followed by DEN1 (15.6%), DEN4 (15.6%), and DEN3 (11.6%). Thirty-six secondary cases (46.8%) presented with dengue fever, which was significantly fewer frequent than primary infection (p-value=0.03). Of the 39 cases of DHF, there were 10, 10, 16 and 3 cases in grades 1, 2, 3 and 4, respectively.

The ratios of primary:secondary infection for each dengue serotype were 0.8:1, 0.2:1, 0.3:1

Table 5
Clinical and laboratory findings for dengue hemorrhagic fever by dengue serotype.

	DEN1 (n=8)	DEN2 (n=29)	DEN3 (n=4)	DEN4 (n=5)	p-value
Duration of fever (day)	6.8 (5-8)	6.1 (5-7)	5.8 (3-8)	7.4 (6-9)	0.420
Bleeding manifestations (%)	50	62.1	75	60	0.861
Positive tourniquet test (%)	100	100	100	100	NA
Hepatomegaly (%)	100	92.3	75	66.6	0.545
Highest hematocrit (%)	45.8 (41-51)	47.8 (45-50)	41.3 (34-49)	46 (40-53)	0.126
Lowest white blood cell count ($X10^9$/l)	3.1 (2-4)	3.4 (3-4)	3.2 (1-5)	4.5 (3-6)	0.155
Lowest platelet count ($X10^9$/l)	45.5 (24-67)	48.5 (20-78)	49 (0-100)	59.8 (40-79)	0.925
Neutrophils ($X10^7$/l)	189.8 (125-255)	187.9 (158-218)	177.9 (0-408)	290.1 (178-360)	0.187
Lymphocytes ($X10^7$/l)	98.6 (78-119)	117.3 (97-137)	219.8 (0-852)	161.6 (47-276)	0.022*
Atypical lymphocytes ($X10^7$/l)	4.1 (0-10)	15.3 (4-27)	15.7 (0-128)	13.6 (0-34)	0.042*
Monocytes ($X10^7$/l)	13 (7-19)	13.8 (2-26)	1.7 (0-23)	4.7 (0-14)	0.832
Eosinophils ($X10^7$/l)	3.1 (0-6)	2.2 (1-4)	0	0	0.479
Basophils ($X10^7$/l)	3.5 (0-8)	2.8 (0-5)	0	3 (0-11)	0.914
Neutrophils (%)	59.5 (52-67)	55.2 (50-61)	43.5 (0-88)	61.2 (43-80)	0.401
Lymphocytes (%)	32.3 (27-38)	35.9 (31-41)	52.5 (21-84)	34 (19-49)	0.232
Atypical lymphocytes (%)	1.2 (0-3)	4 (2-7)	3.5 (0-23)	2.8 (0-6)	0.658
Monocytes (%)	4.5 (3-6)	3.4 (1-5)	0.5 (0-7)	1.2 (0-3)	0.499
Eosinophils (%)	1.1 (0-2)	0.7 (0-1)	0	0	0.482
Basophils (%)	1.3 (0-3)	0.7 (0-1)	0	0.8 (0-3)	0.617

( ) = 95% confidence interval
*p-value<0.05

and 0:1 for DEN1, DEN2, DEN3, and DEN4, respectively, which were significantly different (p-value = 0.03). The ratios of DF:DHF for the different dengue serotypes were 1.6:1, 0.9:1, 2:1 and 1.4:1 for DEN1, DEN2, DEN3, and DEN4, and the ratios for non-DSS:DSS were 4.3:1, 2.6:1 and 5.0:1, respectively, with no DSS found in DEN3.

The clinical manifestations and laboratory findings of dengue fever are presented in Table 4. The mean (SD) duration of fever was 6.2 (1.6) days. The percentages of patients with bleeding manifestations in DEN1, DEN2, DEN3, and DEN4 were 7.6, 25.0, 37.5, and 42.8%, respectively. The percentages of positive tourniquet tests were 42.8, 76.2, 100, and 100%, respec-

tively. Hepatomegaly was found in 33.3-75.0%, with no significant difference among the serotypes. The mean (SD) highest hematocrit was 40.3% (3.2%). The lowest WBC count was in DEN1 (mean, 2.6 x 10^9/l) and the highest was in DEN3 (mean, 5.0 x 10^9/l), with significant differences among the serotypes. None of the nadirs for the platelet counts or differential counts had significant differences among the serotypes.

The clinical manifestations and laboratory findings of dengue hemorrhagic fever are shown in Table 5. The mean (SD) duration of fever was 6.3 (1.8) days. The percentages of patients with bleeding manifestations in DEN1, DEN2, DEN3, and DEN4 were 50.0, 62.1, 75.0, and 60.0%, respectively. All cases had positive tourniquet tests. Hepatomegaly was found in 66.6-100.0% with no significant differences among the serotypes. The mean (SD) peak hematocrit was 46.2% (4.5%). Although the nadirs of the white blood cell counts were not significantly different among the serotypes, the white blood cell count in DEN1 seemed to be lower than the others. The nadirs of the platelet counts were not different among the serotypes. The total lymphocyte (mean, 219.8 x 10^7 /l) and atypical lymphocyte counts (mean, 15.7 x 10^7 /l) in DEN3 were the highest of the dengue serotypes while those of DEN1 were the lowest.

DISCUSSION

This study showed the clinical spectra and compared the clinical findings in 99 dengue patients in northeastern Thailand. Fifty-five patients (55.6%) had dengue fever and 44 (44.4%) had dengue hemorrhagic fever. Although dengue fever was more common than dengue hemorrhagic fever, more cases of dengue fever should be suspected because mild dengue fever usually needs no admission. The patient with secondary infection has more severe symptoms than primary infection, which is proven by the high percentage of dengue hemorrhagic fever. This finding also confirms many previous studies, which have shown a strong association between the occurrence of DHF/DSS and secondary infection (Halstead *et al*, 1970; Halstead, 1988; Hoke *et al*, 1983; Vaugh *et al*, 2000). In this study (2002), DEN2 was the most prevalent serotype, followed by DEN1, DEN3 and DEN4, which is different from the national survey, Department of Medical Science, Ministry of Public Health, Thailand in the year 2000, which found DEN1, DEN2, DEN3, and DEN4 caused 43.5, 26.7, 24.6, and 5.5% of dengue infections, respectively. The dynamics of the 4 dengue serotypes have been published in the literature (Halstead, 1988), and are believed to be a cause of severe dengue disease. Previous studies showed dengue disease severity correlated with high viremia titers, secondary infection (Boonpucknavig *et al*, 1979) and DEN2 infection (Thein *et al*, 1997; Vaughn *et al*, 2000). Our findings found the ratios of DF:DHF and non-DSS:DSS for DEN2 was lower than the others, which implies that severity was related to DEN2 infection. This conclusion should be more closely considered, because a previous dengue infection may influence the severity or the clinical manifestations of a subsequent dengue infection.

The clinical findings for DF and DHF in the serotypes were described. There were no differences among the serotypes, which may be caused by the limitations of the retrospective study and the sample size. However, the bleeding manifestations, including the positive tourniquet tests in DEN2 and DEN4, were more frequent than in DEN1 and DEN2. The blood count profiles for DF and DHF were also compared. We found that the nadirs of the white blood cell counts for the DEN1 were the lowest of the 4 serotypes. The peaks for the lymphocyte and atypical lymphocyte counts for DEN3 were the highest of the 4 serotypes. Several publications have shown a the higher percentage of lymphocytes and atypical lymphocytes in the peripheral blood of dengue patients (Boonpucknavig *et al*, 1979; Kalayanarooj *et al*, 1997), but the comparison was done without determinating the serotype. This is the first publication that shows the different patterns on the peripheral blood among the serotypes proven by PCR. Although this finding can not be applied as evidence for serotype differentiation, it should give new information for further prospective studies, along with the differences in hematological responses of the serotypes after dengue infection.

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