SECONDARY DENGUE VIRUS TYPE 4 INFECTIONS IN VIETNAM

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Abstract. This study was designated to describe clinical and biological features of patients with a suspected diagnosis of dengue fever/dengue hemorrhagic fever during an outbreak in Central Vietnam. One hundred and twenty-five consecutive patients hospitalized at Khanh Hoa and Binh Thuan Provincial hospitals between November 2001 and January 2002 with a diagnosis of suspected dengue infection were included in the present study.Viruses were isolated in C6/36 and VERO E6 cell cultures or detected by RT-PCR. A hemagglutination-inhibition test (HI) was done on each paired sera using dengue antigens type 1-4, Japanese encephalitis (JE) virus antigen, Chickungunya virus antigen and Sindbis virus antigen. Anti-dengue and anti-JE virus IgM were measured by a capture enzyme-linked immunosorbent assay (MAC-ELISA). Anti-dengue and anti-JE virus IgG were measured by an ELISA test. Dengue viruses were isolated in cell culture and/or detected by RT-PCR in 20.8% of blood samples. DEN-4 and DEN-2 serotypes were found in 18.4% and 2.4% of the patients, respectively. A total of 86.4% of individuals had a diagnosis of acute dengue fever by using the HI test and/or dengue virus-specific IgM capture-ELISA and/or virus isolation and/or RT-PCR. The prevalence of primary and secondary acute dengue infection was 4% and 78.4%, respectively. Anti-dengue IgG ELISA test was positive in 88.8% of the patients. In 5 cases (4%), Japanese encephalitis virus infection was positive by serology but the cell culture was negative. No Chickungunya virus or Sindbis virus infection was detected by the HI test. In patients with acute dengue virus infection, the most common presenting symptom was headache, followed by conjunctivitis, petechial rash, muscle and joint pain, nausea and abdominal pain. Four percent of hospitalized patients were classified as dengue hemorrhagic fever. The clinical presentation and blood cell counts were similar between patients hospitalized with acute dengue fever and patients with other febrile illnesses.

INTRODUCTION

Dengue is a mosquito-borne infection that in recent years has become a major international public health concern, particularly in tropical and subtropical regions. There are an estimated 50 million infections annually, including 400,000 cases of dengue hemorrhagic fever (DHF), a potentially lethal complication, which requires hospitalization (WHO, 2000).

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There are 4 dengue virus serotypes: DEN-1, DEN-2, DEN-3, and DEN-4. The viruses are members of the genus *Flavivirus* and family Flaviviridae. They are transmitted by the bite of infective mosquitos, mainly *Aedes aegypti.* An infection with one serotype provides lifelong immunity to only the homologous type. Thus, people may experience multiple dengue infections. Dengue infection can manifest as a clinically inapparent, undifferentiated illness, classic dengue fever, with hemorrhagic manifestations (DHF) or shock syndrome (DSS).

The first epidemic of dengue was recorded in 1635 in the French West Indies, but a disease compatible with dengue had already been reported in China in 992 AD (Gubler, 1997).

The first recognized diagnosis of DHF in the Southeast Asia and the Western Pacific region was in the Philippines in 1953. An exceptionally large outbreak of DHF occurred in Vietnam in 1987 with 354,517 cases (WHO, 1997). In 1998, 119,429 cases of DHF and 342 deaths were reported in South Vietnam (Ha ***et al***, 2000).

Our study was conducted in Central Vietnam in 2001-2002, with the objective of describing the clinical and serological features of dengue in hospitalized patients. Very little data on dengue fever was available for Central Vietnam, although outbreaks are often severe. Most hospitals base the diagnosis of dengue fever solely on clinical grounds because specific serology and virus isolation are not always available or done at the time of hospitalization.

MATERIALS AND METHODS

Patients and materials

Serum specimens were collected in the Infectious Diseases Departments of Khanh Hoa Provincial Hospital (Nha Trang city) and Binh Thuan Provincial Hospital (Phan Thiet city, 200 km to the south of Nha Trang), in Central Vietnam, and sent to the laboratory of virology of the Institut Pasteur in Nha Trang.

All patients hospitalized at both hospitals between November 2001 and January 2002 with a diagnosis of suspected dengue infection were eligible for participating in this study. This period fell in the middle and end of rainy season, and is a usual period for dengue outbreaks in Central Vietnam.

The data collected from the medical records included age, sex, clinical presentation and blood count.

Blood samples were obtained at the time of admission (for virus isolation or RNA detection), and around the 6th and the 14th day of illness (for serology). Samples were stored at -20°C or -70°C prior to assay.

Methods

Dengue viruses were isolated from diluted sera (1:30) injected into C6/36 (*Aedes albopictus*) and VERO E6 cell cultures seeded at 3 x 10^5 cells per ml in 1-ml glass tubes and incubated at 28°C (for C6/36 cells) or 37°C (for VERO cells) for 7 days. Infected cells were harvested and assayed for dengue virus by direct fluorescent antibody technique using four serotype-specific monoclonal antibodies kindly provided by Dr D Gubler (CDC, Atlanta): DEN-1 (Hawaii D2-1F1-3), DEN-2 (New Guinea C 3H5-1-21), DEN-3 (H-87 D6-8A1-12), Den-4 (H-241 1H10-6-7) (Gubler, 1987). Detection of dengue viral RNA was done in some samples by reverse-transcriptase polymerase chain reaction (RT-PCR) according to the Lanciotti procedure (Lanciotti ***et al***, 1992) after silica extraction (Chungue ***et al***, 1993). Suspected sera were tested for JE virus by specific RT-PCR (Raengsakulrach ***et al***, 1999) in a supernatant of AP61 cell cultures. Hemagglutination-inhibition tests (HI) were performed on each paired sera using the procedure of Clarke and Casals (1958), modified to microplates, employing 8 U each of dengue antigens type 1-4, Japanese encephalitis virus antigen, Chickungunya virus antigen and Sindbis virus antigen. Antigens were produced by sucrose acetone extraction of the brains of suckling mice infected with the following virus strains: DEN-1 Hawaii, DEN-2 New Guinea, DEN-3 H-87, DEN-4 H-241, Japanese encephalitis virus Nakayama, Chickungunya C347 and Sindbis N.Y.

IgM antibodies reactive for dengue antigens or Japanese encephalitis (JE) antigens were measured by in-house IgM antibody capture enzyme-linked immunosorbent assay (MAC-ELISA) as described by Rossi and Ksiazek (1998) and adapted for dengue and Japanese encephalitis diagnosis. Anti-dengue and anti-Japanese encephalitis virus IgG were measured by an in-house ELISA test according to the technique proposed by Rossi and Ksiazek (1998); but using dengue and JE virus antigens. The cutoff values for both IgG and IgM immunoassays were calculated with specific control sera provided by AFRIMS, Bangkok (Innis ***et al***, 1989), after adaptation to our techniques.

Serologic definitions of dengue and Japanese encephalitis infections

Dengue virus infection was defined as isolation of dengue virus in the serum (or detection of virus RNA by RT-PCR) and/or serologic evidence of dengue virus infection by HI or MAC-ELISA. HI criteria for dengue infection was a four-

fold rise in antibody titers against any dengue serotype between the acute and convalescent sera. Using MAC-ELISA, acute dengue virus infection was defined as a dengue virus-specific IgM level higher than the cutoff value.

Absence of dengue infection was defined as no detectable dengue virus in acute sera, the absence of IgM in acute and convalescent sera, and stable antibody titers of less than 1:2,560 to all dengue virus antigens without a fourfold rise in titer by the HI test.

Primary and secondary dengue infection were defined by the HI test according World Health Organization criteria (WHO, 1997). Acute primary dengue infection was also defined as an acute dengue infection with an IgM-to-IgG ratio of 1.8 or greater by ELISA in the acute or convalescent sample. Infection with an IgM-to-IgG ratio less than 1.8 was classified as an acute secondary dengue infection (Innis *et al*, 1989).

A ratio of dengue IgM to JE IgM < 0.91 was defined as a probable infection with JE virus (Strickman *et al*, 2000).

Statistical analysis

All calculations were done using the R version 1.7.1 (Ihaka and Gentleman, 1996).

RESULTS

A total of 125 consecutive patients (75 in Nha Trang and 50 in Phan Thiet) hospitalized with a diagnosis of suspected dengue infection were included in the study. Of these, 79 (63%) were males. The median age was 13 years.

The mean body temperature on the day of admission was 38.3°C. The following symptoms and signs were observed: headache (69%), retro-orbital pain (31%), myalgia and/or arthralgia (38%), nausea (35%), vomiting (25%), abdominal pain (26%), conjunctival injection (51%), petechial rash (42%), bleeding (13%) including gastrointestinal bleeding (4.8%), epistaxis (7.2%), and gingival bleeding (3.2%). The tourniquet test was often positive (93%). The mean hematocrit value was 39.7% and the median value for the platelet count was 142,020. The median white blood cell and neutrophil counts were 5,018 cells/mm^3 and 2,321 cells/mm^3, respectively.

Table 1
General clinical and biological presentations of all patients.

	N=125
Age (years)	13.0 (8.0-15.0)
Sex: M (%)	63
Temperature at admission (°C)	38.3 (37.5-39.0)
Headache (%)	69
Retro-orbital pain (%)	31
Myalgia/arthralgia (%)	38
Nausea (%)	35
Vomiting (%)	25
Abdominal pain (%)	26
Petechial rash (%)	42
Conjunctival injection (%)	51
Positive Tourniquet test (%)	93
Bleeding (%)	13
Hematocrit (volume%)	39.7 (37.0-42.75)
Platelet count (/mm^3)	142,020 (102,250-174,500)
White cells count (/mm^3)	5,018 (3,000-6,000)
Neutrophils count (/mm^3)	2,321 (1,500-2,835)
Day of admission (range)	3.9 (2-7)
Day of second sampling (range)	6.4 (4-9)
Day of third sampling (range)	13.7 (9-25)

Except for the day ranges, the numbers in parentheses represent the lower quartiles and upper quartiles, while the numbers not in the parentheses represent the median.

The median length of admission was 3.9 days after reporting the onset of fever. The second and the third blood sample were obtained 6.4 days and 13.7 days, respectively, after the onset of symptoms (Table 1).

No case of circulatory failure was noticed. According to WHO criteria (WHO, 1997), 4% of hospitalized patients (2.7% in Nha Trang and 6% in Phan Thiet) had DHF.

Dengue virus was isolated in cell culture and/or detected by RT-PCR in 20.8% of the patients: 20% (N = 15) in Nha Trang and 22% (N = 11) in Phan Thiet. In Nha Trang, only the DEN-4 serotype was detected. In Phan Thiet, DEN-2 was isolated from 3 blood samples (6%) and DEN-4 from 8 sera (16%).

A total of 86.4% of patients (90.7% in Nha Trang, 80% in Phan Thiet) had a confirmed diagnosis of acute dengue fever by serological tests (dengue virus-specific IgM by MAC-ELISA

Table 2
Clinical and biological presentations of acute dengue infection confirmed by virus isolation.

	Dengue virus isolated (N=26)	Non-dengue acute illness (N= 17)	p-value
Age (years)	11.0 (7.25-14.75)	11.0 (8.0-12.0)	0.941[a]
Sex: M (%)	53	69	0.28[b]
Temperature on admission (°C)	38.7 (38.0-39.5)	38.0 (37.5-39.7)	0.623[a]
Headache (%)	69	76	0.605[a]
Retro-orbital pain (%)	38	41	0.859[a]
Myalgia/arthralgia (%)	38	38	1[b]
Nausea (%)	27	35	0.559[b]
Vomiting (%)	12	41	0.0245[b]
Abdominal pain (%)	24	24	0.972[b]
Petechial rash (%)	46	35	0.48[b]
Conjonctival injection (%)	67	41	0.105[b]
Positive Tourniquet test (%)	88	88	0.982[b]
Bleeding (%)	8	18	0.319[b]
Hematocrit (volume%)	39.0 (36.3-43.7)	39.0 (37.4-40.2)	0.794[a]
Platelet count (/mm^3)	124,000 (101,000-184,000)	159,000 (121,000-217,000)	0.202[a]
Anti-dengue IgG by ELISA test (% of positive)	100	41	<0.001[b]

The numbers in parentheses represent the lower quartiles and upper quartiles, while the numbers not in the parentheses represent the median.
Tests used: [a]Wilcoxon test; [b]Pearson test

and/or HI test) and/or dengue virus isolation in cell culture or PCR. There was non significant difference between the hospitals (p=0.11, Fisher's exact test)

With acute or convalescent sera, using the criteria of the IgM to IgG ratio ≥ 1.8 to define a primary case, 4% of the patients had a primary acute dengue infection (5.3% in Nha Trang, 2% in Phan Thiet) and 78.4% a secondary or tertiary acute dengue infection (81.3% in Nha Trang and 74% in Phan Thiet).

The anti-dengue and anti-JE virus IgG ELISA tests were positive in 88.8% and 82.4%, respectively, of the patients.

Clinical and biological comparisons between non-dengue acute illness and dengue infection confirmed by virus isolation or serological tests and virus isolation are shown in Tables 2 and 3. Table 4 describes the differences between patients with dengue fever hospitalized in Nha Trang and Phan Thiet.

In five patients (4 in Nha Trang, 1 in Phan Thiet), the ratio of dengue IgM to JE IgM was < 0.91 These samples were sent to the Institut Pasteur in Cambodia (Dr J-M Reynes, Virology Unit) for JE virus isolation in cell culture and identification by RT-PCR, but all the results were negative. Table 5 shows the characteristics of these patients.

No Chickungunya or Sindbis virus infections were detected by the HI tests.

DISCUSSION

To our knowledge, our study is unique in Central Vietnam. It describes clinical and biological presentations of patients hospitalized during a dengue outbreak in which DEN-4 was the predominant circulating serotype.

DEN-4 serotype was first detected in 1987 in some Vietnamese provinces but with a low attack rate (3.5%). It was later isolated in 1990, 1991, 1992, 1997, and 1998. In 1999, DEN-4 activity increased to 26% of the investigated cases and spread to several provinces in North and South Vietnam. This DEN-4 outbreak in 2001-2002 was foreseeable because in 1999 most of the children under 12 years of age had never been exposed to DEN-4, and thus were

Table 3
Clinical and biological presentations of acute dengue infection confirmed by serological tests and virus isolation.

	Acute dengue infection (N=108)	Non-dengue acute illness (N=17)	p-value
Age (years)	11.0 (8.0-15.25)	11.0 (8.0-12.0)	0.774[a]
Sex: M	53	65	0.345[b]
Temperature on admission (°C)	38.0 (37.5-39.0)	38.0 (37.5-39.7)	0.453[a]
Headache (%)	68	76	0.493[b]
Retro-orbital pain (%)	30	41	0.353[b]
Myalgia/arthralgia (%)	38	38	0.946[b]
Nausea (%)	35	35	0.993[b]
Vomiting (%)	22	41	0.0925[b]
Abdominal pain (%)	26	24	0.817[b]
Petechial rash (%)	43	35	0.57[b]
Conjunctival injection (%)	52	41	0.391[b]
Positive Tourniquet test (%)	93	88	0.441[b]
Bleeding (%)	12	18	0.551[b]
Hematocrit (volume%)	40.0 (37.0-42.9)	39.0 (37.4-40.2)	0.492[a]
Platelet count (/mm^3)	128,000 (101,000-164,500)	159,000 (121,000-217,000)	0.0686[a]
Anti-dengue IgG by ELISA test (% of positive)	96	41	<0.001[b]

The numbers in parentheses represent the lower quartiles and upper quartiles, while the numbers not in the parentheses represent the median.
Tests used: [a]Wilcoxon test;[b] Pearson test

Table 4
Clinical and biological presentations of acute dengue infection: a comparison between patients from Nha Trang and Phan Thiet's hospitals.

	Nha Trang (N=68)	Phan Thiet (N=40)	p-value
Age (years)	12.0 (9.0-19.25)	9.0 (7.0-12.0)	<0.001[a]
Sex: M (%)	72	52	0.0398[b]
Temperature on admission (ºC)	38.5 (37.97-39.25)	38.0 (37.0-38.25)	<0.001[b]
Headache (%)	87	36	<0.001[b]
Retro-orbital pain (%)	43	8	<0.001[b]
Myalgia/arthralgia (%)	40	34	0.571[b]
Nausea (%)	28	48	0.0398[b]
Vomiting (%)	12	40	<0.001[b]
Abdominal pain (%)	13	49	<0.001[b]
Petechial rash (%)	28	68	<0.001[b]
Conjunctival injection (%)	42	71	0.00391[b]
Positive Tourniquet test (%)	91	97	0.208[b]
Bleeding (%)	10	16	0.379[b]
Hematocrit (volume%)	39.9 (36.07-43.0)	40.0 (37.0-42.0)	0.899[a]
Platelet count (/mm^3)	127,000 (105,750-177,500)	128,000 (100,000-140,000)	0.141[a]
Anti-dengue IgG by ELISA test (% of positive)	96	98	0.611[b]

The numbers in parentheses represent the lower quartiles and upper quartiles, while the numbers not in the parentheses represent the median.
Tests used: [a]Wilcoxon test; [b]Pearson test

Table 5
Characteristics of the patients with suspected Japanese encephalitis virus infection.

	Values
Number of patients	5
Age (years)	17.8 (12-25)
Sex: M (%)	100
Temperature on admission (ºC)	38.3 (37.5-39.5)
Headache (%)	60
Retro-orbital pain (%)	40
Myalgia/arthralgia (%)	60
Nausea (%)	0
Vomiting (%)	0
Abdominal pain (%)	20
Petechial rash (%)	60
Conjunctival injection (%)	100
Positive Tourniquet test (%)	100
Bleeding (%)	0
Hematocrit (volume%)	42.8 (35.7-47)
Platelet count (/mm^3)	129,000 (103,000-197,000)
Ratio DEN virus IgM:JE virus IgM	0.43 (0.1-0.78)
Anti-JE virus IgG ELISA test (% of positive)	100

Values are the medium (range) or number (%)

susceptible to this serotype (Ha, 2000).

In our study, dengue virus was detected in 20.8% of the blood samples obtained at the time of admission. The patients usually waited four days after onset of the fever before presenting to the hospital. By that time, the virus had already disappeared in some patient's sera, but the diagnosis was made by serological tests in over 80% of individuals.

Primary dengue virus infections represented only 4% of confirmed cases. Such a low frequency of primary cases seems to be usual for endemic countries. Endy *et al* (2002) found that in symptomatic acute dengue fever in Thai children, 3.9% were primary cases.

Because of cross-reactivity between anti-dengue and anti-JE virus IgG by ELISA tests (Koraka *et al*, 2002), we can only conclude that most of the patients were previously exposed to a flavivirus infection. The results of the anti-dengue and anti-JE virus IgM by MAC-ELISA tests suggest that dengue fever was more frequent than JE infections in the studied population. Because most DHF cases occur after sequential heterotypic dengue virus infection (secondary infection) (Rothman and Ennis, 1999), it is important to note that 78.4% of individuals had been previously exposed to flavivirus infection.

Since some convalescent sera were missing, the HI test was less efficient in diagnosing dengue infections and distinguishing primary from secondary infections than the ELISA test, which only need one sample.

Patients with acute dengue fever were older (12 *versus* 9 years) and more often male (72% *versus* 52%) when comparing Nha Trang with Phan Thiet, respectively. Even though all the investigators who participated in the study used similar case report forms, the findings in patients with acute dengue fever were sometimes different between the 2 provincial hospitals: nausea, vomiting, abdominal pain, petechial rash and conjunctivitis were more often reported at Binh Thuan Hospital than at Khanh Hoa Hospital. In Phan Thiet, the clinicians observed symptoms of headache and retro-orbital pain less often. This observation suggests the poor specifity of clinical symptoms in the diagnosis of dengue fever.

In addition, we did not find any statistically significant differences with the symptoms, except for vomiting, between the patients infected with dengue virus and with non-dengue febrile illnesses (Table 1). Among the patients with acute dengue virus infection, the most common presenting symptom was headache, followed by conjunctivitis, petechial rash, muscle and joint pains, nausea and abdominal pain. The clinical presentation, tourniquet test, and blood cell counts (data not shown) were similar in patients hospitalized with other febrile illnesses. In Thailand, similar clinical observations have been previously discussed (Endy *et al*, 2002). The tourniquet test, one of the tests proposed by the WHO for the diagnosis of DHF, was found positive in 88% of non-dengue acute illnesses. This test had previously been found to poorly differentiate between DHF and dengue fever in Vietnam (Phuong *et al*, 2002).

During our study, only five DHF cases (4%) and no DSS were registered at the participating hospitals. This number is low compared with the 5 to 10% of DHF and DSS usually reported with

dengue infections (Halstead, 1988; Rigau-Perez *et al*, 1988), specially in a selected population of hospitalized patients. Several factors, including the status of immunity to other viral strains (patients with DEN-2 infections experience more severe disease than those infected with other serotypes) (Vaughn *et al*, 2000; Kalayanarooj and Nimmannitya, 2000) could explain the mild severity of this outbreak.

Five patients (4%) were suspected to have JE virus infection. The absence of neurological symptoms does not exclude the diagnosis of JE virus infection, because infections are 25 to 1,000 times more likely to be asymptomatic than symptomatic (Benenson *et al,* 2000; Solomon *et al*, 2000). In North Thailand, Watt and Jongsakul (2003) diagnosed JE virus infections in 14% adults with undifferentiated fever. Fever, conjunctival infection, a positive tourniquet test, petechial rash, headache, and mild thrombocytopenia were the predominant features of JE infection in our cases. No bleeding was observed, but the small number of cases did not allow us to make any statistically significant conclusion.

All these observations suggest that Vietnamese clinicians who practise in both these provincial hospitals have good experience in the clinical detection of dengue fever and DHF cases.

Nevertheless, dengue fever diagnosis should be improved by using biological tests to complement non-specific clinical symptoms, because early case detection is known to be significantly associated with a reduction in mortality (Dung *et al*, 1999). This is particularly important during inter-epidemic periods when most clinicians are less aware of dengue fever.

In addition, JE virus infection should be considered in the differential diagnosis of dengue fever with or without neurologic signs.

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