DENGUE VIRUS VIRULENCE AND DISEASES SEVERITY

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Abstract. The dengue virus is the causative agent of a wide spectrum of clinical manifestations, ranging from mild acute febrile illness to classical dengue fever, dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). DHF and DSS are the potentially fatal forms of dengue virus infection, which has become an intractable public health problem in many countries. The pathogeneses of DHF/DSS are not clearly understood. One hypothesis concerning virus virulence and the immune enhancement hypothesis has been debated. Although dengue disease severity has been associated with evidence of genetic differences in dengue strains, virus virulence has been difficult to measure because of the lack of *in vivo* and *in vitro* models of the disease.

Keywords: dengue disease severity, dengue pathogenesis, dengue virus virulence

INTRODUCTION

Dengue virus (DENV) is the causative agent of a wide spectrum of clinical manifestations, ranging from mild acute febrile illness to classical dengue fever (DF), dengue hemorrhagic fever (DHF), and dengue shock syndrome (DSS). This virus has four major serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. The major pathophysiologic hallmark that determines disease severity and that distinguishes DHF/DSS from DF is plasma leakage (Hemungkorn *et al*, 2007). DHF and DSS are the potentially fatal forms of dengue virus infection, which has become a serious public health problem in many countries and is still expanding its range (Halstead, 1990).

The pathogenesis of DHF/DSS is not clearly understood (Martina *et al*, 2009). Current hypotheses of risk factors for DHF/DSS include immune enhancement, autoimmune responses against dengue non-structural 1 (NS1) protein, host genetic predisposition, and virus virulence (Prommalikit *et al*, 2004; Lin *et al*, 2006; Halstead, 2007; Waidab *et al*, 2007; Prommalikit and Thisyakorn, 2015). An important finding concerning the immune enhancement from the Phase III efficacy trial for a recombinant, live, attenuated tetravalent dengue vaccine (CYD-TDV) in Asia and Latin America showed the absence of more severe disease attributable to antibody dependent enhancement with an observation time of up to 25 months (Capeding *et al*, 2014; Dengue Vaccine Initiative, 2014).

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The virus virulence hypothesis arose from clinical, epidemiological associative, and entomologic studies that first described DENV virulence differences. This hypothesis suggests that DHF/DSS may result from infection by a more virulent serotype or strain within the serotypes of virus. This was first proposed to explain the recognition of DHF associated with the first isolation of dengue serotype 3 virus in the Philippines (Hammon, 1973).

The risk of DHF/DSS is higher in infections with dengue serotype 2, compared with the other serotypes. In Southeast Asia and the Americas, serotype 2 was associated with the first epidemics of DHF in these regions (Kourí *et al*, 1983; Sangkawibha *et al*, 1984; Burke *et al*, 1988). On the Pacific island of Tonga, an outbreak of dengue serotype 2 virus infection occurred in 1974, and an outbreak of serotype 1 virus occurred in 1975. The 1974 type 2 outbreak was characterized by relatively mild clinical disease with few hemorrhagic manifestations, a low attack rate, and relatively low viremia levels.

The 1975 type 1 outbreak was characterized by relatively severe disease with frequent hemorrhagic manifestations and a high attack rate. A difference in virus virulence was considered as the most likely explanation, because the differences between the outbreaks could not be attributed to differences of the human population in profusion, susceptibility to infection, mosquito vectors, prior immune status, or other characteristics (Gubler *et al*, 1978).

Previous studies were cited as evidence that severe dengue disease accompanies primary dengue infections, and these studies did not support the immune enhancement hypothesis. In 1964, severe illnesses were observed in an outbreak in the rural town of Ubon Ratchathani Province in Thailand, in which some cases had primary dengue virus of serotype 1 antibody responses (Halstead and Yamarat, 1965). In 1972, an outbreak of dengue serotype 2 virus on the small isolated Pacific island of Niue reported 790 cases. The conclusion drawn from this outbreak supported the contention that not all DHF cases are associated with a second dengue infection in an individual older than one year (Barnes and Rosen, 1974). One study (Scott *et al*, 1976) reported 114 patients with DHF admitted to Bangkok Children's Hospital during 1974. Over 40% of these patients had DSS, including three fatalities aged 4, 8, and 12 years of age who had primary dengue infections with shock. Their results suggest that dengue viruses are inherently virulent.

The magnitude and duration of dengue viremia, which did not significantly differ between primary and secondary dengue infection, determines disease severity (Murgue *et al*, 2000). Other researchers have described the role of the virus load in the pathogenesis of DHF and suggested that patients with dengue serotype 2 virus infections experienced more severe disease than those infected with other serotypes (Vaughn *et al*, 2000). Higher peak titers were associated with increased disease severity, with a peak titer identified (mean $10^{7.6}$ for those with DF versus $10^{8.5}$ for patients with DHF, p=0.01). Their study also indicated that viremia during primary infection was prolonged compared to secondary infection, and that the rate

Table 1
Dengue serotype 2 virus strains used for comparative sequence analysis.

Strain code	Clinical severity	Age (years)	Gender	Serologic response
ThNH7/93	DSS	12	Female	Secondary
ThNH28/93	DHF grade II	10	Male	Secondary
ThNH52/93	DHF grade I	7	Male	Secondary
ThNHp11/93	DF	14	Male	Primary

Table 2
Strain specific amino acid replacements among four dengue serotype 2 strains in their structural protein genes and a major nonstructural protein NS1 gene.

Strain code	Clinical severity	PrM		E					NS1				
		130	163	338	396	410	541	614	860	947	1002	1053	1056
ThNH7/93	DSS	I	I	K	C	V	H	K	R	R	R	G	D
ThNH28/93	DHF grade II	I	L	K	R	A	H	R	K	K	R	D	D
ThNH52/93	DHF grade I	I	I	K	C	V	R	R	K	K	K	D	D
ThNHp11/93	DF	R	I	R	C	V	H	K	K	K	K	D	E

I, isoleucine; R, arginine; L, leucine; K, lysine; C, cysteine; V, valine; A, alanine; H, histidine; G, glycine; D, aspartic acid; E, glutamic acid.

of virus clearance was faster in patients with secondary infection than in those with primary infection.

Molecular characterization of the dengue virus suggests that genetic variation between strains may be correlated with clinical manifestation and epidemiological characteristics (Rico-Hesse, 2010). Ribonucleic acid (RNA) nucleotide sequencing techniques and the use of these sequences to generate phylogenetic trees of evolutionary relationships among viruses led to the discovery that specific variant groups or genotypes were more frequently associated with dengue epidemics and with disease severity (Rico-Hesse, 1990; Lanciotti *et al*, 1994; Chungue *et al*, 1995; Lanciotti *et al*, 1997). However, researchers are still attempting to ascertain which genotypes are associated with higher virulence, severe disease, or larger epidemics. For dengue serotypes 2 and 3, genotypes that have undergone greater spread than the others and that have the potential to cause DHF appear to have been identified (Rico-Hesse, 2003).

Igareshi (1997) attempted to find direct evidence for the virus virulence hypothesis by comparative sequence analysis of dengue serotype 2 virus strains isolated from patients in the same epidemic area in the northeast of Thailand during the same

season with different clinical manifestations. All strains were isolated from serum specimens by inoculation into *Aedes albopictus* clone C6/36 cell cultures, and their serotypes were ascertained by reverse transcription polymerase chain reaction (RT-PCR). The envelope (E)/NS1 junctions of these viral genomes were sequenced and showed that all strains belonged to the same genotype II of dengue serotype 2 virus. For the analysis of the structural protein genes [capsid (C), membrane (PrM/M), and E] and noncoding regions, the target sequences were first amplified by rapid RT-PCR with infected C6/36 culture fluid. Virus strains, clinical severity, serological response, age, and gender of the patients are shown in Table 1. The results indicated a DF strain specific amino acid substitution from isoleucine (I) to arginine (R) at amino acid number 130 in the PrM and a DSS strain specific amino acid substitution from aspartic acid (D) to glycine (G) at amino acid number 1053 in the NS1 gene regions. This could significantly alter the nature of these proteins shown in Table 2. Moreover, DF strain specific nucleotide substitutions in the 3´-noncoding region were predicted to alter secondary structure. This study could not detect disease severity related molecular differences among strains isolated from patients with different clinical manifestations. Complete conservation of the 5´ noncoding region in this cluster of dengue serotype 2 virus strains indicated that this region was not related to the disease severity of dengue virus infection.

DHF/DSS was not observed when humans were infected by the American (AM) dengue genotype of serotype 2 as either a primary or a secondary infection. Watts *et al* (1999) reported a major epidemic of dengue serotype 2 virus infection in Peru in 1995, about 5 years after an epidemic of dengue serotype 1 virus infection had occurred in the same population. An estimated 60% of the population experienced a secondary dengue serotype 2 infection after having had a dengue serotype 1 infection. Dengue serotype 2 virus isolates were of the AM genotype. This study showed that secondary infection by the AM genotype of dengue serotype 2 did not cause DHF/DSS. The AM genotype strains may have lacked the properties necessary to cause severe disease. This same sequence of dengue serotype 1 infection followed by dengue serotype 2 infection resulted in a large DHF/DSS outbreak in Cuba in 1981, which was caused by a serotype 2 of Southeast Asian (SEA) origin (Guzmán *et al*, 1990; 1995). An editorial in the *Lancet* suggested the possibility of virulence differences between the Asian and AM dengue serotype 2 viruses (White, 1999).

Rico-Hesse (2003) found structural differences between the SEA and AM dengue serotype 2 in the prM gene, amino acid 390 in the E protein, in nucleotides 68-80 in the 5´ nontranslated region (NTR), and in the upstream 300 nucleotides of the 3´ NTR. They hypothesized that the primary determinants of DHF reside in (1) amino acid 390 of the E protein, which purportedly alters virion binding to host cells; (2) in the downstream loop (nucleotides 68-80) of the 5´ NTR, which may be involved in translation initiation; and (3) in the upstream 300 nucleotides of the 3´ NTR, which may regulate viral replication via the formation of replicative intermediates (Leitmeyer *et al,* 1999). The AM genotype dengue serotype 2 viruses are less transmissible by *Aedes aegypti* mosquitoes than SEA viruses and

AM replicative constraints produce lower titered viremias in humans than do SEA dengue serotype 2 viruses. All dengue serotype 2 viruses from DHF/DSS patients have been shown to belong to the South-east Asian genotype (Rico-Hesse, 2003).

In India, changes in dengue severity have been suggested to be a result of a change in the genotype of dengue serotype 2 virus and a change in the lineage of dengue serotype 1 virus on the basis of the E gene sequence (Kumar *et al*, 2010; Patil *et al*, 2011). Dengue serotype 1 virus was associated with DHF outbreak in Delhi in 1997 and was also implicated during recent outbreaks in 2006 and 2008 (Bharaj *et al*, 2008; Chakravarti *et al*, 2010). Patil *et al* (2011) sequenced the E gene of 13 Indian dengue serotype 1 virus isolates obtained between 1962 and 2005. Those 13 Indian dengue serotype 1 virus isolates were analyzed together with the available sequences of 40 globally representative isolates. The viruses were distributed into five genotypes (Americas/Africa, Malaysia, Thailand, Asia, and South Pacific). All the Indian dengue serotype 1 isolates were found to belong to the American African (Cosmopolitan) genotype and were distributed into four lineages [India I, II, III, and the Africa (Afro-India) lineage].

Patil *et al* (2012) described the population dynamics of the dengue serotype 3 virus (1966-2010) and the dengue serotype 4 virus (1961-2009), from which the E gene was sequenced and analyzed together with global sequences of 97 dengue serotype 3 virus and 43 dengue serotype 4 virus isolates retrieved from GenBank. The isolates obtained before 2000 were procured from the Virus Repository of the National Institute of Virology in Pune, India, at passage level 5 in infant mouse brains. After 2000, the isolates were obtained using C6/36 cells and sequenced at second passage level. Dengue serotype 3 virus isolates were distributed into five genotypes, namely, I-V, with six lineages (A-F) in genotype III. Dengue serotype 4 virus isolates were distributed into two genotypes: genotype I and a new genotype (genotype V). Genotype III of the dengue serotype 3 virus and genotype I of dengue serotype 4 viruses are more virulent and show higher dissemination potential.

Hapuarachchi *et al* (2013) reported a rare case of a fatal dengue serotype 4 virus infection complicated by encephalitis and multiple organ failure. Full genomes of serum and cerebrospinal fluid-derived viruses shared 99.99% similarity, indicating virus dissemination across blood-brain barrier. Although these virus genomes did not reveal any of the neurotrophic substitutions of DENV documented so far, case isolates possessed a combination of eight novel amino acid alterations, predominantly distributed in non-structural genes of dengue serotype 4 virus.

In Vietnam, the complete coding region of 187 dengue serotype 2 genomes and 68 E genes in viruses sampled from Vietnamese patients between 1995 and 2009 were sequenced. An episode of genotype replacement in which Asian 1 lineage viruses entirely displaced the previously dominant Asian/American lineage viruses was observed. A similar scenario in which a region-wide proliferation of Asian 1 lineage viruses appears to have occurred in Thailand and Cambodia. Investigation found that Asian 1 viruses attain higher

virus levels in the blood than viruses of the Asian/American lineage. This difference in virus titer is likely to have a profound impact on viral fitness by increasing the probability of mosquito transmission, thereby providing Asian 1 lineage viruses with a selective advantage (Hang *et al*, 2010).

In Brazil from 1990-2010, partial genome sequencing (genes C/PrM/M/E) was performed in 25 dengue serotype 2 virus strains and full-length genome sequencing (coding region) was performed in 9 strains (Faria *et al*, 2013). From percentage of similarity among the dengue serotype 2 virus strains in this study and of reference strains, two epidemiologically distinct groups were identified. One group represented strains isolated from 1990 to 2003, and the other group represented strains isolated from 2007 to 2010. No consistent differences were observed on the E genes in strains isolated from cases with different clinical manifestations. This suggests that, if the disease severity has a genetic origin, it is not only attributable to the differences observed on the E gene. Phylogeny characterized the dengue serotype 2 virus strains as belonging to the Southeast Asian genotype. Furthermore, all strains presented an asparagine in E_{390} previously identified as a probable genetic marker of virulence.

Antigenic and genetic differences in virus strains have now become evident. The lack of animal models has been the main hindrance to identifying the differences in virulence of dengue viruses (Rico-Hesse, 2003). Only a few of the DENV strains reported so far have elicited a virulent phenotype in mice, which results at best in an acute infection by which mice die within days with no or few clinical manifestations.

Tan *et al* (2010) described a DENV strain, which is highly virulent in mice and reproduces some clinical findings of severe dengue in humans, including the disease kinetics, organ damage/dysfunction, and increased vascular permeability. AG129 mice (129/Sv mice deficient in both interferon alpha/beta and gamma receptors) were administered with 10^7 to 10^2 plaque-forming units of D2Y98P (derived from a 1998 dengue serotype 2 Singapore human isolate that had been exclusively passaged for about 20 rounds in *Aedes albopictus* C6/36 cells) via the intraperitoneal route (0.4 ml in sterile PBS). They found that infection with a high dose of D2Y98P induced cytokine storm, massive organ damage, and severe vascular leakage, leading to hemorrhage and rapid death of the animals at the peak of viremia. In contrast, infection of AG129 mice with a lower dose of D2Y98P led to a transient asymptomatic systemic viral infection followed by death of the animals a few days after viral clearance similar to the disease kinetics described in humans.

Although dengue disease severity is associated with evidence of genetic differences in dengue strains, virus virulence has been difficult to measure because of the lack of *in vivo* and *in vitro* models of the disease. A strong case has been made that the association of the AM genotype dengue serotype 2 virus with mild severity during primary or secondary infections can be explained by low viral virulence.

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