MOLECULAR TYPING OF DENGUE VIRUSES CIRCULATING ON THE EAST COAST OF PENINSULAR MALAYSIA FROM 2005 TO 2009

G Vinomarlini, Tg Rogayah, TS Saraswathy, R Thayan, M Apandi, MK Fauziah and Zainah Saat

Virology Unit, Infectious Diseases Research Centre, Institute for Medical Research, Kuala Lumpur, Malaysia

Abstract. From 2005 to 2009, the Institute for Medical Research (IMR), Kuala Lumpur, Malaysia received 488 serum and blood samples from hospitalized patients on the East Coast of Peninsular Malaysia, suspected of having dengue infection. In this study we determined the prevailing dengue serotypes using a real time polymerase chain reaction assay (RT-PCR). All 4 dengue virus serotypes were found circulating during the study period; however the predominant serotype varied. In 2005 and 2006, the predominant serotypes circulating were DENV-1 and DENV-3, in 2007, DENV-1 and DENV-2 were predominant, and in 2008 and 2009, DENV-3 was the predominant serotype.

Keywords: dengue, serotype, RT-PCR, Malaysia

INTRODUCTION

Dengue fever (DF) is an acute, self limited febrile viral disease of 2-7 days duration, characterized by sudden onset fever and a variety of other symptoms, including headache, joint and muscular pain, and rash (Chow, 1997). Occasionally, hemorrhagic manifestations, such as skin hemorrhages, bleeding gums and gastrointestinal hemorrhage, occur in dengue infected patients (Gubler, 1998).

The incidence of dengue has grown dramatically around the world in recent decades (WHO, 2009). About 2.5 billion people (two fifths of the world's population) are now at risk from dengue (WHO, 2009). The WHO currently estimates there are 50 million dengue infections worldwide each year (Gubler, 1997). The disease is now endemic in more than 100 countries in Africa, the Americas, the Eastern Mediterranean, Southeast Asia and the Western Pacific. Southeast Asia and the Western Pacific are the most seriously affected (WHO, 2008). Before 1970 only nine countries had experienced dengue epidemics, a number that had increased more than four-fold by 1995. Not only has the number of cases increased, the disease is spreading to new areas and explosive outbreaks are occurring. In 2007, Venezuela reported more than 80,000 cases, including more than 6,000 cases of dengue hemorrhagic fever (DHF) (WHO, 2006).

The dengue virus, formal name: Den-

Correspondence: Vinomarlini Gunasagaran, Virology Unit, Infectious Diseases Research Centre, Institute for Medical Research, Jalan Pahang, 50588 Kuala Lumpur, Malaysia.
Tel: 603-2616 2674; Fax: 603- 2696 8094
E-mail: vinomarlini@imr.gov.my

gue virus DENV), belongs to the Flaviviridae family, which includes yellow fever and Japanese encephalitis virus. Dengue virus has four serotypes, Den 1 to Den 4. Infection with one of these serotypes conveys life-long immunity but not cross-protective immunity to the other serotypes (Schwartz *et al*, 1996). Serologic analysis is difficult because of cross-reactivity between the viruses. Humans are infected with DENV by the bite of infective *Aedes* mosquitoes (*Aedes aegypti* and *Aedes albopictus*) (Ramos *et al*, 2008). The most important vector is *Aedes aegypti*, which prefers to feed on humans during daylight hours. The incubation time is 3-14 days, most often 4-7 days. Transmission of DF increases during the rainy season (Sangkawibha *et al*, 1984).

The main objective of this study was to determine the prevalence of DENV subtypes along East Cost Peninsular Malaysia from 2005 to 2009 by Real Time Polymerase Chain Reaction (RT-PCR). The East Coast of Peninsular Malaysia includes the states of Kelantan, Pahang and Terengganu.

MATERIALS AND METHODS

Clinical samples

Serum and plasma samples from suspected dengue patients were obtained from 488 patients admitted to hospitals in East Coast Peninsular Malaysia. Blood or serum samples of patients suspected of having dengue infection from Day 2 until Day 14 from onset of disease were sent to the Virology Unit, Infectious Diseases Research Center for dengue detection and subtyping. All samples were stored at -70ºC until tested.

Primers

Detection and serotyping of the dengue virus was done by RT-PCR and confirmed by direct sequencing. The detection and sub-typing of dengue virus was done using published primers: UTR_F (5′ TTA GAG GAG ACC CCT CCC 3′) and UTR_R (5′ TCT CCT CTA ACC TCT AGT CC 3′) (Chutinimitkul *et al*, 2005).

RNA extraction

Viral RNA was extracted according to the Qiagen QIAamp Viral Mini protocol. One hundred forty microliters of sample was added to 560 µl AVL buffer containing carrier RNA and incubated at room temperature for 10 minutes. Then, 560 µl of 96-100% ethanol was added and centrifuged briefly to remove drops from inside the lid. Next, the solution was centrifuged at 8,000 rpm for 1 minute. This step was repeated with all the solution, then, 500 µl of AW1 buffer followed by AW2 buffer was added and centrifuged at 8,000 rpm. The filter column was centrifuged at 14,000 rpm for 3 minutes. The spin column was then discarded and replaced with a 1.5 ml microcentrifuge tube and 50 µl of AVE buffer was eluted and centrifuged for 1 minute at 8,000 rpm. The eluted RNA was stored at -70ºC until used for the PCR assay.

SYBR Green PCR

The extracted RNA was evaluated with the One-Step SYBR Green RT-PCR kit, using a method modified from Chutinimitkul *et al* (2005). Instead of using RT-PCR on cDNA, we optimized similar primers for a One-Step RT-PCR. This method was optimized using extracted RNA from each Dengue serotype, namely: Dengue 1 strain Hawaii (VR 1254), Dengue 2 strain New Guinea C (VR1584), Dengue 3 strain H87 (VR 1256 CAF) and Dengue 4 strain H241 (VR 1257). Following this, the assay was evaluated using local samples. The assay was performed using a RotoGene 6000 RT-PCR machine using

Table 1
Blood samples from suspected dengue infection cases, East Coast, Peninsular Malaysia 2005-2009.

Year	Positive	Negative	DEN-1	DEN-2	DEN-3	DEN-4	Total samples received
2005	21	103	8	1	3	0	124
2006	4	72	2	0	2	0	76
2007	22	105	6	6	5	2	127
2008	82	71	18	25	31	8	153
2009	5	3	0	0	5	0	8
Total	134	354	34	32	46	12	488

a One-Step QuanTitect SYBR Green kit (Qiagen, Hilden, Germany). After optimization, the RNA sample from the 488 collected specimens were assayed in a 25 µl reaction containing 2X QuantiTect SYBR Green, 2 µl of sample RNA, optimal concentration of primer 10 µM each) and QuantiTect RT. The thermal cycling profile of this assay consisted of 30 minutes RT at 50ºC, 15 minutes of Taq polymerase activation at 95ºC followed by 40 cycles of PCR of denaturation at 95ºC for 20 seconds, annealing at 55ºC for 30 seconds and extension at 72ºC for 40 seconds, extension at 72ºC for 7 minutes and melt curve analysis between 75ºC-95ºC at increments of 0.5ºC for 10 seconds each.

Following amplification, the melting curves were analyzed. This is to verify the specificity of the PCR product by looking at its Tm. The Tm for each sample was used to identify the dengue serotype and the samples sharing the same Tm were interpreted as belonging to the same serotype.

RESULTS

From 2005 to 2009, the Virology Unit, IMR received a total of 488 serum samples from hospitalized patients suspected of having DENV infection. Of these, 134 (37.5%) were positive for DENV infection. Twenty-one patients (18.9%) had DENV infection in 2005, 4 (5.9%) in 2006, 22 (23.9%) in 2007, 82 (63%) in 2008 and 5 (62.6%) in 2009 (Table 1).

Overall, from 2005 to 2009, DENV-3 was the most prevalent serotype with 46 cases (26.9%) followed by DENV-1 with 34 cases and DENV-2 with 32 cases (23.9%).

The largest number of cases in 2008 were from Kelantan State (62 cases; 72.0%) followed by Pahang State, (21 cases; 24.4%). In 2007, Kelantan had the largest number of cases (10; 52.6%) followed by Pahang (5; 26.3%).

The predominant dengue serotype circulating throughout the study was DENV-1. In 2006, the predominant serotypes were DENV-1 and DENV-3, in 2007 they were DENV-1, DENV-2 and DENV-3 and in 2008 and in 2009 was DENV-3.

In 2005, 67% of dengue infected paients were male, in 2006 59% were male, in 2008 88% were male and in 2009 60% were male.

The age group with the largest numbers of cases was the 31-50 years old group (Table 2).

Table 2
Distribution of dengue infection by age group (2005-2009).

Age in years	Number of dengue cases
>10	6
11- 20	31
21-30	32
31-50	40
51- 60	20
> 61	5
Total	134

Table 3
Day of disease sample taken.

Day of disease	Total samples received	PCR positive
1-3	104	31.4%
4-6	217	64.2%
7-10	125	3.7%
11-14	42	0.7%
Total	488	134

The largest number of positive cases were from Day 4 to Day 6 of disease and the smallest number of cases were from Day 11 to Day 14. The number of samples received during the first 6 days of infection was the largest indicating, this was the viremic phase. By the 7th day, antibodies appear contributing to the lower number of positive samples after Day 6.

DISCUSSION

Of the 488 patients from Kelantan, Terengganu and Pahang admitted to the hospital with suspected dengue infection only 27.5% were positive by RT-PCR for dengue infection. This probably reflects the time of collection of specimens. The period for viral RNA detection is usually to 5 to 7 days after onset of infection. After this, antibodies appear in the blood and the viral load drops. PCR specimens should be collected during the viremic phase of acute infection when the viral load is high, but patients often arrive at the hospital too late for viral RNA detection (Vaughn *et al*, 1997; Libarty *et al*, 2002). Some blood samples sent to our laboratory were not sent on ice and had lysed, probably contributing to some PCR inhibition.

In 2005, Pahang had more dengue cases than Kelantan and Terengganu. In 2006, both Kelantan and Pahang had the same number of cases, while in 2007, 2008 and 2009, Kelantan had the largest number of cases. This is probably due a dengue outbreak in 2007 and 2008 in Kelantan. Our study showed that all 4 dengue serotypes were circulating along the east coast of peninsular Malaysia.

Twenty-six percent, 23%, 35% and 7% of cases in our study were positive for the presence of dengue serotypes 1, 2, 3 and 4, respectively. The year 2008 had the greatest number of dengue cases due to a dengue outbreak in the state of Kelantan.

From 2005 to 2009, the male:female ratio of dengue cases was 1.2:1. A male:female ratio > 1 has been reported in other Asian countries, such as Singapore, India and Bangladesh (Guha-Sapir and Schimer, 2005). A study from Singapore showed the likelihood of dengue infection increased with time spent away from home and the majority of the males spend more time away from home than females (Ooi, 2001). However, in many Asian communities lower disease incidence in women may be a statistical artifact related to lower reporting for women who seek care from out-patient clinics or traditional

practitioners who do not report to public surveillance systems.

The most predominant age group infected with dengue from 2005 to 2009 was 31 to 50 years old (29%) followed by 21 to 30 years old (24%). Our samples represent hospitalized cases from government hospitals where patients suspected of dengue were tested as part of the clinical service provided. Persons with severe clinical symptoms are more likely to seek in-patient treatment compared to persons who are asymptomatic or mildly symptomatic. The risk of developing severe disease is increased in patients having a second dengue infection with a different serotype, possibly due to immune enhancement (Halstead, 1989). Severity of disease also depends on serotype, host factors, age and degree of viremia. Dengue incidence among older age groups in our study could reflect a higher risk of second infection.

In summary, our findings show dengue is a health problem along the east coast of peninsular Malaysia with all four serotypes circulating. The greatest number of dengue cases occurred in 2008 in Kelantan.

In 2005, Den 1 was the predominant serotype in 2006, Den 1 and Den 3 were the predominant serotypes, in 2007, Den 1, Den 2 and Den 3 were the predominant serotypes and in 2008 and 2009, Den 3 was the predominant serotype. This study underscores the need for effective dengue control measures.

ACKNOWLEDGEMENTS

The authors would like to thank the Director General of Health, Malaysia, and the Director of the IMR for permission to publish this paper.

REFERENCES

Chow VT. Molecular diagnosis and epidemiology of dengue virus infection. *Ann Acad Med Singapore* 1997; 26: 820-6.

Chutinimitkul S, Payungporn S, Theamboonlers A, Poovorawan Y. Dengue typing assay based on real-time PCR using SYBR Green I. *J Virol Methods* 2005; 129: 8-15.

Gubler DJ. Dengue and dengue hemorrhagic fever: its history and resurgence as global public health problem. In: Gubler DJ, Kuno G, eds. Dengue and hemorrhagic fever. New York: CAB International, 1997: 1-22.

Gubler DJ. Dengue and dengue hemorrhagic fever. *Clin Micobiol Rev* 1998; 11: 480-96.

Guha-Sapir D, Schimer B. Dengue fever: new paradigms for a changing epidemiology. *Emery Themes Epidemiol* 2005; 2: 1.

Halstead SB. Antibody, macrophages, dengue virus infection, shock, and hemorrhage. pathogenetic cascade. *Rev Infect Dis* 1989; 4: 830-9.

Libraty DH, Endy TP, Houng HS, *et al*. Differing influences of virus burden and immune activation on disease severity in secondary dengue -3 virus infections. *J Infect Dis* 2002; 185: 1213-21.

Ooi EE. Changing patterns of dengue transmission in Singapore. *Dengue Bull* 2001; 25: 40-3.

Ramos MM, Mohammed H, Zielinski-Gutierrez E, *et al*. The dengue serosurvey Working Group. Epidemic dengue and dengue hemorrhagic fever at the Texas-Mexico border: results of a household-based seroepidemiologic survey, December 2005. *Am J Trop Med Hyg* 2008; 78: 364-9.

Sangkawibha N, Rojanasuphot S, Ahandrink S, *et al*. Risk factors in dengue shock syndrome: a prospective epidemiologic study in Rayong, Thailand. *Am J Epidemiol* 1984; 120: 653-69.

Schwartz E, Mendelson E, Sidi Y. Dengue fever among travelers. *Am J Med* 1996; 101: 516-20.

Vaughn DW, Green S, Kalayanarooj S, *et al.* Dengue in the early febrile phase: viremia and antibody responses. *J Infect Dis* 1997; 176: 322-30.

World Health Organization (WHO). Fact sheet Nº117. Revised March 2009. [Cited 2010 Feb 5]. Available from: URL: http//www.who.int/mediacentre/factsheets/fs117/en/

World Health Organization (WHO). Report of the scientific working group meeting on dengue. Geneva: WHO, 2006: 1-168.

World Health Organization, Regional Office for South East Asia (WHO SEARO). Dengue status in South East Asia region: an epidemiological perspective. New Delhi: WHO SEARO, 2008. [Cited Jan 26]. Available from: URL: www.searo.who.int/LinkFiles/Dengue_dengue-sear-2008.pdf