DIAGNOSIS OF DENGUE INFECTION USING VARIOUS DIAGNOSTIC TESTS IN THE EARLY STAGE OF ILLNESS

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Abstract. In order to elucidate the usefulness of various tests in the early course of dengue infection, in terms of diagnosis and correlation with clinical severity, blood specimens were collected every 48 hours on 3 occasions from patients with clinical suspicion of dengue infection with fever for less than 4 days. Viral isolation was attempted by mosquito inoculation (MI), tissue culture inoculation (TC), and reverse transcriptase polymerase chain reaction (RT-PCR). Antibodies were detected by hemagglutination inhibition test (HI), an in-house-ELISA (IH-ELISA), and an ELISA by MRL diagnostics Clinical data were collected from the time of enrollment to complete recovery. Of the 40 patients enrolled, 31 were diagnosed as dengue infection and confirmed by either serology or viral isolation. Of these, 12 had primary infection and 19 had secondary infection. Dengue fever occurred in 9 cases. Dengue viruses were isolated from 28 out of 31 patients, and dengue hemorrhagic fever was diagnosed in 22 patients. Viral serotypes identified by viral isolation, and RT-PCR were concordant: DEN1 was isolated in 8, DEN2 in 13, DEN3 in 5, and DEN4 in 2 patients. Viral isolation yielded positive results on blood collected before the 5th day of fever. MI was more sensitive than TC. RT-PCR was less sensitive than viral isolation during the early days of fever, but became more sensitive after the 5th day of fever. RT-PCR was able to detect virus up to day 7-8 of fever, even after defervescence, and in the presence of antibody. During the febrile stage, serological diagnosis on blood samples taken 48 hours apart was carried out by HI, IH-ELISA, and MRL-ELISA, facilitating diagnosis in 3 (10%), 21 (67%), and 27 (87%) of patients, respectively. All of the patients with secondary infection were diagnosed by MRL-ELISA before defervescence. By the 8th day of fever, a serological diagnosis aided to diagnose in 9 (29%), 29 (93%), and 31 (100%) of patients by HI, IH-ELISA, and MRL-ELISA, respectively.

INTRODUCTION

The worldwide incidence of dengue infection is estimated at over 100 million cases annually (Monath, 1994). Each year in Thailand, more than 100,000 children become sick with dengue infections and more than 400 die. Appropriate supportive care and fluid therapy are crucial for a good outcome and should be started as soon as possible. A high index of suspicion and early diagnosis are therefore important. Conventional serologic diagnosis using paired blood samples taken 2 weeks apart can usually be made after defervescense, but this strategy is not helpful for guiding management. Viral detection is possible during the early course of fever. However, this is not useful if the patient presents late. The objective of this study was to evaluate various diagnostic tests during the febrile stage of dengue infection, and whether paired blood samples taken 48 hours apart, during the febrile stage, were able to provide a serologic diagnosis using various methods.

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MATERIALS AND METHODS

This prospective study was conducted at Siriraj Hospital, a tertiary care center in Bangkok, between August 2000 and August 2001. Parents of children who presented with fever for less than 4 days and other symptoms compatible with dengue infection (*ie* facial flushing, hepatomegaly, petechiae, and anorexia) were given information about the study. Only children whose parents signed consent forms were enrolled in the study.

Blood samples were taken at enrollment, and 48 and 96 hours later. The information from the study was available to the attending physician; however, there was no treatment intervention in this study. Each blood sample was tested for the presence of dengue virus by mosquito inoculation (MI) and tissue culture (TC) inoculation techniques. Viral genomic detection was done by reverse transcriptase polymerase chain reaction (RT-PCR), and antibody against dengue virus by the hemagglutination inhibition test (HI), in-house enzyme-linked immunosorbent assay (IH-ELISA) for IgG and IgM (captured), and enzyme-linked immunosorbent assay by MRL Diagnostics (Cypress, California; MRL-ELISA) were conducted at the Center for Vaccine Development, Institute of Sciences and Technology for Research and Development, Mahidol University, Salaya, Thailand. Relevant clinical outcomes were recorded. The Ethics Review Board of the Faculty of Medicine Siriraj Hospital, Mahidol University, Thailand, approved the study.

Viral isolation by MI was carried out by intrathoracic inoculation on laboratory-reared *Toxorhynchites splendens*, as described earlier (Rosen, 1981; Rosen and Shroyer, 1985; Rosen *et al*, 1985). The presence of viral antigens was detected by direct fluorescence assay using anti-dengue fluorescein isothiocyanate (FITC) conjugate. Viral isolation in LLC-MK2 cell line was done by incubation for 7 days at 37ºC. After thawing 3 times, the supernatant was used for plaque assay. Identification of dengue was carried out by an indirect fluorescence assay using serotype-specific monoclonal antibodies. Detection of dengue virus DNA was carried out using serotype specific primers in a semi-nested PCR, as described by Lanciotti *et al* (1992).

The HI antibody against dengue virus types 1-4 and Japanese encephalitis virus (JEV) was performed as described previously (Clarke and Casals, 1958). The Japanese encephalitis and dengue antigens were provided by the National Institute of Health, Department of Medical Sciences, Ministry of Public Health, Thailand. Seroconversion, or high titers (≥1:2560), were suggestive of acute infection. Secondary infection was diagnosed if the titers after 1 week exceeded 1:1280 (WHO, 1986). IH-ELISA antibody against dengue and JEV were carried out as described previously (Innis *et al*, 1989). For single sera IgM titers of >1:40 (with dengue IgM >JEV IgM) and for paired sera, a 2-fold rise in IgM titer to >1:30 was considered evidence of acute dengue infection. The MRL-ELISA was carried out according to the manufacturer's instructions. For single serum, MRL-ELISA IgM titers >3 and an IgG titer >4, and for paired sera, a 2-fold rise of titer to >1, were considered evidence of acute dengue infection. An ELISA IgM/IgG ratio of ≥1.8:1 was considered a primary infection.

RESULTS

Of the 40 children enrolled, 3 were excluded because the final diagnoses were not dengue infection, and 6 were excluded because of inadequate blood samples. The 31 children, 15 boys and 16 girls, included in the study, were 3-13 years old (median 8 years). On enrollment, fever had been present for 2 days in 3 cases, 3 days in 10 cases, and 4 days in 18 cases. The final diagnoses were dengue fever (DF) in 9 (29%) cases and dengue hemorrhagic fever (DHF) in 22 (71%) cases. Complications observed were encephalopathy in 2 cases and upper GI bleeding in 2 cases. During the defervescent period, 2 cases had impending shock.

All 31 children had serologic confirmation of dengue infection; 12 had primary, and 19 had secondary, infections. The patients with secondary infection were more likely to have severe infections (Table 1). Viral isolation or RT-PCR was positive in 28 (90%) cases. Three cases with negative viral isolation and RT-PCR had first blood drawn on the 4th day of fever (Table 2), however they had serological confirmation of acute infection. Of the 25 samples positive for viral isolation by the MI method, only 19 (76%) samples were positive by TC method. In 2 samples, the virus was isolated by TC, but not by MI.

RT-PCR was less sensitive than viral isolation until after the 5th day of fever when RT-PCR became more sensitive. RT-PCR was able to detect viral genome in half the specimens on day 7, when viral isolation was rarely positive (Fig 1). After defervescence, the MI method was able to detect the virus in 2 (6%) cases (both primary infections) and the PCR method was able to detect the virus in 7 (23%) cases (4 primary and 3 secondary infections). The 5th day of fever is the transition day from viral detection to an increase in antibody titer (Fig 1). During the febrile stage, serological diagnosis aided diagnosis using HI,

Table 1
Clinical and serological features of dengue infection in 31 patients.

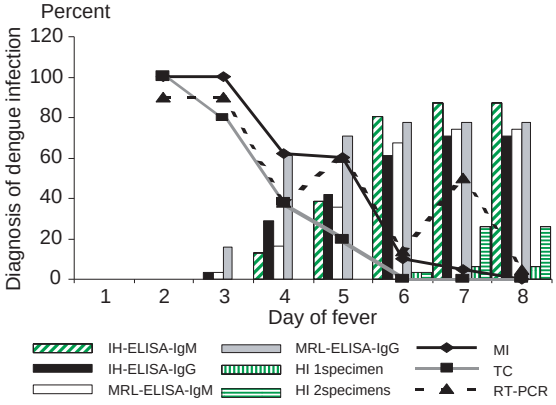
	Primary (N=12)	Secondary (N=19)
DF[a]	5	4
DHF[b] grade 1	3	8
DHF[b] grade 2	4	5
DHF[b] grade 3	-	2
Encephalopathy[c]	1	1
Upper GI bleed	-	2
Dengue serotype 1	5	3
Dengue serotype 2	3	11
Dengue serotype 3	4	1
Dengue serotype 4	0	2
Mean of last day of fever that virus was detected (latest day)	Day 5 (Latest = day 7)	Day 3.6 (Latest = day 5)

[a]DF = Dengue fever; [b]DHF = Dengue hemorrhagic fever; [c]Both were infected by dengue type 2.

Table 2
Performance of serological diagnostic tests during the febrile stage: blood drawn at admission and 48 hours later.

No. of sample available before/on defervescence day	N	No. (%) of patients with a titer level that met the criteria for serologic diagnosis by various methods						
		HI	IH-ELISA			MRL-ELISA		
			IgM alone	IgG alone	IgM or IgG	IgM alone	IgG alone	IgM or IgG
1	13	0	3(23)	6(46)	7(54)	2(15)	10(77)	10(77)
2	18	3(17)	13(72)	8(57)	14(78)	16(88)	11(61)	17(94)
Total	31	3(10)	16(51)	14(45)	21(67)	18(58)	21(67)	27(87)
		(1^o = 0)	(1^o = 7)	(1^o = 1)	(1^o = 7)	(1^o = 8)	(1^o = 2)	(1^o = 8)
		(2^o = 3)	(2^o = 9)	(2^o = 13)	(2^o = 13)	(2^o = 10)	(2^o = 19)	(2^o = 19)

Note: Criteria for diagnosis: HI:>4-fold rise of titer; IH-ELISA: rise of titer to >40; MRL-ELISA: rise of titer to >1 or, in single blood, IgM>3, IgG>4.
Primary infection = IgM: IgG>1.8 or HI titer<1:1280; 1^o = primary infection, 2^o = secondary infection.



*The serological diagnosis after day 5 of fever was determined using knowledge of the antibody titer measured on an earlier day of fever.

Fig 1–Diagnosis of dengue infection by various diagnostic methods according to days of fever.

IH-ELISA, and MRL-ELISA in 3 (10%), 21 (67%), and 27 (87%) of the patients, respectively (Table 2). All the patients with secondary infections were diagnosed by MRL-ELISA before defervescence, as IgG rose very rapidly within 3-4 days of fever (Fig 2). Up to day 8 of fever, regardless of defervescence, serological diagnosis was possible by using serial sera 48 hours apart in 9 (29%), 29 (93%), and 31 (100%) patients by HI, HM-ELISA, and MRL-ELISA, respectively (Fig 3). The cost and turn-around time for each test is shown in Table 3.

DISCUSSION

Although 90% of dengue infections are mild or asymptomatic (Burke *et al*, 1988), a significant

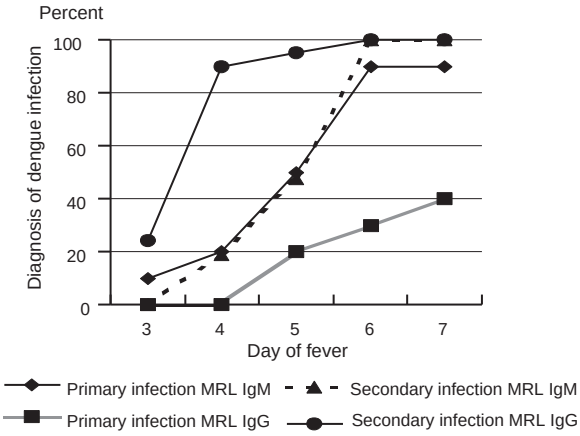


Fig 2–Diagnosis of dengue infection made by MRL-ELISA IgG or IgM according to days of fever.

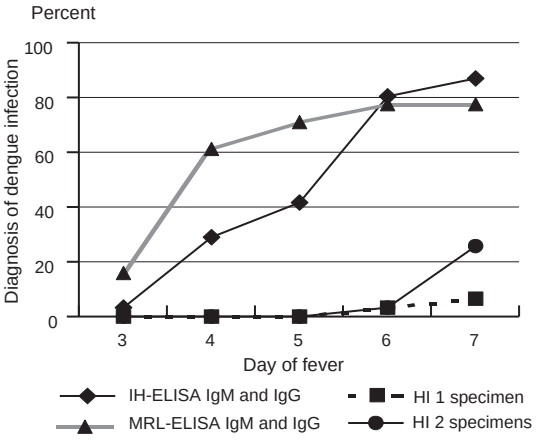


Fig 3–Diagnosis of dengue infection by various serologic methods according to days of fever.

Table 3
Cost and turn-around time of various diagnostic tests.

Test	Cost per test (US$)	Turn-around time
Viral isolation: mosquito inoculation	6	14 days
Viral isolation: tissue culture	10	7 days
Reverse transcriptase-polymerase chain reaction	25	12 hours
IH-ELISA IgG and IgM	5 (2.5 each)	5 hours
MRL-ELISA IgG and IgM	15 (7.5each)	4 hours
Hemagglutination inhibition	2	2 days

IH-ELISA = in-house-enzyme-linked immunosorbent assay; MRL-ELISA = enzyme-link immunosorbent assay by MRL Diagnostics (Cypress, California).

number of children in hyper-endemic areas are hospitalized for fear of DHF and its severe, mortal outcome. Early diagnosis is crucial for appropriate fluid management. Unawareness of the disease may result in missing the chance to resuscitate patients during the circulatory shock stage or in giving inappropriate fluid therapy causing fluid over-load and complications. Some patients may present atypically with encephalitis. A diagnosis of dengue encephalitis generally has a better prognosis than other causes of encephalitis (Chokephaibulkit *et al*, 2001). An accurate and timely diagnostic method for dengue infection is needed. The ideal test should be simple, easy to perform, cheap, and able to differentiate recent from acute infections. The latter is particularly important in a hyper-endemic area where dengue infection may occur in almost every child throughout the year but may not be the cause of the acute illness that presents. A mistaken diagnosis of dengue may cause parental alarm and over-treatment.

The diagnostic test will help management only if it can diagnose during the febrile stage, and before a potential massive leakage of intravascular fluid occurs. The ideal test would be viral detection, which is generally positive in the early phase of illness. Dengue virus can be isolated from almost all patients if the blood is taken early enough, *ie* before defervescense (Vaughn *et al*, 1997). Primary infection has a longer duration of viremia than secondary infection. The rapid rise of antibody in secondary infection clears the virus from the blood within 24 hours (Vaughn *et al*, 1997). Therefore, viral isolation may yield negative results if blood is drawn late in the course of fever. Our study confirmed the high rate of viremia during the febrile stage.

RT-PCR is a more sensitive test for the presence of virus. Earlier studies found RT-PCR to be more sensitive than viral isolation (Chan *et al*, 1994; Thayan *et al*, 1995), especially in sera with measurable antibody that could neutralize the vi-

rus. We found that during the early days of illness, viral isolation by MI was more sensitive than RT-PCR. However, after day 5 of fever or defervescence, RT-PCR was more sensitive. We also found that viral isolation using a tissue culture technique, (LLC-MK2 cell), was not as sensitive as MI. However, the turn-around time was much shorter and the methodology was easier than the MI method. Viral isolation and RT-PCR are complementary for detecting virus in the febrile stage. The data from our study confirm that a combination of both tests can detect virus in 90% of patients with fever for less than 4 days.

Serology seems to be the most reliable test for the evidence of infection (Vaughn *et al*, 1997). It is much cheaper than viral isolation and PCR, but requires a late blood sample to show the rise of antibody. Traditionally, paired blood samples, 1-2 weeks apart, are needed. It is the appropriate confirmatory test in patients who present late in the illness. The serological results, however, are usually available too late to guide treatment. In the non-research setting, a test that performs well at any stage of fever, before defervescense, is the most helpful. Our study attempts to make a serological diagnosis during the febrile stage. We found that a strategy of paired blood samples drawn on admission and 48 hours later could make a diagnosis in 87% of patients, and all patients with secondary infection if MRL-ELISA was used. This test is useful due to its short turnaround time, but it is relatively expensive. The IH-ELISA performed quite well on days 7-8 of fever, but could only diagnose half the patients before defervescence, consistent with a previous study (Vaughn *et al*, 1997). IH -ELISA is cheap but requires a longer turn-around time, making it less useful in guiding treatment. HI is the least useful method for diagnosis during the febrile stage. The HI antibody rose in only one-third of patients on days 7-8 of fever.

Finally, we did not find any correlation between the onset or duration of positivity, or level of titers of the diagnostic tests and the severity of disease. In conclusion, our study demonstrated that viral detection by MI and PCR were both useful at different times during the early febrile stage. ELISA was able to make a serological diagnosis with paired blood samples taken 48 hours apart, particularly in the cases of secondary infection, which may be potentially severe. MRL-ELISA outperformed IH-ELISA, and HI was not useful for making a diagnosis during the febrile stage of dengue infection.

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